AAPS Advances in the Pharmaceutical Sciences Series 38

Nicholas W. Warne
Hanns-Christian Mahler
Editors

Challenges in Protein Product Development

AAPS Advances in the Pharmaceutical Sciences Series

Volume 38

Series Editor

Yvonne Perrie, Strathclyde Institute of Pharmacy, University of Strathclyde,
Bearsden, Dunbartonshire, UK

The AAPS Advances in the Pharmaceutical Sciences Series, published in partnership with the American Association of Pharmaceutical Scientists, is designed to deliver volumes authored by opinion leaders and authorities from around the globe, addressing innovations in drug research and development, and best practice for scientists and industry professionals in the pharma and biotech industries.

More information about this series at http://www.springer.com/series/8825

Nicholas W. Warne · Hanns-Christian Mahler
Editors

Challenges in Protein Product Development

Editors
Nicholas W. Warne
BioTherapeutics Research and Development
Pharmaceutics Research and Development
Pfizer Inc.
Andover, MA
USA

Hanns-Christian Mahler
Lonza AG
Drug Product Services
Basel, Basel Stadt
Switzerland

ISSN 2210-7371 ISSN 2210-738X (electronic)
AAPS Advances in the Pharmaceutical Sciences Series
ISBN 978-3-319-90601-0 ISBN 978-3-319-90603-4 (eBook)
https://doi.org/10.1007/978-3-319-90603-4

Library of Congress Control Number: 2018939307

© American Association of Pharmaceutical Scientists 2018
This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
The publisher, the authors and the editors are safe to assume that the advice and information in this book are believed to be true and accurate at the date of publication. Neither the publisher nor the authors or the editors give a warranty, express or implied, with respect to the material contained herein or for any errors or omissions that may have been made. The publisher remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Printed on acid-free paper

This Springer imprint is published by the registered company Springer International Publishing AG part of Springer Nature
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

The use of therapeutic proteins continues to increase, with most of these protein drug products being used to treat severe diseases such as various types of cancer or inflammation. As the field of recombinant therapeutic proteins enters its fourth decade and the market for biopharmaceuticals becomes increasingly competitive, companies are increasingly dedicating resources to develop innovative biopharmaceuticals to address unmet medical needs.

Often, the pharmaceutical development scientist encounters challenging pharmaceutical properties of a given protein, the demands placed on the product by stability, manufacturing and preclinical or clinical expectations, as well as the evolving regulatory expectations and competitive landscape. Further, there have been new findings that require close assessment, for example, related to excipient quality, processing, viscosity and device compatibility and administration, solubility and opalescence and container-closure selection. The literature varies widely in its discussion of these critical elements, and consensus does not exist. This book aims to serve to provide a broad overview, yet deep insight, into these different topics. We seek to discuss several of these and other challenges currently faced in biotechnology dosage form development to provide guidance, shared experience and thoughtful reflection on how best to address these potential concerns.

In order to develop an adequate drug product of a therapeutic protein, significant considerations need to be made to the formulation, container-closure system, and processing. Only the combination of all yields an acceptable drug product. Firstly, Part 2 provides some insights into the formulation development of biologics. Recently, the degradation and quality of surfactants such as polysorbates have received significant attention. Furthermore, the use of excipients for formulations will impact not only protein stability but also the products' pharmaceutical parameters such as pH, viscosity, and osmolality. Excipients can crystallize under frozen conditions and may subsequently lead to challenges related to protein stability in the frozen state. In order to enable self-administration or improve convenience, high-concentration protein formulations are often required. Part 3 will discuss high-concentration protein formulations, related theoretical considerations, product considerations, including opalescence, appearance, and particulate matter,

but also analytical and predictive tools as well as impact and practical considerations related to protein concentration, such as viscosity and relation to device functionality. In most R&D portfolios, there are increasingly more "novel constructs" in research and development and less focus on "traditional" monoclonal antibodies. Part 4 will introduce different categories of novel therapeutic protein constructs, including antibody drug conjugates, fusion proteins, and strategies for half-life extension.

Significant focus during the development of drug products includes the development and selection of the container closure, required to provide protection and possible functionality for a given product. Historically, glass vials, rubber stoppers, and aluminum crimp caps are used for therapeutic protein injectables where self-administration is not desired or required. Increasingly, combination products are used, including syringes with needle-safety devices or autoinjector, injection pens or infusion (mini) pumps. Part 5 will introduce various technologies and discuss possible challenges related to primary packaging and device, including container-closure integrity testing, delamination, functionality of syringes and clogging, impact of silicone and tungsten and related analytics. Finally, the stability of a given product also needs to be ensured during preparation and administration and some considerations to in-use stability, including microbiological stability, will be provided.

Finally, processing is being introduced and discussed in Part 6. Challenges for the "seemingly simple fill/finish process" are many: extractables and leachables, hold times, material (in)compatibility, sterile filtration, processing residuals (e.g., vaporized hydrogen peroxide) impact on stability, QbD considerations, tech transfer challenges, and GMP drug product manufacturing segregation concerns, e.g., ADCs, cytotoxics, growth hormones, or some antibiotics.

The final section, Part 7, will discuss strategies and options for life-cycle management. This may include changing the route of administration (e.g., IV to SC), changing the formulation or dosage form (e.g., replacing excipients, changes from lyophilisate to liquid), or the use of devices.

Given the span of topics, we believe this book is of significant interest for colleagues in the biotechnology and pharmaceutical industry, as well as academic researchers and regulatory agencies globally, may it be business or project leaders, researchers, scientists in formulation and process development, analytical scientists, QC/QA colleagues, regulatory staff, and manufacturing leaders.

Enjoy reading!

Cambridge, USA Nicholas W. Warne
Basel, Switzerland Hanns-Christian Mahler

Contents

Part I Formulation Development of Biologics

1 Introduction into Formulation Development of Biologics 3
Daniel Weinbuch, Andrea Hawe, Wim Jiskoot and Wolfgang Friess

Part II Challenges with Excipients

2 Polysorbate Degradation and Quality 25
Kishore S. K. Ravuri

3 Sucrose and Trehalose in Therapeutic Protein Formulations 63
Satish K. Singh

Part III High Concentration Proteins

4 Introduction to High-Concentration Proteins 99
Wei Wang, Arun Alphonse Ignatius, Satoshi Ohtake
and Teng-Chieh Yang

5 Solubility, Opalescence, and Particulate Matter 125
Hanns-Christian Mahler and Anja Matter

6 Analytical Characterization and Predictive Tools for Highly Concentrated Protein Formulations 139
Andrea Allmendinger, Stefan Fischer and Robert Mueller

7 Practical Considerations for High Concentration Protein Formulations 163
Deirdre Murphy Piedmonte, Jian Hua Gu, Stephen R. Brych
and Monica M. Goss

Part IV Container-Closure Systems

8 Parenteral Container Closure Systems 191
Roman Mathaes and Alexander Streubel

9 Development of Prefilled Syringe Combination Products for Biologics 203
Mariana N. Dimitrova, Jared S. Bee, Ling Lu and Jason E. Fernandez

10 Special Topics in Analytics of Pre-filled Syringes 225
Atanas Koulov

11 C Mini-pumps 235
Dirk Gläser, Jürg Liniger and Daniel Peter

12 Container Closure Integrity Testing of Primary Containers for Parenteral Products 257
Shu-Chen Chen

13 Chemical Durability of Glass—Delamination 291
Holger Roehl, Philippe Lam and Dominique Ditter

14 Fogging 305
Holger Roehl, Philippe Lam and Dominique Ditter

Part V Processing Considerations

15 Bulk Protein Solution: Freeze–Thaw Process, Storage and Shipping Considerations 313
Parag Kolhe and Sumit Goswami

16 Leachables and Extractables: From Regulatory Expectations to Laboratory Assessment 337
Michael Jahn

17 Biotherapeutic Drug Product Manufacturing and Process Development 353
Daniel Dixon and Anthony Gudinas

18 Line Sterilization Considerations and VHP 385
Ulla Grauschopf, Katherine Thomas, Joerg Luemkemann, Sebastian Schneider, Ada Hui, Y. John Wang and Kirk Eppler

19 Lyophilization: Process Design, Robustness, and Risk Management 407
Daniel Dixon, Serguei Tchessalov and Bakul Bhatnagar

20 Scientific Approaches for the Application of QbD Principles in Lyophilization Process Development 441
Vinay Radhakrishnan, Penny Davis and David Hiebert

21 Manufacturing of Highly Potent Drug Product in a Clinical Multi-Product Aseptic Facility and Transfer of Principles to Antibiotic Drug Product 473
Karoline Bechtold-Peters and Silke Mohl

Part VI Novel Constructs

22 Introduction into Novel Constructs 497
Susanne Joerg, Kapil Gupta and Margarida Rodrigues

23 Novel Constructs—Half-Life Extensions 527
Jeonghoon Sun and Mark Michaels

24 "Fc Fusion Proteins" 545
Carole Heath and Dean Pettit

Part VII Lifecycle Management

25 Lifecycle Management of Biotherapeutic Dosage Forms 561
Nicholas Warne, Bryan Balthazor and William Parr

26 Switching from an IV to an SC Formulation—Considerations for Formulation Development and Formulation Bridging 579
Claudia Mueller and Michael Adler

Index 591

Contributors

Michael Adler Pharmaceutical Development and Supplies, PTD Biologics Europe (PTDE-P), F. Hoffmann-La Roche Ltd., Basel, Switzerland

Andrea Allmendinger Pharmaceutical Development & Supplies, PTD Biologics Europe, F. Hoffmann-La Roche Ltd., Basel, Switzerland

Arun Alphonse Ignatius Pfizer, Chesterfield, MO, USA

Bryan Balthazor BioTherapeutics Pharmaceutical Sciences, Pharmaceutical R&D, Pfizer Inc., Chesterfield, MO, USA

Karoline Bechtold-Peters Novartis Pharma AG, Basel, Switzerland

Jared S. Bee Analytical Sciences, MacroGenics Inc, Rockville, MD, USA

Bakul Bhatnagar BioTherapeutics Pharmaceutical Sciences, Pharmaceutical R&D, Pfizer, Inc, Andover, MA, USA

Stephen R. Brych Amgen, Inc., Process Development, One Amgen Center Drive, Thousand Oaks, CA, USA

Shu-Chen Chen Agoura Hills, USA

Penny Davis Pfizer Inc., Andover, MA, USA

Mariana N. Dimitrova Technical Operations, Jazz Pharmaceuticals, Philadelphia, PA, USA

Dominique Ditter Site Manufacturing Science and Technology, Validation Engineering & Filling, Roche Diagnostics GmbH, Mannheim, Germany

Daniel Dixon BioTherapeutics Pharmaceutical Sciences, Pharmaceutical R&D, Pfizer, Inc, Andover, MA, USA

Kirk Eppler Process Development Engineering, Genentech, South San Francisco, USA

Jason E. Fernandez Technical Development, Biogen, Cambridge, MA, USA

Stefan Fischer Pharmaceutical Development & Supplies, PTD Biologics Europe, F. Hoffmann-La Roche Ltd., Basel, Switzerland

Wolfgang Friess Department of Pharmacy—Pharmaceutical Technology and Biopharmaceutics, Ludwig-Maximilians-Universität München, Munich, Germany

Dirk Gläser Device Development, F. Hoffmann-La Roche Ltd, Basel, Switzerland

Monica M. Goss Amgen, Inc., Process Development, One Amgen Center Drive, Thousand Oaks, CA, USA

Sumit Goswami BioTherapeutics Pharmaceutical Sciences, Pharmaceutical R&D, St. Louis, MO, USA

Ulla Grauschopf Device Development, F. Hoffmann La Roche, Basel, Switzerland

Jian Hua Gu Amgen, Inc., Process Development, One Amgen Center Drive, Thousand Oaks, CA, USA

Anthony Gudinas BioTherapeutics Pharmaceutical Sciences, Pharmaceutical R&D, Andover, MA, USA

Kapil Gupta Biologics Process Research and Development, Integrated Biologics Profiling, Novartis Pharma AG, Basel, Switzerland

Andrea Hawe Coriolis Pharma Research GmbH, Martinsried, Germany

Carole Heath Process Development, Amgen, Thousand Oaks, CA, USA

David Hiebert Pfizer Inc, Peapack, NJ, USA

Ada Hui Late Stage Formulation & Process Development, Genentech, South San Francisco, USA

Michael Jahn Lonza Drug Product Services—Forensic Chemistry, Basel, Switzerland

Wim Jiskoot Division of BioTherapeutics, Leiden Academic Centre for Drug Research, Leiden University, Leiden, The Netherlands

Susanne Joerg Biologics Process Research and Development, Early Phase Development, Pharmaceutical Development, Novartis Pharma AG, Basel, Switzerland; Pharma and Biotech Drug Product Services, Lonza AG, Basel, Switzerland

Y. John Wang Late Stage Formulation & Process Development, Genentech, South San Francisco, USA

Parag Kolhe BioTherapeutics Pharmaceutical Sciences, Pharmaceutical R&D, Pfizer Inc., St. Louis, MO, USA

Atanas Koulov Drug Product Services, Analytical Development and Quality Control, Lonza AG, Basel, Switzerland

Philippe Lam Pharmaceutical Processing and Tech. Dev., Genentech Inc., South San Francisco, CA, USA

Jürg Liniger Device Development, F. Hoffmann-La Roche Ltd, Basel, Switzerland

Ling Lu BioTherapeutics Pharmaceutical Sciences, Pharmaceutical R&D, Pfizer, Andover, MA, USA

Joerg Luemkemann Pharmaceutical Development & Supplies, F. Hoffmann La Roche, Basel, Switzerland

Hanns-Christian Mahler Lonza AG, Drug Product Services, Basel, Switzerland

Roman Mathaes Hochbergerstrasse, Basel, Switzerland

Anja Matter Lonza AG, Drug Product Services, Basel, Switzerland

Mark Michaels Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA, USA

Silke Mohl F. Hoffmann-La Roche Ltd, Basel, Switzerland

Claudia Mueller Pharmaceutical Development and Supplies, PTD Biologics Europe (PTDE-P), F. Hoffmann-La Roche Ltd., Basel, Switzerland

Robert Mueller Pharmaceutical Development & Supplies, PTD Biologics Europe, F. Hoffmann-La Roche Ltd., Basel, Switzerland

Satoshi Ohtake BioTherapeutics Pharmaceutical Sciences, Pharmaceutical R&D, Pfizer, Chesterfield, MO, USA

William Parr Global marketing, Pfizer Innovative Health, Pfizer Inc., Collegeville, PA, USA

Daniel Peter Device Development, F. Hoffmann-La Roche Ltd, Basel, Switzerland

Dean Pettit Just Biotherapeutics, Seattle, WA, USA

Deirdre Murphy Piedmonte Amgen, Inc., Process Development, One Amgen Center Drive, Thousand Oaks, CA, USA

Vinay Radhakrishnan Pfizer Inc., Chesterfield, MO, USA

Kishore S. K. Ravuri Pharmaceutical Development, F. Hoffmann-La Roche, Ltd., Basel, Switzerland

Margarida Rodrigues Biologics Process Research and Development, Early Phase Development, Pharmaceutical Development, Novartis Pharma AG, Basel, Switzerland

Holger Roehl Late Stage Pharmaceutical and Processing Development, Pharmaceutical Development & Supplies, Pharma Technical Development Biologics EU, F. Hoffmann-La Roche Ltd., Basel, Switzerland

Sebastian Schneider Pharmaceutical Development & Supplies, F. Hoffmann La Roche, Basel, Switzerland

Satish K. Singh Lonza AG, Basel, Switzerland

Alexander Streubel Hochbergerstrasse, Basel, Switzerland

Jeonghoon Sun Department of Biotherapeutics, Celgene Corporation, San Diego, CA, USA

Serguei Tchessalov BioTherapeutics Pharmaceutical Sciences, Pharmaceutical R&D, Pfizer, Inc, Andover, MA, USA

Katherine Thomas Pharmaceutical Development & Supplies, F. Hoffmann La Roche, Basel, Switzerland

Wei Wang Pfizer, Chesterfield, MO, USA; Bayer U.S. LLC, Berkeley, CA, USA; Regeneron Pharmaceuticals, Tarrytown, NY, USA

Nicholas Warne Biotherapeutic Pharmaceutical Research and Development, Pharmaceutical R&D, Pfizer Inc., Andover, MA, USA

Daniel Weinbuch Coriolis Pharma Research GmbH, Martinsried, Germany; Division of BioTherapeutics, Leiden Academic Centre for Drug Research, Leiden University, Leiden, The Netherlands

Teng-Chieh Yang Pfizer, Chesterfield, MO, USA; Regeneron Pharmaceuticals, Tarrytown, NY, USA

Abbreviations

AAC	Antibody-Antibiotic-Conjugate
ADC	Antibody-drug-conjugate
ADE/PDE	Acceptable or permitted daily exposure
AF4	Asymmetric-flow field-flow fractionation
API	Active pharmaceutical ingredient
AUC	Analytical ultracentrifugation
CFU	Colony forming units
CIP	Cleaning in place
CIP/SIP/DIP	Cleaning in place, sterilization in place, drying in place
CMC	Critical micelle concentration
DLS	Dynamic light scattering
DOE	Design of experiment
DP	Drug product
DS	Drug substance
DSC	Differential scanning calorimetry
DSF	Differential scanning fluorimetry
ELISA	Enzyme-linked immunosorbent assay
EU	Endotoxin unit
FFA	Free fatty acids
FID	Flame ionization detector
FTIR	Fourier-transform infrared
GMP	Good manufacturing practice
HPLC	High-performance liquid chromatography
HTF	High-throughput formulation
HSA	Human serum albumin
ICH	International conference on harmonization
LC-MS	Liquid chromatography-coupled mass spectrometry
LTP	Liquid transfer port
MAC	Maximum allowable carry-over

MALD-TOF	Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry
MBS	Minimum batch size
MDD	Maximum daily dose of the subsequent product
OEL	Operational exposure level
pKa	Acid dissociation constant
ppb	Parts per billion
ppm	Parts per million
PS	Polysorbates
PS20	Polysorbate 20
PS80	Polysorbate 80
QC	Quality control
RTP	Rapid transfer port
STD	Smallest therapeutic dose
SEC	Size exclusion chromatography
UPLC	Ultra-high-performance liquid chromatography
UV	Ultraviolet
VHP	Vaporized hydrogen peroxide
WIP	Washing in place

Part I
Formulation Development of Biologics

Chapter 1
Introduction into Formulation Development of Biologics

Daniel Weinbuch, Andrea Hawe, Wim Jiskoot and Wolfgang Friess

Abstract Formulation development is an essential part of every biopharmaceutical development program and important for the therapeutic and commercial success of a protein drug product by assuring the quality, safety, and efficacy. The multiple phases of formulation development interact with other product development exercises as early as discovery research all the way until and beyond market approval. Every drug product demands a tailor-made formulation, due to the complexity of different degradation pathways potentially affecting product stability, the specific characteristics of the individual drug molecule, special patient needs, and even marketing considerations. Formulation development can be approached using various strategies, based on a rational design, relying on scientific knowledge in low or medium throughput, or high-throughput formulation screening of hundreds or even thousands of conditions employing miniaturized analytical methods. In this chapter, an introduction to the field of protein formulation development is given, the literature on current protein formulation development strategies is reviewed, and current challenges are summarized.

Keywords Protein stability · Stability testing · Preformulation
High throughput screening · Excipients · Development strategy

---

W. Friess (✉)
Department of Pharmacy—Pharmaceutical Technology and Biopharmaceutics,
Ludwig-Maximilians-Universität München, Butenandtstr. 5-13, 81377 Munich, Germany
e-mail: wolfgang.friess@lrz.uni-muenchen.de

D. Weinbuch · A. Hawe
Coriolis Pharma Research GmbH, Fraunhoferstr. 18B, 82152 Martinsried, Germany
e-mail: daniel.weinbuch@coriolis-pharma.com

A. Hawe
e-mail: Andrea.Hawe@coriolis-pharma.com

D. Weinbuch · W. Jiskoot
Division of BioTherapeutics, Leiden Academic Centre for Drug Research,
Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands
e-mail: w.jiskoot@lacdr.leidenuniv.nl

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_1

List of Abbreviations

AF4	Asymmetrical flow field-flow fractionation
API	Active pharmaceutical ingredient
AUC	Analytical ultracentrifugation
CTA	Clinical trial authorization
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DOE	Design of experiment
DP	Drug product
DS	Drug substance
DSC	Differential scanning calorimetry
DSF	Differential scanning fluorimetry
FTIR	Fourier-transform infrared
Glu	Glutamic acid
GMP	Good manufacturing practice
h	Hour(s)
H_2O_2	Hydrogen peroxide
His	Histidine
HPLC	High-performance liquid chromatography
HTF	High-throughput formulation
ICH	International conference on harmonization
LA	License application
LC-MS	Liquid chromatography-coupled mass spectrometry
mg/ml	Milligram per milliliter
mM	Millimolar
µm	Micrometer
nm	Nanometer
pH	Potential of hydrogen
pI	Isoelectric point
pKa	Acid dissociation constant
QC	Quality control
RH	Relative humidity
RNA	Ribonucleic acid
rpm	Rounds per minute
SEC	Size exclusion chromatography
SLS	Static light scattering
Tg’	Glass transition temperature
UPLC	Ultrahigh-performance liquid chromatography
USP	United States Pharmacopeia
UV	Ultraviolet
°C	Degrees Celsius
%	Percent

1.1 Introduction

Protein formulation development aims to render a therapeutic protein product robust for manufacturing, storage, handling, and administration to patients. So, formulation development is essential for the therapeutic and commercial success of a promising protein molecule: "it is a medicine, not a molecule, that we are giving to the patient" [4]. With this chapter, we aim to introduce the reader into general concepts related to formulation development of biologics. The focus is on liquid and lyophilized protein formulations for parenteral use, as those comprise the vast majority of our current arsenal of marketed biologics. Nevertheless, most of the concepts described in this chapter also apply to other biologics, such as vaccines and DNA- and RNA-based products. Issues specific for the challenges of protein delivery systems for non-invasive administration and particles for sustained release and targeting are beyond the scope of this chapter; the interested reader is referred to the literature [2, 17, 30, 35, 40].

Within this chapter, we discuss various elements of protein formulation development, formulation strategies during several stages of development and challenges that can be encountered. Rather than going into great detail, our intention is presenting the complexity of the topic and important aspects that should be considered during formulation development (see Table 1.1). Details about several of the topics briefly touched upon in this chapter, such as high-concentration formulation, excipient selection, lyophilization development, and in-use stability testing, will be presented in more detail elsewhere in this book.

1.2 Formulation Development Strategies and Approaches

1.2.1 Protein Formulation: Beyond Stabilization

One of the major challenges in the formulation of therapeutic proteins is to assure their stability, not only during storage but also during manufacturing, shipment, handling, and administration. Nevertheless, it should be realized that the "optimal" formulation is not necessarily the one that is most stable, but rather should fit the purpose depending on several factors. These include, besides sufficient stability, the stage of development, clinical requirements, regulatory requirements, packaging and device configuration, economical issues, marketing considerations, or the freedom to operate within the patent landscape (Table 1.1). As an example, what is "best" in terms of a product's stability is not necessarily good from a patient's or economical perspective. For instance, suppose a certain product would be most stable in 50 mM sodium citrate, pH 4.0. If the product is meant for subcutaneous administration, this formulation probably would be not preferred, because the unfavorable combination of low pH and hypotonicity may cause pain at the

Table 1.1 Critical factors to be considered during formulation development

Factor	Description/attributes/examples
Analytical methods	High- versus low-throughput, stability-indicating, QC, extended characterization
API	Type of protein, physicochemical properties, e.g., molecular weight, pi, hydrophobicity, solubility, post-translational modifications, pegylation
Clinical factors	Patient population (e.g., age, indication, concomitant medication), therapeutic window, self-administration versus administration by professional, compatibility with infusion solution
Competitive landscape	Originator versus biosimilar product, patent situation, competitive drugs
Dosage form	Single- or multi-dose, prefilled syringe, dual chamber cartridge, pen cartridge
Drug substance	API concentration, formulation composition, available amount, purity
Excipients	Pharmaceutical quality, safety record (for intended administration route and dose), manufacturer, tested for critical impurities, stability
Manufacturing capabilities	Disposable/non-disposable technologies, dedicated equipment, filling line/pumping
Other factors	Budget, time(lines), manufacturability, company policy, marketing strategy, regulatory requirements
Phase of development	Phase of development Preclinical, early clinical, late clinical, commercial
Primary packaging material	Glass, polymers, rubber, silicone oil, metals, leachables (anti-oxidants, plasticizers, etc.)
Route of administration	Subcutaneous, intravenous injection of infusion, intramuscular, intravitreal, intraarticular, intradermal
Target dose and dosing regime	Concentration, volume, indication (e.g., one-time application or chronical application)
Type of formulation	Liquid, lyophilizate, frozen liquid

injection site [26]. The same formulation might, however, be acceptable if the product were intended to be diluted in an infusion liquid prior to intravenous administration, provided that the product is stable in use and compatible with the infusion system. Another example: If a lyophilizate in a vial would be stable for five years but the same molecule could be formulated as an aqueous solution in a prefilled syringe with two years shelf life, the latter might be preferred over the more stable formulation for economical and marketing reasons and due to easier patient self-administration.

Since a liquid formulation is often faster and cheaper to produce and is more user-friendly, generally it is preferred over a lyophilizate. However, it may be impossible to develop a sufficiently stable liquid formulation, either because of time constraints during (early) product development or because the molecule turns out to be insufficiently stable even after extensive formulation development exercises.

The obvious alternative in such cases is a dry formulation (apart from an early stage frozen liquid formulation), which is almost exclusively achieved by lyophilization, a process requiring dedicated formulation development.

From a formulation scientist's perspective, in an ideal world already at the earliest stage of development the final dosage form, the required stability profile as well as other needs (see Table 1.1) have been defined, high-throughput, stability-indicating analytical methods are in place, and material, time and resources are available in unlimited amounts. However, the real world is quite different. Consequently, the first formulation used during preclinical studies (e.g., toxicity studies) is likely going to be different from the formulation applied during later clinical phases and the final formulation used for commercialization. This may be explained, besides by the above-mentioned reasons, by changes in the dosing regime, the route of administration or the primary packaging material (e.g., switch from vials to syringes) or by instabilities occurring in a not-yet-optimized formulation as well as additional insight gained into the stability of the protein molecule and/or the excipients. Nevertheless, it is highly favored to have the final formulation composition defined as early as possible during drug product (DP) development to avoid additional studies, regulatory efforts and to align drug substance (DS) and DP composition. To this end, it is imperative that the formulation scientist acquires knowledge about the clinical needs, marketing considerations as well as regulatory requirements. Moreover, the more is known about the physical and chemical stability of a protein molecule as function of major formulation variables and external stress factors (temperature, mechanical stress, freezing, and thawing, etc.) early in the development, the less complex, costly, and time-consuming, it will be in a later stage of development to accommodate a formulation to the needs of the molecule and the product.

"There isn't just one way of doing it" holds true for formulation development of biologics, and there are numerous ways and philosophies how to come to a stable and robust formulation. No matter which approach is followed for achieving a satisfactory formulation, the selection of analytical methods plays a crucial role. Already early in the process, the critical routes of instability need to be identified in order to establish the important stability-indicating analytical methods as well as the appropriate formulation strategy to tackle the instability issues. Formulation development usually evolves during a drug development program, and often thereafter, and can generally be divided into the following activities: preformulation, formulation development for DS, DP formulation development for preclinical phases, for early clinical phases, for late stage/commercialization, and finally formulation activities during the life cycle of a product (Fig. 1.1). Of course, there certainly is an overlap between these phases, and wherever applicable, considerations for a later stage should be reflected as early in the development process as possible. In the following sections, we describe first what typically forms part of a protein formulation and then discuss several phases and approaches of formulation development.

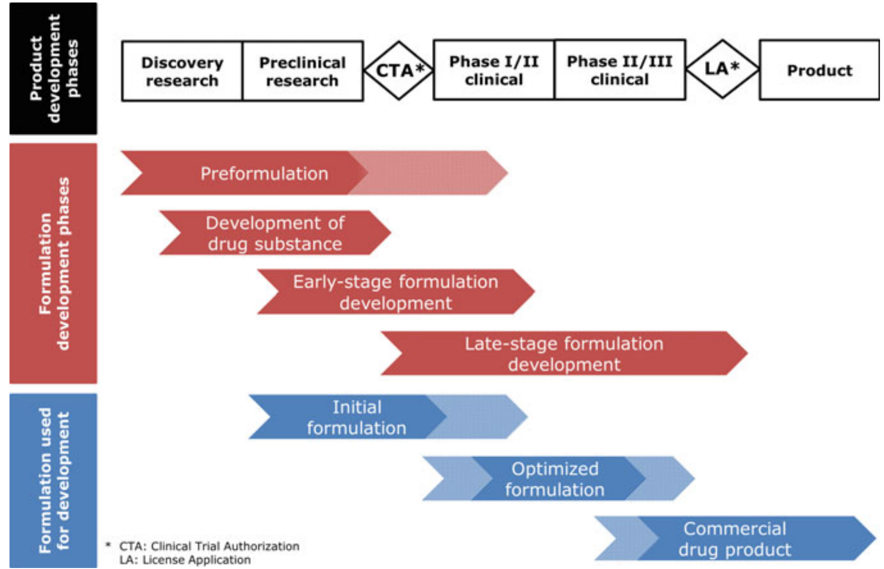


Fig. 1.1 Diagram of a formulation development process (modified from Chang and Hershenson [8])

1.2.2 Components of a Protein Formulation

1.2.2.1 Active Pharmaceutical Ingredient and Drug Substance

The term active pharmaceutical ingredient (API) refers to the molecule of interest, e.g., a peptide, monoclonal antibody, or enzyme. In a pure state, the API would typically be a solid powder, as often found for peptides. This state, however, is extremely impractical to obtain and/or presents an unstable state for most biologics. Therefore, a DS, a (sometimes frozen) liquid formulation containing the API is used for purified bulk storage. A DS typically results from a chromatographic or ultra-diafiltration step at the end of a purification process. In commercial-scale production, the formulation composition of the DS is often very similar to that of the final DP, but this can obviously not be the case when formulation development has yet to be completed. This may have consequences for DP formulation screening, as discussed in the Sects. 1.2.4 and 1.3.1.

1.2.2.2 Excipients

One rule in formulation development is to avoid putting anything into the formulation that is not needed. In other words, a formulation should be kept as simple as possible, and each excipient, as well as its quantity, should be justified. Having mentioned this, it is not an easy task to combine the right excipients in the right

concentration, because a stabilizing excipient potentially exhibits a destabilizing effect on a different protein instability pathway, and excipients potentially influence each other's action. For instance, polysorbates added for protection against interface-related protein aggregation may contain oxidizing species, which may promote chemical instability [21]. Whereas sodium chloride could help reducing a formulation's viscosity, it may negatively affect a protein's colloidal stability and also be detrimental upon freezing or lyophilization as upconcentrated in the freeze-concentrated solution [39]. Finally, the most frequently used excipient, water for injection, is a natural solvent for proteins but at the same time mediates most if not all possible protein degradation reactions, reason why many products are lyophilized to reduce the water content to minimal amounts.

Table 1.2 gives an overview of the most commonly used excipient classes and their functions in protein formulations. Importantly, it is common practice to choose among excipients that are approved and commonly used in protein formulations (see examples in Table 1.2) in comparable doses and dosing frequencies for the intended route of administration. Although it would be interesting to explore novel excipients in order to expand the options for a formulation scientist, including a new excipient in a formulation is often a "no go." The reason is that it would greatly increase development time and cost, because—besides the need for a justification to use it instead of a more common excipient—its safety would have to be evaluated in order to get the product approved for clinical trials and registration. The same may hold true for unusually high doses of a certain excipient. Furthermore, the quality of excipients should be considered critically, and their stability in the specific formulation should be assessed. For instance, sucrose might not be included

Table 1.2 Common excipients encountered in protein formulations

Excipient class	Function	Examples
Solvents	Dissolution	Water for injection
Buffers	pH control, tonicity	Acetate, citrate, glutamate, histidine, phosphate, succinate, glycine, aspartate
Salts	Tonicity, solubilization, stabilization, viscosity reduction	Sodium chloride
Sugars, polyols	Tonicity, stabilization, cryoprotection, lyoprotection[a], bulking agent[a]	Mannitol, sorbitol, sucrose, trehalose
Surfactants	Solubilization, stabilization, adsorption prevention, reconstitution improvement[a]	Polysorbate 20, polysorbate 80, Poloxamer
Amino acids	Solubilization, stabilization, tonicity, viscosity reduction, pH control, bulking agent[a]	Arginine, glycine, glutamate, histidine, lysine, succinate
Anti-oxidants	Oxidation prevention	Methionine, sodium edetate
Preservatives	Antibacterial action (multi-dose formulations)	Benzyl alcohol, meta-cresol, phenol

[a]Specifically in lyophilized products, but only if sugar or polyol stays amorphous

in liquid formulations below pH 6, because its hydrolysis rate during storage may become significant, leading to the formation of fructose and glucose; the latter degradant can form glycation products with the protein via the Maillard reaction [31]. While excipients preferably should comply with compendial standards, additional requirements may apply for specific protein formulations, as further illustrated.

Excipients can exert several functions; e.g., glycine can act as stabilizer, buffer, and tonicity modifier and may have several modes of action. The need for their inclusion in a protein formulation mainly depends on the critical instability pathways of the protein and other not protein stability-related needs, such as tonicity requirements and lyophilizate appearance. Furthermore, certain excipients that may be useful in liquid formulations should be avoided in lyophilizates (e.g., volatile buffers such as acetate, or salts that lower the glass transition temperature of the maximally freeze-concentrated solution (Tg') of amorphous formulation), whereas some excipient functions are specific for lyophilized products, e.g., bulking agent, lyoprotector.

Buffer species may have specific destabilizing or stabilizing effects on proteins, besides offering buffer capacity. So, buffer type and concentration should be carefully selected during formulations screening, and the decision depends not only on the desired pH (typically well within about ± 1 unit from the pKa of the buffer species) but also on the protein, the route of administration, and whether it is a liquid or a lyophilized formulation. Furthermore, in high-concentration protein formulations, one could consider not to include any buffer. Especially in slightly acidic, highly concentrated (>50 mg/ml) antibody formulations, the total number of His, Glu, and Asp residues in the API may provide sufficient buffer capacity to provide a stable pH value [13].

1.2.2.3 Primary Packaging Material

Since the primary packaging material may affect the quality of the DP, it is an important and integral part of the formulation development program. Obviously, the primary packaging material depends on the dosage form (see Table 1.1 for some examples), which in turn impacts the way a drug is administered and its user-friendliness. Implications of the primary container on formulation development, e.g., the setup of mechanical stress studies, are addressed in Sect. 1.3.2 of this chapter. For more information, the reader is referred to Chap. 5 of this book.

1.2.3 Preformulation

Preformulation studies are a prerequisite "to know your molecule," which is vital for the entire development cycle of a therapeutic protein. On the short term, preformulation studies may be used for candidate selection and will help in the

optimization of upstream and downstream processes for the selected candidate molecule as well as in the development of a sufficiently stable formulation for DS, preclinical and first-in-human clinical trials. At later stages of development and after commercialization, the fundamental knowledge acquired with preformulation activities will support the rational design of (an) optimized formulation(s) and the assessment of the shelf life under appropriate storage conditions.

The term preformulation is used rather flexible and differently among research groups with respect to its transition to, or its position within, formulation development. Preformulation studies are performed in close collaboration with discovery research and should start as early as a promising drug candidate has been obtained. Preformulation studies are meant to gain insight into critical physicochemical properties of the protein drug candidate (see Table 1.1), such as primary, secondary, and higher-order structure, molecular weight, extinction coefficient, isoelectric point, post-translational modifications, hydrophobicity, and biophysical properties, such as conformational and colloidal stability. Moreover, they are aimed to determine the criticality of various environmental factors, such as pH, ionic strength and buffer species, and the API's sensitivity to pharmaceutically relevant stress conditions (Table 1.3). The latter involves assessment of the predominant degradation pathways. The critical predominant degradation pathways, as well as the sensitivity to pH and ionic strength, may be quite different between proteins, even for relatively similar ones such as monoclonal antibodies [10, 33, 43, 45]. Preformulation should ultimately lead to the development of suitable stress conditions and a toolbox of stability—indicating analytical methods, enabling the differentiation between good and bad formulations in upcoming, more comprehensive formulation development studies. In some cases, selected excipients may already be screened to improve the stability of the molecule against critical stress factors.

Table 1.3 Accelerated stability and forced-degradation studies used in protein formulation screening

Stress type	Exemplary stress conditions	Anticipated instability types
Temperature	Real-time/intended temperature, e.g., at 2–8 °C Accelerated testing, e.g., at 15, 25, or 40 °C	Aggregation, conformational changes, chemical changes
Mechanical, shaking	50–500 rpm, 2 h to >48 h	Aggregation, adsorption, conformational changes
Mechanical, stirring	50–500 rpm, <1 h to 48 h	Aggregation, adsorption, conformational changes
Mechanical, freeze-thawing	1–5 cycles, e.g., between 25 °C and −20 to −80 °C	Aggregation, adsorption, conformational changes
Oxidation	H_2O_2, 1–5% for 1–2 days, oxygen purge	Chemical changes (oxidation), aggregation, conformational changes
Humidity[a]	0–100% RH	Aggregation, conformational changes, chemical changes, moisture content

[a]Specifically for lyophilized products

Preformulation includes the testing of the thermal stability, e.g., by (micro-) differential scanning calorimetry (DSC) or dynamic scanning fluorimetry (DSF) as well as the testing of colloidal stability, including aggregation propensity and viscosity, e.g., by determination of the second virial coefficient or the interaction parameter k_d by static light scattering (SLS), dynamic light scattering (DLS) or analytical ultracentrifugation (AUC) [37, 44]. DSC and DSF are often applied to assess thermal events, such as unfolding, which is helpful to define relevant conditions for accelerated stability studies (see Sect. 3.2). However, although thermal stability studies are routinely used in formulation screening, for several reasons, thermal stability may not correlate with storage stability. For example, Bam et al. observed an excellent stabilization against agitation by polysorbates, although DSC experiments showed lower unfolding temperatures in presence of the surfactant [3]. Furthermore, the ranking of melting temperatures does not always predict the order of conformational stability at storage temperature [12, 36]. Therefore, preformulation should include mechanical stress, e.g., by shaking or stirring at temperatures, far below the Tm value (Table 1.3). Moreover, chemical degradation can arise from the fully native structure even without the application of thermal or mechanical stress and might in specific cases be more problematic than conformational or colloidal instability [29]. Preformulation should thus test for such pathways, e.g., by forced oxidation (Table 1.3).

1.2.4 Formulation Development

1.2.4.1 Formulation Development Strategies

Formulation development involves studying the influence of formulation variables on potential critical quality attributes upon intended storage, accelerated, and forced-degradation conditions in order to identify a stable and robust formulation based on previous experience with the same API or similar molecules and the preformulation work. There are several ways and philosophies to reach a stable and robust formulation. One is a rational design methodology testing well-selected formulation conditions in low or medium throughput and a defined number of excipients based 13 on the properties of the molecule, as established in preformulation studies. The alternative high-throughput formulation (HTF) approach involves the empirical screening of hundreds or even thousands of different formulations under accelerated conditions preferably employing miniaturized analytical methods. Finally, for some well-known molecule formats (e.g., monoclonal antibodies), platform approaches might be suitable by applying standard formulation conditions with a high chance, but no guarantee of success. For novel protein molecule designs, such a fast-track formulation approach may not be feasible, as a better understanding of the physicochemical properties and the routes of instability is required to identify appropriate formulation conditions.

Independent of the formulation strategy followed, once a suitable formulation has been identified, its shelf life must be confirmed in real-time and accelerated stability studies and its robustness assessed under relevant stress conditions. Accelerated stability studies can never replace real-time stability assessment, because rates of the degradation routes may have different temperature dependency potentially affected by a change in protein conformation with temperature [8]. Consequently, the predominant degradation pathway at elevated temperature, e.g., 25/60 °C, could differ from that under refrigerated conditions (2–8 °C). Therefore, because protein degradation processes can mutually influence each other in a complex fashion, Arrhenius kinetics often do not apply to protein formulations [15].

1.2.4.2 Early Stage Formulation Development

Time pressure, limited resources, the risk of a drug to drop out during the development program, or plans to sell a drug candidate after clinical phase 1, are only some arguments to define an early stage DP for preclinical phase or clinical phase 1 without extensive formulation development. In this case, within a relatively short-time frame, the formulation scientist should aim to deliver such an initial formulation that can be reproducibly manufactured with a standard container closure system, while leaving enough flexibility to, e.g., alter the dosage regime and the route of administration at later development stages. Lyophilization and reconstitution with a different volume is one approach to allow dosing flexibility and setting up different protein concentrations [1, 24]. The shelf life requirement of this early DP is mainly determined by the logistics of supplying the drug for clinical trials. Stability of the API in the DP until at least the end of the trial must be supported by stability data. Importantly, the more is known at this stage about the intended commercial formulation (e.g., administration route, dosage form, and primary packaging material), the better.

In preformulation and early formulation development, HTF screening can be beneficial, especially if there is no or very limited pre-existing knowledge about the sensitivity of the API to formulation and stress conditions. The high number of test formulations can be handled when working with automated pipetting systems or robots ideally combined with stress testing/stability testing in plates and plate-reader based analytics requiring low sample volumes. Typical analytical methods for this purpose are UV spectroscopy (protein content, turbidity), fluorescence spectroscopy (intrinsic or extrinsic with dyes), and DLS, all of which can be performed fully automated in multi-well plates. Moreover, intermediate-throughput methods, such as HPLC/UPLC and DSC, when performed with autosampler devices, can be conveniently used [5, 32, 38].

1.2.4.3 Late-Stage Formulation Development

While the protein in its initial formulation is tested in clinical trials, the formulation scientist will already be working on an optimized, commercially viable formulation. This formulation should, beyond the stability required for the initial formulation, ultimately be robust against external stresses during the desired shelf life, administration (sometimes using product specific application devices) and to potential protein-specific degradation pathways. In order to test robustness, forced-degradation studies at relevant stress conditions (Table 1.3) combined with a tailored set of stability indicating analytical methods, defined during preformulation, are employed. In this context, design of experiment (DOE) approaches can be applied to optimize experimental setups and reduce the number of required sample measurements [14]. While forced-degradation studies do not reflect real-life conditions, they are useful to reveal differences in stability between formulations and to give justification on why excipients are added and at which quantity. In late-stage formulation development, tasks of the preformulation phase might still be ongoing and specific molecule characterization tasks may be intensified. Since the DS is at this stage available in larger quantities (and often higher purity), the formulation scientist is not anymore tied to low-volume analytical methods used in early stage development, but can also employ resource consuming or high-volume methods, e.g., AF4, AUC, FTIR-spectroscopy, MS, particle characterization [11] to test the stability of the protein more in detail. Knowledge from clinical trials on application route, dosage regime, and the potential use of an application device will also influence the formulation design. The investigation of processing stability should include filter tests, tubing tests, handling test, and fill-finish tests to assure robustness toward stresses during manufacturing, if not already, at least in parts, performed during early stage development. Finally, real-time stability studies at relevant storage conditions (e.g., 2–8 °C) using the DP in its primary container system from different production batches are to be conducted to define and justify the product's shelf life. This is stated in the ICH guideline QC5, and for most DPs, a shelf life of at least 18–24 months is desired.

1.2.4.4 Formulation Development After Commercialization

When a commercial DP has successfully entered the market, formulation development might still be needed, e.g., for life cycle management to change protein concentration, packaging material, or route of administration and to support changes in the manufacturing process. In this case, knowledge from pre-, early stage, and late-stage formulation activities is key to enable fast and effective formulation change and comparability studies. Since slight changes in formulation conditions potentially affect the safety and efficacy of the DP, it is necessary to perform detailed studies to assure that product quality and degradation profile have not quantitatively worsened or even qualitatively altered. If analytical characterization and non-clinical comparability studies are not sufficient for this claim, the ICH Guideline Q5E demands additional clinical comparability studies.

1.3 Challenges During Formulation Development

1.3.1 Amount and Quality of DS

One challenge in preformulation and early stage formulation studies is the typically limited availability of API. The required amount depends in part on the product development stage as well as on the formulation strategy. Vice versa, if substantially limited amounts are available, this may unavoidably lead to a change in formulation strategy and/or a reduction of the number of stress testing methods applied, formulations screened and analytical methods used [11]. Obviously, analytical methods that require little sample are preferred, including well-plated-based spectroscopic and light scattering-based methods as well as electrophoretic and chromatographic techniques [38].

Another challenge is the potential variation in DS quality during product development, which may be due to coinciding development and changes in production cell line, cultivation conditions, and downstream processes. In particular during early stages of product development, the quality of the DS may not reflect that of later-stage (pilot or full-scale production) batches. In particular, aggregate and particle levels in pre-GMP technical batches do not always meet the minimum standards, such as those defined by the USP Chapter 787, which will impede proper assessment of a formulation's capability to avoid aggregation. Moreover, the level of impurities or contaminants may have major effects on product stability [42]. For instance, variations in residual protease activity will especially affect the stability of the API in a liquid DP. Similarly, a relatively high residual lipase activity may lead to unexpectedly rapid degradation rates of polysorbates [22, 25]. If the root cause of such degradation processes would be identified in an early stage, one could choose to first develop a frozen liquid or lyophilized DP for early stage (pre)clinical development, while optimizing the upstream and downstream processes in the meantime. This, however, would take additional resources and time. Ultimately, there is the risk that formulation development is focused on inhibiting a degradation process that turns out to be irrelevant as soon as higher-quality DS batches become available.

For DP formulation screening, the available DS formulation will have to be exchanged with the formulations of interest, e.g., by column chromatography, dialysis, or ultra-/diafiltration. Such processes, which may also involve dilution or concentration of the API, pose stress upon the molecule. Consequently, it should be investigated whether the chosen method compromises the protein quality. Furthermore, in buffer exchange and concentration procedures using a semi-permeable membrane, especially at high protein concentrations, the final formulation composition may significantly differ from the intended one because of unequal partitioning of excipients. This may be due to volume exclusion, non-specific interactions, and for ionic solutes, such as salts and buffer components, the Donnan effect [41]. The presence of a surfactant such as polysorbates in the DS formulation, e.g., introduced in the downstream process to protect the API against

interfacial stress, would pose a particular challenge, as it is practically impossible to remove surfactants quantitatively and they may accumulate in an unpredictable way during membrane concentration processes [27]. Thus, quantification methods for each of the excipients that are part of DS and DP should be in place for guiding the proper design of formulation screening methodologies. Furthermore, once a suitable final DP formulation is chosen, the polishing step in the downstream process can be adjusted to bring the DS formulation in line with that of the DP.

1.3.2 Selection of Analytical Methods and Stress Conditions

The paradigm "formulation is characterization" refers to the fact that only with a proper analytical toolbox one can differentiate between good and poor formulations within the limited time frame of a short accelerated stability and stress program. But how should one set up the analytical package and appropriate stress conditions?

1.3.2.1 Analytical Methods

No matter which formulation approach is followed, the availability of low-volume, high-throughput methods is advantageous, especially in preformulation and early stage formulation studies. Techniques used in these stages preferable provide a general indicator for stability, such as melting temperature by DSF or DSC, or colloidal stability by light scattering. Since proteins can undergo a variety of degradation reactions [29], complementary analytical methods should be used for monitoring the formation of all potential degradation products when performing stability and forced-degradation studies. Filipe et al. gave an excellent overview of commonly used analytical methods outlining their measurement parameter, their sample requirement, and whether they can be operated in high throughput [11]. The interested reader is also referred to books by Jiskoot and Crommelin [18], and by Houde and Berkowitz [16] providing details about analytical methods beyond the scope of this chapter. Especially in later stages of formulation development, orthogonal methods should be used to verify the validity of specific methods. For instance, size-exclusion chromatography (SEC) methods only cover a limited size range of relatively small protein aggregates (up to about 100 nm) and may not detect reversible aggregates within this range [7, 34]. Consequently, regulatory agencies expect SEC data to be confirmed by orthogonal methods, such as AUC and AF4 [9, 11]. In addition, until recently the use of compendial methods such as light obscuration has been focused on the analysis of subvisible particles larger than 10 micron.

However, safety concern with respect to protein aggregates and other particulates in the size range of 2–10 μm and more recently also the submicron size range has facilitated the development of new particle analysis methods, e.g., microflow imaging, nanoparticle tracking analysis, and resonant mass measurement that are

now increasingly being applied in formulation development [6, 9, 11, 28, 46]. This has also been acknowledged by regulatory bodies and has lead to new and updated guidelines such as the USP <787> and the educational chapter USP <1787>, suggesting quantification and qualitative characterization of particles in this size range by orthogonal methods [23]. With the analytical methods comes the challenge of setting specifications and their justification. For many quality attributes assessed throughout the whole manufacturing process of a DP like appearance, color, pH, sterility, osmolarity, visible particles, or subvisible particles, the pharmacopoeial monographs apply. Other specifications, e.g., the SEC monomer content, are not ultimately defined at early stage. A specification of more than e.g., 95% monomer can be accepted at early stage development, may be set in accordance with platform technology experience and revised reflecting experience and stability data gathered on the way to commercialization.

1.3.2.2 Stability Testing and Forced-Degradation Studies

How to select appropriate stress conditions? The answer to this question is not straightforward, because it depends, among others, on the purpose, the protein, the formulation, the dosage form, and the development stage [15]. For formulation screening, the stress conditions should be discriminative and allow ranking of formulations, which implies that they should be harsh enough to induce detectable changes, but at the same time not so harsh that all formulations show similar, nearly complete degradation. Pre-existing knowledge from the literature and in-house experience with similar molecules may be extremely valuable to set up appropriate stress conditions. Moreover, the relevance of the stress conditions should be kept in mind. For instance, exposing a protein to a temperature above its unfolding temperature over a longer storage period would be as irrelevant as pyrolyzing a small molecule; and if a formulation is shown to be resistant to rigorous shaking for several days, rather than continuing the applied stress for another few weeks, one may conclude that the formulation is robust toward this mechanical stress factor.

Setting up appropriate stress conditions may be part of preformulation and could be done with the DS. Typical stressors include thermal, freeze-thawing, mechanical, and oxidation stress. Table 1.3 gives some rough indications of possible conditions that could be applied for each of these stress factors. Although extreme pH and ionic strength are sometimes mentioned as stress factors, those are in fact formulation variables that are typically studied in preformulation studies, often in combination with exposure to elevated temperatures. The outcome of such extreme pH/ionic strength exposure studies is relevant to define the design space not only in formulation development but also in downstream processing steps, such as elution conditions in chromatographic procedures, viral inactivation, hold times, and conditions between purification steps.

Light stress may be added at later-stage formulation studies, and essential protection is finally provided by the secondary packaging material. One may consider using also less harsh conditions than those according to ICH, in order to assess

subtle differences between formulations. If the final container is known, this may be advantageous, especially for mechanical stress studies. For instance, the influence of shaking stress (conditions) is highly dependent on not only the shaking frequency and the incubation temperature, but on container dimensions, filling volume and solution viscosity as well.

For lyophilized formulations, storage of lyophilizates with different residual moistures levels under accelerated testing conditions needs to be considered. Moreover, the effect of freeze-thawing stress to the corresponding liquid formulation (with conditions used during lyophilization) needs to be studied. Furthermore, stress stability testing after reconstitution is highly valuable to reflect light, temperature, and mechanical stress, which the liquid could potentially be exposed to in the clinics and by the patients.

While forced-degradation or accelerated stress studies are valid means to compare formulation conditions during development and are recommended by the ICH Q1 guidelines, they have limited predicting value to the stability of a protein at real-time storage conditions. Thus, one can use these data to understand degradation pathways and to define and justify formulation conditions, for instance, the use of an excipient in a certain concentration, but one should not 22 exaggerate forced-degradation studies. Instead, a promising formulation should be tested by long-term studies testing at relevant storage conditions as early as possible since these studies are the basis for the determination of the product's shelf life and demonstrate the relevance of the different degradation pathways.

1.3.3 Manufacturability and Formulability

Formulation development has the goal to obtain a DP that serves the patient's needs and promotes stability of the protein. However, manufacturability should also play a role when defining final formulations, because the product needs to be manufactured at large scale and commercially viable. Some steps and procedures that can be performed with ease in small scale or on a laboratory bench might be difficult to implement in a large-scale production facility. For example, filtration steps using very low pore size filters are easily performed in the laboratory, but low-volume throughput and the costs of industry-sized filter systems might make implementation problematic in production scale. Also, high-concentration and viscous formulations could be difficult to handle during manufacturing and might cause problems during release testing by required compendial methods such as light obscuration. Contrary, low-concentration formulations might face the problem of protein loss through surface absorption, a factor that can become more relevant in a production facility. The same holds true for excipients in low concentrations; e.g., substantial loss of polysorbate to filters at the beginning of a filling process can occur. The scale-up to a commercial facility can create additional problems not observed in small scale. For example, mixing solutions in a large stainless steel tank, pumping solutions through stainless steel tubing, filtration, and filling through

a high-speed filling machine can introduce unexpected stresses to the protein. In addition, the introduction of particles, e.g., by pump systems, has been observed. Therefore, the relevance of such scale-up-related problems should be assessed early during process development and should be considered during formulation development. This aspect will be the subject of several following book chapters. Since some, if not all, of the factors mentioned above can show a certain batch-to-batch variability, regulators require stability data from multiple production batches before approval of the final DP.

1.3.4 Data Handling and Analysis

From the above, it should be clear that protein formulation screening will involve the generation, analysis, and interpretation of huge data sets. The two goals of the formulation scientist should be to make analytical data manageable as well as interpretable. For the first, a streamlined data analysis is important, which should include standardized export and analysis templates for each analytical technique (either using standard office software or dedicated data analysis programs). In addition, meaningful data folder and file structures as well as traceable sample names are crucial when handling huge data sets. For the second goal, singular-value decomposition analysis can help to condense complex data sets, e.g., spectroscopic data, by vector algorithms to a few descriptive values without loosing information. Further visualization tools, such as empirical phase diagrams and radar plots [19, 20], will improve data interpretation and will allow the formulation scientist to identify the best formulation more quickly.

1.4 Conclusions

Protein formulation activities are an important part of a protein drug development process. Formulation development should start early in product development. Selecting "the right" formulation requires extensive exercises, including analytical method development, forced-degradation studies, and accelerated and real-time stability studies. Moreover, clinical needs, company policy, and marketing strategy should be taken into consideration during formulation development. Knowledge gained during preformulation activities will help the scientist to identify potential hurdles in the subsequent formulation development program and to design a formulation to overcome those, by selecting a limited number of required excipients in appropriate amounts. Since the definition of "the right" formulation depends in part on the development stage, early stage formulations typically differ from late-stage and commercial formulations. Despite its complexity, if formulation development is done properly, the final result is often a simple liquid or lyophilized formulation in a dosage form for parenteral administration.

References

1. Andya J, Cleland JL, Hsu CC, Lam XM, Overcashier DE, Shire SJ, Yang JYF, Wu SSY. Stable isotonic lyophilized protein formulation, WO 9704801. 1997.
2. Antosova Z, Mackova M, Kral V, Macek T. Therapeutic application of peptides and proteins: parenteral forever? Trends Biotechnol. 2009;27(11):628–35.
3. Bam NB, et al. Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions. J Pharm Sci. 1998;87(12):1554–9.
4. Brown MB. The lost science of formulation. Drug Discovery Today. 2005;10(21):1405–7.
5. Capelle MAH, Gurny R, Arvinte T. High throughput screening of protein formulation stability: practical considerations. Eur J Pharm Biopharm. 2007;65(2):131–48.
6. Carpenter JF, et al. Overlooking subvisible particles in therapeutic protein products: gaps that may compromise product quality. J Pharm Sci. 2009;98(4):1201–5.
7. Carpenter JF, et al. Potential inaccurate quantitation and sizing of protein aggregates by size exclusion chromatography: essential need to use orthogonal methods to assure the quality of therapeutic protein products. J Pharm Sci. 2010;99(5):2200–8.
8. Chang BS, Hershenson S. Practical approaches to protein formulation development. In: Carpenter JF, Manning MC, editors. Rationale design of stable protein formulations-theory and practice. New York: Kluwer Academic/Plenum; 2002. p. 1–20.
9. Den Engelsman J, et al. Strategies for the assessment of protein aggregates in pharmaceutical biotech product development. Pharm Res. 2011;28(4):920–33.
10. Fesinmeyer RM, et al. Effect of ions on agitation- and temperature-induced aggregation reactions of antibodies. Pharm Res. 2009;26(4):903–13.
11. Filipe V, et al. Analytical approaches to assess the degradation of therapeutic proteins. TrAC Trends Anal Chem. 2013;49:118–25.
12. Freire E, et al. Chemical denaturation as a tool in the formulation optimization of biologics. Drug Discovery Today. 2013;18(19–20):1007–13.
13. Gokarn YR, et al. Self-buffering antibody formulations. J Pharm Sci. 2008;97(8):3051–66.
14. Grillo AO. Late-stage formulation development and characterization of biopharmaceuticals. In: Jameel F, Hershenson S, editors. Formulation and process development strategies for manufacturing biopharmaceuticals. Hoboken, NJ, USA: Wiley; 2010.
15. Hawe A, et al. Forced degradation of therapeutic proteins. J Pharm Sci. 2012;101(3):895–913.
16. Houde D, Berkowitz S, editors. Biophysical characterization of proteins in developing biopharmaceuticals. Elsevier B.V. 1st edn. 2014.
17. Jiskoot W, et al. Protein instability and immunogenicity: roadblocks to clinical application of injectable protein delivery systems for sustained release. J Pharm Sci. 2012;101(3):946–54.
18. Jiskoot W, Crommelin D, editors. Methods for structural analysis of protein pharmaceuticals. Scientists, Arlington, VA: American Association of Pharm; 2005.
19. Joshi SB, et al. An empirical phase diagram-high-throughput screening approach to the characterization and formulation of biopharmaceuticals. In: Jameel F, Hershenson S, editors. Formulation and process development strategies for manufacturing biopharmaceuticals. USA: Hoboken, NJ; 2010.
20. Kalonia C, et al. Radar chart array analysis to visualize effects of formulation variables on IgG1 particle formation as measured by multiple analytical techniques. J Pharm Sci. 2013;102 (12):4256–67.
21. Kerwin BA. Polysorbates 20 and 80 used in the formulation of protein biotherapeutics: structure and degradation pathways. J Pharm Sci. 2008;97(8):2924–35.
22. Khossravi M, Kao Y-H, Mrsny RJ, Sweeney TD. Analysis methods of polysorbate 20: a new method to assess the stability of polysorbate 20 and established methods that may overlook degraded polysorbate 20. Pharm Res. 2002;19(5):634–9.
23. Kirshner LS. Regulatory expectations for analysis of aggregates and particles. In: Colorado protein stability conference. Breckenridge, CO. 2012.

24. Krishnan S, Pallitto MM, Ricci MS. Development of formulations for therapeutic monoclonal antibodies and Fc fusion proteins. In: Jameel J, Hershenson S, editors. Formulation and process development strategies for manufacturing biopharmaceuticals. New Jersey, NJ: Wiley; 2010. p. 383–427.
25. Labrenz SR. Ester hydrolysis of polysorbate 80 in mAb drug product: evidence in support of the hypothesized risk after the observation of visible particulate in mAb formulations. J Pharm Sci. 2014;103(8):2268–77.
26. Laursen T, Hansen B, Fisker S. Pain perception after subcutaneous injections of media containing different buffers. Basic Clin Pharm Toxycology. 2006;98:218–21.
27. Mahler H-C, et al. Behaviour of polysorbate 20 during dialysis, concentration and filtration using membrane separation techniques. J Pharm Sci. 2008;97(2):764–74.
28. Mahler HC, Jiskoot W, editors. Analysis of aggregates and particles in protein pharmaceuticals. New York, NY: Wiley; 2011.
29. Manning MC, et al. Stability of protein pharmaceuticals: an update. Pharm Res. 2010;27(4): 544–75.
30. Mitragotri S, Burke PA, Langer R. Overcoming the challenges in administering biopharmaceuticals: formulation and delivery strategies. Nat Rev Drug Discovery. 2014;13(9):655–72.
31. Monnier VM. Nonenzymatic glycosylation, the maillard reaction and the aging process. J Gerontol. 1990;45(4):B105–11.
32. Nayar R, Manning MC. High throughput formulation: strategies for rapid development of stable protein products. In: Carpenter JF, Manning MC, editors. Rationale design of stable protein formulations-theory and practice. Kluwer Academic/Plenum: New York, NY; 2002. p. 177–99.
33. Neergaard MS, et al. Viscosity of high concentration protein formulations of monoclonal antibodies of the IgG1 and IgG4 subclass—prediction of viscosity through protein-protein interaction measurements. Eur J Pharm Sci. 2013;49(3):400–10.
34. Philo JS. A critical review of methods for size characterization of non-particulate protein aggregates. Curr Pharm Biotechnol. 2009;10(4):359–72.
35. Pisal DS, Kosloski MP, Balu-Iyer SV. Delivery of therapeutic proteins. J Pharm Sci. 2010; 99(6):2557–75.
36. Privalov PL, Khechinashvili NN. A thermodynamic approach to the problem of stabilization of globular protein structure: a calorimetric study. J Mol Biol. 1974;86(3):665–84.
37. Saito S, et al. Behavior of monoclonal antibodies: relation between the second virial coefficient (B (2)) at low concentrations and aggregation propensity and viscosity at high concentrations. Pharm Res. 2012;29(2):397–410.
38. Samra HS, He F. Advancements in high throughput biophysical technologies: applications for characterization and screening during early formulation development of monoclonal antibodies. Mol Pharm. 2012;9(4):696–707.
39. Sarciaux JM, Mansour S, Hageman MJ, Nail SL. Effects of buffer composition and processing conditions on aggregation of bovine IgG during freeze-drying. J Pharm Sci. 1999; 88(12): 1354–1361.
40. Schweizer D, Serno T, Goepferich A. Controlled release of therapeutic antibody formats. Eur J Pharm Biopharm. 2014;88(2):291–309.
41. Stoner MR, et al. Protein-solute interactions affect the outcome of ultrafiltration/diafiltration operations. J Pharm Sci. 2004;93(9):2332–42.
42. Wang W, Ignatius AA, Thakkar SV. Impact of residual impurities and contaminants on protein stability. J Pharm Sci. 2014;103(5):1315–30.
43. Yadav S, Sreedhara A, et al. Establishing a link between amino acid sequences and self-associating and viscoelastic behavior of two closely related monoclonal antibodies. Pharm Res. 2011;28(7):1750–64.
44. Yadav S, Scherer TM, et al. Use of dynamic light scattering to determine second virial coefficient in a semidilute concentration regime. Anal Biochem. 2011;411(2):292–6.

45. Yadav S, Shire SJ, Kalonia DS. Viscosity behavior of high-concentration monoclonal antibody solutions: correlation with interaction parameter and electroviscous effects. J Pharm Sci. 2012;101(3):998–1011.
46. Zolls S, et al. Particles in therapeutic protein formulations, Part 1: overview of analytical methods. J Pharm Sci. 2012;101(3):914–35.

Further Reading

47. Formulation and Process Development Strategies for Manufacturing Biopharmaceuticals. *F. Jameel, S. Hershenson (Editors), 2010*. Hoboken, NJ, USA: John Wiley & Sons. ISBN 978-0-470-111812-2.
48. Rationale Design of stable protein formulations-theory and practice. *J.F. Carpenter, M.C. Manning (Editors), 2002*. New York, NY, USA. Kluwer Academic/Plenum. ISBN 978-1-4615-0557-0.
49. Development and Manufacture of Protein Pharmaceuticals. *S.L. Nail, M.J. Akers (Editors), 2002*. New York, NY, USA. Kluwer Academic/Plenum. ISBN 978-1-4615-0549-5.

Part II
Challenges with Excipients

Chapter 2
Polysorbate Degradation and Quality

Kishore S. K. Ravuri

Abstract Among many other applications, polysorbates (PSs) are used as the most common surfactants in biopharmaceutical products in particular to protect proteins against interfacial stress. Structural heterogeneity, presence of degradants and other impurities, and tendency for degradation are interrelated features found in commercial PSs with a direct impact on their functional properties in biopharmaceutical products. These pose a challenge for the analytical characterization of PSs at different stages of product development. This review article focuses on methods and strategies reported in the recent years for the analytical characterization of PSs, their degradants and other impurities within neat PS (i.e., PS raw materials), diluted PS solutions, as well as in biopharmaceutical formulations. The use of versatile and complementary methods applied in a systematic approach is crucial to understand the impact of the concentration, composition, and degradation of PSs on the quality of biopharmaceutical products.

Keywords Excipients · Protein formulation · Stability · Surfactants

Abbreviations Used

CMC	Critical micelle concentration
HSA	Human serum albumin
FFA	Free fatty acids
FID	Flame ionization detector
ICH	International Conference for Harmonization
MALDI-TOF	Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry
PS	Polysorbates
PS20	Polysorbate 20
PS80	Polysorbate 80

K. S. K. Ravuri (✉)
Pharmaceutical Development, F. Hoffmann-La Roche, Ltd.,
4070 Basel, Switzerland
e-mail: satya_krishna_kishore.ravuri@roche.com

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_2

2.1 Introduction

Nonionic surfactants are widely used in protein formulations, especially parenteral monoclonal antibody formulations in both liquid and dried (e.g., lyophilized) forms. Virtually, all marketed parenteral biotherapeutics contains varying concentrations of surfactants. In general, nonionic surfactants have been favored for use with biotherapeutics over ionic surfactants due to their proven safety profile, primarily based on prior use for other products [1–3]. Ionic surfactants on the other hand are also known to possibly denature the protein [4]. The primary role of nonionic surfactants in a protein formulation is to protect the protein against interfacial stresses and related interactions/degradation, including interface-induced protein aggregation [5], protein precipitation (particle formation), and/or surface adsorption [5–12]. Surfactants are effective against various stresses such as agitation, for example, shaking or stirring (air/water interfaces) [6, 13–15], freezing and thawing (ice/water interfaces), and stresses that can occur during lyophilization [16, 17]).

The most extensively used surfactants in biotherapeutic formulations are the polyoxyethylene (PEO)-based surfactants, like polysorbates 20 and 80 and poloxamer 188.

Polysorbates are a family of nonionic surfactants widely used as excipients in food and pharmaceutical products. Polysorbate 20 (PS20) and polysorbate 80 (PS80) are preferred surfactants used to protect therapeutic proteins against adsorption to interfaces. The use of PS20 and PS80 in biopharmaceutical products is well accepted, and accepted excipients for parenteral administration by IV, SC, and IVT are approved by regulatory agencies. About 80% of biopharmaceutical products containing peptides, proteins, antibodies, and vaccines are formulated with polysorbates. Their high hydrophilic–lipophilic balance value and low critical micelle concentration (CMC) account for their high surface activity even at low concentrations. PS20 and PS80 not only have been reported to prevent interface-induced protein aggregation [18–23] and freeze thaw stress [5, 8, 18, 20, 24–26], but also prevent adsorption of the protein to various surfaces [19] such as sterile filters [27] and primary packaging. Prevention of aggregation by PS20 has been reported previously including porcine growth hormone [28], recombinant human growth hormone (rHGH) [29], and recombinant human growth factor XIII [30]. Similarly, there have been reports of the benefit of PS80 in preventing aggregation induced by vortexing (rHGH [31]) and freeze–thaw-induced denaturation (lactate dehydrogenase (LDH) [5], recombinant hemoglobin [20], etc. [18].

Typical polysorbate concentrations in biopharmaceuticals are between 0.001 and 0.1% (w/v), corresponding to 0.01 and 1 mg/mL. The choice and concentrations of the surfactant are usually determined by screening for the lowest effective concentration which stabilizes the therapeutic protein upon interfacial stress. These concentrations are determined by stress studies generating air–water and/or ice–water interfaces, such as shaking, stirring, or freezing/thawing at varied surfactant concentrations followed by aggregate and particulate analysis. The concentration chosen is ideally one level above the edge of failure to provide a sufficient safety margin and

protection during real-time stress such as transportation, stirring, freeze–thaw. During commercial production, the surfactant is usually added after ultrafiltration–diafiltration (UF-DF) and prior to a bulk freezing step in the bioprocess (drug substance) manufacturing since the UF-DF step can alter the concentration of polysorbate in a nonreproducible manner due to membrane adsorption. Since drug substance bulk is typically stored frozen prior to drug product manufacturing, the presence of surfactant is important to protect the protein from ice–water interfaces formed during freeze–thaw. Thus, the preferential point of surfactant addition is post-UF-DF, but prior to freezing of the drug substance.

2.2 Chemical Structure, Synthesis and Composition

Chemically, polysorbates are polyoxyethylene-1,4-sorbitan-monoesters of fatty acid. Commercially available PS20 and PS80 are diverse mixtures containing mainly sorbitan POE fatty acid esters (Fig. 2.1).

However, this is just an idealized structure, and commercially available polysorbates consist of a mixture of structurally related molecules. Additionally, substantial amounts of POE, sorbitan POE, and isosorbide POE fatty acid esters are present [32–34]. For instance, the theoretical expected structure for PS20 and PS80, according to their formal names polyoxyethylene (20) sorbitan monolaurate and polyoxyethylene (20) sorbitan monooleate, was reported to only account for about 20% (w/w) of the total PS material.

2.2.1 *Structure Heterogeneity in PS20 and PS80*

The first step in the synthesis of polysorbates is the dehydration and esterification with fatty acids. This step occurs in a single sequence at very high temperatures in anhydrous basic conditions [35]. It is not clear whether esterification occurs before epoxylation [34, 36, 37] because vendors usually do not specify which route is used for the synthesis. The reverse order (epoxylation first) has also been reported [38]. The average PEG subunits on each polysorbate molecule (w + x + y + z) is supposed to be 20. Typical side products expected in the synthesis is the formation of isosorbides as observed in the HPLC-CAD profiles of polysorbates 20 and 80 [39] (b) Further heterogeneity arises from the composition of the fatty acids in PS20 and

Tween 80 Tween 20

Fig. 2.1 Chemical structure of commonly used surfactants polysorbate 80, polysorbate 20 (top); x + y + z = 20 and poloxamer188 (bottom); a = 80 and b = 27

PS80 according to Ph. Eur. 8.5 and 8.0 and USP. Thus, the heterogeneity of polysorbate arises from (1) the polydispersity of the PEG, (2) the degree of esterification, (3) the uncontrolled dehydration of the sorbitol, and (4) the fatty acid composition. The polydispersity of PEG leads to a distribution typically expected from PEG-containing molecules [40]. While the main compound is supposed to be a monoester, mixtures of isomeric di-, tri-, and tetra-esters are commonly found (Fig. 2.2). The polysorbate sugar core is a mixture of sorbitans and isosorbides, depending on the degree of dehydration of the sorbitol as well as the conditions of the reaction [33]. Typically, PEG sorbitans are also present to a considerable extent based on unfinished esterification [37]. Up to (<17%) of PS20 corresponded to nonesterified sorbitan-PEG has been reported [41]. While the main fatty acid is either lauric acid for PS20 and oleic acid for PS80, the distribution of fatty acids brings additional complexity [36, 42, 43]. Traces of free fatty acids can also be found in commercial neat polysorbates. Altogether, this structural heterogeneity affords the surfactant like properties of this molecule [33] but also tends to be a significant liability. This leads to a significant degree of batch-to-batch variability requiring a close scrutiny of each batch in order to ensure uniform behavior [44]. The presence of residual levels of peroxide in bulk polysorbate is also a concern. Although the European Pharmacopoeia (Ph.Eur.) specifies a limit of "not more than 10 ppm," there have been reports of rapid buildup of peroxides in bulk as well as in aqueous solutions of polysorbate, when exposed to ambient oxygen and light [45]. Depending on handling and storage conditions, varying concentrations of peroxides were noted among different lots of polysorbates [46]. The buildup of peroxides can be detrimental not only to the stability of polysorbate itself but also to the protein therapeutic, which it is supposed to stabilize [47]. Based on this consideration, Harmon et al. have also developed an oxidation stress test relying on peroxides formed in PS80 in the presence of Fe(III) [48]. The Amplex Red-based fluorescence assay has also been used extensively to measure peroxides in polysorbates.

2.3 Use in Biotherapeutics

The use of nonionic surfactants in therapeutic protein formulations is governed by a number of properties like solubility, stability, and most importantly low toxicity. The more extensively used surfactants in this regard are the polyoxyethylene-based surfactants like polysorbates 20 and 80. Along production, distribution, storage, and administration to the patient, protein pharmaceutical solutions are constantly exposed to a plethora of interfaces (e.g., glass, plastic polymers, stainless steel, air, ice crystals, silicone oil) which can lead to adsorption, denaturation, aggregation, and decrease of the effective protein concentration. When combined with mechanical stress, [49–52] interfacial adsorption might act as a trigger for aggregation and particle formation. This is particularly crucial for hydrophobic proteins [53] formulated at low-concentration doses in which the loss of protein due to surface adsorption can be significant [49–52].

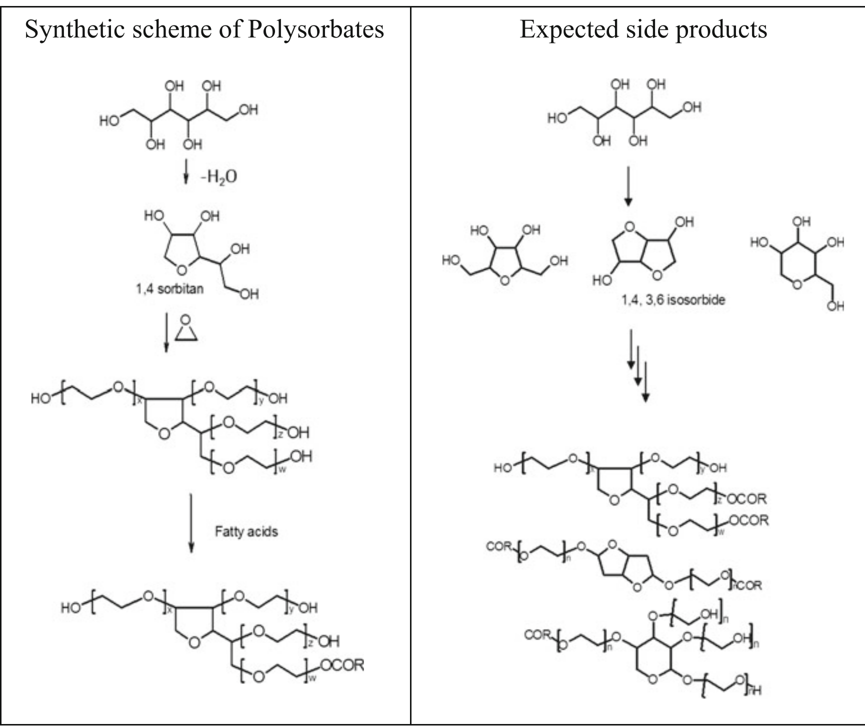


Fig. 2.2 A synthetic scheme and the expected side products

Although the molecular details of stabilization are not yet fully understood, polysorbates are known to contribute to protein stability by two different mechanisms, that is, through (1) competitive adsorption to the hydrophobic interfaces and (2) direct binding to the protein [54]. In the first case, polysorbates show higher adsorption energies per unit area than proteins, thereby efficiently competing with proteins and protecting them against adsorption to hydrophobic interfaces [55]. Moreover, polysorbates may interact directly with the protein increasing its stability in solution by binding to and protecting exposed hydrophobic regions [56]. This prevents aggregation by reducing protein–protein interactions. Which of these two mechanisms prevails within the stabilization of a particular protein is protein dependent and PS dependent. For instance, the binding between PS20 or PS80 and several mAbs was reported to be negligible, indicating that mAb stabilization by PS occurs mainly via a competition mechanism [57].

2.4 Interaction of Nonionic Surfactants with Proteins

Proteins, being amphiphilic, high-molecular-weight molecules, tend to fold or assemble into globular or micellar structures in aqueous solutions, in order to expose hydrophilic parts to the exterior and hide the hydrophobic part in the core of

the structure. Their interaction with surfactants is driven by the various forces governing their stability in solution as well as at the interface. In the following sections, these two aspects are discussed in detail.

Ideally, the nature of interactions between nonionic surfactants and proteins can best be described by understanding the thermodynamic drivers such as changes in enthalpy and entropy, in relation to properties (polar, charged, or hydrophobic) of regions on the surfactant and protein molecule themselves. Unfortunately, detailed thermodynamic and site-specific interaction behavior is difficult to measure and quantify due to the generally low-affinity interactions [58]. Lee et al. [3] have recently comprehensively reviewed the interactions of polysorbate 20, polysorbate 80, and poloxamer 188 with proteins and have summarized the contributions of the protein, the surfactant, and surfactant–protein complexes relating to protein adsorption and implications in aggregation behavior. Table 2.1 details a fairly comprehensive listing of work related to interaction of proteins with surfactants. A few of these examples of interaction have been highlighted below.

In investigating protein–surfactant interactions, considerable work has been reported with bovine serum albumin (BSA) as a model protein, establishing interactions between polysorbates 20/80 and other nonionic surfactants with BSA. For instance, Perez et al. investigated the interactions between nonionic surfactants and bovine serum albumin (BSA), using fluorescence, surface tension measurements, and computational simulations [59]. Based on their studies, they demonstrated that upon addition of the protein, the surface tension behavior of the surfactants was modified, and the apparent CMC values of polysorbates 20 and 80 in the presence of protein increased compared to the CMC values of the surfactants themselves. These results also suggested that despite structural similarities of polysorbate 20 and polysorbate 80, their interaction with BSA in solution seems to be different in nature, potentially with differing interaction sites. Further studies by varying BSA concentrations also suggested that these interactions are different at distinct aggregated states of the protein in solution. Changes observed in the tryptophan emission indicated the surfactants first preferentially associate within cavities in the tertiary structure; once saturated, additional surfactant molecules are able to interact with hydrophobic patches on the protein surface. Nielsen et al. used isothermal calorimetry (ITC) to understand the binding of BSA to nonionic surfactants with C12 acyl chains along with ionic surfactants [60]. Their data demonstrated that the binding of low-affinity sites generates energetic parameters, such as a large negative change in heat capacity, characteristic of hydrophobic interactions. In this study, it was found that nonionic surfactants bind to proteins with association constants several orders of magnitude lower than sodium dodecyl sulfate. It was also concluded that large exothermic enthalpy changes occur along with large increases in heat capacity during the binding process. These changes associated with nonionic surfactants were greater than that observed for anionic surfactants.

Hoffmann et al. [61] reported similar findings when they assessed binding by ITC and thermal stability of the proteins in the presence of polysorbates 20 and 80

by differential scanning calorimetry (DSC). Here, ITC provided the binding constants and thermodynamic parameters, while DSC yielded thermal protein stability information. The results show that both polysorbate 20 and polysorbate 80 bound to BSA with a binding constant of 8–12 $\times$ 10^{-3} M^{-1} and a ΔH −50 to −60 kJ/mol (25 °C). Stoichiometrically, it was deduced that one to two detergent molecules bound to BSA. The large exothermic enthalpic changes could be considered as an outcome of the interaction of the PEO chain with the protein by hydrogen bonding. It was also recently shown that PEO chains (polyethylene glycol) can also exhibit affinity for proteins like BSA [62]. Delgado et al. [63] support this hypothesis with their molecular dynamics simulation results, which suggest enhanced stabilization of BSA by cooperative self-assembly with polysorbate molecules. However, Hoffman et al. observed that the interactions of polysorbates 20 and 80 with an immunoglobulin and lysozyme were negligible. ITC studies showed that very low binding was observed even at higher titrated concentrations.

In another highly similar analysis performed by Garidel et al. [57], only weak interactions were found between polysorbates 20 and 80 with several immunoglobulins. However, this study did find a measurable interaction with human serum albumin (HSA). This finding was interpreted as the fatty acid chains of the polysorbates being sterically poorly matched for the fatty acid binding pocket of HSA. The results showed that binding constants of polysorbates to human serum albumin were in the range of 10^3 M^{-1}, a rather negligible value, leading to the conclusion that direct surfactant interaction is not the main factor for stabilization of the protein. McAuley et al. [64] also employed ITC to investigate the interaction between polysorbates and lactate dehydrogenase (LDH). Additionally, surface tension measurements and interfacial rheology were used in order to understand the mechanism of prevention of protein adsorption to the air–water interface by the surfactant. No significant interaction between polysorbate 20 and LDH could be found using ITC. It was apparent that surface pressure effects dominated the prevention of LDH adsorption to the air–water interface by polysorbates. Interfacial rheology suggested that the concentration of polysorbate 20 needed to displace LDH was well below the CMC. The majority of studies based on ITC have revealed primarily weak binding between polysorbates and therapeutic proteins (in particular mAbs), and those with albumins suggest a significant interaction component driven by van der Waals interactions and hydrogen bonding.

In another study, Chou et al. characterized the binding interactions between the surfactants and Albutropin™ (human growth hormone genetically fused to human albumin) based on fluorescence spectroscopy and ITC [65]. The authors concluded that polysorbates had saturable binding to Albutropin with a molar binding stoichiometry of approximately 10:1 (surfactant:protein) and binding of the surfactants to Albutropin led to an increase in free energy of unfolding. This increased free energy of unfolding was thought to be responsible for stabilizing the protein even in concentrations of surfactants well below their CMC. The belief of stabilizing monomeric proteins from aggregation has previously been described in terms of molecular chaperones [19]. Earlier studies used chemically denatured protein to evaluate protein–surfactant interactions, protein structure, and enzymatic activity by

electron paramagnetic resonance, circular dichroism spectroscopy, and activity assays [66, 67]. These studies suggested polysorbate 20, as well as other surfactants, may temporarily occupy hydrophobic regions exposed on partially denatured proteins, enabling them to refold and displace the polysorbate prior to the formation of an aggregation event.

Kim et al. [68] demonstrated based on optical waveguide light mode spectroscopy that polysorbates 20 and 80 are able to prevent protein adsorption to a hydrophilic surface exclusively by their preferential location at the interface due to higher affinity. It was also concluded that no significant surfactant–protein associations occurs in solution. On the other hand, poloxamer 188 was thought to have surfactant–protein associations in solution, independent of its affinity for the interface, thereby inhibiting protein adsorption to the interface. A variety of techniques has been employed to study surfactant–protein interactions in solutions. Ideal measurement methods are based on measuring direct interactions, are capable of providing insight into the mechanistic drivers, and determine the site-specific locations of molecular recognition. However, the weak interactions in play often require more indirect methods to be used, but are still able to provide insight and offer relative levels of interactions between various molecules.

2.5 Interactions of Protein–Surfactant Mixtures at Interfaces

As discussed in the previous section, interactions of nonionic surfactants with proteins vary according to the nature of the protein. It is also evident that the surfactant interaction at interfaces typically dominates (e.g., water–air, water–container, water–silicone oil) as the mechanism stabilizing therapeutic proteins. The complex behavior of surfactant adsorption is governed by many factors such as the bulk concentration, the chemical potentials in the bulk solution and at the interface, the thickness of the adsorption layer, the maximum possible adsorption, and temperature. In a typical therapeutic protein formulation mixture containing both protein and surfactant, upon exposure to a new interface, different species compete to adsorb at the interface which leads to lowering of the surface tension. A dynamic equilibrium is established due to the adsorption and desorption processes [80–82]. The ability to adsorb at the interface would depend on the relative surface activity and also mutual interaction in solution [68, 83].

Early experiments evaluated the ability of polysorbate 20 to desorb proteins previously adsorbed to hydrophilic and hydrophobic solid surfaces [84]. It was found that the polysorbates had little effect at displacing proteins (fibrinogen and human gamma globulin) adsorbed on the hydrophilic surfaces. However, polysorbate 20 was effective at removing the protein molecules from a hydrophobic surface. The ability of the polysorbates to desorb protein from hydrophobic surfaces was reduced when the proteins had been incubated for extended periods of time at

Table 2. A review of studies investigating surfactant–protein interactions

Sl. No.	Surfactant investigated	Protein investigated	Nature of investigation	Techniques implemented	Interaction proposed	Authors	Ref.
1	16-doxyl stearic acid, Brij, Tween 20, 40, 80	rHGH, Inf g	binding stoichiometries	spin labeled partition curves via EPR	na	Bam et al.	[69]
2	Tween 20, Brij 35, Brij 78	α and β-lactoglobulin	Electrophoretic behavior	CE	na	Xu et al.	[70]
3	C12E8	rhTF220, 243	Binding stoichiometries	AUC, EPR	na	Jones et al.	[71]
4	Tween 20 and Tween 80	Albutropin	Albutropin–surfactant interactions	Fluorescence spectroscopy and ITC	Interaction leading to increase of free energy of unfolding	Chou et al.	[65]
5	Tween 80	LDH	Interfacial behavior at ice liquid interface	DSC and IR	Tween competes with protein at interface	Hillgren et al.	[72]
6	Tween 20 and Tween 80	IgG and HSA	Interaction and binding	ITC and DSC	Nonspecific interactions	Garidel et al.	[57]
	Tween 20 and Tween 80	BSA, lysozyme, IgG	Interaction and binding	ITC and DSC	Negligible binding with igG	Hoffmann et al.	[57]
7	Tween 20, Tween 40, Tween 80	LDH	Interfacial behavior at air-liquid interface	ITC, calorimetry, surface tension, and interfacial rheometry	Competitive displacement mechanism driven by surface pressure	Kett et al.	[73]
8	Dodecyl dimethyl phosphine oxide	β-caesin	Interfacial behavior at air-liquid interface	Tensiometry and rheometry	Competitive displacement at interface	Kotsmar et al.	[74]

(continued)

Table 2. (continued)

Sl. No.	Surfactant investigated	Protein investigated	Nature of investigation	Techniques implemented	Interaction proposed	Authors	Ref.
9	Tween 20, Tween 40, Tween 80	LDH	Binding and interfacial behavior	ITC	Weak hydrophobic interaction	McAuley et al.	[64]
10	Tween 20, Tween80	BSA	Surfactant interaction and binding	Surface tension, fluorescence, and computational analysis	Binding model for BSA–surfactant binding	Perez-Gramatges et al.	[59]
11	Triton-X	Gelatin	Surfactant–protein binding	Density, adiabatic compressibility	Hydrophobic binding model	Chauhan et al.	[75]
12	C12EO7, C12EO5	BSA	Surfactant interaction and binding	ITC	Binding model for BSA-surfactant binding	Nielsen et al.	[60]
13	Tween 20, Tween 40	lysosome	Adsorption at solid–water interface	CD and adsorption kinetics	Hydrophobicity-driven preferential tween adsorption	Joshi et al.	[76]
14	Tween 80	Recombinant factor FVIII	Adsorption at air-water interface	Surface tension	Competitive displacement at the interface	Joshi et al.	[77]
15	Tween 20, Tween 40	fibrinogen	Adsorption and binding	SPR	Adsorption and packing phenomenon	Shen et al.	[78]
16	Tween 80	BSA	Aggregation prevention	CD and native PAGE	Aggregation inhibition of partially or fully denatured monomers	Arakawa et al.	[79]

elevated temperatures. These conditions likely increased the hydrophobic interaction surface area involved increasing the entropic barrier for solvation, beyond the level the aliphatic chains of the polysorbate could stabilize. Joshi et al. [85] later demonstrated that at the liquid–solid interface, the adsorption propensity of nonionic surfactants depends on the hydrophobicity of the surface. They also showed that the pretreatment of hydrophilic surfaces with polysorbate 80 did not have an effect on subsequent protein (lysozyme) adsorption, while the precoating of polysorbate 80 on hydrophobic surfaces dramatically decreased the ability of a protein film to form on the surface, measured by ellipsometry. The trends for preventing protein adsorption on hydrophobic surfaces held true when both the polysorbate and protein were added in the same solution, compared to protein alone. While the mechanisms involved in protein surface adsorption are complex and are determined by multiple factors (e.g., size, structural stability, and exposed hydrophobic patch distribution [86, 87]), the literature in general suggests greater adsorption due to hydrophobic surfaces (regardless of protein properties) and by more hydrophobic proteins (regardless of surface properties [84, 88]). Polysorbates appear to be most effective at competing and desorbing proteins from moderately hydrophobic surfaces [84, 85].

Among the many hydrophobic surface which therapeutic proteins encounter, silicone oil is a very relevant one. Silicone oil is typically applied to the surface of prefilled syringes and represents an additional pharmaceutically relevant interface. Previous work has evaluated the ability of polysorbate 20 to compete with BSA for interfacial sites on water–oil emulsion droplets [89–91]. These studies relied on front-face fluorescence spectroscopy, based on tryptophan fluorescence, to demonstrate a concentration-dependent behavior of polysorbate to effectively displace protein from the water–oil interface. Mass adsorption measurements using quartz crystal microbalance (QCM) have been used in evaluating the competition behavior of surfactants and proteins to silicon oil surfaces [92, 93]. In one study, it was determined by QCM and surface tension that polysorbate 80 was more effective than poloxamer 188 at inhibiting the adsorption of an Fc-fusion protein to the water–silicone oil interface [92]. This was attributed to polysorbate 80, being more hydrophobic, lower HLB value, than poloxamer 188. In another study, it was found that both polysorbates (20 and 80) and poloxamer 188 were effective at inhibiting the adsorption of an Fc-fusion when preadsorbed, but rinsing the surface after applying the preadsorbed surfactants enabled a greater amount of protein to be adsorbed for the poloxamer 188 case, also being attributed to a weaker poloxamer 188–silicone oil interaction creating more unoccupied surface after rinsing. It was also determined that when surfactant and protein were coadsorbed, all surfactants reduced the amount of protein adsorbed the interface. However, it was also shown that all surfactants were not effective at displacing a preexisting protein-adsorbed layer.

A common method for determining the CMC of polysorbates in solution is based upon the stabilization of the surface tension after the concentration of the surfactant exceeds that of the CMC [94]. This is due to the previously mentioned propensity of the polysorbate molecules to associate and orient themselves with the air–liquid interface. This trend of concentration-dependent reduction (up to the

CMC) of the surface tension remains for polysorbates in the presence of protein solutions, although often altered by the surface activity of the protein itself [95]. A similar study based on dynamic surface tension also demonstrated surface tension increases of polysorbate 80 solutions in the presence of protein (HSA) suggesting the lack of surface active molecules present at the air–water interface was attributed to surfactant–protein interactions in solution [96]. This phenomenon helps displace proteins away from the air–liquid interface, much in the same way as the above-described competition for solid–liquid interfaces. Interestingly, the dynamic surface tension may be reduced when interactions take place below the CMC, as seen for solutions of GCSF with polysorbates and poloxamer 188 [80]. This behavior has been attributed to protein-disrupting surfactant aggregates unable to readily adsorb to the interface, increasing the amount of surfactant monomer near the interface and enhancing the rate of adsorption. Such behavior may improve formulation stability in shake-stress scenarios by enabling more surfactant to compete for the interface. However, the proposed aggregate interaction may also destabilize the protein itself by inducing a partially denatured state.

Mackie et al. [97] propose an "orogenic mechanism" to elucidate the ability of nonionic surfactants to displace proteins from the interface. They explain how small quantities of surfactant added to a protein-adsorbed interface can break away the protein network. This alteration of the interface is thought to be due to different mechanisms in which surfactants and proteins behave at interfaces. Proteins tend to form strong interactions with one another, likely caused by hydrophobic interactions caused by partial denaturation. In contrast, surfactants move in the direction of surface tension gradients, described by the Gibbs–Marangoni effect [98]. The protein network inhibits surfactant lateral mobility, and a surface pressure is induced on the protein network leading to competition and displacement of proteins from the interface [97, 99]. Korsmar et al. and Kett et al. [73, 74] demonstrate evidence for polysorbate 20-driven disruption of surface shear viscosity of β-lactoglobulin. While some of the orogenic studies may not be fully translatable to formulation studies with biologics, they provide insight regarding the need to prevent protein adsorption at interfaces and implications for drug product manufacturing.

The various studies performed to date provide different perspectives as to possible mechanisms surfactants could be contributing to protein stabilization. Although studies conducted with BSA demonstrate the presence of binding propensity of surfactant to the protein [59, 60], it could be considered that such binding is a special situation for BSA and not representative of most other proteins. The few studies on IgG, published by Garidel et al. and others, show that the binding energy (from ITC measurements) are weak and likely play no significant role for stabilization [57]. The presence of surfactant also is not expected to impact on the pharmacokinetic parameters associated with biologics, especially in the case for mAbs dominated by FcRn-mediated recycling [100], in contrast to the use of nonionic surfactants in small molecules where the impacts on pharmacokinetics can be dramatic [101, 102].

The following are the various postulated mechanisms of interaction of nonionic surfactants to proteins: (a) a competitive mechanism at the interface where the occupancy of the interface by the nonionic surfactant is more favored

thermodynamically compared to the protein [18, 31, 83, 97, 103–105]. This competitive adsorption of the surfactant at the interface is correlated to an increased surface pressure by the surfactant [106]. (b) Direct binding of hydrophobic portion of the surfactant on the exposed hydrophilic surfaces of the native protein, thereby increasing the protein's colloidal stability [29]. This would lead to thermodynamic stabilization of the native state by preferential binding of the surfactant [107]. (c) "Molecular chaperone"-like function of the surfactant, similar to the "b," but stabilizing partially denatured forms of the protein long enough to enabling refolding of the protein [67].

To date, based on the published data and from our own experience, the primary mechanism of stabilization of proteins such as mAbs by nonionic surfactants against aggregation is mainly contributed by competition of (surface active) polysorbate or poloxamer molecules against proteins at interfaces (e.g., air–water). The competitive adsorption of nonionic surfactants at interfaces would generally be thermodynamically favoured [31, 103, 104, 108] over adsorption of the protein at these interfaces. With the decrease of the protein concentration locally at the interface, a decrease in local concentration of proteins is expected, thereby minimizing risk of protein aggregation and/or precipitation (protein particle formation). The connection between protein concentration, collision rate, and thus their aggregation of self-association is known from studies of nonideal solutions [109, 110]. Surface tension and rheology experiments [73, 74] provide evidence that the prevention of adsorption of the protein (LDH in the present case) at the interface is more related to surface energy and surface pressure rather than to the CMC. Our own studies with mAbs showed that surfactants stabilize antibodies against mechanical stress and found that polysorbates and poloxamers have the ability to prevent aggregation or particle formation against shaking at concentrations well below their CMC [95, 111].

The presence of stipulated amounts of polysorbates is important for the protein drug product within its shelf life. Despite their successful use in marketed therapeutic protein products, polysorbate can undergo a variety of degradation reactions [20]. This may result in a likely loss of the functional properties of polysorbate in the formulation as well as formation of degradants that may induce protein instability [112]. The last is a key area of concern because aggregates and chemically modified protein molecules are considered critical quality attributes and may be related to enhanced immunogenicity [113]. Moreover, PS degradation may lead to unwanted PS-related particles. Therefore, it is crucial to set up analytical approaches to characterize and quantify polysorbate and their degradation products during formulation development, manufacturing, and the shelf life of the product.

2.6 Pharmacopoeia Requirements and Commercially Available Grades of Polysorbate

The international pharmacopoeias describe the minimum criteria that commercially available polysorbates intended for pharmaceutical purposes should adhere to (Table 2.2). The pharmacopoeia of the ICH countries usually agrees on the key

Table 2.2 Possible markers from the major routes of PS degradation under pharmaceutically relevant conditions

Oxidation	Chemical/Enzymatic hydrolysis
Aldehydes	Free fatty acids
Alkanes	
Epoxides	
Free fatty acids	
Ketones	
PEG esters	
Peroxides	
Oxidized fatty acids	
Short-chain organic acids	

requirements, such as the fatty acid distribution. Based on these specifications, the main manufacturers offer polysorbates for pharmaceutical applications. The grades offered typically comply with the specifications from a particular pharmacopoeia (USP, EP, or JP) are available for example at ACROS Organics, Avantor-J.T. Backer, CRODA, NOF, and Sigma-Aldrich and SEPPIC. Some polysorbate providers also offer multicompendial polysorbates, e.g., Avantor-J.T.Backer, CRODA, and NOF. Avantor-J.T.Backer defines multicompendial as a grade of products meeting the criteria specified by the US (USP), the European (Ph. Eur.), the British (BP), and the Japanese (JP) pharmacopoeias. Multicompendial polysorbates from CRODA and NOF comply with USP, Ph. Eur., and JP. The suppliers, having recognized some liabilities of polysorbates, have begun to offer polysorbates at claimed higher purity, sometimes referred to as "super-refined" or "ultrapure" depending on the vendor. These are claimed to contain low peroxide, low endotoxin, and low impurity levels. The Chinese pharmacopoeia recently established a set of stricter requirements for PS80 intended for injection (Table 2.3). For instance, the content of oleic acid ($\geq 98\%$) and the specified limit for the endotoxin level (0.012 EU) are tighter specifications compared to other pharmacopoeias. While the use of a customized grade of PS20 and PS80 containing $\sim 99\%$ laurate (BASF) and $\sim 98\%$ oleate (NOF) fatty acid esters is observed in some in recent publications, these polysorbates are not yet commercially produced in the US or European regions.

Given the correlation between PS quality and long-term stability of protein formulations, it is a common practice in pharmaceutical development to use the highest purity grade of polysorbates in parenteral formulations. Nevertheless, beyond the grade of the starting material, it is crucial to control handling and storage conditions for polysorbates at each manufacturing stage to minimize degradation and impurities and to maintain a low peroxide level. Neat polysorbates are recommended to be stored at low temperature, protected from light and in a tightly closed container under a nitrogen atmosphere to avoid exposure to oxygen. A caveat to this is the practice of the vendor storing the bulk at room temperature. Long-term storage of diluted PS solutions should be avoided because a higher diffusion coefficient facilitates the oxidation cascade propagation. Finally, regular

Table 2.3 Requirements of different pharmacopoeias regarding PS20 and PS80 for injection

Tests	Ph. Eur.		USP		JP		ChP	
	PS20	PS80	PS20	PS80	PS20	PS80	PS20	PS80
Acid value (mg KOH/g)	≤ 2	≤ 2	≤ 2	≤ 2	≤ 4	≤ 2	≤ 2	≤ 1
Hydroxyl value (mg KOH/$g_{acetylated}$)	96–108	65–80	96–108	65–80	–	65–80	96–108	65–80
Peroxide value (mEq/Kg)	≤ 10	≤ 10	≤ 10	≤ 10	–	≤ 10	≤ 10	≤ 3
Saponification value (mg KOH/g)	40–50	45–55	40–50	45–55	43–55	45–55	40–50	45–55
Composition of FA (%)								
Capric acid	≤ 10	–	≤ 10	–	–	–	≤ 10	–
Caproic acid	≤ 1	–	≤ 1	–	–	–	≤ 1	–
Caprylic acid	≤ 10	–	≤ 10	–	–	–	≤ 10	–
Lauric acid	40–60	–	40–60	–	–	–	40–60	–
Linoleic acid	≤ 3	≤ 18	≤ 3	≤ 18	–	≤ 18	≤ 3	≤ 0.5
Linolenic acid	–	≤ 4	–	≤ 4	–	≤ 4	–	≤ 0.5
Myristic acid	14–25	≤ 5	14–25	≤ 5	–	≤ 5	14–25	≤ 0.5
Oleic acid	≤ 11	≥ 58	≤ 11	≥ 58	–	≥ 58	≤ 11	≥ 98
Palmitic acid	7–15	≤ 16	7–15	≤ 16	–	≤ 16	7–15	≤ 0.5
Palmitoleic acid	–	≤ 8	–	≤ 8	–	≤ 8	–	≤ 0.5
Stearic acid	≤ 7	≤ 6	≤ 7	≤ 6	–	≤ 6	≤ 7	≤ 0.5
Ethylene oxide (ppm)	≤ 1	≤ 1	≤ 1	≤ 1	–	≤ 1	≤ 1	≤ 1
Dioxane (ppm)	≤ 10	≤ 10	≤ 10	≤ 10	–	≤ 10	≤ 10	≤ 10
Heavy metal (ppm)	≤ 10	≤ 10	≤ 10	≤ 10	–	≤ 20	≤ 10	≤ 10
Water (%)	≤ 3	≤ 3	≤ 3	≤ 3	≤ 3	≤ 3	≤ 3	≤ 0.5
Total ash (%)	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 1	≤ 0.25	≤ 0.25	≤ 0.1
pH	–	–	–	–	–	–	4–7.5	5–7.5
Relative density (kg/m^3)	~ 1.1	~ 1.1	–	1.06–1.09	–	~ 1.1	1.09–1.12	1.06–1.09

(continued)

Table 2.3 (continued)

Tests	Ph. Eur.		USP		JP		ChP	
	PS20	PS80	PS20	PS80	PS20	PS80	PS20	PS80
Viscosity (mPa·s, 25°C)	~ 400	~ 400	–	–	–	~ 400	250–400	350–450
Iodine value	–	–	–	–	–	–	–	18–24
Arsenic content (ppm)	–	–	–	–	–	–	≤ 2	≤ 2
Ethylene glycol (%)	–	–	–	–	–	–	≤ 0.01	≤ 0.01
Diethylene glycol (%)	–	–	–	–	–	–	≤ 0.01	≤ 0.01
Triethylene glycol (%)	–	–	–	–	–	–	–	≤ 0.01
Endotoxin (EU)	–	–	–	–	–	–	–	0.012

Ph. Eur.—European Pharmacopoeia 8.5 and 8.0 versions for PS20 and PS80. USP—United States Pharmacopoeial Convention from 2014 and 2015 for PS20 and PS80, respectively. JP—Japanese Pharmacopoeia, 17th edition, for PS80. JP has no monograph specific for PS20. The information shown here corresponds to PS20 mention under General Test 9.41 (Reagents, Test solutions). ChP—Chinese Pharmacopoeia, 2015.—Indicates value not reported in the corresponding pharmacopoeia

characterization controls (e.g., in-process controls) and the determination of appropriate expiration dates are recommended to preserve the integrity of neat polysorbates.

2.7 Stability and Degradation in Formulations

For the pharmaceutical industry, the characterization of neat polysorbate (i.e., raw material) or aqueous diluted PS solutions—and as part of a biopharmaceutical formulations—that is, in the presence of active pharmaceutical ingredient and other excipients—is highly relevant. The inherent complexity of polysorbates makes their analytical characterization challenging.

The study of PS degradation is complex due to the heterogeneity of the species inherently present in polysorbates. Several recent publications have contributed to a better understanding of the complexity of polysorbates and their degradation mechanisms, in particular with regard to pharmaceutically relevant conditions [112, 114–120].

The degradation of polysorbates has been also studied in the past. Donbrow et al. in their seminal work, researched various parameters such as pH, peroxide number, and surface tension in aqueous solutions of polysorbate and proposed a degradation profile based on mechanisms of hydrolysis and auto-oxidation [121]. In another study by Bates et al. [38] the hydrolysis of PS80 was studied and pseudo-first-order rates were calculated for degradation by hydrolysis. It was also observed that the rate of hydrolysis was dependent on factors such as pH, temperature, and the presence of micelles. It was also found that degradation of PS80 in an aqueous formulation was different using different rubber stoppers [122]. In recent studies, Yao et al. have looked at the auto-oxidation scenario by following the rate of oxidizability of PS80 and PS20 in the presence of a radical initiator [123]. They followed the oxygen consumption in the headspace and used the Hammond's approach to assess the radical chain initiation and thus evaluated the oxidizability of PS20 and PS80. It was found that apart from the known route of initiation of auto-oxidation at the ethylene oxide, radical initiation also occurred majorly at the site of the unsaturation (especially linoleate and linolenate moeities), thus rendering the rate of oxidizability higher in PS80. The complexity of the PS constituents was reduced in recent publications by using customized PS20 and PS80 containing $\sim$99% and $\sim$98% lauric and oleic acid esters, respectively [39, 120]. In one of these publications, unique degradant patterns of all-laurate PS20 were observed which, in combination with ^{18}O-labeling, provided a direct approach to differentiate the mechanism of PS degradation [124].

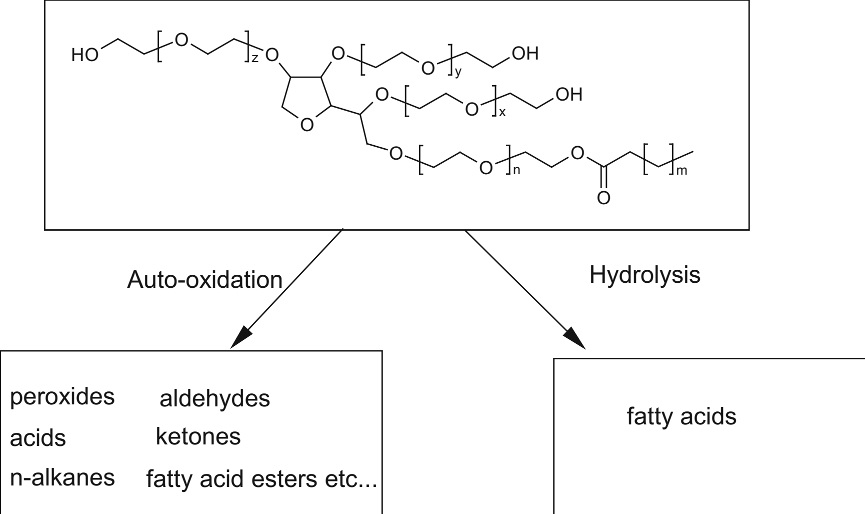
The above study and work by others [38, 121, 123, 125–132] throw light on the cause and nature of initiation [127] of auto-oxidation and rates of hydrolysis [38]—mechanisms which operate in degradation of polysorbates.

2.8 Mechanistic Pathways of Polysorbate Degradation

The two known mechanistic pathways of degradation of polysorbate are hydrolysis and auto-oxidation [129]. Scheme 2.1 provides a general outline of the degradants formed from the different pathways.

Oxidative mechanisms:

Oxidation was found to be the most common degradation pathway under pharmaceutically relevant conditions (e.g., pH 5.5 and 5 °C–25 °C) [112]. It is initiated in the presence of oxygen by UV light or metal catalysis, leading to the formation of peroxides as the main product of the reaction. The highly reactive peroxides promote the formation of other secondary oxygenated products which trigger the auto-oxidation reaction [119]. The susceptible positions of oxidation within the PS structure are (1) the PEG, [121] (2) the ester bond, [120], and (3) the unsaturation site in the alkyl chains [123]. Oxidation in PS80 results preferentially from the attack of the double bond of the fatty acid chain, whereas in PS20, it takes place at the α-carbons of the PEG (ether bond scission). The higher content in unsaturated alkyl side chains makes PS80 more prone to oxidation than PS20. In addition, peroxides propagate the oxidation cascade not only to other PS-related molecules but also to the protein, compromising its chemical stability. Oxidation is a very common degradation pathway for therapeutic proteins, [133], and it is known that peroxides preferentially react with Met, His, and Trp residues, whereas acetaldehydes mainly react with primary amino groups (N-terminus or lysine side chain). Within a biopharmaceutical formulation, the oxidation cascade is likely to



Scheme 2.1 Main routes of degradation in polysorbate

proceed also through the protein, which acts as a scavenger of oxidative reactive species. In addition, the mass concentration of the protein can be several orders of magnitude higher than that of the polysorbates. Thus, in biopharmaceutical formulations, the extent of polysorbates and protein oxidation might be presumably lower than expected according to forced degradation experiments. Present in low concentrations, some PS oxidation products can be also difficult to detect.

The mechanism of auto-oxidation follows the classical radical initiation, propagation, and termination reactions and has been discussed previously by various groups and us [129, 134–136]. The initiation of auto-oxidation in polysorbates could occur by various means. Residual peroxides, metal traces, and light may induce radical initiation in the presence of oxygen. The auto-oxidation of polysorbate adopts a similar pathway as has been described for most polyoxyethylene systems [137]. Radical initiation on the POE chain is followed by insertion of atmospheric oxygen and subsequently a fast intermolecular hydrogen abstraction leading to the formation of a hydroperoxide species. As proposed by Decker et al. [134], homolytic cleavage of the peroxide leads to the formation of alkoxy radicals which trigger subsequent reactions such as β-cleavage or radical disproportionation reactions. β-scission of alkoxy radicals would lead to formates and formic acid. Alternately, a six-membered intramolecular decomposition would also lead to the formation of formaldehyde and formic acid (Scheme 2.2). Formation of acetaldehyde and acetic acid has been explained by Dulog et al. [135] to occur via an epoxy intermediate (Scheme 2.2).

In the isolation of insoluble degradants of polysorbate, the major component was found to be short-chain POE esters of fatty acids. Some of the lower analogues were also characterized in the SBSE-GC-MS. Their formation could be explained by scission triggered by peroxide formation on the ester-containing arm of the molecule. Oxygen insertion followed alkoxy radical formation and β-scission at the C–O bond on the POE chain would lead to the formation of a fatty acid ester (Scheme 2.3).

The presence of unsaturation in the fatty acid chain is always a concern with respect to auto-oxidation processes because of their sensitivity toward degradation by radical reactions [123]. PS80 contains between 50 and 87% of oleic acid and up to 20% of linoleic and linolenic acid. Even PS20 contains around 1% of oleic and 0.3% linoleic acid. The oxidation potential of unsaturated fatty acids decreases with increasing unsaturation, thus becoming more susceptible to radical initiation reactions.

Auto-oxidation of oleic acid begins by peroxide formation at the allylic site. Subsequently formed alkoxy radicals undergo hemolytic cleavage of the adjacent C–C bond, giving rise to an alkanes or aldehydes. Based on the position of peroxidation and the C–C bond scission, a number of alkanes are expected as shown in Scheme 2.4. Studies with the headspaces GC-MS and SBSS-GC-MS show formation of a number of aldehydes and alkanes which can be explained by possible double-bond migration prior to radical reaction.

The formation of a series of 5-alkyl dihydro 2-furanones can be traced back to secondary reactions on unsaturated fatty acids. Lactonization of oleic acids in acidic

Scheme 2.2 Auto-oxidation of PEG

conditions has been reported [138, 139]. The reaction is an acid-catalyzed reaction occurring with or without migration of the double bond. The reaction thus yields 5-alkyl lactones, the alkyl chain length varying due to migration of the double bond.

Polysorbates 20 and 80 show differences in the degradants formed based on their initial constitution. While the fatty acids obtained in hydrolysis reflect the initial constitution, differences were also seen with unsaturated fatty acids. PS20 has 1% of oleic acid and thus showed degradants related to oleic acid in headspace GC-MS. Hexane and heptanal are secondary products of β-scission of alkoxy radical at C7. Traces of pentane (a β-scission product at C13 of linoleic acid) were also observed. In SBSE-GC-MS of PS80, undecenal, 3-nonenal, and 2-heptenal were observed, these being degradation products of oleic and linoleic acid.

The observations in the UV absorption spectra correspond very well with the observed degradants. An increase in UV absorption was observed upon aging of PS20 and PS80 at 257 nm with a broad shoulder at 300 nm. PS80 contains a double bond (C = C) which gives rise to a $\pi \rightarrow \pi^*$ transition (λmax = 195 nm), an ester (C = O) and an ether (C–O–C) both giving $n \rightarrow \pi^*$ transition (λmax = ~210 nm). Thus, the UV spectrum of PS80 should have a λ not exceeding 230 nm.

Fatty acid ester of POE

Scheme 2.3 Auto-oxidation of polysorbate via chain breaking of the ester-containing arm

Scheme 2.4 Auto-oxidation at the site of unsaturation leading to aldehydes and ketones

The absorption beyond 200 nm could be ascribed to the presence of linoleic acid (λmax = ~ 250 nm) [140]. The decrease in the absorption peak at ~ 250 nm in the PS80 sample measured after 2 weeks [112] points to the breakdown of the linoleic acid moiety. Subsequent measurements after 1, 2, and 6 months show a gradual increase of a peak at λmax = ~257 nm and a broad shoulder at λmax = ~300 nm. The peak at 257 nm indicates accumulation of molecules with n → π*

transition such as aldehydes and ketones, which according to Woodward–Feiser rules would produce $n \rightarrow \pi^*$ transitions at 293 and 279 nm, respectively. Conjugated ketones would further shift the maximum to longer wavelengths fitting with the appearance of a broad shoulder at ~300 nm. PS20 displays a similar behavior. It must be noted that auto-oxidation also occurs in histidine, and thus, the increase in absorption could also be ascribed to the formation of degradation products of photo- or thermal oxidation of histidine [124, 141] which is present in the characterized formulations.

2.9 Hydrolysis

The presence of fatty acids as degradants points to the occurrence of ester hydrolysis as the degradation mechanisms. Since the formulations are in very mildly acidic conditions (pH 6 or 5.5), the mechanism can be thought to be an acid-catalyzed AAC2 reaction which is known to be a thermodynamically driven reaction with the SN2 attack of the H2O at the ester carbonyl being a bimolecular rate-determining step [142]. The rates of the reaction can be obtained by treating the reaction as a pseudo-first-order reaction as has been done by Bates et al. [38]. This mechanism has some influence on the overall degradation only at higher temperatures (40 °C) and has very slow rates at 5 °C and 25 °C [119] (Scheme 2.5).

Thus, chemical hydrolysis of PS catalyzed by basic or acidic conditions is almost negligible under pharmaceutically relevant conditions and was found mainly during forced degradation studies [114]. In contrast, recent publications show evidences of enzymatically driven hydrolysis of polysorbates in protein formulations [115–117, 143]. Host cell proteins, 2 of which have been identified, were copurified in trace amounts with the therapeutic protein of interest and found to catalyze the hydrolysis of the fatty ester bond. Therefore, the enzymatically driven hydrolytic degradation pathway is considered to be more relevant for protein-based products as compared to the nonenzymatic hydrolytic pathway.

These unwanted reactions give rise to a wide range of possible PS degradation products, namely peroxides and short-chain organic acids, aldehydes, alkanes, ketones, and short-chain esterified sorbitan/isosorbide-PEG species (fatty acid esters [FAEs]) are the main degradants resulting from PS oxidation (see Table 2.2)

Scheme 2.5 Hydrolysis in polysorbate

[112, 144]. The hydrolytic pathway is characterized by the presence of free fatty acids (FFAs) and unesterified sorbitan/isosorbide-PEG species (see Table 2.3) [112]. The identification of the underlying degradation mechanism, oxidation or hydrolysis, is important to develop a mitigation strategy. However, the identification of specific markers to differentiate between the degradation pathways represents an analytical challenge. Peroxides are the primary intermediate product of oxidation; however, they get consumed within the oxidation cascade and are not always reliable markers of oxidation. Other degradants are difficult to detect due to their low concentration and high volatility, such as short-chain organic aldehydes. Apart from identifying polysorbate degradants, other observations, such as monitoring of protein oxidation and peroxide formation or the presence of host cell proteins with enzymatic activity, will help to determine the prevalent polysorbate degradation pathway (Table 2.4).

Some of the polysorbate degradants, such as FFA [148] and nonesterified sorbitan/isosorbide-PEG species, [43] are also found in neat polysorbates as by-products from the manufacturing process. Their initial presence together with the ongoing degradation impacts the final product quality and stability. Some of the impurities and degradants, such as FFA, show low solubility in aqueous buffers. Although some of these components can lead to the formation of insoluble particles, others may get solubilized by the intact polysorbates in a temperature-dependent or pH-dependent manner. For instance, FFAs with a long hydrocarbon chain, such as myristic, palmitic, or stearic acid, are known to be less soluble in aqueous buffers, depending on pH, compared to, for example, lauric acid [158].

Analytical Characterization of polysorbates in Biopharmaceutical Product Development

It is necessary to have characterization and monitoring methods for the polysorbate bulk, the stock solutions, and the final drug product. The analytical methods developed for neat polysorbates (and diluted PS solutions) respond to three main purposes: (1) the quantification of the total amount of polysorbates; (2) the characterization of the PS composition; and (3) purity by revealing the distribution of the different species present (i.e., PS-related molecules, degradants, and other impurities). For biopharmaceutical formulations, the PS functionality assessment plays a role next to assessing its concentration and composition.

The minimum concentration of polysorbates required to protect the protein of interest within a formulation is determined experimentally in a functional test during formulation development, for example, testing formulations with varying PS type and concentration in forced degradation and (accelerated) stability studies. For example, a minimal functional level of PS20 or PS80, namely 0.01% w/v, was reported to be required to maintain the stability of 2 mAbs in case of PS degradation [112]. It is a common practice to add an excess of polysorbates above the concentration required to assure that enough functional surfactant is left in solution at

Table 2.4 Summary of methods reported for the detection and quantification of PS and PS-related species, adapted from {Martos, 2017 #287}

Methods	Analyte	PS	Short description	Presence of protein	Dynamic range (mg/mL)	Ref.
Methods based on UV/Vis and fluorescence spectroscopy						
Quantification of fatty acids (from FFA and FAE)						
Derivatization/HPLC/ fluorescence N/F/C/S	PDAM-lauric ac.	PS20	Free lauric acid is derivatized with 1-pyrenyl diazomethane (PDAM) and detected after HPLC by fluorescence (340/395 nm)	Nonreported	Nonreported	[145]
Derivatization/RP-HPLC/ UV-vis or fluorescence N/F/C/S	ADAM-lauric ac.	PS20	Free lauric acid is derivatized with 9-anthryldiazomethane (ADAM) and detected after RP-HPLC by absorption (254 nm) or fluorescence (365/412 nm)	Nonreported	LOD nm range	[146]
RP-HPLC/UV-vis N/F/C/S	Oleic ac.	PS80	Base hydrolysis is used to release the oleic acid from PS80. After separation by RP-HPLC, the oleic acid is detected directly by absorbance at 195 nm. MS is used to confirm the peak of oleic acid in the chromatogram	$\leq$ 75 mg/mL	LOD 0.004 LOQ 0.013 L 0.005–1	[147]
SPE/Derivatization/ RP-UPLC N/F/C/S	PDAM-fatty ac.	PS20	The protein is removed by SPE followed by the extraction of fatty acids and PS-related species. The FFAs are derivatized using PDAM. After RP-UPLC, the PDAM-fatty acid complexes are detected by absorbance at 241 nm	60 mg/mL	LOD given per FFA L 0.0005–0.0125	[143]
Quantification of free fatty acids (insoluble fraction)						
Derivatization/HPLC/ fluorescence N/C/S	PDAM-fatty ac.	PS80 PS20	Free fatty acids in isolated PS-related particles are derivatized with PDAM and detected after HPLC by fluorescence (340/395 nm)	No	–	[148]

(continued)

Table 2.4 (continued)

Methods	Analyte	PS	Short description	Presence of protein	Dynamic range (mg/mL)	Ref.
Particle filtration/ Methanol/Derivatization/ RP-UPLC N/F/C/S	PDAM-fatty ac.	PS20	The sample, containing FFA particles, is filtered and the retained particles solubilized in methanol. The FFAs are derivatized using PDAM and detected after RP-UPLC by absorbance at 241 nm	60 mg/mL	–	[143]
Quantification of PEG-sorbitan						
Complexation/UV-vis N/F/C	PEG-Co complex	PS20	PEG groups are complexed with cobalt thiocyanate and detected by absorbance at 620 nm	Nonreported	L 0.025–0.1	[146, 149]
SPE/Complexation/ UV-vis N/F/C	PEG-Co complex	PS20 PS80	The PS is extracted from protein and other excipients by SPE and complexed with cobalt thiocyanate. Detection of the complex is carried out by absorbance at 620 nm	≤ 70 mg/ mL	LOD 0.003 LOQ 0.01 L 0.01–0.08	[68]
SPE/Complexation/ UV-vis N/F/C	PEG complex	PS80	The protein fraction is removed by SPE before complexation. PEG groups are complexed with ferric thiocyanate and detected by absorbance at 510 nm	≤ 140 mg/ mL	LOQ 0.001 L 0.001–0.08	[150]
Fluorescence micelle-based assays						
Incubation/Fluorescence N/F	NPN in micelles	PS20	N-phenyl-1-naphthylamine (NPN) gets incorporated into the micelles and is detected by fluorescence (~356/410 nm)	Nonreported	L 0.01–12.3	[146, 149]
Incubation/Fluorescence N/F	bis-ANS in micelles	PS80	4,4′-dianilino-1,1’binaphthyl-5,5’-disulfonic acid potassium salt (bis-ANS) interacts with micelles and is detected by fluorescence (~380/500 nm)	≤ 10 mg/ mL	LOD 0.0015 LOQ 0.0047 L 0–0.04	

(continued)

Table 2.4 (continued)

Methods	Analyte	PS	Short description	Presence of protein	Dynamic range (mg/mL)	Ref.
Quantification of peroxides						
Incubation/UV-vis N/F/S	H_2O_2 Organic-hydroperoxides	PS80 PS20	Hydroperoxides mediate the oxidation of Fe(II) to Fe (III) which complexes with xylenol orange. The complex is detected by absorbance at 560 nm	–	LOD 1 μM	[45, 151]
Incubation/Fluorescence N/F/S	Hydroperoxides	PS20	Amplex® Red react with hydroperoxide to result in the formation of resorufin, which can be detected by fluorescence (570/585 nm)	–	LOD 50 nM	
Methods based on MS						
Identification of free fatty acids						
Saponification/ Extraction/RP-HPLC/ ESI-MS N/S	FFA	PS80	The FFA is isolated by saponification and extraction and then analyzed via RP-HPLC-MS	No	–	[144]
Identification of free fatty acids (insoluble fraction)						
Particle filtration/ Methanol/RP-HPLC/ ESI-MS N/C/S	Ions (m/z)	PS20 PS80	The sample containing FFA particles is filtered and the retained particles solubilized in methanol. After separation by RP-HPLC, the elution profile is analyzed by MS. Molecular structures are assigned to the peaks observed in the ion chromatograms with support of MS/MS	No	–	[112]

(continued)

Table 2.4 (continued)

Methods	Analyte	PS	Short description	Presence of protein	Dynamic range (mg/mL)	Ref.
Quantification of free fatty acids and fatty acid esters						
RP-(U)HPLC/ESI-MS N/F/C/S	Ions (m/z) Dioxalanylium ions	PS20 PS80	Protein and PS-related species are separated by RP-UPLC. The identification of the different PS-related species reveals the PS profile. The dioxalanylium ions of the different fatty acids generated by "in-source" fragmentation can be used for relative quantification of PS-esterified species.22; 47	153 mg/mL	–	[117]
Protein precipitation/ RP-HPLC-ESI-MS N/F/C/S	Ions (m/z)	PS20	The protein fraction is precipitated with ethanol in a previous preparative step. PS-related components in the supernatant are separated by RP-HPLC. MS in positive and negative modes are run to identify the different peaks, which are verified by MS/MS	Unknown	–	[115]
Quantification of small molecules, volatiles						
GC-MS N/S	Volatiles	PS20 PS80	The diluted PS solution is directly used for ionization and MS analysis. Acetaldehyde can be detected as the main degradation product for both PS20 and PS80	No	–	[119]
SBSE/GC-MS N/F/C/S	Volatiles	PS20 PS80	The degradants, at ppm levels, in the sample are absorbed onto a Twister™ and then transferred into the GC-MS for analysis. Small organic molecules, aldehydes, ketones, alkanes, fatty acids, and FAE can be detected	No	–	[112]

(continued)

Table 2.4 (continued)

Methods	Analyte	PS	Short description	Presence of protein	Dynamic range (mg/mL)	Ref.
Methods based on ELSD and CAD						
Detection and quantification of PS-related species						
RP-HPLC/ELSD N/F/C	PS constituents	PS80	Gradient used in RP-HPLC allows separation of the protein fraction and subsequent elution of PS80 as a single peak, which then is detected by ELSD	Unknown	LOD 0.015 LOQ 0.04 L 2–6	[152]
RP-HPLC/ELSD N/F/C	PS constituents	PS20	Gradient used in RP-HPLC allows separation of the protein fraction and subsequent elution of PS20 as a single peak, which then is detected by ELSD	30–50 mg/mL	–	
Mixed-mode-SPE-HPLC/ELSD N/F/C/S	PEG-sorbitan ester	PS80	The protein fraction is removed by a mixed-mode resin in HPLC. MS is used to assign the peaks observed in the chromatogram. Elution of sorbitan-PEG esters as a single peak is then detected and quantified by ELSD detection	≤ 100 mg/mL	LOD 0.008 LOQ 0.013 L 0.1–0.4	[41, 43]
SPE/RP-HPLC/ELSD N/F/C/S	PS constituents	PS80 PS20	The protein fraction is removed and the sample concentrated by SPE before HPLC analysis. Elution of PS80 as a single and sharp peak is then quantified by ELSD detection	Unknown	LOD 0.006 LOQ 0.013 L 0.013–1	[153]
RP-HPLC/CAD N/F/C	PS constituents	PS80	The protein fraction is separated by a Poroshell column before detection of a main peak of PS80 compounds by CAD. MS is used to assign the peaks observed in the chromatogram	≤ 1 mg/mL	LOD 0.005 LOQ 0.01 L 0.01–0.06	[154]
Protein precipitation/RP-HPLC-CAD N/F/C/S	Sorbitan/isosorbitan monoester C12:0	PS20	The protein fraction is precipitated with ethanol in a previous preparative step. RP-HPLC of the supernatant separates the lauric acid-containing isosorbide/sorbitan-(PEG)n ester peak which is used to quantify PS20	Unknown	PS20 between 0.01 and 0.05	[115]

(continued)

Table 2.4 (continued)

Methods	Analyte	PS	Short description	Presence of protein	Dynamic range (mg/mL)	Ref.
Quantification of fatty acids (from FFA and FAE)						
Saponification/ Extraction/ RP-HPLC-CAD N/S	FFA	PS20 PS80	Sample requires fatty acids to be released by forced hydrolysis when starting from FAE. The FFAs are extracted and analyzed via RP-HPLC-CAD. ESI-MS is used for the identification of unknown peaks. Dynamic range values given are for fatty acids with a chain length of 14 or greater	No	LOQ (2–6) $\times 10^{-4}$ L 0.001–0.1	[42]
Methods based on NMR						
Identification of PS-related components and relative quantification						
NMR (^{1}H) N/S	PS constituents	PS20	To monitor hydrolysis, the decrease of the proton signals of the fatty acid esters is followed over time	No	–	[112]
NMR (^{1}H, ^{13}C) N/S	PS constituents	PS20	After isolation by filtration of the particles, FAEs of short PEG chains are identified as the major constituents of the insoluble matter. The relative amounts of the major components can be obtained	No	–	[119]
NMR (^{1}H, ^{13}C, ^{1}H, COSY, HMBC) N/S	PS constituents	PS60	The relative amounts of the major components can be obtained. Content on fatty acids and isosorbide is addressed	No	–	[36]
Diffusion ^{1}H NMR N/S	PS constituents	PS20	It provides discrimination between nonesterified and esterified PEG species. It differentiates between water-soluble components and those incorporated into micelles	No	–	[155]
NMR (^{1}H) N/S	PS constituents	PS80	^{1}H NMR was used to verify the identification of components by MS	No	–	[144]

(continued)

Table 2.4 (continued)

Methods	Analyte	PS	Short description	Presence of protein	Dynamic range (mg/mL)	Ref.
NMR (^{1}H) N/S	PS constituents	PS20	The sum of all PS20-related peaks is integrated and used for relative quantification of PS. The method is suggested to be comparable to mixed-mode-HPLC-ELSD in terms of LOD and dynamic range	No	LOD 0.05 L 0.05–50	[156]
NMR (^{1}H, ^{13}C) N/S	PS constituents	PS80	Besides identification, it facilitates the relative quantification of the major components	No	–	[157]

The following terms and abbreviations were used: *Unknown* The protein concentration was not specified; *Non-reported* The presence of protein and the dynamic range were not mentioned; *No* experiments performed in the absence of protein; *LOD* limit of detection; *LOQ* limit of quantification; *L* linearity; *N* method suitable for neat PS (i.e., raw materials) and diluted PS solutions; *F* method suitable for PS within a formulation, that is, in the presence of protein; *C* method suitable for determination of PS concentration; *S* method suitable for profiling PS constituents

the end of a product's shelf life. However, this needs to be balanced out with the possibility of accelerate product degradation.

It should be noted that surfactant loss can occur not only due to degradation but also due to unspecific binding to surfaces during filtration or processing [159]. The content of polysorbates within a biopharmaceutical product is monitored as in-process test to confirm the correct PS concentration during and after manufacturing and in some cases measured at the time of release. In addition, real-time stability studies help to define an appropriate control strategy for polysorbates in the formulation. Besides monitoring the purity of neat polysorbates, a characterization of its degradation profile within the drug product allows one to understand whether PS needs to be periodically monitored or an in-process test is sufficient to verify the correct amount in the drug product.

In conclusion, polysorbates are excellent surfactants which have proven to be highly beneficial to biotherapeutic formulation in the past decade. It is likely that polysorbates will continue to be used in the coming years as well given their wide acceptance and low toxicity. Moreover, despite many new surfactants being available for trial, it is unlikely that a replacement is available for extensive use in the coming 5–10 years. Thus, it is important to understand the various facets of polysorbate and implement controls for polysorbate and measures in place along the manufacturing path of a biotherapeutic to ensure safe and effective use of these surfactants.

References

1. Frokjaer S, Otzen DE. Protein drug stability: a formulation challenge. Nat Rev Drug Discov. 2005;4(4):298–306.
2. Kamerzell TJ, et al. Protein–excipient interactions: mechanisms and biophysical characterization applied to protein formulation development. Adv Drug Deliv Rev. 2011;63 (13):1118–59.
3. Lee HJ, et al. Molecular origins of surfactant-mediated stabilization of protein drugs. Adv Drug Deliv Rev. 2011;63(13):1160–71.
4. Cleland JL, Powell MF, Shire SJ. The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation. Crit Rev Ther Drug Carrier Syst. 1993;10(4):307–77.
5. Hillgren A, Lindgren J, Alden M. Protection mechanism of Tween 80 during freeze-thawing of a model protein. LDH. Int J Pharm. 2002;237(1–2):57–69.
6. Kiese S, et al. Shaken, not stirred: mechanical stress testing of an IgG1 antibody. J Pharm Sci. 2008;97(10):4347–66.
7. Jones LS, Bam NB, Randolph TW. Surfactant-stabilized protein formulations: a review of protein-surfactant interactions and novel analytical methodologies. In: Shahrokh Z, Cleland JL, Shire SJ, editors. Therapeutic proteins and protein formulation and delivery. Washington, DC.; 1997. pp. 206–22.
8. Kreilgaard L, et al. Effect of Tween 20 on freeze-thawing- and agitation-induced aggregation of recombinant human factor XIII. J Pharm Sci. 1998;87(12):1597–603.
9. Wang W. Protein aggregation and its inhibition in biopharmaceutics. Int J Pharm. 2005;289 (1–2):1–30.

10. Mahler HC, et al. Protein aggregation: pathways, induction factors and analysis. J Pharm Sci. 2009;98(9):2909–34.
11. FDA. Approved biologics [Internet]. 2008. Available from: http://www.fda.gov/cder/biologics/biologics_table.htm.
12. EMEA. European Public Assessment Reports [Internet]. 2009 [cited 2008; Available from: http://www.emea.europa.eu/htms/human/epar.
13. Mahler H-C, et al. Induction and analysis of aggregates in a liquid IgG1-antibody formulation. Eur J Pharm Biopharm. 2005;59(3):407–17.
14. Maa Y-F, Hsu CC. Protein denaturation by combined effect of shear and air-liquid interface. Biotechnol Bioeng. 1997;54(6):503–12.
15. Cromwell MEM, Hilario E, Jacobson F. Protein aggregation and bioprocessing. Aaps Journal. 2006;8(3):E572–9.
16. Carpenter JF, et al. Rational design of stable lyophilized protein formulations: theory and practice. Pharm Biotechnol. 2002;13(Rational Design of Stable Protein Formulations):109–33.
17. Carpenter JF, et al. Rational design of stable lyophilized protein formulations: some practical advice. Pharm Res. 1997;14(8):969–75.
18. Chang BS, Kendrick BS, Carpenter JF. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. J Pharm Sci. 1996;85(12):1325–30.
19. Randolph TW, Jones LS. Surfactant-protein interactions, in rational design of stable protein formulations—theory and practice. In: Carpenter J, Manning MC, editors. New York: Kluwer Academic/Plenum Publishers; 2002. p. 159–175.
20. Kerwin BA, et al. Effects of Tween 80 and sucrose on acute short-term stability and long-term storage at −20 degrees C of a recombinant hemoglobin. J Pharm Sci. 1998;87(9):1062–8.
21. Vidanovic D, et al. Effects of nonionic surfactants on the physical stability of immunoglobulin G in aqueous solution during mechanical agitation. Pharmazie. 2003;58(6):399–404.
22. Chou DK, et al. Effects of Tween 20 and Tween 80 on the stability of Albutropin during agitation. J Pharm Sci. 2005;94(6):1368–81.
23. Mahler H-C, et al. Induction and analysis of aggregates in a liquid IgG1-antibody formulation. Eur J Pharm Biopharm. 2005;59(3):407–17.
24. Carpenter JF, Arakawa T, Crowe JH. Interactions of stabilizing additives with proteins during freeze-thawing and freeze-drying. Dev Biol Stand. 1992;74:225–38; discussion 238–9.
25. Jones LS, et al. The effects of Tween 20 and sucrose on the stability of anti-L-selectin during lyophilization and reconstitution. J Pharm Sci. 2001;90(10):1466–77.
26. Liu W, Wang DQ, Nail SL. Freeze-drying of proteins from a sucrose-glycine excipient system: effect of formulation composition on the initial recovery of protein activity. AAPS PharmSciTech. 2005;6(2):E150–7.
27. Mahler HC, et al. Adsorption behavior of a surfactant and a monoclonal antibody to sterilizing-grade filters. J Pharm Sci. 2010;99(6):2620–7.
28. Charman SA, Mason KL, Charman W.N. Techniques for assessing the effects of pharmaceutical excipients on the aggregation of porcine growth hormone. Pharmaceut Res. 1993; V10(7):954–62.
29. Bam NB, et al. Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions. J Pharm Sci. 1998;87(12):1554–9.
30. Kreilgaard L, et al. Effects of additives on the stability of recombinant human factor XIII during freeze-drying and storage in the dried solid. Arch Biochem Biophys. 1998;360(1):121–34.
31. Katakam M, Bell LN, Banga AK. Effect of surfactants on the physical stability of recombinant human growth hormone. J Pharm Sci. 1995;84(6):713–6.
32. Ayorinde FO, et al. Analysis of some commercial polysorbate formulations using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom. 2000;14(22):2116–24.

33. Brandner JD. The composition of NF-defined emulsifiers: sorbitan monolaurate, monopalmitate, monostearate, monooleate, polysorbate 20, polysorbate 40, polysorbate 60, and polysorbate 80. Drug Dev Ind Pharm. 1998;24(11):1049–54.
34. Frison-Norrie S, Sporns P. Investigating the molecular heterogeneity of polysorbate emulsifiers by MALDI-TOF MS. J Agric Food Chem. 2001;49(7):3335–40.
35. Smidrkal J, Cervenkova R, Filip V. Two-stage synthesis of sorbitan esters, and physical properties of the products. Eur J Lipid Sci Technol. 2004;106(12):851–5.
36. Vu Dang H, et al. Composition analysis of two batches of polysorbate 60 using MS and NMR techniques. J Pharm Biomed Anal. 2006;40(5):1155–65.
37. Zhang R, et al. Analysis of polysorbate 80 and its related compounds by RP-HPLC with ELSD and MS detection. J Chromatogr Sci. 2012;50(7):598–607.
38. Bates TR, Nightingale CH, Dixon E. Kinetics of hydrolysis of poly(oxyethylene) (20) sorbitan fatty acid ester surfactants. J Pharm Pharmacol. FIELD Full Journal Title: J Pharm Pharmacol. 1973;25(6):470–7.
39. Lippold S, et al. Impact of mono- and poly-ester fractions on polysorbate quantitation using mixed-mode HPLC-CAD/ELSD and the fluorescence micelle assay. J Pharm Biomed Anal. 2017;132:24–34.
40. Tani TH, Moore JM, Patapoff TW. Single step method for the accurate concentration determination of polysorbate 80. J Chromatogr A. 1997;786(1):99–106.
41. Hewitt D, Zhang T, Kao Y-H. Quantitation of polysorbate 20 in protein solutions using mixed-mode chromatography and evaporative light scattering detection. J Chromatogr A. 2008;1215(1–2):156–60.
42. Ilko D, et al. Fatty acid composition analysis in polysorbate 80 with high performance liquid chromatography coupled to charged aerosol detection. Eur J Pharm Biopharm. 2015;94:569–74.
43. Hewitt D, et al. Mixed-mode and reversed-phase liquid chromatography-tandem mass spectrometry methodologies to study composition and base hydrolysis of polysorbate 20 and 80. J Chromatogr A. 2011;1218(15):2138–45.
44. Kerwin BA. Polysorbates 20 and 80 used in the formulation of protein biotherapeutics: structure and degradation pathways. J Pharm Sci. 2008;97(8):2924–35.
45. Ha E, Wang W, Wang YJ. Peroxide formation in polysorbate 80 and protein stability. J Pharm Sci. 2002;91(10):2252–64.
46. Wasylaschuk WR, et al. Evaluation of hydroperoxides in common pharmaceutical excipients. J Pharm Sci. 2007;96(1):106–16.
47. Lam X, et al. Site-specific tryptophan oxidation induced by autocatalytic reaction of polysorbate 20 in protein formulation. Pharm Res. 2011:1–13.
48. Harmon PA, et al. A novel peroxy radical based oxidative stressing system for ranking the oxidizability of drug substances. J Pharm Sci. 2006;95(9):2014–28.
49. Gerhardt A, et al. Protein aggregation and particle formation in prefilled glass syringes. J Pharm Sci. 2014;103(6):1601–12.
50. Ludwig DB, et al. Protein adsorption and excipient effects on kinetic stability of silicone oil emulsions. J Pharm Sci. 2010;99(4):1721–33.
51. Britt KA, et al. Excipient effects on humanized monoclonal antibody interactions with silicone oil emulsions. J Pharm Sci. 2012;101(12):4419–32.
52. Basu P, et al. IgG1 aggregation and particle formation induced by silicone-water interfaces on siliconized borosilicate glass beads: a model for siliconized primary containers. J Pharm Sci. 2013;102(3):852–65.
53. Hawe A, Friess W. Formulation development for hydrophobic therapeutic proteins. Pharm Dev Technol. 2007;12(3):223–37.
54. Khan TA, Mahler HC, Kishore RS. Key interactions of surfactants in therapeutic protein formulations: a review. Eur J Pharm Biopharm. 2015;97(Pt A):60–7.
55. Bos MA, van Vliet T. Interfacial rheological properties of adsorbed protein layers and surfactants: a review. Adv Colloid Interface Sci. 2001;91(3):437–71.

56. Bam NB, et al. Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions. J Pharm Sci. 1998;87(12):1554–9.
57. Garidel P, Hoffmann C, Blume A. A thermodynamic analysis of the binding interaction between polysorbate 20 and 80 with human serum albumins and immunoglobulins: a contribution to understand colloidal protein stabilisation. Biophys Chem. 2009;143(1–2):70–8.
58. Jones LS, Bam NB, Randolph TW. Surfactant-stabilized protein formulations: A review of protein-surfactants interactions and novel analytical methodologies. Ther Protein Pept Formulation Deliv. 1997;675:206–22.
59. Perez-Gramatges A, et al. Physico-chemical studies of molecular interactions between non-ionic surfactants and bovine serum albumin. Colloids Surf B-Biointerfaces. 2010;75 (1):282–9.
60. Nielsen AD, Borch K, Westh P. Thermochemistry of the specific binding of C12 surfactants to bovine serum albumin. Biochim Biophys Acta. 2000;1479(1–2):321–31.
61. Hoffmann C, et al. Insights into protein-polysorbate interactions analysed by means of isothermal titration and differential scanning calorimetry. Eur Biophys J Biophys Lett. 2009;38(5):557–68.
62. Wu J, et al. Binding characteristics between polyethylene glycol (PEG) and proteins in aqueous solution. J Mater Chem B. 2014;2(20):2983–92.
63. Delgado-Magnero KH, et al. Unraveling the binding mechanism of polyoxyethylene sorbitan esters with bovine serum albumin: a novel theoretical model based on molecular dynamic simulations. Colloids Surf B-Biointerfaces. 2014;116:720–6.
64. McAuley WJ, Jones DS, Kett VL. Characterisation of the interaction of lactate dehydrogenase with tween-20 using isothermal titration calorimetry, interfacial rheometry and surface tension measurements. J Pharm Sci. 2009;98(8):2659–69.
65. Chou DK, et al. Effects of tween 20 (R) and tween 80 (R) on the stability of albutropin during agitation. J Pharm Sci. 2005;94(6):1368–81.
66. Tandon S, Horowitz PM. Detergent-assisted refolding of guanidinium chloride-denatured rhodanese the effects of the concentration and type of detergent. J Biol Chem. 1987;262:4486–91.
67. Bam NB, Cleland JL, Randolph TW. Molten globule intermediate of recombinant human growth hormone: stabilization with surfactants. Biotechnol Prog. 1996;12(6):801–9.
68. Kim HL, et al. Modulation of protein adsorption by poloxamer 188 in relation to polysorbates 80 and 20 at solid surfaces. J Pharm Sci. 2014;103(4):1043–9.
69. Bam NB, Randolph TW, Cleland JL. Stability of protein formulations—investigation of surfactant effects by a novel EPR Spectroscopic technique. Pharm Res. 1995;12(1):2–11.
70. Xu RJ, Vidal-Madjar C, Sebille B. Capillary electrophoretic behavior of milk proteins in the presence of non-ionic surfactants. J Chromatogr B. 1998;706(1):3–11.
71. Jones LS, et al. Investigation of protein-surfactant interactions by analytical ultracentrifugation and electron paramagnetic resonance: the use of recombinant human tissue factor as an example. Pharm Res. 1999;16(6):808–12.
72. Hillgren A, Lindgren J, Alden M. Protection mechanism of tween 80 during freeze-thawing of a model protein, LDH. Int J Pharm. 2002;237(1–2):57–69.
73. Kett VL, Mcauley WJ, Jones DS. Characterisation of the interaction of lactate dehydrogenase with tween-20 using isothermal titration calorimetry, interfacial rheometry and surface tension measurements. J Pharm Sci. 2009;98(8):2659–69.
74. Kotsmar C, et al. Thermodynamics, adsorption kinetics and rheology of mixed protein-surfactant interfacial layers. Adv Colloid Interface Sci. 2009;150(1):41–54.
75. Chauhan S, Jyoti J, Kumar G. Non-ionic surfactant interactions in aqueous gelatin solution: a physico-chemical investigation. J Mol Liq. 2011;159(3):196–200.
76. McGuire J, Joshi O. Adsorption behavior of lysozyme and tween 80 at hydrophilic and hydrophobic silica-water interfaces. Appl Biochem Biotechnol. 2009;152(2):235–48.
77. McGuire J, et al. Adsorption and function of recombinant factor VIII at the air-water interface in the presence of tween 80. J Pharm Sci. 2009;98(9):3099–107.

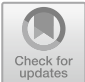
78. Zhu XY, Shen L, Guo A. Tween surfactants: adsorption, self-organization, and protein resistance. Surf Sci. 2011;605(5–6):494–9.
79. Arakawa T, Kita Y. Protection of bovine serum albumin from aggregation by tween 80. J Pharm Sci. 2000;89(5):646–51.
80. Kim HL, Mcauley A, Mcguire J. Protein effects on surfactant adsorption suggest the dominant mode of surfactant-mediated stabilization of protein. J Pharm Sci. 2014;103 (5):1337–45.
81. Lin SY, Lu TL, Hwang WB. Adsorption-kinetics of decanol at the air-water-interface. Langmuir. 1995;11(2):555–62.
82. Wustneck R, et al. Dynamic surface tension and adsorption properties of beta-casein and beta-lactoglobulin. Food Hydrocolloids. 1996;10(4):395–405.
83. Gunning PA, et al. Effect of surfactant type on surfactant-protein interactions at the air-water interface. Biomacromol. 2004;5(3):984–91.
84. Elwing H, et al. A wettability gradient method for studies of macromolecular interactions at the liquid/solid interface. J Colloid Interface Sci. 1987;119(1):203–10.
85. Joshi O, McGuire J. Adsorption behavior of lysozyme and tween 80 at hydrophilic and hydrophobic silica-water interfaces. Appl Biochem Biotechnol. 2009;152(2):235–48.
86. Rabe M, Verdes D, Seeger S. Understanding protein adsorption phenomena at solid surfaces. Adv Coll Interface Sci. 2011;162(1–2):87–106.
87. Sammond DW, et al. Predicting enzyme adsorption to lignin films by calculating enzyme surface hydrophobicity. J Biol Chem. 2014;289(30):20960–9.
88. Absolom DR, Zingg W, Neumann AW. Protein adsorption to polymer particles—role of surface-properties. J Biomed Mater Res. 1987;21(2):161–71.
89. Rampon V, et al. Evidence that homogenization of BSA-stabilized hexadecane-in-water emulsions induces structure modification of the nonadsorbed protein. J Agric Food Chem. 2003;51(20):5900–5.
90. Rampon V, et al. Front-face fluorescence spectroscopy study of globular proteins in emulsions: displacement of BSA by a nonionic surfactant. J Agric Food Chem. 2003;51 (9):2482–9.
91. Rampon V, et al. Front-face fluorescence spectroscopy study of globular proteins in emulsions: influence of droplet flocculation. J Agric Food Chem. 2003;51(9):2490–5.
92. Li JJ, et al. Mechanistic understanding of protein-silicone oil interactions. Pharm Res. 2012;29(6):1689–97.
93. Dixit N, Maloney KM, Kalonia DS. Protein-silicone oil interactions: comparative effect of nonionic surfactants on the interfacial behavior of a fusion protein. Pharm Res. 2013;30 (7):1848–59.
94. Wan LSC, Lee PFS. CMC of polysorbates. J Pharm Sci. 1974;63(1):136–7.
95. Mahler HC, et al. Surface activity of a monoclonal antibody. J Pharm Sci. 2009;98 (12):4525–33.
96. Dixit N, Zeng DL, Kalonia DS. Application of maximum bubble pressure surface tensiometer to study protein-surfactant interactions. Int J Pharm. 2012;439(1–2):317–23.
97. Mackie AR, et al. *Orogenic displacement of protein from the air/water interface by competitive adsorption*. J Colloid Interface Sci. 1999;210(1):157–66.
98. Gibbs JW. On the equilibrium of heterogeneous substances. Am J Sci. 1878; Series 3 Vol. 16(96):441–58.
99. Mackie AR, et al. Orogenic displacement of protein from the oil/water interface. Langmuir. 2000;16(5):2242–7.
100. Wang W, Wang EQ, Balthasar JP. Monoclonal antibody pharmacokinetics and pharmacodynamics. Clin Pharmacol Ther. 2008;84(5):548–58.
101. Loos WJ, et al. Clinical pharmacokinetics of unbound docetaxel: role of polysorbate 80 and serum proteins. Clin Pharmacol Ther. 2003;74(4):364–71.
102. ten Tije A, et al. Pharmacological effects of formulation vehicles. Clin Pharmacokinet. 2003;42(7):665–85.

103. Levine HL, et al. The use of surface tension measurements in the design of antibody-based product formulations. J Parenter Sci Technol. 1991;45(3):160.
104. Dickinson E. Proteins at interfaces and in emulsions—stability, rheology and interactions. J Chem Soc—Faraday Trans. 1998;94(12):1657–69.
105. Pugnaloni LA, et al. Competitive adsorption of proteins and low-molecular-weight surfactants: computer simulation and microscopic imaging. Adv Coll Interface Sci. 2004;107(1):27–49.
106. Wang Y-CJ, Hanson MA. Parenteral formulations of proteins and peptides: stability and stabilizers. PDA J Pharm Sci Technol. 1988;42:S1–25.
107. Timasheff N. The control of protein stability and association by weak interactions with water: how do solvents affect these processes? Annu Rev Biophys Biomol Struct. 1993;22:67–97.
108. Mahler HC. Stabilisierung und Analyse pharmazeutischer Proteinformulierungen. In: Fachbereich Chemische und Pharmazeutische Wissenschaften. Frankfurt: Johann Wolfgang Goethe-Universität; 2009.
109. Zimmerman SB, Minton AP. Macromolecular crowding: biochemical, biophysical, and physiological consequences. Annu Rev Biophys Biomol Struct. 1993;22:27–65.
110. Hall D, Minton AP. Macromolecular crowding: qualitative and semiquantitative successes, quantitative challenges. Biochim Biophys Acta. 2003;1649(2):127–39.
111. Kiese S, et al. Equilibrium studies of protein aggregates and homogeneous nucleation in protein formulation. J Pharm Sci. 2010;99(2):632–44.
112. Kishore RSK, et al. The degradation of polysorbate 20 and 80 and its potential impact on the stability of biotherapeutics. Pharm Res. 2011;28(5):1194–210.
113. Rosenberg AS. Effects of protein aggregates: an immunologic perspective. AAPS J. 2006;8 (3):E501–7.
114. Saggu M, Liu J, Patel A. Identification of subvisible particles in biopharmaceutical formulations using raman spectroscopy provides insight into polysorbate 20 degradation pathway. Pharm Res. 2015;32(9):2877–88.
115. Dixit N, et al. Residual host cell protein promotes polysorbate 20 degradation in a sulfatase drug product leading to free fatty acid particles. J Pharm Sci. 2016;105(5):1657–66.
116. Labrenz SR. Ester hydrolysis of polysorbate 80 in mab drug product: evidence in support of the hypothesized risk after the observation of visible particulate in mAb formulations. J Pharm Sci. 2014;103(8):2268–77.
117. Hall T, et al. Polysorbates 20 and 80 degradation by group XV lysosomal phospholipase A2 isomer X1 in monoclonal antibody formulations. J Pharm Sci. 2016;105(5):1633–42.
118. Borisov OV, Ji JA, Wang YJ. Oxidative degradation of polysorbate surfactants studied by liquid chromatography-mass spectrometry. J Pharm Sci. 2015;104(3):1005–18.
119. Kishore RSK, et al. Degradation of polysorbates 20 and 80: studies on thermal autoxidation and hydrolysis. J Pharm Sci. 2011;100(2):721–31.
120. Zhang L, et al. Degradation mechanisms of polysorbate 20 differentiated by 18O-labeling and mass spectrometry. Pharm Res. 2017;34(1):84–100.
121. Donbrow M, Azaz E, Pillersdorf A. Autoxidation of polysorbates. J. Pharm. Sci. FIELD Full Journal Title: J Pharm Sci. 1978;67(12):1676–81.
122. Mueller R, et al. Evaluation of the immuno-stimulatory potential of stopper extractables and leachables by using dendritic cells as readout. 2009;98(10):3548–61.
123. Yao J, et al. A quantitative kinetic study of polysorbate autoxidation: the role of unsaturated fatty acid ester substituents. Pharm Res. 2009;26(10):2303–13.
124. Li S, Schöneich C, Borchardt RT. Chemical instability of protein pharmaceuticals: mechanisms of oxidation and strategies for stabilization. Wiley; 2004, pp. 490–500.
125. Donbrow M, Hamburger R, Azaz E. Surface tension and cloud point changes of polyoxyethylenic nonionic surfactants during autoxidation. J Pharm Pharmacol. FIELD Full Journal Title: J Pharmacy and Pharmacology. 1975;27(3):160–6.

126. Donbrow M, et al. Development of acidity in nonionic surfactants: formic and acetic acid. Analyst (London) FIELD Full Journal Title: Analyst (Cambridge, United Kingdom). 1978;103(1225):400–2.
127. Ha E, Wang W, Wang YJ. Peroxide formation in polysorbate 80 and protein stability. J. Pharm. Sci. FIELD Full Journal Title: J Pharm Sci. 2002;91(10):2252–64.
128. Hamburger R, Azaz E, Donbrow M. Autoxidation of polyoxyethylenic nonionic surfactants and of polyethylene glycols. Pharm Acta Helv. FIELD Full Journal Title: Pharmaceutica Acta Helvetiae. 1975;50(1–2):10–17.
129. Kerwin BA. Polysorbates 20 and 80 used in the formulation of protein biotherapeutics: structure and degradation pathways. J Pharm Sci. 2008;97(8):2924–35.
130. Magill A, Becker AR. Spectrophotometric method for quantitation of peroxides in sorbitan monooleate and monostearate. J Pharm Sci. FIELD Full Journal Title: J Pharm Sci. 1984;73 (11):1663–4.
131. Segal R, Azaz E, Donbrow M. Peroxide removal from nonionic surfactants. J Pharm Pharmacol. FIELD Full Journal Title: J Pharm Pharmacol. 1979;31(1):39–40.
132. Wasylaschuk WR, et al. Evaluation of hydroperoxides in common pharmaceutical excipients. J Pharm Sci. 2007;96(1):106–16.
133. Li S, Schoneich C, Borchardt RT. Chemical instability of protein pharmaceuticals: mechanism of oxidation and strategies for stabilization. Biotechnol Bioeng. 1995;48 (5):490–500.
134. Decker C, Marchal J. Autoxydation radio-induite du poly (oxyéthylène) en solution aqueuse, 7. Cinétique de la consommation d'oxygène, pp. 3531–40.
135. Dulog VL, Storck G. Die oxydation von polyepoxiden mit molekularem sauerstoff. 1966:50–73.
136. Kishore RSK, Pappenberger A, Dauphin IB, Ross A, Buergi B, Staempfli A, Mahler H-C. An assessment of degradation of polysorbate 20 and 80: studies on thermal auto-oxidation in bulk and hydrolysis in formulations. J Pharm Sci. 2010.
137. Donbrow M. Stability of polyoxyethylene chain in non ionic surfactants. In: Schick MJ, editor. Nonionic surfactants: physical chemistry. New york: CRC Press; 1987. p. 1135.
138. Zhou Y, Woo LK, Angelici RJ. Solid acid catalysis of tandem isomerization-lactonization of olefinic acids. Elsevier; 2007.
139. Shepherd IS, Showell JS. The mechanism of the aqueous perchloric acid isomerization of oleic acid to -stearolactone. Springer; 1969. p. 479–81.
140. Arudi RL, Sutherland MW, Bielski BH. Purification of oleic acid and linoleic acid. 1983. p. 485.
141. Tomita M, Irie M, Ukita T. Sensitized photooxidation of histidine and its derivatives. Products and mechanism of the reaction. Am Chem Soc. 1969:5149–5160.
142. Carey FA, Sundberg RJ. Advanced organic chemistry. Springer Verlag; 2007.
143. Tomlinson A, et al. Polysorbate 20 degradation in biopharmaceutical formulations: quantification of free fatty acids, characterization of particulates, and insights into the degradation mechanism. Mol Pharm. 2015;12(11):3805–15.
144. Hvattum E, et al. Characterization of polysorbate 80 with liquid chromatography mass spectrometry and nuclear magnetic resonance spectroscopy: specific determination of oxidation products of thermally oxidized polysorbate 80. J Pharm Biomed Anal. 2012;62:7–16.
145. Nimura N, et al. 1-Pyrenyldiazomethane as a fluorescent labeling reagent for liquid chromatographic determination of carboxylic acids. Anal Chem. 1988;60(19):2067–70.
146. Khossravi M, et al. Analysis methods of polysorbate 20: a new method to assess the stability of polysorbate 20 and established methods that may overlook degraded polysorbate 20. Pharm Res. 2002;19(5):634–9.
147. Adamo M, et al. A simple reversed phaseHPLCaphy method for polysorbate 80 quantitation in monoclonal antibody drug products. J Chromatogr B: Anal Technol Biomed Life Sci. 2010; 878(21):1865–70.
148. Siska CC, et al. Free fatty acid particles in protein formulations. Part 2: contribution of polysorbate raw material. J Pharm Sci. 2015;104(2):447–56.

149. Daniels DH, Warner CR, Selim S. Determination of polysorbate 60 in salad dressings by colorimetric and thin layer chromatographic techniques. J Assoc Off Anal Chem. 1982;65 (1):162–5.
150. Savjani N, et al. Use of ferric thiocyanate derivatization for quantification of polysorbate 80 in high concentration protein formulations. Talanta. 2014;130:542–6.
151. Wasylaschuk WR, et al. Evaluation of hydroperoxides in common pharmaceutical excipients. J Pharm Sci. 2006;96(1):106–16.
152. Nair LM, et al. Determination of polysorbate 80 in parenteral formulations by high-performance liquid chromatography and evaporative light scattering detection. J Chromatogr A. 2003;1012(1):81–6.
153. Nayak VS, et al. Evaporative light scattering detection based HPLC method for the determination of polysorbate 80 in therapeutic protein formulations. J Chromatogr Sci. 2012;50(1):21–5.
154. Fekete S, Ganzler K, Fekete J. Fast and sensitive determination of Polysorbate 80 in solutions containing proteins. J Pharm Biomed Anal. 2010;52(5):672–9.
155. Verbrugghe M, et al. Quantification of hydrophilic ethoxylates in polysorbate surfactants using diffusion 1H NMR spectroscopy. J Pharm Biomed Anal. 2010;51(3):583–9.
156. Lei M, et al. The effects of membrane filters used in biopharmaceutical processes on the concentration and composition of polysorbate 20. Biotechnol Prog. 2013;29(6):1503–11.
157. Zhang Q, et al. NMR method for accurate quantification of polysorbate 80 copolymer composition. Anal Chem (Washington, DC, U. S.). 2015;87(19):9810–16.
158. Doshi N, Demeule B, Yadav S. Understanding particle formation: solubility of free fatty acids as polysorbate 20 degradation byproducts in therapeutic monoclonal antibody formulations. Mol. Pharmaceutics. 2015;12(11):3792–804.
159. Mahler H-C, et al. Behaviour of polysorbate 20 during dialysis, concentration and filtration using membrane separation techniques. J Pharm Sci. 2007;97(2):764–74.

Chapter 3
Sucrose and Trehalose in Therapeutic Protein Formulations



Satish K. Singh

Abstract Sucrose and trehalose are key ingredients in the formulation and stabilization of biotherapeutics. Their utility and function is driven by their unique chemical and physical properties, especially in aqueous solutions which are summarized in this chapter. There are commonalities as well as differences in these properties that arise from their conformation, H-bonding characteristics, water-binding ability, glass transition temperatures, polymorphic behavior, solubility, chemical stability etc. Both sugars are well suited to provide solution-state stabilization, as well as cryo- and lyo-protection, for therapeutic proteins as excipients in the formulations. Compendial monographs are available for both sugars, and the safety and tolerability have been well documented. Although the final assessment is dependent on the individual biotherapeutic, experience suggests that sucrose can generally be considered as suitable in most cases, unless constrained by a low-pH formulation.

Keywords Sucrose · Trehalose · Stabilizers · Biopharmaceuticals
Excipients

3.1 Introduction

Sucrose and trehalose are key ingredients in the formulation and stabilization of biotherapeutics. Their utility and function is driven by their unique chemical and physical properties. This chapter reviews some of these properties in the context of their use in protein products. Other saccharides such as mannitol and sorbitol are also commonly used in biotherapeutic products, but are outside the scope of the current chapter.

S. K. Singh (✉)
Lonza AG, Hochbergerstrasse 60A, 4057 Basel, Switzerland
e-mail: satish.singh@lonza.com

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_3

3.2 Physical Properties

3.2.1 Introduction

The structures of sucrose and trehalose are provided in Fig. 3.1. Sucrose is a disaccharide (glucose + fructose) with the molecular formula $C_{12}H_{22}O_{11}$. Its systematic name is α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside. In sucrose, the components glucose and fructose are linked via an ether bond between C1 on the glucosyl subunit and C2 on the fructosyl unit. The glycosidic bond is formed between the reducing ends of both glucose and fructose which inhibits further bonding to other saccharide units. Since it contains no anomeric hydroxyl groups, it is classified as a non-reducing sugar. The molecule exists as a single isomer.

Trehalose is a disaccharide (glucose + glucose) with the same overall molecular formula $C_{12}H_{22}O_{11}$ for the anhydride. Its systematic name is α-D-glucopyranosyl-(1 → 1)-α-D-glucopyranoside. In trehalose, the constituent glucoses are linked via an ether bond between C1s on the glucosyl subunits. The glycosidic bond is formed between the reducing ends of both glucoses which inhibits further bonding to other saccharide units. Since it contains no anomeric hydroxyl groups, it is classified as a non-reducing sugar. The molecule exists as a single isomer. The (1–1)-α bonding makes trehalose very resistant to acid hydrolysis and therefore is stable in solution at high temperatures, even under acidic conditions.

Fig. 3.1 Structural diagrams of sucrose and trehalose

3.2.2 Solution-State Properties

The solution properties of trehalose and sucrose show significant differences that are related to the conformational flexibility and hydrogen-bonding behavior of the disaccharides.

Hexose rings have greater conformational flexibility than pentose rings. Disaccharides that contain a furanose ring (sucrose and others) have lower conformational flexibility than those composed of two pyranose residues (trehalose). The α-(1 → 1)-α glycosidic linkage, however, confers a high degree of rigidity (low dynamic flexibility) to the molecule despite the lack of intramolecular H-bonds [1, 2]. Furthermore, according to Lerbret et al. [3], fluctuations of the radius of gyration and glycosidic dihedral angles of trehalose indicate a greater flexibility for trehalose compared to sucrose, reflecting fewer intramolecular H-bonds (=1) compared to sucrose (=2). The result of the greater rigidity of sucrose and greater number of intramolecular H-bonds (compared to trehalose) results in reduced ability to form H-bonds with water and thus a less efficient packing in the solid state and consequently, a greater free volume and lower glass transition temperature (T_g) (for sucrose compared to trehalose). The T_g value of the disaccharides is related to the extent of intermolecular H-bonds. With adsorbed moisture, some of the saccharide–saccharide H-bonds are replaced by saccharide–water interaction, thus reducing the T_g significantly with water content (Fig. 3.2; Table 3.1). In solution, this property gives trehalose a lower partial molar volume and a high thermal expansion coefficient [4]. It is also reflected in the greater heat of solution of amorphous trehalose (−27.2 kJ/mol) compared to amorphous sucrose (−16.3 kJ/mol). Since this heat of solution reflects the sum of enthalpy of breaking water–water H-bonds, enthalpy of forming H-bonds with water in the solvated state as well as enthalpy of conformational change induced by solvation in water, it is an approximate measure of H-bonding ability of the disaccharide [4]. Since Trehalose forms only one intramolecular H-bond as opposed to two in sucrose, there are more sites available in trehalose for H-bond formation with water, resulting in a higher hydration number [5]. At concentrations approx. 50% w/w or greater, both trehalose and sucrose assume structures that are analogous to that in the crystalline state. Aqueous sucrose solutions retain the two intramolecular H-bonds that are found in the crystalline sucrose, while trehalose retains its single H-bond [5, 6]. However, the greater probability of sucrose molecules to form intramolecular H-bonds [7], strongly reduces their interaction with water or other sugar molecules. Capability of trehalose molecules to interact with water or with other trehalose molecules is similar, which makes trehalose–water systems more homogenous from a structural perspective. At high concentrations (33–66% w/w), trehalose molecules can thus also form large interconnected clusters making them less mobile, while sucrose arranges itself into smaller isolated clusters [3]. All-atom molecular dynamics simulations have shown that when reaching a concentration of 1.5–2.2 molar, the trehalose molecular clusters percolate and form large, continuous aggregates within the system [8].

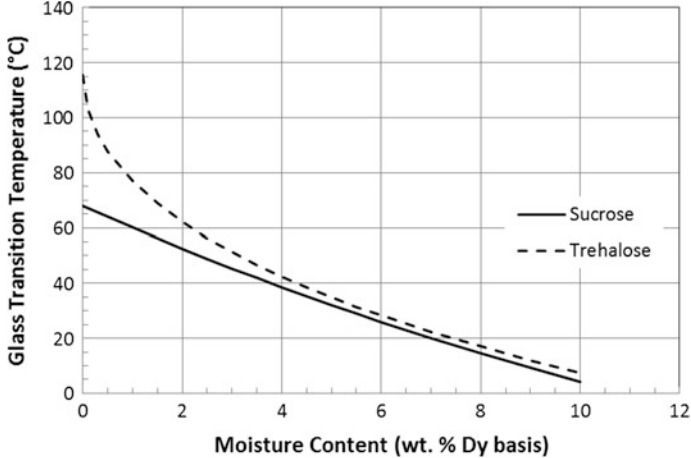


Fig. 3.2 Glass transition temperature of amorphous sucrose and trehalose as a function of moisture content shown as best-fit curves. The equation used for sucrose is taken from Sun et al. [124] and is based on a composite data set from various sources. The equation used for trehalose is taken from Crowe et al. [125]. Best-fit curve equations are given in Table 3.1

Disaccharides in solution obstruct the crystallization of ice, reducing the amount of freezable water and destroying the network of water compatible with ice. Trehalose with its higher H-bonding ability has the greater de-structuring impact on the tetrahedral H-bond network of pure water compared to sucrose [9–12], i.e., the greater inhibition to the formation of ice. Trehalose de-structures the water network and orders the water molecules around itself as a kosmotrope. The amount of impact of trehalose on the water dynamics and structures is however subject to some controversy since the results reported are variable. Magazu et al. [13, 14] show that the dynamics of water is significantly impacted by trehalose, but Pagnotta et al. [15] show that the H-bonding between trehalose and water is limited and the influence of solvation of trehalose on the water network is small relative to that of bulk water [2, 16], and in line with that of other organic solutes [2]. Lee et al. [17] also report that no significant differences were found between trehalose and sucrose in their overall effects on the structural order, translational diffusion coefficient and rotational correlational time of the surrounding water.

Trehalose and sucrose slow the dynamics of water molecules (their diffusion) in their hydration shells to similar extents, and not significantly [2]. Trehalose is reported to have slightly higher hydration number compared to sucrose at all temperatures in the range (10–60 °C) [3, 12]. Hydration numbers for both disaccharides decline with concentration as sugar–sugar contacts become important. (Note though that reported absolute hydration numbers can differ dependent on the method or assumptions involved; [2, 18, 19]).

The number of equatorial-OH groups correlates well with the movement of water molecules and thus is an index of water structure in saccharide solutions. This structure, indicative of solute–solvent interaction, reflects the viscosity B-coefficient

[20, 21]. Trehalose has a higher number of equatorial-OH groups (8) than sucrose (6.3) and has a greater number of unfreezable water molecules associated with it [22], and therefore its solutions show higher viscosity than that of sucrose. Trehalose forms transient clusters (self-association) at all concentrations, generally through a single H-bond [2] which along with its higher hydration number may also explain its higher viscosity in solution. The viscosity of trehalose solutions is higher than that of sucrose with the difference increasing at higher concentrations and lower temperatures [12, 23] (Fig. 3.3; Table 3.1).

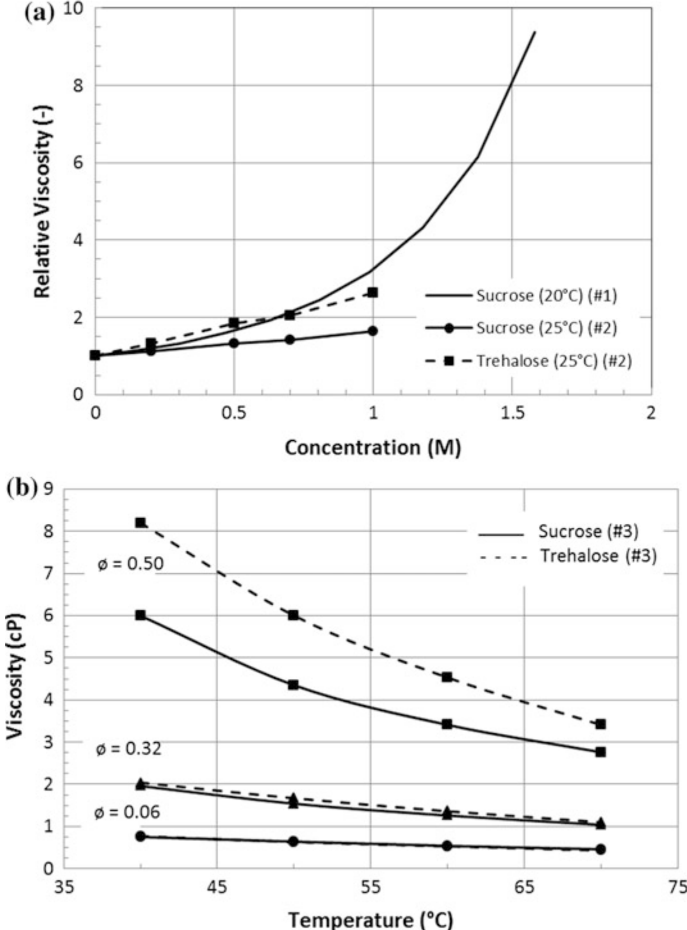


Fig. 3.3 Viscosities of aqueous solutions of sucrose and trehalose at various concentrations and temperatures. **a** Relative viscosity of sucrose at 20 °C (#1, data from Hoffman [126], shown as a best-fit curve with equation in Table 3.1), and sucrose and trehalose at 25 °C (#2, from Sola-Penna and Meyer-Fernandes [23]; with permission from Elsevier); **b** viscosity (measured by capillary viscometry) at three levels of concentration (ø, weight fraction) for sucrose and trehalose at various temperatures (#3, from Branca et al. [12]; with permission from American Chemical Society) showing the strong impact of concentration on the differences in the viscosities

Table 3.1 Some key aqueous solution-state properties of sucrose and trehalose

Glass transition temperature *Y* (°C) as a function of moisture content *X* (wt% dry basis) as shown in Fig. 3.2
Sucrose: $Y = -2042.2X^3 + 1865.3X^2 - 803.3X + 67.9$ ($R2 = 0.967$) (Sun et al. [124]; fitted over 0 to >35% moisture) Trehalose: $Y = -405.6X^{0.5} + 202.5X + 115.5$ ($R2 = 0.99$) (Crowe et al. [125]; fitted over 0 to >50% moisture)
Relative viscosity (−) as a function of concentration *C* (M) at 20 °C; range as shown in Fig. 3.3
Sucrose: $Y = 3.4514 * C^3 - 3.7035 * C^2 + 2.509 * C + 0.9119$ ($R^2 = 0.9986$) (data from [126]) *Other correlation for Viscosity* Viscosity (Y mPas) measured by DLS, as a function of concentration C (wt%) at 27.3 °C Trehalose: $Y = 0.000208C^3 - 0.00868C^2 + 0.141C + 0.467$ ($R^2 = 0.99$) (Uchida et al. [127]) Also see Longinotti and Corti [128]
Density *Y* (g/mL) as a function of concentration of solution *C* (g solute/g solution) at 20 °C; range as shown in Fig. 3.4
Sucrose: $Y = 0.1752C^2 + 0.3749C + 0.9986$ ($R^2 = 1$) (data from [126]) Trehalose: Solution density can be taken to be similar to that of sucrose as a first approximation
Aqueous solubility *Y* (% w/w) as a function of temperature *T* (°C); range as shown in Fig. 3.7
Sucrose: $Y = 64.447 + 0.08222T + 1.6168 * 10^{-3} * T^2 - 1.558 * 10^{-6} T^3 - 4.63 * 10^{-8} T^4$ (Mathlouthi and Reiser, [60]) (−13 to 100 °C) Trehalose: $Y = -2E\text{-}05T^3 + 0.0041T^2 + 0.3897T + 31.511$ ($R^2 = 0.9999$) (data from [24]) *Other correlations for Solubility* Trehalose: $\mathrm{Ln}[S] = \mathrm{Ln}(0.1223) - (1330/T)$ where S is aqueous saturation solubility (mM) at temperature T (Kelvin) (Mehl [129])

Viscosity measurements extrapolated to high concentrations and temperatures near T_g also suggest that trehalose forms slightly stronger glass compared to sucrose, although more importantly, both are significantly fragile compared to a conventional strong systems such as of SiO_2 [12, 24, 25]. Thus, their viscosities are strongly influenced (increased) by (reduction of) temperatures below T_g, improving their protective ability through vitrification, as discussed later. Most glassy systems relax toward the equilibrium state over time with the relaxation times dependent on the molecular mobility at the "storage" temperature (below T_g). The amount of relaxation in a glassy system can be estimated by measuring enthalpic relaxation (in DSC experiments) for material that has been aged at different temperatures (below T_g). Trehalose was found to have significantly higher activation energy for enthalpic relaxation compared to sucrose proving that its glass is "more stable" [26]. Another related concept is the so-called temperature of zero mobility where the relaxational movements become negligible, i.e., configurational entropy reaches zero. This point of zero mobility is considered the safest high temperature to prevent changes over significant (to pharmaceutical applications) timescales. This temperature is estimated as 44 °C for trehalose and 3.5 °C for sucrose, showing that trehalose would be a more robust excipient for long-term storage of freeze-dried products and would also handle temperature variations better [27].

Gibbs free energy change for the (solid state) amorphous to crystalline transformation at 25 °C is −17 kJ/mol for trehalose, almost double that of sucrose (−9.6 kJ/mol). Trehalose will therefore have a greater propensity to crystallize out of solution (although rate processes such as nucleation and crystal growth may govern the ultimate outcome) [4]. The crystallization tendency is probably aided by the observation (from MD simulations) that in solution, one water molecule is very often in resident in a position that corresponds to that of one of the two water molecules in the crystalline dihydrate (Th) form of trehalose, and would ease the appearance of crystalline Th form at relatively moderate concentrations (seen as low solubility) [28]. The solid forms of sucrose and trehalose and their transformations are discussed in greater detail later.

Diffusion coefficients of sucrose and trehalose are similar at lower concentrations but begin to differ from each other at higher concentrations (>50% w/w) and lower temperatures. Sucrose diffusivity is significantly higher than that of trehalose (almost 3X higher at 74 wt% and 323 K) reflecting its smaller hydration number and thus more compact shape. The corresponding water diffusion coefficients are similar for both systems below 72 wt% solute, but at higher disaccharide concentrations, water diffuses faster in sucrose than in trehalose [6], as a consequence of the size of the (intervening) sugar–water clusters discussed above.

Measurements of the partial specific volumes of solid trehalose (crystalline dihydrate or amorphous) compared to trehalose in solution (extrapolated to infinite dilution) show that there is a slight contraction of trehalose when it dissolves in water. This is speculated to be related to an internal H-bond between OH(6) and OH (2) of the different pyranose rings through partial folding of the rings along the glycosidic linkage [24]. The partial molar volume (at infinite dilution) reported for sucrose at 25 °C is slightly higher than that for trehalose [20, 24], but other values at 20 °C [29] are lower suggesting differences in measurement/errors, etc.

The density of trehalose and sucrose solutions is provided in Fig. 3.4 and Table 3.1, showing close equivalency and similar dependence on temperature.

Biotherapeutics, such as monoclonal antibodies are often buffer exchanged and concentrated into the final buffer after an ultrafiltration/diafiltration step. Stabilizing excipients such as sucrose or trehalose are subsequently added as concentrated stock solutions to the protein pool in a compounding step, in order to achieve their target final concentration in the formulation. The higher solubility and lower viscosity of sucrose makes this process step easier to execute than with trehalose.

3.2.3 Solid-State Properties

Polymorphic forms and phases of the disaccharides are important for their utility and functions. Schematics of the phases and transitions in the disaccharide–water system caused by water exchange and/or temperature changes are shown in Fig. 3.5.

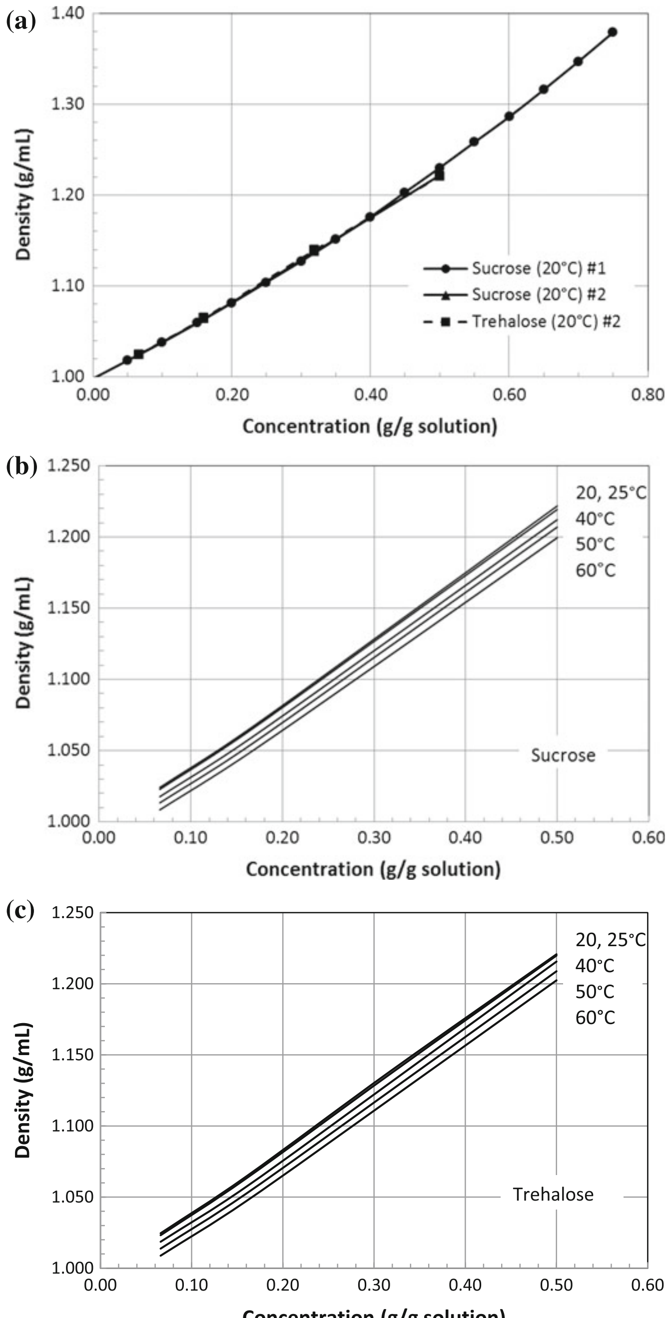
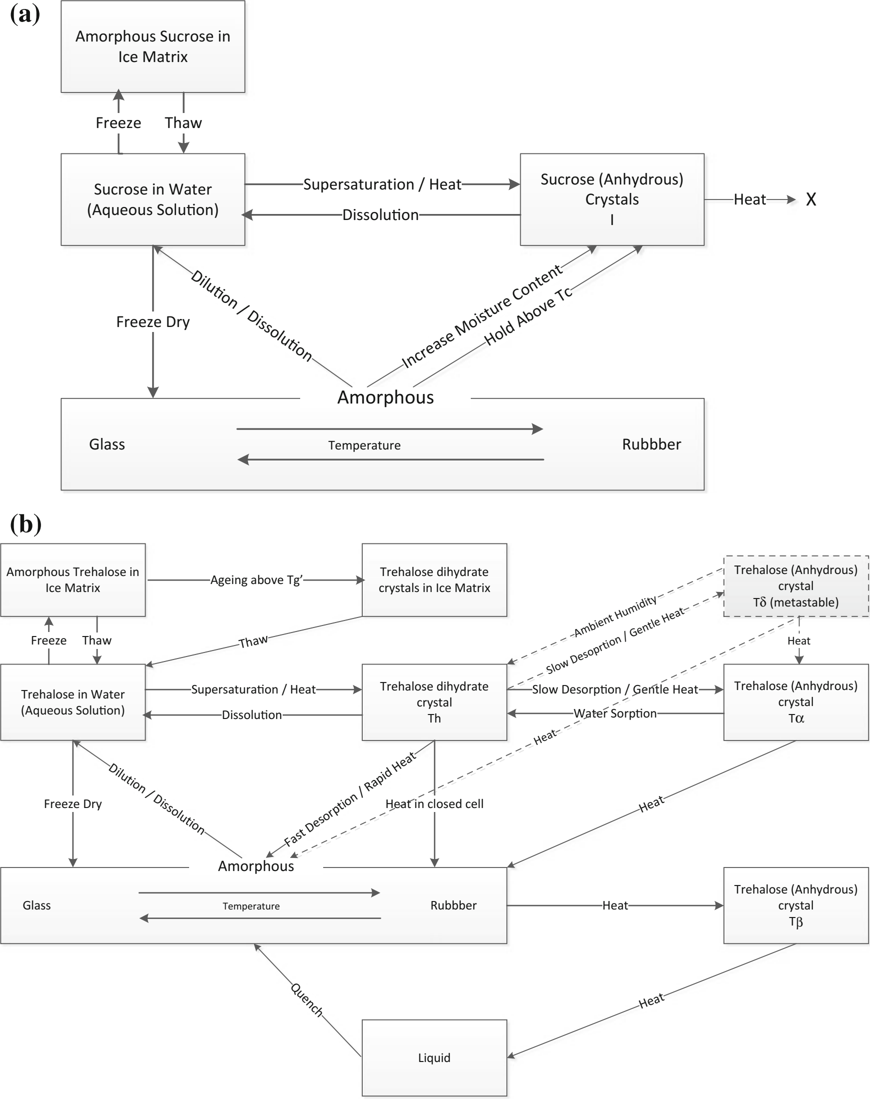


Fig. 3.4 Densities of aqueous solutions of sucrose and trehalose at various temperatures. **a** Density of sucrose (#1) (data from Hoffman [126]) and sucrose and trehalose (#2) (data from Branca et al. [12]) showing the similarity in densities of the two disaccharides; **b** density of sucrose from 20 to 60 °C (data from Branca et al. [12]); **c** density of trehalose from 20 to 60 °C (data from Branca et al. [12])



The phase transitions of sucrose in the solid state are quite simple, going between anhydrous crystal (Form 1) and glass through heat and/or moisture changes (Fig. 3.4a). A properly designed freeze-drying process for sucrose solutions will generally result in the amorphous form, since the promotion of crystallization of Form 1 in the lyophilizer is difficult. This amorphous form will crystallize when raised above its T_g, the value of which is dependent on the moisture content. On crystallization, the absorbed water is expelled from the crystal into the matrix as free water [30–33]. Thus sucrose does not provide a sink for

◂**Fig. 3.5** Schematic diagram of phase transitions in the disaccharide–water systems as a consequence of water exchange and/or temperature changes. **a** Sucrose—Drying or supersaturation of a sucrose–water solution results in sucrose crystals (Form I) which on further heating toward melting point (~188 °C) leads to decomposition along with the melting. Freeze drying from the solution leads to the anhydrous amorphous form, which can be crystallized (to Form 1) by exposure to moisture or by heating above crystallization temperature (~130 °C), but the crystallization temperature depends on the moisture content of the amorphous form (Saleki-Gerhardt and Zografi [32]); **b** trehalose—gentle drying or supersaturation of a trehalose–water solution results in the dihydrate crystalline form (Th) which on further gentle drying forms the α-crystalline form. (A metastable form Tδ may also be formed on dehydration around or below 100 °C.) Rapid desorption of moisture from the dihydrate above ~110 °C under vacuum or dry nitrogen leads to the anhydrous amorphous form. Other ways to form the anhydrous amorphous form are to freeze-dry from the solution or to melt and cool the α-crystals. Heating the anhydrous amorphous form above 160–170 °C results in the anhydrous β-crystals. Freezing the trehalose solution also results in the formation of amorphous trehalose, which if held above the glass transition temperature (~−32 °C) can result in the crystallization of the dihydrate crystals dispersed in the ice matrix. Note that the transitions for trehalose may be impacted by variations in starting phase sample properties (particle size, surface area, morphology, etc., [41, 130]) and by variations in heating rate and water activity [35]. Trehalose diagram is modified with permission from Kilburn and Sokol [35]) Copyright 2009 American Chemical Society

moisture as in the case of trehalose (see discussion below). Heating of the crystalline form will lead to a melt followed by decomposition. (Only recently has a second Form II crystalline polymorph been identified for sucrose, formed under high pressures [34].)

The phase transitions of trehalose in the solid state are, on the other hand, quite complex and are depicted in Fig. 3.4b [35]. The outcomes are strongly dependent on rates of transformation (rate of temperature change, rate of moisture removal, etc.) [25, 35–37]. The main polymorphic forms are the dihydrate crystal (Th) and the anhydrous crystals Tα and Tβ. Another crystalline form Tγ has been suggested to be combination of distinct domains of Th and Tβ, possibly embedded in an anhydrous crystal matrix [25]. Of particular mechanistic interest are the reversible Th ↔ Tα transformation which can occur under gentle dehydration/hydration without major changes in the main crystal motif. Kilburn and Sokol [35] suggest that the Tα could, in a strict sense, not be an equilibrium phase of dehydrated trehalose but a metastable collapsed form of dehydrated Th. Irrespective of the polymorphic details, the channels in the crystal (that lead to the surface) allow diffusion of water into and out of the lattice and thus act as a source/sink for water, and thus a platform for water immobilization [25, 35, 36, 38]. Jones et al. [39], however, point out that the Th to Tα transformation changes the surface morphological characteristics of the powder (cracks, roughness) which are not fully reversible. This can have implications for the use of trehalose where the surface (or flow) characteristics are important, e.g., dry powder inhalation carrier, spray-dried powders. (More recently, a new solid form Tδ has been identified by solid-state NMR. It is formed by dehydration of Th at or below 100 °C and is generated concurrently with Tα and amorphous trehalose; Tβ was also observed in some cases during the dehydration. The Tδ form is metastable, converting to Tα and amorphous trehalose above 80 °C, and to Th on exposure to ambient humidity [40].)

The ability of trehalose glass to form a dihydrate crystal has been proposed as an important mechanism in its protective ability (in anhydrobiosis) by allowing it to remain structurally sound under gently changing humidity levels. Increase in humidity allows local hydrate crystalline regions (of Th) to form and act as sinks of water molecules, thus regulating the rest of the matrix to low levels of moisture and amorphicity [38]. This ability of amorphous trehalose to capture moisture in crystallites results in a high T_g even in the presence of residual water (Fig. 3.2, Table 3.1). The transformation from glassy to crystalline state is influenced by the method of preparation (as assessed by enthalpic relaxation and onset of crystallization in thermal analysis while the T_g is not impacted). Resistance to crystallization (thermal and moisture mediated) can be rank-ordered on the basis of method of preparation of amorphous form: dehydration $<$ freeze-dried $\approx$ spray-dried $<$ melt-quenched [41]. Moisture adsorption isotherm data is shown in Fig. 3.6, with trehalose taking up greater amounts as per the sink model discussed above. Sucrose held below about 12% relative humidity at 25 °C can remain in the amorphous state for long periods of time [30]. Higher storage temperatures require higher levels of moisture for the amorphous to crystalline transformation to occur (while remaining below the T_g) [32]. Eventual crystallization at higher hydration levels results in the formation of anhydrous crystals (Form 1, Fig. 3.5) and a release of the adsorbed

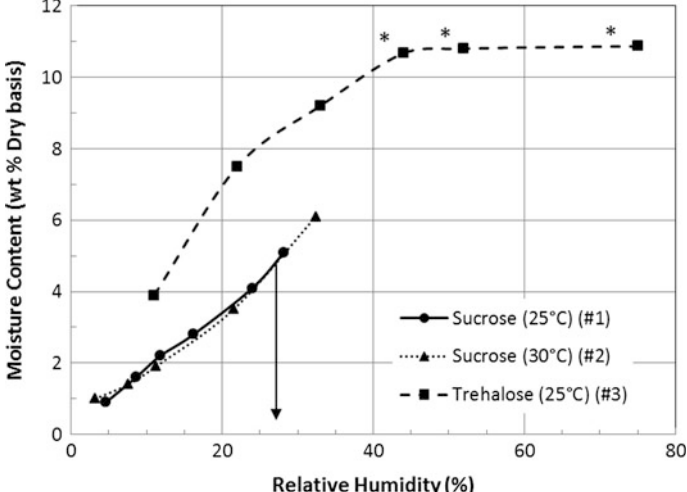


Fig. 3.6 Equilibrium moisture content for amorphous sucrose and trehalose on exposure to different relative humidities prior to crystallization. Temperatures of exposure are indicated on the plot. The downward arrow (↓) highlights the data point for sucrose at 25 °C that will crystallize rapidly on equilibration and release the adsorbed moisture. The data set for sucrose at 30 °C covers a range where crystallization does not occur before equilibration. (*) represent points on the trehalose curve where crystallization to the dihydrate (Th) form will occur with an equilibrium moisture content close to 10.5% on dry basis. Sucrose data is from (#1) Makower and Dye [30] and (#2) Saleki-Gerhardt and Zografi [32]. Trehalose data is from (#3) Igelsias et al. [42]

water into the matrix. Trehalose equilibrates to approximately 10.5% moisture (dry basis) which corresponds to the dihydrate form (Th, Fig. 3.4) [42].

Of particular interest for biopharmaceutical applications is the observation reported by Sundaramurthi et al. [43] on the freeze-drying of trehalose solution. They observed formation of the dihydrate Th in the freezing stage upon annealing of the frozen solution. Formation of the crystalline form represents a phase separation. This Th form then subsequently converted to the anhydrous amorphous state during drying. The implication of this transformation is discussed later.

The aqueous solubility of both disaccharides is reasonably high (Fig. 3.7; Table 3.1). Sucrose has the greatest solubility of the disaccharide sugars. At 80 °C, solubilities of trehalose and sucrose are similar (∼78% w/w) but the solubility of trehalose drops rapidly with temperature, reaching ∼30% at 0 °C while sucrose remains at ∼64.5% (Fig. 3.5; Table 3.1). This has important implications for the use of these materials in biotherapeutic applications as will be discussed later. Congruously, the water activity lowering behavior of trehalose was determined to be identical to that of sucrose at the same concentrations, but the lower overall solubility of trehalose results in higher water activity in saturated solutions compared to sucrose [44].

Most of the above described solution research has been performed in disaccharide–water systems. Addition of buffers/salts has a significant impact on the thermophysical behavior of these solutions. For example, phosphate-buffered saline added to trehalose (mixed in solution state and dried) reduces the T_g and a decrease in the H-bonding capacity of trehalose glass in a concentration-dependent manner [45].

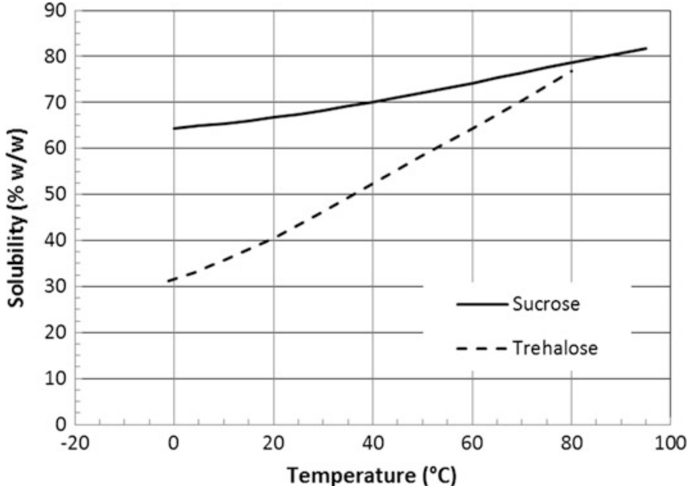


Fig. 3.7 Solubility of sucrose and trehalose as a function of temperature shown as best-fit curves. The data for sucrose used for the curve-fit was taken from Mathlouthi and Reiser [60], and for trehalose from Miller et al. [24]. Best-fit curve equations are given in Table 3.1

3.2.4 Chemical Properties

Trehalose and sucrose are both non-reducing sugars since their constituent glycosidic bonds link anomeric carbons in their monosaccharides. However, the α-(1 → 1)-α linkage in trehalose is significantly more resistant to hydrolysis than the α-(1 → 2)-β bond in sucrose. The hydrolysis proceeds by protonation of the glycosidic oxygen followed by heterolysis of the resultant oxonium ion into a monosaccharide and a carbenium ion. The carbenium ion subsequently reacts with water to produce the second monosaccharide. Protonation of the glycosidic oxygen creates steric crowding which is relieved by the bond breakage in sucrose, while in trehalose, there are few steric barriers to rotation about the glycosidic bond. The anomeric carbon of the glucopyranose carbenium ion is also more electropositive than that of fructofuranose, making the latter a better leaving group in the heterolysis and may explain the instability of sucrose [46]. Activation energy for acid-catalyzed hydrolysis is reported as 26.3 kcal/mol (110 kJ/mol) for sucrose and 40.2 kcal/mol (168.2 kJ/mol) for trehalose although the pH is not reported (summarized by O'Brien [47]). Torres et al. [48] determined that the activation energy for acid hydrolysis (pH 2.5 and below) of sucrose is independent of pH (~99 kJ/mol) with the pre-exponential factor dependent on the pH. Even under alkaline conditions (0.1 M potassium phosphate buffer, pH 8.1), the rate constant for trehalose hydrolysis was found to be ~10,000X slower than that for sucrose [49]. For biopharmaceutical formulation purposes, a pH of 5.0 or below should be considered a risk factor for the hydrolysis of sucrose. Accordingly, a sodium phosphate buffer with sucrose may be considered a risk if used in a freezing or lyophilization process due to the drop in pH of this buffer due to precipitation of the basic salt. The high local concentration due to cryo-concentration combined, with the low pHs (down to pH ~ 3) may lead to hydrolysis of the sucrose even if the temperature and/or moisture content is low (see discussion and references in [50]).

Hydrolysis of sucrose leads to the formation of glucose and fructose, both of which are reducing sugars, and which in turn can cause glycation of proteins through the Maillard reaction. Glycation occurs as a condensation reaction between the aldehyde groups of reducing sugars and the primary or secondary amines in proteins. Glucose in solution exists as a stable pyranose ring structure in equilibrium with the open-chain aldehyde form. The Maillard reaction is initiated through a reversible Schiff's base (glycosylamine) formed between the amine group and the carbonyl carbon aldehyde, and then converts into a more stable ketoamine form through an Amadori rearrangement. The formation of the Amadori products is an early stage reversible glycation that is dependent on concentration and exposure time [51]. The primary residues that are impacted are the ε-amino groups on lysine residues and the α-amino on the N-terminus. Glycation of proteins leads to the formation of a sugar adduct and can result in loss of structure and function. Fructose has greater reactivity than glucose for Schiff's base formation [52]. Participation of fructose (fructosylation) can lead to cross-linking and aggregation [53]. Glycation has been reported in liquid formulations of proteins with sucrose in the formulation

at pH 5 or 5.5 at elevated temperatures [54, 55]. The impact of Maillard reaction has also been observed in low-pH freeze-dried systems containing sucrose, while not with trehalose [47, 56]. Hydrolysis of sucrose in this system occurred during the freezing/dehydration/storage steps due to the high H+ ion concentration, despite the significantly lower mobility compared to liquid state. Karel and Labuza [57] reported acid-catalyzed hydrolysis of sucrose at water contents below 1%, when held at 55 °C. The higher concentration of reactants compensates for the reduced mobility.

Interestingly, trehalose has also been found to have an antioxidant effect, but not through any ability to scavenge free radicals, etc. The mechanism proposed for protection of unsaturated fatty acids is direct interaction of trehalose with the oxidation-sensitive parts of the fatty acid. The OH-6′ group binds with the double bond through O–H…π interactions while the OH-2 hydroxyl group binds to one of the CH groups on the 2-butene through CH…O hydrogen-bond. The intermolecular complex raises the activation energy for hydrogen abstraction, lowering the reaction rate by a factor of 10^8 [58]. This effect was not found with sucrose, and it is proposed that intramolecular H-bonds in sucrose may discourage this type of interaction. However, sucrose is also considered to have antioxidant properties (e.g., in protecting foods) due to its effect on reducing water activity, and the potential for hydrolysis products (glucose and fructose) to block reactive sites of ions such as copper, iron, and to a lesser extent, cobalt, thus impeding catalytic oxidation reactions [59, 60].

3.3 Use in Formulations and Stabilization Mechanisms

Trehalose and sucrose are frequently used as stabilizers for protein biotherapeutics. Stabilization of protein in this context refers to the maintenance of protein structure (conformational stability) and the ability to resist aggregation (colloidal stability) [61].

3.3.1 Liquid State

In the liquid state, the main mechanism cited for the usefulness of sugars (along with that of other osmolytes) to stabilize proteins is that of preferential exclusion [62]. Osmolytes are excluded from the first hydration shell of the protein, leading to a preferential hydration of the protein. The chemical potential of the system is increased, which drives the protein to take on a more compact, i.e., folded state, since the unfolded state has a greater surface area—this enhances the conformation stability of the proteins. As an example, the water of preferential hydration (molecules of water/molecule of protein RNase) is estimated as 350 for sucrose and 400 for trehalose [62]. This is an immediate result of the significant effect of the

sugars on distortion of the water tetrahedral coordination, and therefore on the solvation properties of the water molecules around the protein. The extensive sugar–sugar and sugar–water interaction leads to an enhancement of intra-protein interactions at the expense of protein–solvent interactions [63–66]. The slightly higher water of preferential hydration of trehalose over sucrose may be a reflection of the greater interaction of the former with the water [14], as discussed above. Greater trehalose–water H-bonding and the more rigid configuration of trehalose molecule also give it a greater size in solution compared to sucrose (as seen above in the higher diffusion coefficient of sucrose). The hydrated trehalose molecule is larger than the sucrose ($\sim$2.5X), and therefore, it has a larger excluded—volume effect than sucrose simply by dint of steric exclusion. In summary, trehalose can provide the same preferential hydration (of the protein) at lower concentrations compared to sucrose [23]. Overall, although both trehalose and sucrose are suitable stabilizers of a protein in the liquid state (not accounting for any chemical effects due to sucrose hydrolysis), trehalose can be expected to be somewhat more effective at the same molar concentration. In practice for biotherapeutics, this difference may however not be perceptible over the expected shelf-life of the product.

The specificity for solute (sugar) requirements for stabilization by the preferential exclusion mechanism is low. A number of solutes stabilize a variety of proteins by this mechanism.

As expected from the conformational stabilization due to preferential exclusion, sugars increase the melting points and thermodynamic parameters (Gibbs free energy and the melting enthalpy and entropy) in a concentration-dependent manner over a range of pH [67–69]. Trehalose with slightly greater exclusion ability should be more effective in raising the melting point but the differences would be minor and would require extremely careful experiments to discern (see, e.g., data reported in [63, 66]). Rubin et al. [70] also observed slightly greater increase in melting point of protein with 500 mM sucrose over trehalose for an aglycosylated antibody.

More interestingly, sugars can also improve the colloidal stability of proteins. The normalized mutual diffusion coefficient for the protein was increased (implying greater mutual repulsion) by the addition of sugars for an aglycosylated antibody. Disaccharides were more effective than monosaccharides, and sucrose was slightly more effective than trehalose [70, 71]. However, the colloidal stabilization by sugars may be perceptible only if the inherent colloidal stability due to electrostatic repulsion is poor, i.e., the hydrophobic interactions dominate [71]. Addition of sugar reduces the dielectric constant and thus enhances charge–charge repulsion between adjacent protein molecules [69, 72]. Saito et al. [69] also report that sucrose and trehalose (10% w/w) could reduce zeta potential and thus (the calculated) net charge on a mAb, with the magnitude varying by the antibody. The mechanism is not clear. Reduction of net charge in general will tend to increase the probability of self-association.

Preferential exclusion is enhanced at high protein concentrations, because self-association is favored due to the greater reduction of excluded surface area afforded [62, 73, 74], and reduced by increasing temperatures [14]. Furthermore, during freezing and during thawing, the preferential exclusion mechanism is still

applicable [75], as long as there is unfrozen water present, i.e., prior to glass formation (further discussion below). Therefore, solutes (such as sucrose and trehalose) that work by the preferential exclusion mechanism, are effective protectants as they stabilize over a wide range of conditions.

3.3.2 Frozen State

Once in the frozen state itself, additional factors come into play. The solutes are excluded from the growing ice crystals and thus undergo cryoconcentration. The concentration of maximally freeze-concentrated sucrose and trehalose is approximately 80% w/w [76–78]. Trehalose due to its stronger water de-structuring effect is more effective in preventing the formation of ice, but in practical non-equilibrium cooled systems, the difference will not have a significant impact. At solute concentrations near 80% w/w, the water available for H-bonding with the concentrated protein for maintenance of structure may be restricted, although still considerably more than normal water of hydration of proteins [79]. The sugars, now compressed into closer contact with the proteins, may participate in some of the H-bonds, thus providing stabilization through the water replacement mechanism. In parallel, the cryo-concentrated sugar glass leads to the inhibition of molecular mobility and a reduction in all reaction rates and therefore an improvement in storage stability. The fragile glasses of sucrose and trehalose show a significant increase in viscosity under T'_g, rapidly enhancing the stabilization effect with decreasing temperature. This latter mechanism is referred to as the glassy state-vitrification mechanism. Both water replacement (an enthalpic) mechanism and glassy state-vitrification (an entropic) mechanism are likely active simultaneously in the system.

As discussed earlier, trehalose with a greater impact on the tetrahedral H-bonds of water causes a greater degree of disruption and thus the ability of water to form ice. It interacts strongly and with more molecules of water (compared to sucrose), creating a conformation which is locally more ordered, and encapsulates the protein in a rigid environment. This has been termed “cryptocrystalline” by Magazu et al. [14], who propose that the cryoprotectant action of trehalose arises more from its strong interaction with water and not directly with the protein.

The water replacement and the glassy state-vitrification mechanisms (in the frozen state under discussion) clearly require the sugar to remain in the amorphous state to achieve stabilization of the protein. Crystallized sugars will effectively phase separate from the protein, thus leading to a loss of protection. In the frozen glassy state, trehalose with its lower solubility, and its tendency to form interconnected clusters at high concentrations, shows a greater propensity to crystallize out of the glass if temperatures rise above the T'_g. The fragility of the trehalose glass showing a rapid reduction in viscosity above T'_g aids in this process. Long-term frozen storage of proteins above T'_g with trehalose as cryoprotectant has been shown to result in aggregation due to this crystallization effect on storage at −20 °C; [80].

Crystallization likely begins at some nucleation hot spots and propagates throughout the matrix due to the long-range clusters (percolation) of the trehalose molecules. The protective effect of trehalose is thus lost in a large region of the frozen matrix. Sucrose, on the other hand, due to its high solubility has not been reported to crystallize and thus is a safer alternative for frozen state storage, being more robust to choice of storage temperature (e.g., −20 °C) despite a T_g' similar to that of trehalose. Furthermore, due to its smaller clusters, crystallites of sucrose, if formed, would have difficulty propagating and would therefore require multiple nucleation events to cause damage to the whole matrix.

Increasing concentrations of protein raises the T_g' of sugar matrices since the T_g' of proteins reportedly lies around −13 °C [81] (although more data is needed on this topic), with lower molecular weight proteins providing a greater increase than a higher molecular weight protein for an equivalent mass concentration; larger molecular weight proteins are more effective than smaller proteins at similar molar ratios [82–84]. Raising the T_g' closer to −20 °C by increasing the protein concentration thus reduces the risk of crystallization of the sugar at −20 °C. This is particularly relevant for trehalose, although no confirmatory data is available in the literature to date. Another observation reported on the use of trehalose in frozen storage relates to the impact of process conditions, viz. the rate of freezing. Fast freezing enables more uniform spatial distribution/entrapment of solute (trehalose), instead of being pushed toward the center of the matrix by the moving ice front as in the case of slow freezing. The more dispersed trehalose is able to resist crystallization (or crystallize in pockets and not spread throughout the whole matrix) and thus provide protection to the protein even when stored above T_g' [85].

3.3.3 Lyophilized State

As proteins are lyophilized, there is a further loss of water (compared to the frozen state). Protein molecules are faced with loss of H-bonds which can destabilize the structure. Loss of water significantly lowers the dielectric constant that enhances the intermolecular interactions, with attractive interactions/orientations being favored as the intermolecular distance decreases [72]. These stresses generally result in conformational changes on drying or even aggregation [86]. Sucrose and trehalose again function as lyoprotectants by replacing the water–protein H-bonds with sugar–protein H-bonds (water replacement), and by intercalating between the protein molecules thus creating a glassy matrix in which the molecules are immobilized (vitrification). In the freeze-dried state, the H-bond replacement is particularly important, so that there is a greater degree of specificity in the structural requirements of the lyoprotectant over the cryoprotectant. As in the frozen state, the sugars have to be in an amorphous state to allow effective H-bonding with the protein; crystallized sugars will form a separate phase, losing their effectiveness as stabilizers.

A certain minimum amount of protein—sugar is required for effective protection. Low excipient to protein ratios may simply enhance risk for high moisture levels on storage, due to low solids content and potential moisture transfer from container/closures. However, more importantly, a certain mass of the protectant is needed for creating a suitable functional matrix, even though the sugar is often in significant molar excess. A certain minimum level of the excipient is needed to satisfy the surface accessible hydrogen-bonding requirements of the protein and preserve a native-like structure (the water replacement component). Additional sugar then helps to provide dilution of the protein, thus reducing protein–protein contact and also creating a glassy matrix for immobilization (vitrification mechanism) [87–89]. Heljo et al. [90] found that at a 2:1 w/w (730:1 mol/mol) excipient to protein ratio, trehalose was more effective than sucrose at protecting the activity of β-glucosidase on storage at 45 °C. However, at higher ratios tested (20:1 and 40:1 w/w or 7300:1 and 14,600:1 mol/mol) the stability was less dependent on amount and type of excipients. For a mAb, the minimum stabilizing ratio of sugar to protein was 360:1 mol/mol, but further benefits were seen even above 500:1 mol/mol. No differences were observed between the stabilization effects of sucrose versus trehalose [87, 89, 91]. The minimum amount of sugar required for stabilization does not clearly correlate to the size of the protein [91]. A starting 500:1 mol/mol ratio is a reasonable minimum level to test. The differences in preservation efficacy among various sugars as reported in the literature probably reflect the varying conditions of the experiment including the protein itself, the assay (e.g., use of enzyme activity as opposed to actual degradation rates), and the storage conditions tested, etc. An additional stabilizing effect in the lyophilized matrix would arise from the ability of sugars to reduce the dielectric constant, thus enhancing the charge–charge repulsion between adjacent protein molecules and preventing them from aggregating [72]. Sucrose or trehalose in a proper pH-buffer system will likely provide adequate stability to biopharmaceutical products.

For stable lyophilized matrices, a high T_g is preferred, as it is more resistant to temperature fluctuations. There is more room to tolerate increases in moisture content. In this respect, trehalose with its high T_g in the amorphous state is the better lyoprotectant. The trehalose matrix is better able to resist crystallization (and collapse) during temperature excursions or storage at warmer temperatures compared to sucrose matrices. The ability of trehalose to "absorb" moisture by converting to the dihydrate Th state also increases the robustness of the matrix to moisture content. However, the high T_g is not the only factor. In matrices (1:10 w/w) of glucose/sucrose and glucose/trehalose which had similar T_g ($\sim$24 °C), the glucose/trehalose system was still better able to preserve the activity of an enzyme to high temperature (44 and 60 °C) storage stress [92]. Trehalose has superior glass-related properties such as lower free volume and restricted molecular mobility, along with being resistant to phase separation and crystallization (even above T_g).

Delving into the details of the sugar–protein interactions, however, reveals that the picture is more nuanced than the simple T_g would predict. Storage stability of proteins in the lyophilized form does not always directly correlate with the glass transition temperature of the excipients (see, e.g., [93–96]). There are two parts to

the stabilization mechanism—the H-bonding water replacement and the immobilization vitrification aspect. How these are individually involved through the nominal glass transition phenomenon is discussed below.

Addition of protein raises the T_g of the amorphous matrix [97] since the "pure" protein itself, in the amorphous dry state, has a high T_g (see, e.g., [98]). The T_g of the mixture of sugar and protein, however, does not follow ideal mixing behavior (≡complete miscibility and additivity of free volume) since specific H-bonding interactions formed between the sugar and protein molecules are not numerically and energetically equivalent to the sugar–sugar interactions that are lost [97, 99, 100]. A negative deviation from ideality is seen for both sucrose and trehalose systems. Assessment of excess mixing enthalpies shows that sucrose–protein H-bonding becomes more favorable compared to sucrose–sucrose interactions at weight fraction above 0.5 (Sucrose:RNase system; equivalent to a 40:1 mol/mol ratio). The same holds true for trehalose but the excess enthalpies are higher since the trehalose–trehalose H-bond is stronger (≡high T_g) and the replacement trehalose–protein bonds may be fewer than between sucrose–protein [97, 99–101]. Mixing or miscibility in these systems is thus driven by entropy gain (through net loss of H-bonds and/or lower strengths of the new H-bonds). In simpler terms, it is likely that a glassy matrix with a lower T_g (sucrose compared to trehalose) provides a greater ability to incorporate protein molecules through sugar–protein interactions and therefore better preservation of the protein structure. A high T_g matrix will have stronger sugar–sugar interactions and will preferentially interact with itself instead of the protein [88]. The difference between sucrose–protein and trehalose–protein interaction is probably small as the non-ideality interaction parameter was found to be slightly higher for the sucrose–protein, but not statistically different than for the trehalose–protein system [97]. Based on FTIR spectra, Katayama et al. [97] also speculate that the trehalose, compared to sucrose, interacts more favorably with the (small amount of) water present in the system which likely resides around the charged and polar residues of the protein. Could this be an additional reason for the more favorable sucrose–protein interactions?

Stabilization in the glassy state through vitrification is related to the molecular dynamics in the glassy state. Glassy matrices exhibit dynamics over a broad range of timescales. The global structural relaxation processes (α process) are most directly related to the storage temperature *vis-a-vis* the glass transition temperature (represented by $T - T_g$); however, other factors such as fragility and thermal history also play a role. Viscous flow of/in the matrix is closely linked to the global motions related to the α process. At storage temperatures near T_g, degradation rates are correlated to ($T - T_g$), as global motions are significant and determine the rates of denaturation or chemical reactions. Fragility is important as it determines the extent of change of viscosity at temperatures near T_g. Therefore at high storage temperatures, sucrose matrices will provide less protection than trehalose matrices due to the differences in their T_g (see, e.g., [95]).

However, along with the large timescale motions in glassy matrices, there are present faster secondary relaxation processes, comprising small-amplitude local motions (β process). Pikal, Cicerone, and coworkers [93, 96, 102, 103] have shown

that the stabilization of protein (against denaturation and chemical degradation) in glassy matrices stored well below their T_g, is directly linked to these β processes, and does not correlate to the $(T - T_g)$ difference. They propose that the high frequency β relaxation processes in the glass affect protein stability through coupling of these motions to local small-amplitude protein domain conformational changes which enables aggregation to be nucleated by local unfolding. Chemical degradation reactions such as deamidation can also be occurred due to local domain motions. Local matrix mobility allows diffusion of small molecules and gases which can enable oxidation and hydrolysis reactions. Sucrose is better able to suppress these fast dynamics than trehalose, probably because of its smaller free volume in the glassy state. Pikal et al. [96] found that at equivalent levels of disaccharide to protein, sucrose systems were about a factor of two more stable than trehalose formulations. By adding small amounts of additives (called anti-plasticizers), these local fast dynamics can be suppressed, improving the stability of the encapsulated protein. The low T_g anti-plasticizer additive (e.g., 5–10% glycerol) functions by filling the small voids in the host glass (the free volume), restricting motion and thereby slowing the fast dynamics. This additive simultaneously depresses the T_g and speeds up the global α motions, but the gain in stability from suppression of fast dynamics dominates. However, if an excess of the anti-plasticizer over what is needed for occupation of the original free volume is added, the plasticizer begins to lubricate and speed up all dynamics resulting in a loss of stability. One of the requirements of the anti-plasticizer is that it should have a T_g close to that of the host glass. A small amount of residual water may actually be beneficial from this perspective [93].

The discussion above raises the question of the relative importance of H-bonding needs of the protein (better supplied by low T_g protectant) and the need for immobilization (better provided by a high T_g protectant), for long-term stability of the protein. The bottom line is that in practical systems, as long as there is sufficient amount of the sugar, either disaccharide will be suitable (especially to fulfill the H-bond requirements). The high T_g trehalose (Fig. 3.1) may then not be a big advantage from a practical perspective because general storage temperatures are far below T_g. A note of caution is warranted though in that the T_g of a sugar–protein system is highly dependent on the moisture content and reports of measured values must be reviewed in this context. Bellavia et al. [82, 104], for example, report that addition of protein decreases the T_g of ternary sucrose/trehalose–water–protein systems at low hydration. However, their mixtures were created by blow-drying and the lowest water content tested of 2:1 mol water/mol sugar corresponds to approximately 5% w/w water, which is very high for lyophilized biopharmaceutical systems.

The relative ease with which trehalose (unlike sucrose) crystallizes out in the frozen state (when annealed above T_g') suggests that the process development for such systems must be carefully considered [43, 80]. As shown by Suryanarayanan and coworkers, the subsequent sublimation of water in the freeze-drying cycle dehydrates the trehalose into the desired amorphous state. However, the question is whether the phase separation between protein and trehalose due to crystallization of the latter (while frozen) is eliminated by this conversion of trehalose to the

amorphous state in the lyophilized matrix. Sundaramurthi et al. [43] propose otherwise but currently no data, direct or indirect, has been published on this question. The number of reports of excellent lyoprotection with trehalose proves that it is an effective excipient, but since these reports likely did not explore the lyophilization cycle as was done by Sury and coworkers, appropriate caution is warranted. An annealing step should not be incorporated in a cycle with trehalose, due to the risk for crystallization of trehalose.

From a practical cycle development perspective, the low T_g' of both sucrose and trehalose solutions implies low primary drying temperatures and thus long cycles. In both cases, higher concentrations of proteins increase the T_g' and thus also the collapse temperatures. Although out of scope of this chapter, increasing the protein concentrations also results in a separation of temperatures of onset of collapse and full collapse, allowing aggressive drying cycles to be utilized, shortening the overall process time [84, 105, 106]. For low protein concentration (e.g., 20 mg/mL or lower of monoclonal antibodies) combinations of sucrose as stabilizer and mannitol as a crystalline bulking agent can be used to enable fast drying after ensuring crystallization of mannitol during the freezing step [107]. Mannitol in a solution with a ratio of 3:1 w/w mannitol: sucrose can be safely crystallized. However, keeping the requirement for a certain minimum level of stabilizer (sucrose) to protein ratio in mind, the overall solids load in the solution can become high, leading to higher cake resistance. Lower ratios down to 2:1 may also be crystallized but may require the aid of an annealing step, especially since the protein will also hinder crystallization. It may be expected that a trehalose + mannitol system would behave similarly, but the above caution against annealing a trehalose system must be kept in mind.

3.4 Regulatory and Safety Aspects

3.4.1 Regulatory Aspects

Monographs for both sucrose and trehalose are available in the major pharmacopoeia. Tables 3.2 and 3.3 summarize the tests/specifications in the US, European, and Japanese pharmacopoeia. Sucrose, having been around longer, has fewer tests and is significantly harmonized. There is a bacterial endotoxin limit but no limit for microbiological bioburden, despite its significant use in parenterals. However, most vendors of parenteral grade material test for bioburden although acceptance limits seem to vary (for TAMC and TYMC and/or testing for absence of identified microorganism).

Trehalose monographs on the other hand have greater differences. Bacterial endotoxin limits are specified significantly differently between United States Pharmacopoeia (USP) and European Pharmacopoeia (Ph.Eur.), while the Japanese Pharmacopoeia (JP) does not have any criteria. The USP lets the level float to enable the product requirements to be met while the Ph.Eur. sets two limits based

Table 3.2 Summary of compendial requirements for sucrose[a]

Test/ characteristic	NF 33	Ph.Eur 01/2015:0204	JP XVI
Appearance		White or almost white, crystalline powder, colorless or white or almost white crystals	White crystalline powder, or lustrous colorless or white crystals
Solubility		Very soluble in water, slightly soluble in ethanol, practically insoluble in anhydrous ethanol	Very soluble in water and slightly soluble in ethanol
Identification	Meets test		
Appearance of solution	Solution is clear; opalescence NMT Reference Standard I for 500 mg/mL solution in water		
Conductivity	NMT 35 μs cm^{-1} at 20 °C for a 313 mg/mL solution		
Specific optical rotation	+66.3 to + 67.0 @ 20 °C, 260 mg/mL, 100 mm		
Acidity or alkalinity	NA	NA	Meets test
Color value	NMT 45 (parenteral grade) and NMT 75 (non-parenteral grade) for 1.0 g/mL solution at 420 nm	NMT 45 for 1.0 g/mL solution at 420 nm	
Dextrins	Meets test		
Reducing sugars	Meets test		
Sulfites	Meets test; maximum 10 ppm calculated as SO_2		NMT 15 ppm as SO_2
Loss on drying	NMT 0.1% for 2.000 g, 105 °C 3 h		
Bacterial endotoxins	Less than 0.25 IU/mg if intended for use in manufacture of large-volume parenteral preparations		
Lead	NA	NA	NMT 0.5 ppm

[a]The current version of the pharmacopoeia should always be consulted for the latest revision of the monograph

on amount of trehalose added to the product. The microbiological bioburden limits are similar between the USP and Ph.Eur., while the JP does not have any criteria. The Impurities/Related Substances limits are similar but specified with somewhat greater detail in Ph.Eur. Heavy Metals limits are specified for trehalose but not for sucrose. Vendors typically use NMT 5 ppm (as lead). However, more detailed profiling of metal content should be requested as trace metals (especially Fe, Cu) can have a significant impact on stability of proteins. For illustration, Fe and Cu limits are currently 130 and 10 ppm, respectively, for both sugars from one vendor.

Table 3.3 Summary of compendial requirements for trehalose[a]

Test/ characteristic	NF 33 trehalose	Ph.Eur 8.0 07/2010:2297 trehalose dihydrate	JP XVI trehalose hydrate
Appearance	NA	White or almost white, crystalline powder	White crystals or white crystalline powder
Solubility	NA	Freely soluble in water, slightly soluble in methanol, practically insoluble in ethanol (96%)	Freely soluble in water and slightly soluble in methanol and in ethanol (99.5%)
Identification	Meets test		
Color and clarity of solution	Meets test		
Appearance of solution		Solution is clear; opalescence NMT Reference Standard I for 100.0 mg/mL solution in water	
pH	4.5–6.5 for 100.0 mg/mL solution in water		
Specific optical rotation	+197 to +201 @ 20 °C, 100.0 mg/mL, 100 mm		
Chlorides	NMT 125 ppm		NMT 0.018%
Sulfates	NMT 200 ppm		NMT 0.024%
Nitrogen content	NMT 0.005%		NMT 0.005%
Dextrin	NA	NA	Meets test
Sulfite	NA	NA	Meets test
Soluble starch	Meets test		
Water	Anhydrous form, NMT 1.0%; dihydrate form 9.0–11.0%	9.0–11.0%	9.0–11.0%
Heavy metals	NA; (NMT 5 ppm to be official December 1, 2015)	Maximum 5 ppm	NMT 5 ppm
Sulfated ash		Maximum 0.1% on 1.0 g	
Residue on ignition	NMT 0.1% on 2.0 g		NMT 0.1% on 2.0 g
Bacterial endotoxins	If labeled for use in preparing parenteral dosage forms: The level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which trehalose is used can be met. Where the label	Less than 4 IU/g for parenteral preparations of 100 g/L or less of trehalose dihydrate; Less than 2.5 IU/g for parenteral preparations of more than 100 g/L of trehalose dihydrate	

(continued)

Table 3.3 (continued)

Test/ characteristic	NF 33 trehalose	Ph.Eur 8.0 07/2010:2297 trehalose dihydrate	JP XVI trehalose hydrate
	states that trehalose must be subject to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement (s) in which trehalose is used can be met		
Microbial contamination	TAMC is NMT 100 cfu/g (<61>) TYMC is NMT 100 cfu/g (<61>) – Meets test for absence of *Escherichia Coil* (<62>) – Meets test for absence of Salmonella (<62>)	If intended for use in parenteral products: – TAMC acceptance criterion 100 CFU/g (2.6.12) If not intended for use in parenteral products: – TAMC acceptance criterion 1000 CFU/g (2.6.12) – TYMC acceptance criterion 100 CFU/g (2.6.12) – absence of *E. Coil* (2.6.13) – absence of Salmonella (2.6.13)	
Assay	97.0–102.0% on the anhydrous basis reference to USP *trehalose RS*	Percentage content of trehalose with reference to *trehalose dihydrate CRS*	98.0–101.0% on the anhydrous basis ($C_{12}H_{22}O_{11}$: 342.30)
Impurities/ related substances	Peaks corresponding to Glucose and eluting after trehalose are NMT 0.5%; Peaks corresponding to maltotriose and other polysaccharides and eluting before trehalose are NMT 0.5%	Specified Impurities: Glucose (NMT 0.5%) and oligosaccharides (mainly glucosyl trehalose) (NMT 0.5%); Unspecified Impurities: For each impurity, NMT 0.2% Total Impurities: NMT 1.0% Disregard Limit: 0.1%	Peaks eluting after trehalose and eluting before trehalose are each NMT 0.5%

[a]The current version of the pharmacopoeia should always be consulted for the latest revision of the monograph

Interestingly, the monographs do not specify limits for residual solvents. Typical vendor testing uses a limit of 5000 ppm (0.5%) for ethanol and 3000 ppm (0.3%) for methanol. At a typical use level in liquid products corresponding to isotonic levels (approx. 85 mg/mL), this could correspond to a solvent load of up to 0.7 mg/mL. These levels of solvents can have an impact on the stability/solubility/opalescence behavior of sensitive protein products and may also become apparent in extractable/leachables studies. This issue will likely not arise in lyophilized products where the lyophilization process should help to eliminate these solvents, but would be present in the solution leading up to the lyophilization.

More recently, nanoparticulate impurities have been identified in sucrose (through a dynamic light scattering signal in solutions) in the 100–200 nm size range [108]. These impurities suspected to be arising from the process/raw materials comprise dextrans, ash comprising metals and minerals, and other aromatic colorants that are not completely removed from the purification process. The level of these impurities varies between vendors and likely between batches of the excipient since it is not controlled actively. Although the study only looked at sucrose, it is likely that the same types of nanoparticles are present in trehalose also. The specific exact impact of these nanoparticles on protein stability has not been currently reported, but is baked into the quality attributes of the products where these excipients are currently used.

3.4.2 Safety Aspects

Trehalose, despite its widespread presence in nature in many organisms, does not occur in mammalian cells. However, humans have the enzyme trehalase in intestinal villi cells and in kidney brush border cells, liver and plasma, and are able to hydrolyze ingested trehalose [109].

Trehalose dihydrate is present in a number of marketed products for intravenous administration. Among the current approved products, Avastin (bevacizumab) [110] contains one of the highest level of trehalose (2.4 mg trehalose dihydrate/mg active). At the highest indicated dose of 15 mg/kg and fastest infusion time, this results in an IV dose of 36 mg trehalose dihydrate/kg, over 30 min (72 mg/kg/h) every 3 weeks. However, larger total amounts of trehalose have been dosed in clinical trials (unpublished data).

Despite bypassing the intestinal and first-pass liver trehalases, intravenous administration of trehalose can increase systemic glucose due to hydrolysis by plasma or liver trehalase, or due to hydrolysis by trehalase in the proximal kidney tubules and renal reabsorption of the resulting glucose. It can be surmised that the fate of parenterally administered (or ingested) trehalose corresponds to that of glucose since trehalose is rapidly hydrolyzed to glucose by the enzyme trehalase. In humans, the maximum rate of glucose utilization has been estimated to be 500–800 mg/kg/h [111]. It is known that dextrose solutions should be used with caution in patients with overt or known subclinical diabetes mellitus or with carbohydrate intolerance for any reason [112]. The amount of glucose infused via Cipro IV at maximum

recommended dose can reach 30 g/day. The Japanese Pharmaceutical Excipients Directory reports a maximum precedented daily doses of 8000 mg glucose by IV, 380 mg by IM, and 300 mg by SC routes [113].

Trehalase deficiency is found in certain individuals and is more prevalent in certain ethnic groups (e.g., Inuits). Orally ingested trehalose in these individuals can result in bloating, cramps, or diarrhea. No specific information about IV tolerance of these individuals for trehalose could be found, but trehalose is excreted in urine. When rats were given trehalose IV at doses of 0.5 or 1 g/kg, 87% of dose was recovered from the urine indicating little or no trehalase activity in the liver and kidneys. On the other hand, for the same doses given to guinea pigs and rabbits 7–9% of dose was recovered in urine. Infusion of a 10% solution of trehalose to rabbits for 90 min at 6.7 mL/kg/h led to a rapid increase in serum glucose concentration that returned to normal 90 min after cessation of infusion. Only 1% of the infused trehalose was recovered in the urine [114].

In acute toxicity studies with trehalose, the IV 50% lethal dose (LD50) in mice, rats, and dogs was greater than 1000 mg/kg (the only tested dose), with no signs of toxicity in any species [114]. In all species, trehalose was detected in plasma and urine after administration. Repeat-dose IV studies with trehalose in mice and dogs at 1000 mg/kg/day for 14 days were also well tolerated, with increase in serum glucose concentration during the first hour after treatment [114]. In diabetic rabbits, the serum glucose elevation persisted for longer periods [115].

Sucrose is a common additive in stabilization of IV drugs. On oral ingestion, sucrose is readily digested by acid hydrolysis and the enzyme sucrase in the small intestine. Glucose and fructose are then rapidly absorbed into the blood stream. However, IV administered sucrose is not metabolized by the liver and is thus excreted virtually unchanged in urine. The Japanese Pharmaceutical Excipients Directory reports a maximum precedented daily dose of sucrose as 39.84 g by IV and 25 mg by SC routes [113]. Among the many parenteral products that contain sucrose, AmBisome [116] has one of the highest sucrose concentration (900 mg/vial; 72 mg/mL after reconstitution) which corresponds to an intravenous administration of sucrose of approximately 108 mg/kg/day at the high dose of 6 mg active/kg/day. Furthermore, the solution is diluted (from 4 mg active/mL to 1 or 2 mg active/mL) in 5% dextrose prior to infusion. Infusion is recommended to be carried out in 120 min or 60 min if tolerated. No significant adverse effects similar to those reported for IVIG (discussed below) have been reported for AmBisome. Among products dosed via the SC route, Orencia [117] has one of the highest levels of sucrose (170 mg/mL).

High doses of sucrose have been administered in intravenous immunoglobulin (IVIG) products and have been linked to development of acute renal failure. Histology shows vacuolation of the proximal tubules, swelling of the tubular lamina, and occlusion. Sucrose appears to be pinocytosed into renal tubular cells and stored in lysosomes. Renal cells also cannot metabolize sucrose. Accumulation of sucrose in renal tubular cells causes an osmotic gradient with the resulting flow of water in to the cells causing cellular welling and vacuole formation. Swelling leads to narrowing of the tubular lumen, causing intratubular obstruction and acute

renal failure. Susceptibility to osmotic nephritis is greater in older individuals (>65 years) and those with diabetes, renal insufficiency, hypertension, hypovolemia, or those receiving concomitant nephrotoxic medications. IVIG-associated acute renal failure is observed to be reversible, though there have been reports of death in some circumstances. It is not clear whether the acute renal failure results from the presence of sucrose in the formulation itself or the osmolality of the product, or both. A high percentage of sucrose-containing formulations of IVIG reported to cause acute renal failure (73%) also had high osmolality (e.g., 700 mOsm or greater), though it is clear that acute renal failure was also reported in those sucrose-containing formulations that had low osmolality (e.g., 309 mOsm) as well [118–121]. As a precaution, in the context of IVIG therapy, FDA recommends a maximum infusion rate of 3 mg/sucrose/kg/min [122], which may be a useful limit to adhere to for other products also. Formulation scientists should be aware of the potential adverse effects of intravenous sucrose and take steps to avoid them (e.g., evaluate formulation osmolality, knowledge of patient population comorbidities, and concomitant medications).

Sucrose (along with sorbitol, maltitol, and fructose) may also be contraindicated in patients with hereditary fructose intolerance, or in the rare cases of glucose–galactose malabsorption syndrome or congenital sucrose–isomaltase deficiency [123].

3.5 Summary and Recommendations

This chapter has summarized structural, physical, and chemical properties of sucrose and trehalose and of their aqueous solutions. There are commonalities as well as differences in these properties that arise from their conformation, H-bonding characteristics, water-binding ability, polymorphic behavior, solubility, chemical stability etc. Both sugars are well suited to provide solution-state stabilization, as well as cryo- and lyo-protection, for therapeutic proteins as excipients in the formulations. Table 3.4 summarizes some salient aspects of sucrose and trehalose for their use in biotherapeutics, as discussed in this chapter. Although the final assessment is dependent on the individual biotherapeutic, experience suggests that sucrose can generally be considered as suitable in most cases, unless constrained by the pH of the formulation.

Table 3.4 Summary assessment of the use of sucrose or trehalose in biopharmaceutical products (DS = Drug Substance; DP = Drug Product)

	Sucrose	Trehalose
DS	• Caution for solution pH below 5 • High solubility and lower viscosity enable easier processing during compounding (via a stock solution) step after ultrafiltration/diafiltration • Good stabilizer in frozen state • Protein concentration-dependent T'_g • Negligible risk for crystallization even if stored above T'_g	• Negligible risk for acid hydrolysis • Lower solubility and higher viscosity (at higher concentrations compared to sucrose) constrain compounding (via a stock solution) step after ultrafiltration/diafiltration • Good stabilizer in frozen state • Protein concentration-dependent T'_g • Risk for crystallization if stored above T'_g
DP	• Caution for solution pH below 5 • Good stabilizer in liquid or lyophilized formulation • Certain minimum ratio of protein: sucrose is required for stabilization in lyophilized state • Cycles may be long unless protein concentration is high or a crystalline bulking agent such as mannitol is added • Lyophilized state T_g is dependent on moisture content but generally lower than for trehalose • Consideration of dose of sucrose in diabetic patients	• Negligible risk for acid hydrolysis • Good stabilizer in liquid or lyophilized formulation • Certain minimum ratio of protein: trehalose is required for stabilization in lyophilized state • Cycles may be long unless protein concentration is high • Bulking agent requiring crystallization by annealing should be added with caution • Lyophilized state T_g is dependent on moisture content but generally higher than for sucrose • Consideration of dose of resultant glucose in diabetic patients

References

1. Peric-Hassler L, et al. Conformational properties of glucose-based disaccharides investigated using molecular dynamics simulations with local elevation umbrella sampling. Carbohydr Res. 2010;345(12):1781–801.
2. Winther LR, Qvist J, Halle B. Hydration and mobility of trehalose in aqueous solution. J Phys Chem B. 2012;116(30):9196–207.
3. Lerbret A, et al. How homogeneous are the trehalose, maltose, and sucrose water solutions? An insight from molecular dynamics simulations. J Phys Chem B. 2005;109(21):11046–57.
4. Miller DP, de Pablo JJ. Calorimetric solution properties of simple saccharides and their significance for the stabilization of biological structures. J Phys Chem B. 2000;104:8876–83.
5. Ekdawi-Sever NC, Conrad PB, de Pablo JJ. Molecular simulations of sucrose solutions near the glass transition temperatures. J Phys Chem A. 2001;105:734–42.
6. Ekdawi-Sever NC, et al. Diffusion of sucrose and α, α-trehalose in aqueous solutions. J Phys Chem A. 2003;107:936–43.
7. Mathlouthi M. X-ray diffraction study of the molecular association in aqueous solutions of D-fructose, D-glucose and Sucrose. Carbohydr Res. 1981;91:113–23.
8. Sapir L, Harries D. Linking trehalose self-association with binary aqueous solution equation of state. J Phys Chem B. 2011;115(4):624–34.
9. Bordat P, et al. Comparative study of trehalose, sucrose and maltose in water solutions by molecular modelling. Europhys Lett. 2004;65(1):41–7.

10. Branca C, et al. Tetrahedral order in homologous disaccharide-water mixtures. J Chem Phys. 2005;122(17):174513.
11. Branca C, et al. Vibtrational studies on disaccharides/H_2O systems by inelastic neutron scattering, Raman, and IR spectroscopy. J Phys Chem B. 2003;107(6):1444–51.
12. Branca C, et al. α,α-Trehalose/water solutions. 5. Hydration and viscosity in dilute and semidilute disaccharide solutions. J Phys Chem B. 2001; 105:10140–45.
13. Magazu S, Migliardo F, Telling MT. Alpha, alpha-trehalose-water solutions. VIII. Study of the diffusive dynamics of water by high-resolution quasi elastic neutron scattering. J Phys Chem B. 2006;110(2):1020–5.
14. Magazu S, Migliardo F, Telling MT. Study of the dynamical properties of water in disaccharide solutions. Eur Biophys J. 2007;36(2):163–71.
15. Pagnotta SE, et al. Water and trehalose: how much do they interact with each other? J Phys Chem B. 2010;114(14):4904–8.
16. Kan Z, Yan X, Ma J. Conformation dynamics and polarization effect of alpha, alpha-trehalose in a vacuum and in aqueous and salt solutions. J Phys Chem A. 2015;119 (9):1573–89.
17. Lee SL, Debenedetti PG, Errington JR. A computational study of hydration, solution structure, and dynamics in dilute carbohydrate solutions. J Chem Phys. 2005;122 (20):204511.
18. Lupi L, et al. Hydration and aggregation in mono- and disaccharide aqueous solutions by gigahertz-to-terahertz light scattering and molecular dynamics simulations. J Phys Chem B. 2012;116(51):14760–7.
19. Burakowski A, Glinski J. Hydration numbers of nonelectrolytes from acoustic methods. Chem Rev. 2012;112(4):2059–81.
20. Dey PC, et al. Apparent molar volume and viscosity studies on some carbohydrates in solution. Montashefte fur Chemie. 2003;134:797–809.
21. Sato Y, Miyawaki O. Relationship between proton NMR relaxation time and viscosity of saccharide solutions. Food Sci Technol. 2000;6(2):136–9.
22. Kawai H, et al. Hydration of oligosaccharides: anomalous hydration ability of trehalose. Cryobiology. 1992;29(5):599–606.
23. Sola-Penna M, Meyer-Fernandes JR. Stabilization against thermal inactivation promoted by sugars on enzyme structure and function: why is trehalose more effective than other sugars? Arch Biochem Biophys. 1998;360(1):10–4.
24. Miller DP, de Pablo JJ, Corti H. Thermophysical properties of trehalose and its concentrated aqueous solutions. Pharm Res. 1997;14(5):578–90.
25. Sussich F, et al. Reversible dehydration of trehalose and anhydrobiosis: from solution state to an exotic crystal? Carbohydr Res. 2001;334(3):165–76.
26. Kawai K, et al. Comparative investigation by two analytical approaches of enthalpy relaxation for glassy glucose, sucrose, maltose, and trehalose. Pharm Res. 2005;22(3):490–5.
27. Hatley RH. Glass fragility and the stability of pharmaceutical preparations–excipient selection. Pharm Dev Technol. 1997;2(3):257–64.
28. Cesaro A, De Giacomo O, Sussich F. Water interplay in trehalose polymorphism. Food Chem. 2008;106:1318–28.
29. Mathpal R, et al. Intermolecular forces of sugars in water. Montashefte fur Chemie. 2006;137:375–9.
30. Makower B, Dye WB. Equilibrium moisture content and crystallization of amorphous sucrose and glucose. Agric Food Chem. 1956;4(1):72–7.
31. Roos YH, et al. Melting and crystallization of sugars in high-solids systems. J Agric Food Chem. 2013;61(13):3167–78.
32. Saleki-Gerhardt A, Zografi G. Non-isothermal and isothermal crystallization of sucrose from the amorphous state. Pharm Res. 1994;11(8):1166–73.
33. te Booy MPWM, de Ruiter RA, de Meere ALJ. Evaluation of the physical stability of freeze-dried sucrose-containing formulaiton by differential scanning calorimetry. Pharm Res. 1992;9(1):109–14.

34. Patyk E, et al. High-pressure (+)-sucrose polymorph. Angew Chem Int Ed Engl. 2012;51 (9):2146–50.
35. Kilburn D, Sokol PE. Structural evolution of the dihydrate to anhydrate crystalline transition of trehalose as measured by wide-angle X-ray scattering. J Phys Chem B. 2009;113 (7):2201–6.
36. Kilburn D, et al. Organization and mobility of water in amorphous and crystalline trehalose. Nat Mater. 2006;5(8):632–5.
37. Willart JF, et al. Vitrification and polymorphism of trehalose induced by dehydration of trehalose dihydrate. J Phys Chem B. 2002;106(13):3365–70.
38. Cesaro A. Carbohydrates: all dried up. Nat Mater. 2006;5(8):593–4.
39. Jones MD, et al. Dehydration of trehalose dihydrate at low relative humidity and ambient temperature. Int J Pharm. 2006;313(1–2):87–98.
40. Pyszczynski SJ, Munson EJ. Generation and characterization of a new solid form of trehalose. Mol Pharm. 2013;10(9):3323–32.
41. Surana R, Pyne A, Suryanarayanan R. Effect of preparation method on physical properties of amorphous trehalose. Pharm Res. 2004;21(7):1167–76.
42. Iglesias HA, Chirife J, Buera MP. Adsorption isotherm of amorphous trehalose. J Sci Food Agric. 1997;75:183–6.
43. Sundaramurthi P, Patapoff TW, Suryanarayanan R. Crystallization of trehalose in frozen solutions and its phase behavior during drying. Pharm Res. 2010;27(11):2374–83.
44. Galmirini MV, et al. Determination and correlation of the water activity of unsaturated, supersaturated and saturated trehalose solutions. LWT. 2008;41:628–31.
45. Sitaula R, Bhowmick S. Moisture sorption characteristics and thermophysical properties of trehalose-PBS mixtures. Cryobiology. 2006;52(3):369–85.
46. O'Brien J, Bladon P. Molecular modelling study of the mechanism of acid-catalyzed disaccharide hydrolysis: implications for nonenzymatic browning reactions. In: O'Brien J et al., editors. The Maillard reaction in foods and medicine. Cambridge, Great Britain: Royal Society of Chemistry; 1998. p. 147–53.
47. O'Brien J. Stability of trehalose, sucrose and glucose to nonenzymatic browning in model systems. J Food Sci. 1996;61(4):679–82.
48. Torres AP, et al. The influence of pH on the kinetics of acid hydrolysis of sucrose. J Food Process Eng. 1994;17:191–208.
49. Wolfenden R, Yuan Y. Rates of spontaneous cleavage of glucose, fructose, sucrose, and trehalose in water, and the catalytic proficiencies of invertase and trehalas. J Am Chem Soc. 2008;130(24):7548–9.
50. Singh SK, Nema S. Freezing and thawing of protein solutions, in formulation and process development strategies for manufacturing biopharmaceuticals. In: Jameel F, Hershenson S, editors. New York: Wiley; 2010. p. 625–75.
51. Suji G, Sivakami S. Glucose, glycation and aging. Biogerontology. 2004;5(6):365–73.
52. Bunn HF, Higgins PJ. Reaction of monosaccharides with proteins: possible evolutionary significance. Science. 1981;213(4504):222–4.
53. McPherson JD, Shilton BH, Walton DJ. Role of fructose in glycation and cross-linking of proteins. Biochemistry. 1988;27(6):1901–7.
54. Fischer S, Hoernschemeyer J, Mahler HC. Glycation during storage and administration of monoclonal antibody formulations. Eur J Pharm Biopharm. 2008;70(1):42–50.
55. Gadgil HS, et al. The LC/MS analysis of glycation of IgG molecules in sucrose containing formulations. J Pharm Sci. 2007;96(10):2607–21.
56. Li S, et al. Effects of reducing sugars on the chemical stability of human relaxin in the lyophilized state. J Pharm Sci. 1996;85(8):873–7.
57. Karel M, Labuza TP. Nonenzymatic browning in model systems containing sucrose. J Agric Food Chem. 1968;16(5):717–9.
58. Oku K, et al. NMR and quantum chemical study on the OH…pi and CH…O interactions between trehalose and unsaturated fatty acids: implication for the mechanism of antioxidant function of trehalose. J Am Chem Soc. 2003;125(42):12739–48.

59. Anonymus. Functional properties of sugar. [cited 2017 March]; Available from: http://www.sugar.ca/Nutrition-Information-Service/Health-professionals/Functional-Properties-of-Sugar.aspx.
60. Mathlouthi M, Reiser P. Sucrose: Properties and Applications. London, UK: Chapman and Hall.
61. Chi EY, et al. Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation. Pharm Res. 2003;20(9):1325–36.
62. Timasheff SN. Protein-solvent preferential interactions, protein hydration, and the modulation of biochemical reactions by solvent components. Proc Natl Acad Sci USA. 2002;99 (15):9721–6.
63. Hedoux A, et al. Analysis of sugar bioprotective mechanisms on the thermal denaturation of lysozyme from Raman scattering and differential scanning calorimetry investigations. J Phys Chem B. 2006;110(45):22886–93.
64. Lins RD, Pereira CS, Hunenberger PH. Trehalose-protein interaction in aqueous solution. Proteins. 2004;55(1):177–86.
65. Shukla D, Schneider CP, Trout BL. Molecular level insight into intra-solvent interaction effects on protein stability and aggregation. Adv Drug Deliv Rev. 2011;63(13):1074–85.
66. Hedoux A, et al. Evidence of a two-stage thermal denaturation process in lysozyme: a Raman scattering and differential scanning calorimetry investigation. J Chem Phys. 2006;124(1):14703.
67. Kaushik JK, Bhat R. Why is trehalose an exceptional protein stabilizer? An analysis of the thermal stability of proteins in the presence of the compatible osmolyte trehalose. J Biol Chem. 2003;278(29):26458–65.
68. Kim YS, et al. Effects of sucrose on conformational equilibria and fluctuations within the native-state ensemble of proteins. Protein Sci. 2003;12(6):1252–61.
69. Saito S, et al. Effects of ionic strength and sugars on the aggregation propensity of monoclonal antibodies: influence of colloidal and conformational stabilities. Pharm Res. 2013;30(5):1263–80.
70. Rubin J, et al. Gauging colloidal and thermal stability in human IgG1-sugar solutions through diffusivity measurements. J Phys Chem B. 2014;118(11):2803–9.
71. James S, McManus JJ. Thermal and solution stability of lysozyme in the presence of sucrose, glucose, and trehalose. J Phys Chem B. 2012;116(34):10182–8.
72. Laue T. Proximity energies: a framework for understanding concentrated solutions. J Mol Recognit. 2012;25(3):165–73.
73. He F, et al. Screening of monoclonal antibody formulations based on high-throughput thermostability and viscosity measurements: design of experiment and statistical analysis. J Pharm Sci. 2011;100(4):1330–40.
74. He F, et al. Effect of sugar molecules on the viscosity of high concentration monoclonal antibody solutions. Pharm Res. 2011;28(7):1552–60.
75. Arakawa T, Kita Y, Carpenter JF. Protein–solvent interactions in pharmaceutical formulations. Pharm Res. 1991;8(3):285–91.
76. Roos YH, Karel M. Water and molecular weight effects on glass transitions in amorphous carbohydrates and carbohydrate solutions. J Food Sci. 1991;56(6):1676–81.
77. Singh KJ, Roos YH. State transitions and freeze concentration in trehalose-protein-cornstarch mixtures. LWT. 2006;39:930–8.
78. Roos YH. Melting and glass transitions of low molecular weight carbohydrates. Carbohydr Res. 1993;238:39–48.
79. Crowe JH, et al. Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes and biomolecules. Cryobiology. 1990;27:219–31.
80. Singh SK, et al. Frozen state storage instability of a monoclonal antibody: aggregation as a consequence of trehalose crystallization and protein unfolding. Pharm Res. 2011;28(4):873–85.
81. Chang BS, Randall CS. Use of subambient thermal analysis to optimize protein lyophilization. Cryobiology. 1992;29:632–56.

82. Bellavia G, et al. Protein thermal denaturation and matrix glass transition in different protein-trehalose-water systems. J Phys Chem B. 2011;115(19):6340–6.
83. Colandene JD, et al. Lyophilization cycle development for a high-concentration monoclonal antibody formulation lacking a crystalline bulking agent. J Pharm Sci. 2007;96(6):1598–608.
84. Lewis LM, et al. Rational design of freeze-dried formulation for a biologic. Am Pharm Rev; 2007.
85. Miller MA, et al. Frozen-state storage stability of a monoclonal antibody: aggregation is impacted by freezing rate and solute distribution. J Pharm Sci. 2013;102(4):1194–208.
86. Prestrelski SJ, et al. Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. Biophys J. 1993;65(2):661–71.
87. Cleland JL, et al. A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody. J Pharm Sci. 2001;90(3):310–21.
88. Imamura K, et al. Effects of types of sugar on the stabilization of protein in the dried state. J Pharm Sci. 2003;92(2):266–74.
89. Andya JD, Hsu CC, Shire SJ. Mechanisms of aggregate formation and carbohydrate excipient stabilization of lyophilized humanized monoclonal antibody formulations. AAPS Pharmsci. 2003;5(2):E10.
90. Heljo VP, et al. The use of disaccharides in inhibiting enzymatic activity loss and secondary structure changes in freeze-dried beta-galactosidase during storage. Pharm Res. 2011;28(3):540–52.
91. Chang L, et al. Mechanism of protein stabilization by sugars during freeze-drying and storage: native structure preservation, specific interaction, and/or immobilization in a glassy matrix? J Pharm Sci. 2005;94(7):1427–44.
92. Sun WQ, Davidson P, Chan HS. Protein stability in the amorphous carbohydrate matrix: relevance to anhydrobiosis. Biochim Biophys Acta. 1998;1425(1):245–54.
93. Cicerone MT, et al. Substantially improved stability of biological agents in dried form. The role of glassy dynamics in preservation of biopharmaceuticals. Bioprocess Int. 2003;1:36–47.
94. Davidson P, Sun WQ. Effect of sucrose/raffinose mass ratios on the stability of co-lyophilized protein during storage above the Tg. Pharm Res. 2001;18(4):474–9.
95. Duddu SP, Dal Monte PR. Effect of glass transition temperature on the stability of lyophilized formulations containing a chimeric therapeutic monoclonal antibody. Pharm Res. 1997;14(5):591–5.
96. Pikal MJ, et al. Solid state chemistry of proteins: II. The correlation of storage stability of freeze-dried human growth hormone (hGH) with structure and dynamics in the glassy solid. J Pharm Sci. 2008;97(12):5106–21.
97. Katayama DS, et al. Mixing properties of lyophilized protein systems: a spectroscopic and calorimetric study. J Pharm Sci. 2009;98(9):2954–69.
98. Pikal MJ, Rigsbee DR, Roy ML. Solid state chemistry of proteins: I. glass transition behavior in freeze dried disaccharide formulations of human growth hormone (hGH). J Pharm Sci. 2007;96(10):2765–76.
99. Shamblin SL, Zografi G. Enthalpy relaxation in binary amorphous mixtures containing sucrose. Pharm Res. 1998;15(12):1828–34.
100. Taylor LS, Zografi G. Sugar-polymer hydrogen bond interactions in lyophilized amorphous mixtures. J Pharm Sci. 1998;87(12):1615–21.
101. Allison SD, et al. Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. Arch Biochem Biophys. 1999;365(2):289–98.
102. Cicerone MT, Douglas JF. Beta-relaxation governs protein stability in sugar-glass matrices. Soft Matter. 2012;8:2983–91.
103. Cicerone MT, Soles CL. Fast dynamics and stabilization of proteins: binary glasses of trehalose and glycerol. Biophys J. 2004;86(6):3836–45.
104. Bellavia G, et al. Thermal denaturation of myoglobin in water–disaccharide matrixes: relation with the glass transition of the system. J Phys Chem B. 2009;113(33):11543–9.

105. Bhatnagar BS, et al. Freeze drying of biologics. In: Encyclopedia of pharmaceutical science and technology. New York: Taylor and Francis; 2013. p. 1673–1722.
106. Parker A, et al. Determination of the influence of primary drying rates on the microscale structural attributes and physicochemical properties of protein containing lyophilized products. J Pharm Sci. 2010;99(11):4616–29.
107. Johnson RE, Kirchhoff CF, Gaud HT. Mannitol-sucrose mixtures–versatile formulations for protein lyophilization. J Pharm Sci. 2002;91(4):914–22.
108. Weinbuch D, et al. Nanoparticulate impurities in pharmaceutical-grade sugars and their interference with light scattering-based analysis of protein formulations. Pharm Res. 2015;32(7):2419–27.
109. Elbein AD, et al. New insights on trehalose: a multifunctional molecule. Glycobiology. 2003;13(4):17R–27R.
110. Avastin. Prescribing information. 2014.
111. Brayfield A. Martindale: the complete drug reference. Pharmaceutical Press; 2001.
112. Krajicek BJ, Kudva YC, Hurley HA. Potentially important contribution of dextrose used as diluent to hyperglycemia in hospitalized patients. Diabet Care. 2005;28(4):981–2.
113. JPED. Japanese pharmaceutical excipients directory.
114. Abbott PJ, Chen J. Safety evaluation of certain food additives and contaminants: trehalose F.-f.M.o.t.J.F.W.E.C.o.F.A. (JECFA); 2001.
115. Sato S, et al. Trehalose can be used as a parenteral saccharide source in rabbits. J Nutr. 1999;129(1):158–64.
116. AmBisome. Prescribing information; 2012.
117. Orencia. Prescribing information; 2016.
118. Chapman SA, et al. Acute renal failure and intravenous immune globulin: occurs with sucrose-stabilized, but not with D-sorbitol-stabilized, formulation. Ann Pharmacother. 2004;38(12):2059–67.
119. Dantal J. Intravenous immunoglobulins: in-depth review of excipients and acute kidney injury risk. Am J Nephrol. 2013;38(4):275–84.
120. Itkin YM, Trujillo TC. Intravenous immunoglobulin-associated acute renal failure: case series and literature review. Pharmacotherapy. 2005;25(6):886–92.
121. Katz U, et al. Safety of intravenous immunoglobulin (IVIG) therapy. Autoimmun Rev. 2007;6(4):257–9.
122. Epstein JS, Zoon KC. Dear doctor letter—important drug warning: immune globulin intravenous (human). 1998.
123. Anonymus. Fructose and sorbitol containing parenteral solutions should not be used. Curr Prob Pharmacovigilance. 2001; 27:13.
124. Sun WQ, et al. Stability of dry liposomes in sugar glasses. Biophys J. 1996;70(4):1769–76.
125. Crowe LM, Reid DS, Crowe JH. Is trehalose special for preserving dry biomaterials? Biophys J. 1996;71(4):2087–93.
126. Hofmann G, editor. Iscotables: a handbook of data for biological and physical scientists. 8th ed. Lincoln, Nebraska: Instrumentation Supply Company; 1982.
127. Uchida T, Nagayama M, Gohara K. Trehalose solution viscosity at low temperatures measured by dynamic light scattering method: trehalose depresses molecular transportation for ice crystal growth. J Crystal Growth. 2009;311(23–24):4747–52.
128. Longinotti MP, Corti HR. Viscosity of concentrated sucrose and trehalose aqueous solutions including the supercooled regime. J Phys Chem Ref Data. 2008;37(3):1503–15.
129. Mehl PM. Solubility and glass transition in the system alpha-D-trehalose/water. J Therm Anal. 1997;49:817–22.
130. Surana R, Pyne A, Suryanarayanan R. Effect of aging on the physical properties of amorphous trehalose. Pharm Res. 2004;21(5):867–74.

Part III
High Concentration Proteins

Chapter 4
Introduction to High-Concentration Proteins

Wei Wang, Arun Alphonse Ignatius, Satoshi Ohtake and Teng-Chieh Yang

Abstract Monoclonal antibodies (mAbs) have become a major class in protein therapeutics. Many mAbs require development at high concentrations, due to the high dose required for efficacy and patient-preferred self-administration through subcutaneous injection, which is oftentimes limited by dosing volume. Development of high-concentration protein products, however, has been associated with two major challenges—high solution viscosity and enhanced aggregation tendency. These challenges and the relevant strategies to overcome them are discussed herein, and future directions in the development of high-concentration products are proposed.

Keywords Formulation · Viscosity · Aggregation · Stability · Administration

4.1 Overview of Recombinant Therapeutic Proteins

The advent of the recombinant DNA technology in the 1980s led to a rapid expansion of commercial biologic products over the past 30 years, with a growth rate twice as that of the entire pharmaceutical industry. The biologics market

W. Wang (✉) · A. Alphonse Ignatius · T.-C. Yang
Pfizer, 700 Chesterfield Parkway West, Chesterfield, MO 63017, USA
e-mail: wei.wang9@bayer.com

A. Alphonse Ignatius
e-mail: arun.alphonseignatius@Pfizer.com

T.-C. Yang
e-mail: tcyang@uw.edu

W. Wang
Bayer U.S. LLC, 800 Dwight Way, Berkeley, CA 94710, USA

W. Wang · T.-C. Yang
Regeneron Pharmaceuticals, 777 Old Saw Mill River Rd, Tarrytown, NY 10591, USA

S. Ohtake
BioTherapeutics Pharmaceutical Sciences, Pharmaceutical R&D, Pfizer,
700 Chesterfield Parkway West, Chesterfield, MO 63017, USA
e-mail: Satoshi.Ohtake@Pfizer.com

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_4

accounted for about $170 billion in sales in 2012, nearly 18% percent of the overall pharmaceutical market [26]. In regard to the sales volume, there are five biologics in the top 10 global pharmaceutical products. IMS Health forecasts that the global biologics market will reach $200 billion in 2016–2017 and $250 billion by 2020. More importantly, among the predicted top ten selling drugs in 2018, seven are likely to be biologics [26, 92].

Human insulin (Humulin) was the first recombinant protein pharmaceutical products approved by US FDA for diabetic mellitus in 1982, followed by growth hormone (Somatotropin) and several variants of insulin. The following years was a rapid growth period, when many important human protein products were commercialized, including cytokines, interferons, growth factors, blood factors, and enzymes (Table 4.1).

4.1.1 Antibody Therapeutics

Among all protein biotherapeutics, monoclonal antibodies (mAbs) have become a major class—transforming from murine to completely human type and treating various types of diseases, including cancers, chronic inflammatory diseases, infectious diseases, cardiovascular diseases, and several medical conditions such as transplantation [90, 113]. Today, mAbs are the fastest growing class of biotechnology-derived molecules with over 300 candidates in development [2]. As of November 2014, 47 mAbs had been approved by the FDA and EU. Importantly, 70 or more mAb-associated products are expected to be in the market by 2020. mAbs are relatively easy to produce with platform expression systems and purification schemes, and their quality attributes have been standardized relative to other biotechnology products. As therapeutics, mAbs have high target specificity, allowing minimal side effects. In addition, they are relatively stable and easy to engineer to be stand-alone drug molecules, or platforms for other valuable therapeutic agents such as antibody-drug conjugates.

4.1.2 Concentration Ranges of Marketed Biotherapeutics

The concentration ranges of biotherapeutics vary widely. As shown in Table 4.1, the concentration range is 0.25–14.2 mg/mL for growth hormones and growth factors; 0.1–10 mg/mL for most cytokines, except for Kineret at 149 mg/mL; 175–1000 IU/mL for blood factors (different protein concentrations depending on the specificity); 0.58–5 mg/mL for recombinant enzymes; and 1–200 mg/mL for antibodies.

It is obvious that mAbs may need to be developed as high-concentration products. Of the 42 mAbs currently in the market, 14 are high-concentration formulations (>50 mg/mL; see Table 4.1) and 11 of the 14 high-concentration mAbs are

Table 4.1 A list of commercial protein products, administration information, and product concentration

Major class	Trade name	Nonproprietary name	Administration route	Dosage form	Drug volume (mL)	Concentration (mg/mL)
mAb-based	Cimzia	Certolizumab pegol	SC	Liquid (PFS)	1	200
	Actmera	Tociluzimab	IV/SC	Liquid (PFS)	0.9	180
	Ilaris	Canakinumab	SC	Lyophilized	1.2	150
	Xolair	Omalizumab	SC	Liquid (PFS)/ lyophilized	1/0.9	150/144
	Orencia	Abatacept	SC/IV	Liquid (PFS)/ lyophilized	10/1	25/125
	MabThera/ Rituxan	Rituximab	IV/SC	Liquid	10/11.7	10/120
	Herceptin	Trastuzumab	IV	Lyophilized/liquid	3/5	20/120
	Synagis	Palivizumab	IM	Liquid/lyophilized	0.5/1	100/100
	Raptiva	Efalizumab	SC	Lyophilized	1.5	100
	Simponi	Golimumab	SC	Liquid (PFS)	0.5	100
	Stelara	Ustekinumab	SC	Liquid (PFS)	0.5	90
	Benlysta	Belimumab	IV	Lyophilized	1.5	80
	Ranmark	Denosumab	SC	Liquid (PFS)	1.7	70
	Prolia	Denosumab	SC	Liquid (PFS)	1	60
	Humira	Adalimumab	SC	Liquid (PFS)	0.4	50
	Raxibacumab	Raxibacumab	SC	Liquid	50	50
	Enbrel	Etanercept	SC	Liquid (PFS)	0.5	50
	Eylea	Aflibercept	IVT	Liquid	0.278	40
	Amevive	Alefacept	IV/IM	Lyophilized	0.5	30
	Campath	Alemtuzumab/Abatacept	IV	Liquid	1	30

(continued)

Table 4.1 (continued)

Major class	Trade name	Nonproprietary name	Administration route	Dosage form	Drug volume (mL)	Concentration (mg/mL)
	Perjeta	Pertuzumab	IV	Liquid	14	30
	Avastin	Bevacizumab	IV	Liquid	4	25
	Gazyva	Obinutuzumab	IV	Liquid	40	25
	Kadcyla	Ado-trastuzumab emtansine	IV	Lyophilized	5	20
	Tysabri	Natalizumab	IV	Liquid	15	20
	Arzerra	Ofatumumab	IV	Liquid	5	20
	Vectibix	Panitumumab	IV	Liquid	5	20
	Actemra	Tocilizumab	IV	Liquid	4	20
	Bexxar	Tositumomab	IV	Liquid	2.5	14
	Soliris	Eculizumab	IV	Lyophilized	30	10
	Lucentis	Ranibizumab	IVT	Liquid	0.23	10
	Yervoy	Ipilimumab	IV	Liquid	40	5
	Simulect	Basiliximab	IV	Lyophilized	5	4
	Vectibix	Mogamulizumab	IV	Liquid	5	4
	Zevalin	Ibritumomab tiuxetan	IV	Liquid	2	2
	ReoPro	Abciximab	IV	Liquid	5	2
	Mylotarg	Gemtuzumab ozogamicin	IV	Lyophilized	5	1
Hormones, growth factors	Levemir	Insulin detemir	SC	Liquid (PFS)	3	14.2
	Increlex	Mecasermin (IGF-1)	SC	Lyophilized	4	10
	Zorbtive	Somatropin	SC	Lyophilized	1	8.8
	Omnitrope	Somatropin	SC	Lyophilized	1.14	5
	Accretropin	Somatropin	SC	Liquid	1	5

(continued)

Table 4.1 (continued)

Major class	Trade name	Nonproprietary name	Administration route	Dosage form	Drug volume (mL)	Concentration (mg/mL)
	Kepivance	Palifermin (KGF)	IV	Lyophilized	1.2	5
	Novolog	Insulin aspart	SC	Liquid (PFS)	3	3.5
	Apidra	Insulin glulisine	SC	Liquid (PFS)	3	3.5
	Lantus	Insulin glargine	SC	Liquid (cartridge)	3	3.5
	Valtropin	Somatropin	SC	Lyophilized	1.5	3.33
	Infuse	Dibotermin alfa (BMP-2)	Graft	Lyophilized	1	1.5
	Ovidrel	Choriogonadotropin alpha	SC	Liquid (PFS)	0.51	0.5
	Natrecor	Nesiritide (BNP)	IV	Lyophilized	5	0.3
	Forteo	Teriparatide (PTH 1-34)		Liquid (PFS)	2.4	0.25
Cytokines	Kineret	Anakinra (IL-1Ra)		Liquid (PFS)	0.67	149.3
	Neulasta	Pegfilgrastin (G-GCSF PEG)	SC	Liquid (PFS)	0.6	10
	Neumega	Oprelvekin (rhIL-11)	SC	Lyophilized	1	5
	Rebif	Interferon beta-1a	SC	Liquid (PFS)	0.5	0.88
	Aranesp	Darbepoetin alfa	IV/SC	Liquid (PFS)	1	0.5
	Pegasys	Peginterferon alfa-2a	SC	Liquid (PFS)	0.5	0.36
	Pegintron	Peginterferon alfa-2b	SC	Liquid (PFS)	0.5	0.3
	Mircera	Methoxypolyethylene glycol epoetin beta	IV/SC	Liquid (PFS)	0.6	0.13
	Dynepo	Epoetin delta	IV/SC	Liquid (PFS)	0.5	10,000 IU
Blood factors	TNKase	Tenecteplase	IV	Lyophilized	10	5
	Xigris	Drotrecogin alfa	IV	Lyophilized	10	2
	Nplate	Romiplostim	SC	Lyophilized	1.2	0.5
	Benefix	Factor IX	IV	Lyophilized	5	3000 IU/mL

(continued)

Table 4.1 (continued)

Major class	Trade name	Nonproprietary name	Administration route	Dosage form	Drug volume (mL)	Concentration (mg/mL)
	Recothrom	Thrombin, dermal	Topical	Lyophilized	5	1000 units/mL
	Advate	Antihemophilic factor (FVIII plasma/ albumin free)	IV	Lyophilized	5	800 IU/mL
	Refacto	Antihemophilic factor (FVIII B deleted)	IV	Lyophilized	4	500 IU/mL
	Xyntha	Antihemophilic factor (FVIII B deleted)	IV	Lyophilized	4	500 IU/mL
	Atryn	Antithrombin III	IV	Lyophilized	10	175 IU/mL
Enzymes	Fabrazyme	Agalsidase beta	IV	Lyophilized	7	5
	Myozyme	Alglucosidase alfa	IV	Lyophilized	10	5
	Elaprase	Idursulfase	IV	Liquid	3	2
	Elitek	Rasburicase (Urate oxidase)	IV	Lyophilized	5	1.5
	Naglazyme	Galsulfase	IV	Liquid	5	1
	Aldurazyme	Laronidase (alpha-L-Iduronidase)	IV	Liquid	5	0.58
	Hylenex	Hyaluronidase	SC	Liquid	1	150 units/mL

administered by s.c., one by i.v. and one by both i.v. and s.c. To date, certolizumab is the highest concentration product (200 mg/mL) available in the market as a liquid product in a prefilled syringe for the treatment of Crohn's disease by s.c. injection.

4.2 Need for High-Concentration Protein Products

There has been a growing need for developing high-concentration protein products. The need is mainly driven by the following considerations—route of administration, high dose requirement, long-term drug administration, convenience (and compliance), and manufacturing cost.

4.2.1 Routes of Administration

As shown in Table 4.1, the administration routes for protein therapeutics are essentially either intravenous (i.v.) or subcutaneous (s.c.) [80]. Between these two routes, s.c. administration is the preferred route of administration. While i.v. infusion or injection typically requires drug administration by a health care practitioner (HCP), therapeutics deliverable via s.c. route can be provided to patients for convenient self-administration. Since delivery via the s.c. route is generally limited to small volumes for ease of administration and patient acceptance, high-concentration protein products may have to be developed to minimize the product volume to accommodate this more patient-friendly administration route.

In addition to the two common administration routes, the biologics industry continues to explore alternative, non- or less-invasive and patient-friendly delivery approaches, including oral [25, 74, 93], pulmonary [10, 39, 89], nasal [3, 59], buccal [5, 81], rectal [27, 112], and transdermal delivery [45, 77] for proteins, as summarized in Table 4.2. The goal for exploring these alternatives is to use the therapeutic proteins in the most efficient/convenient manner possible (route of administration) while minimizing the impact on the quality of life of the patients (frequency of drug administration and injection-related discomfort). Owing to the large size and relatively hydrophilic nature of proteins, however, membrane permeation of proteins is extremely low, leading to low bioavailability, a key hurdle for successful enablement of these alternative delivery options [80]. Many approaches were tried to improve the low bioavailability of proteins via alternative routes, such as the use of penetration enhancers [121]. It is well known that the membrane permeability of diffusive drugs can be significantly enhanced if the local drug concentration is high (high-concentration gradient). It is conceivable that a high local protein concentration at the administration site would be required to improve the protein permeability and bioavailability. Hence, development of high-concentration protein products may be required for achieving alternative protein delivery.

Table 4.2 Alternative delivery/administration routes for proteins (adapted from Moeller 2008)

Delivery pathway	Oral	Pulmonary	Nasal	Buccal	Transdermal
Potential benefits	Non-invasive; ease of administration	Non-invasive; ease of administration; rapid on-set of action	Non-invasive; ease of administration; rapid on-set of action; circumvents hepatic first-pass metabolism; gateway to brain delivery	Non-invasive; ease of administration; rapid on-set of action; circumvents hepatic first-pass metabolism	Ease of administration
Challenges	Large doses necessary if low bioavailability	Possible toxicity or safety problems for particulate formulation; inconsistent dosing	Enhancers in the formulation often necessary	Enhancers in the formulation often necessary	Can be relatively invasive; enhancers in the formulation often necessary
Example of technologies (associated companies)	Eligen (Emisphere) [51] LPM ™ (DOR Biopharm) [50]	Promaxx (Baxter Healthcare Corporation) [47, 50] Technosphere (ManKind Corporation) [53]	CPE-215® (CPEX Pharmaceuticals) [48, 49] Intravail (Aegis Therapeutics) [46, 55]	Rapidmist TM (Generex) [52] Thiomers (ThioMatrix) [55]	TDS® (TransDermal Technologies, Inc) [56] TPM-02 (Phosphagenics) [54]

4.2.2 High Dose Requirement

Many proteins require a high dose to be effective. This is especially true for mAbs. A high blood mAb concentration is often needed to drive their diffusion to the target tissue and interact adequately with the target antigens (e.g., tumor-associated surface antigens). Frequently, a dose for a mAb exceeds 1 mg/kg patient weight. If the s.c. route is preferred, a higher dose is needed to achieve a desired plasma mAb concentration and to compensate for the relatively poor bioavailability of mAbs upon administration in the subcutaneous space due to their large size and relative hydrophilicity [32]. The actual dose requirement may mandate a mAb product concentration exceeding 100 mg/mL, assuming a preferable injection volume of 1 mL or less. A higher injection volume may be required to accommodate a concentration-dependent limitation on solution viscosity or stability of the protein. An injection volume >2.0 mL is generally not preferred due to major concerns on injection volume-induced pain [63] and possible back pressure-induced product leakage at the injection site [37]. The s.c. route of administration, along with its volume limitation, is a critical factor to be considered for product development.

Alternative options such as the use of a patch pump could allow delivery of a significantly higher volume [67, 102]. Although such an alternative delivery method may not require a high-concentration protein product, a patch pump may require sophisticated control on the drug delivery mechanism, a complex drug reservoir or loading procedure, and the device may be costly relative to vials or prefilled syringes. It should be mentioned that recombinant hyaluronidase could be used in a protein formulation to facilitate tissue diffusion, allowing administration of a larger-than-normal injection volume [11]. Detailed discussion of these alternative administration methods is outside the scope of this chapter.

4.2.3 Long-Term Drug Administration

Management of chronic diseases often requires frequent and long-term drug administration. Both long-term i.v. and s.c. therapies require sacrifice and commitment from patients than would be required for an oral therapeutic, as is typical for a small molecule drug. Patient acceptance and compliance are important factors to consider, particularly for chronic use of protein drugs, as this will impact their quality of life. While protein biotherapeutics are more difficult to develop for non-parenteral administration, s.c. administration is generally favored over i.v. infusion because it allows easier administration and often can be performed at home or anywhere while traveling. Patient compliance can be significantly improved, if a user-friendly prefilled syringe or an auto-injector is provided for drug administration. The availability of a variety of insulin products for self-administration is a successful story for diabetic patients [71]. A high-concentration protein product with a low injection volume for s.c. administration is highly preferred. i.v.

administration may also benefit from high-concentration protein formulations because the lower infusion volume affords a shorter time for infusion (assuming no tolerance issue) and also provides convenience for long-term drug administration.

4.2.4 Manufacturing Cost

Drug product manufacturing processes often include freezing/storage/shipping of a drug substance, thawing of the drug substance, compounding, filling, and possibly freeze-drying and reconstitution. Preparation of a high-concentration drug substance in a lower volume may facilitate freezing/storage/shipping of a large quantity of drug substance. Thawing/filtration/filling/freeze-drying of a higher-concentration protein product may take less time, thus potentially reducing the total cost of drug product manufacturing. On the other hand, protein concentration through UF/DF operation can facilitate protein aggregation for membrane- and/or shear-sensitive proteins. Sampling volumes may need to be balanced to minimize the loss of product and to meet the analytical assay requirement.

4.3 Overall Challenges in Developing High-Concentration Products

Proteins are generally not stable relative to small molecules. The physical and chemical behavior of a protein product may change dramatically with increasing concentrations. This section discusses the theoretical aspects of high-concentration proteins, consequences of protein–protein interactions at high concentrations, influencing factors and the overall challenges.

4.3.1 Theoretical Considerations

As discussed above, a high-concentration product is often needed to minimize the injection volume and thereby enable s.c. administration and its inherent advantages. Theoretically, what is the maximum protein concentration one could achieve in a solution? This question can be addressed by making several assumptions. Assuming proteins can be packed as densely as a single protein with no free space, the maximum concentration would be 1350 mg/mL, equivalent to the average protein density reported [34]. Assuming proteins are hard spheres, the maximum concentration would be $1350 \times 63.4\% = 856$ mg/mL, where 63.4% is the maximum packing density of hard spheres [107]. A slightly lower protein concentration of approximately 700 mg/mL was found in dense but colloidally stable nanoclusters in

solutions [62]. If all protein molecules are dispersed with a gyration volume, the maximum concentration of a mAb would be ~476 mg/mL, based on a radius of 5 nm as determined [127]. In reality, high protein concentrations have been achieved up to 350 mg/mL for lysozyme [40], 380 mg/mL for IgGs [15], and 400 mg/mL for bovine serum albumin [28].

Protein–protein interactions can become significant when the distance between proteins falls into the range of the molecular size at high protein concentrations. Yadav et al. [125] calculated the average surface-to-surface separation distance in mAbs to be a few (1–5 nm) nanometers at 100–200 mg/mL. This essentially brings the molecules to close proximity for possible interactions. The type of protein–protein interactions and their distance dependency have been summarized [69]. In general, the major types of interactions include electrostatic, hydrophobic, steric, and hydrogen-bonded interactions [69, 75, 82, 101]. Dipole–dipole interactions were also shown to play an important role [106].

Combination of these different types of interactions can lead to either an overall attractive or repulsive interaction. These two opposite types of interactions can be measured by two major parameters—osmotic second virial coefficient (B_{22}) and diffusion interaction parameter (k_D). Theoretically, the second virial coefficient, B_{22}, can be calculated based on potential protein–protein interaction energies [29]. These interaction energies are contributed mainly by—(i) electrostatic interaction based on the effective charges, (ii) electrostatic desolvation when charges are buried by an approaching group, and (iii) van der Waals and hydrophobic interactions. In calculating the theoretical B_{22} values, the steric interaction (orientational dependence) can contribute significantly in addition to the above-mentioned molecular forces [82]. B_{22} and k_D are theoretically linked, and clear relationships were also established as shown in several studies [113]. It is argued that B_{22} may be more predictive in assessing colloidal stability [113] and high-concentration-induced viscosity [98], but in reality, both parameters have limitations in predicting high-concentration behavior, as they were generally acquired within a low concentration range and may change its sign as the concentration increases [98]. Indeed, the relative B_{22} values of ovalbumin did not correlate well with aggregation tendency at 37 °C at different pH values [6, 14]. The third virial coefficient representing multi-body interactions was rarely evaluated and could make a significant contribution in protein aggregation [9].

Attractive protein–protein interactions can lead to self-association. Many studies have shown that proteins can self-associate into reversible oligomers of different sizes. These sizes range from dimers [18, 60, 103], trimers [30, 61], 4–6mers [103], or variable sizes of protein clusters consisting of $\geq$6mers [30, 31], 2–9ers IgG1 [73], and 5–45mers [19], depending on the concentration and solution conditions. It is a combination of short-range attraction and long-range repulsion that result in the formation of small equilibrium clusters of limited sizes [38, 109]. A clear link was demonstrated between the undesirable high viscosity of some mAb solutions and the formation of reversible clusters with extended open structures [127]. Stronger self-associations, especially in the presence of hydrophobic interactions, would lead to irreversible aggregation/precipitation with limited solubility, such as an IgG1 [8].

4.3.2 Physical Properties and Influencing Factors of High-Concentration Proteins

As discussed above, increasing protein concentration also increases the chance of protein–protein interactions, especially attractive interactions, leading to significantly different physical and chemical properties of the protein [101]. Three important physical properties of protein solutions are solution viscosity, protein conformation, and aggregation tendency.

4.3.2.1 Solution Viscosity

Proteins are polymers of a variety of amino acids. As the protein concentration increases, an observable property of the solution is the increased solution viscosity. Concentration-dependent solution viscosity can be described by Eq. 4.1 [68]:

$$\eta = [\eta]C + k_h[\eta]^2C^2, \tag{4.1}$$

where η is the specific viscosity, $[\eta]$ is the intrinsic viscosity, C is the polymer concentration, and k_h is the Huggins constant. As shown in Eq. 4.1, increasing the concentration can increase the viscosity exponentially. The exponential concentration dependency is demonstrated in many studies in the absence of apparent protein association [72, 75, 127]. The concentration dependency of solution viscosity is equally applicable for protein mixtures [35].

Protein self-association can occur at a relatively low concentration. An IgG1 antibody was shown to self-associate (to oligomers) above a critical concentration of 16.5 mg/mL at a low-ionic strength [85]. Self-association leads to a dramatic increase in solution viscosity due to formation of a network in solution [64] and, in extreme cases, gelation [17]. The concentration dependency of solution viscosity of two mAbs is shown in Fig. 4.1, where two mAbs showed clear exponential increase in viscosity with increasing concentrations. The difference in viscosity is likely due to a difference in the degree of self-association.

The self-association-induced dramatic increase in solution viscosity can be attributed to changes in these two parameters—(1) alteration of the Huggins constant, which is proportional to the apparent protein–protein association constant [87], and (2) alternation of the apparent molecular weight of the protein. The intrinsic viscosity $[\eta]$ is exponentially proportional to the molecular weight of a polymer according to the following Mark–Houwink equation [68]:

$$[\eta] = KM^{\alpha} \tag{4.2}$$

where K and α are constants for a specific polymer and solvent system, respectively. For a semi-flexible polymer like proteins, α is likely >0.8. In reality, many studies have shown that protein self-association or cluster formation is linked to higher

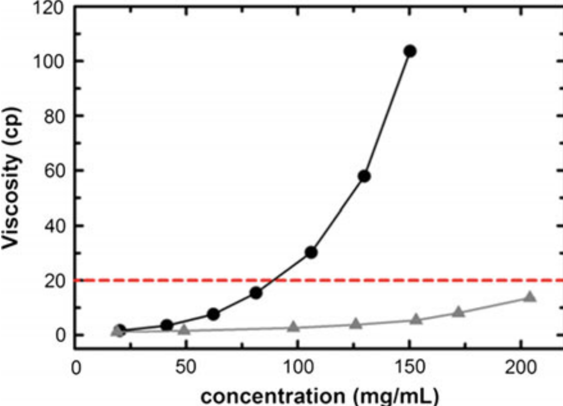


Fig. 4.1 Concentration dependency of two mAbs (adapted from Li et al. [72])

solution viscosity [64, 88, 117, 127]. The size of protein clusters appears to correlate with the solution viscosity in mAbs [73, 129]. Recent data showed that an acidic Fv region could facilitate self-association [72].

On the other hand, repulsive interactions (positive B_{22}) could still lead to high viscosity potentially due to contribution of electroviscous forces, as observed for a IgG1 mAb at a low-ionic strength [99]. Although electroviscous forces consist of different levels of interactions—primary (zeta potential and particle size), secondary (overlapping electrical double layer), and tertiary (change in particle size/shape) [97], this effect has not been fully recognized as an important factor for high protein viscosity, as such effects alone failed to explain the viscosity differences for many mAbs [22, 124].

4.3.2.2 Protein Conformation

A protein solution at high concentrations is a crowded environment. Theoretically, molecular crowding should favor formation of compact conformations over extended conformations, promoting protein stabilization. On the other hand, such an environment should favor both specific (formation of well-defined oligomers) and non-specific (formation of large aggregates) macromolecular associations [79]. In reality, protein–protein interactions may or may not alter the protein conformation. Both repulsive and attractive interactions in mAbs have been found to have no significant effect on protein conformations within the concentration range from 50 to 150 mg/mL [128]. Increasing the concentration of two IgG1's has very little effect on the structure of the antibodies in a solution containing 0.1 M NaCl and 40 mM citrate-phosphate buffer at pH 6 [42]. In comparison, the T_m of several protein candidates was significantly increased, e.g., by 2 °C for a mAb at 177 mg/mL in the presence of a sucrose crowder [43], 2–3 °C for two IgG1's at 100 mg/mL [42], and ~8 °C for hemoglobin at 245 mg/mL [40]. Similarly, hemoglobin and fibrinogen demonstrated higher transition midpoints (T_ms) in more concentrated

solutions (ΔT_m approximately 2–10 °C), while lysozyme and BSA in concentrated solutions exhibited a lower T_m than in dilute solutions (ΔT_m approximately 2–20 °C) [40].

4.3.2.3 Aggregation Tendency

In general, the aggregation tendency of a protein would be enhanced due to the increased protein–protein interactions at high protein concentrations. It is reported that the collision frequency in high-concentration protein solutions is $\sim 10^9$–10^{10} collisions per second [69]. In addition, formation of reversible oligomers was regarded as the initiation step for formation of irreversible aggregates [101]. Many studies have shown that aggregation is accelerated at higher protein concentrations such as thermally induced aggregation and gelation of BSA at 330 mg/mL [40], and the aggregation of two IgG1's at 100 mg/mL [42]. On the other hand, concentration-dependent stabilization of protein conformation may lead to a reduction in protein aggregation tendency at higher protein concentrations, as observed for the temperature-induced protein aggregation of hemoglobin at 245 mg/mL relative to 0.3 mg/mL [40].

Enhanced aggregation may lead to increased formation of particulates. However, the contribution of particulates on solution viscosity should be insignificant as revealed by the following relationship between the amount of particles and the viscosity of a suspension [97]:

$$\eta = \eta_0(1 + 2.5\phi)$$

where η_0 is the solvent viscosity and ϕ is the volume fraction of particles.

4.3.2.4 Factors Influencing Protein–Protein Interactions

The type of protein–protein interactions is mainly dictated by the protein's structure but can be strongly influenced by the solution conditions. Major solution factors include pH, ionic strength, and presence of potential interacting excipients [122]. Solution pH dictates the type and number of charges on the protein surface, influencing mainly the electrostatic interactions. For example, the formation of reversible dimer in IgG preparations increases with an increasing pH from acidic to neutral pH [84]. All three IgG1 monoclonal antibodies (pI = 7.6–8.6) are more associative at pH 6 than at pH 4.4 at a low-ionic strength (<25 mM) [110], and an IgG1 increased self-association with an increasing pH from 5 to 8 (pI > 9) [31]. A more complex pH-dependent self-association behavior was found for three IgG4 antibodies [111]. It was also shown that varying pH (and ionic strength) can influence B_{22} by influencing protein configurations [82].

Ionic strength is another important factor affecting the protein–protein interactions. In general, higher ionic strengths weaken interactions at a long range

proportionally more than shorter-ranged interactions [69]. Due to this screening effect, the Debye length (k^{-1}) of 9.6 nm at 1 mM ionic strength was reduced to 2.5 nm at 15 mM ionic strength in mAb solutions [125]. Increasing NaCl concentration can, therefore, minimize both attractive and repulsive interactions in mAbs [103].

Such effects can lead to a change in the degree of self-association or the apparent molecular size of IgGs [31, 86, 99, 110]. Different ionic strengths can be achieved through the use of different salts or different buffering agents [31, 122]. The effect can be so significant that addition of NaCl resulted in a change from repulsive to attractive interactions [83, 125]. Increasing the ionic strength (>100 mM) actually reversed the pH-dependent trend of antibody self-association [110, 111]. Reduction of the ionic strength of an IgG1 mAb solution led to formation of opalescence and liquid–liquid phase separation (>10X difference in light and heavy protein phases) [85].

4.3.3 Major Challenges

The challenges in the development of high-concentration protein products were widely recognized [23, 105, 120]. These challenges can impact three major product development stages/aspects—formulation development, process development, and drug administration.

4.3.3.1 Formulation Development

Three major issues are associated with formulation of high-concentration protein products—enhanced aggregation, limited solubility, and phase separation. As discussed above, protein self-association at high concentrations is the major cause for these issues. Protein aggregation should be strictly controlled, as such aggregates have a potential to alter the product's activity, toxicity, and immunogenicity [13, 16, 95, 119]. Limited protein solubility may also present a significant challenge, especially for mAb products, which often require a higher dose. For example, the solubility of a particular IgG1 was found only to be ~13 mg/mL at pH 7.2 (pI = 7.4–7.5) [123].

Traditional formulation approaches can be effectively used to minimize protein self-association or aggregation, e.g., selection of proper formulation pH, ionic strength, buffering agents, and excipients. For example, the self-association-induced opalescence of mAb's can be minimized through pH and salt adjustment [70, 85]. Several buffering agents, citrate, succinate, glutamate, propionate, and acetate, were shown to lower the interaction parameter k_D and the relative degree of effect matched the relative rates of protein aggregation [100]. Many non-electrolyte excipients could reduce the attractive protein–protein interactions such as sucrose, trehalose, mannitol, and sorbitol [21, 114–116]. More drastic approaches for minimizing these issues include protein engineering [91] or conjugation [44].

4.3.3.2 Process Development

As discussed above, self-association may lead to significantly higher solution viscosity. A viscous solution is difficult to handle during routine product manufacturing processes, such as solution transfer, filtration, and filling [104]. Similar formulation approaches can be used to minimize self-association or viscosity. Selection of a formulation pH several units away from its pI theoretically increases the charge density of a protein and generally leads to enhanced repulsive or reduced attractive protein–protein interactions, minimizing solution viscosity. Indeed, the highest viscosity of an IgG1 mAb was found at a pH near its pI at low-ionic strength conditions [75]. A similar pH-dependent trend of viscosity was also observed for an IgG2 at 150 mg/mL [20]. As shown above, salts have a significant effect on solution viscosity. The viscosity of two mAbs could be reduced roughly by half in the presence of 150 mM NaCl [43].

If the high viscosity is caused by attractive protein–protein interactions contributed at least partly through hydrophobic interactions [20, 28, 31], the use of hydrophobic salts in a mAb or other protein formulations can be useful [28, 41, 108]. Even at moderate concentrations, some of the hydrophobic salts reduced the viscosities of concentrated mAb solutions over 10-fold at room temperature [41]. It should be noted that some formulation excipients may potentially increase the formulation viscosity at a high protein concentration (>90 mg/mL), such as sugar molecules [43]. Excipients, which could potentially interact with proteins, may have a dramatic effect. It was demonstrated that the use of arginine hydrochloride (ArgHCl) reduced the high viscosities of bovine gamma globulin (BGG) solution at 250 mg/mL (60 cP) and human gamma globulin (HGG) solution at 292 mg/mL to an acceptable level (below 50 cP) [57]. Arginine also reduced the viscosity of both BSA and HSA solutions at pH 7.4 at 25 °C [58]. In general, repulsive interactions are preferred to minimize protein self-association or higher viscosity.

A practical approach to achieve high protein concentration is to reconstitute a lyophilized product to a volume lower than the fill volume. This approach may allow manufacturing of drug products at a lower protein concentration, mitigating high-concentration-associated process challenges and potentially bypassing high-concentration-associated stability issues during long-term storage. Nonetheless, the reconstituted drug product still needs to be handled successfully without difficulty through the administration process.

Other non-traditional approaches can also be used to deal with high viscosity of proteins at high concentrations. Batch crystallization of three mAbs was successfully achieved with full retention of their biological activity in vitro. At 150 mg/mL, the viscosity of an infliximab solution was at 275 cP, but the equivalent crystalline suspension had a viscosity of less than 40 cP [126]. The use of organic solvents was also found to be an effective approach in recent investigations [13, 78, 108]. It was shown that the non-aqueous suspensions of γ-globulin at 300 mg/mL reduced the viscosity of the aqueous solution by 38X [108].

4.3.3.3 Drug Administration

Most protein drug products are administered through i.v. or s.c. injection. In this case, a product solution needs to be withdrawn into a syringe and injected through a needle (syringeability). High viscosity of protein solutions may present delivery or administration challenges. A solution viscosity of higher than 50 cP may present practical difficulty with a 27 or 29 G needle normally used for subcutaneous administration especially for patients who may have challenging dexterity (e.g., rheumatoid arthritis patients) [78]. Assuming the force of 30 Newtons as a limit for manual injection, the viscosity of an IgG solution should not exceed approximately 25 cP to achieve an injection rate of 4 mL/min through a 27G needle [15]. If one would like to achieve a faster injection rate or use a smaller needle size, a less viscous solution would be required. The theoretical relationship between the injection force and these syringe and needle-related parameters can be described by the following equation for Newtonian fluids [15]:

$$F = 32 D^2 l Q \eta / d^4$$

where F is the injection force, D is the syringe diameter, l is the length of the needle, Q is the injection flow rate, η is the viscosity, and d is the inside diameter of the needle.

Both Newtonian and non-Newtonian viscosity behaviors have been reported for mAb protein solutions in the literature [1, 85, 88, 129]. It appears that less viscous protein solutions tend to be Newtonian, while more viscous solutions tend to exhibit strong shear-thinning behaviors [1, 94, 129]. The dividing point may depend on the protein. Shear thinning appears to be observed only at a higher shearing rate for mAb solutions [1, 94, 129]. The shear thinning could be due to disruption of protein oligomers/clusters [129]. The estimated shear rate was about 180,000 s^{-1} for delivering a 1 mL solution through a 27G needle with an inside diameter of 0.222 mm in 6 s [94]. This is significantly higher than what was used to test shear thinning, e.g., 10^4/s [129]. In any case, a recent study has shown that syringeability can also be predicted accurately based on the property of non-Newtonian mAb solutions [1].

In the dose administration process, the protein may experience significant shearing. It has been shown that a concentrated IgG1 mAb solution (>100 mg/mL) can tolerate a shear between 20,000 and 250,000 s^{-1} for between 5 min and 30 ms (far in excess of those expected during normal processing operation) [7].

4.4 Future Directions

The challenges associated with development of a high-concentration protein product justify the continued efforts in the following areas—minimization of viscosity and protein aggregation, development of advanced analytical tools, and better understanding of in vivo product performance.

4.4.1 Minimization of Viscosity and Protein Aggregation

As discussed above, although traditional formulation approaches can reduce the solution viscosity significantly, the degree of reduction may still be inadequate. Recent investigations have shown the potential of significant viscosity reduction by clustering protein molecules in solutions. High protein concentrations up to 320 mg/mL were prepared by formation of dense, reversible, colloidally stable nanoclusters of 35–80 nm driven by small molecule crowders [12]. The homogeneity and stability of these solutions may need further evaluations. Other novel alternatives, such as preparation of crystalline or non-aqueous suspensions, would need similar types of evaluation. In addition, significant evaluation may be required on the safety of the non-aqueous solvents.

Protein aggregation has been recognized as a key product development issue for a long time [76, 118]. Although traditional approaches can be used to mitigate protein aggregation at high concentrations, the conditions for favorable mitigation of protein aggregation may not align with those for minimization of high viscosity. For example, certain formulation excipients, such as sugars, that protect protein from aggregation can increase the viscosity of proteins [43]. Any novel approaches designed for reduction of viscosity should ideally be able to mitigate the aggregation tendency or vice versa.

4.4.2 Development of New Analytical Methodologies

A variety of analytical methods are available to characterize antibody self-association [36]. In many cases, these assays are not in high-throughput format such as analytical ultracentrifugation. Some parameters can only be determined at low concentrations, such as the interaction parameter k_D determined by DLS. The type/dominance of protein–protein interactions, reflected in these parameters, may change with increasing concentrations [98, 101, 110]. Although AUC can be used to probe the protein association at high concentrations, protein self-association may not be detectable by sedimentation velocity experiments, if the association is not strong enough [75]. Some routine assays, such as SEC-HPLC, may require sample preparations, including dilution, so the results may not reflect what is really in the original product solution. In general, the future development would be focused on high-throughput assays, which should cover a wide concentration range, reveal what is in the original product solution, and/or predict protein behavior at higher concentrations.

Protein–protein interactions are the cause of characteristic and sometimes challenging protein behaviors at high concentrations. Although both electrostatic and hydrophobic interactions are apparently involved, specific interaction sites or residues have not been clearly identified. A limited number of studies did point to the dominant role of Fab–Fab interactions [18, 31, 64, 70, 96, 127]. On the other

hand, Fc participation in protein–protein interactions was involved in mAb protein–protein interactions [18]. In fact, Fc was found to be the only part of IgG1, which is responsible for an IgG1 association, leading to phase separation under a low-ionic state [86]. This is possibly due to the hydrophobic and flexible nature of the Fc region [24, 84]. In this regard, more complex instrumentation may be needed to detect local and subtle energetic and/or conformational change in proteins.

4.4.3 Understanding of In Vivo Performance

A desired in vivo performance of a protein product is the final goal in product development. In recent years, pharmaceutical product development scientists increased their attention to the fate of protein products after administration—both at the site of injection and in the blood stream. The pH and composition of different tissue fluids can be significantly different from that of a protein product [66]. Therefore, a high-concentration product with a reversible formation of protein oligomers may not show reversibility upon subcutaneous injection and subsequent dilution with tissue fluid. It has been shown that proteins can aggregate easily after administration or mixing with plasma due to the difference in pH, and different components in different tissues [4]. Protein aggregates at the injection sites were shown to have a slower elimination than monomeric proteins after subcutaneous injection in mice [33, 65].

Even though a high-concentration protein product can be developed with acceptable viscosity and aggregation tendency, the crowded protein product may present additional challenges, altering the in vivo behavior. For example, subcutaneous injection of high-concentration protein with reversible clusters into mice results in indistinguishable pharmacokinetics versus a standard antibody solution [62].

In comparison, the half-life of crystalline infliximab suspension is 777 h, significantly longer than that (390 h) after subcutaneous injection in rats [126]. Understanding the behavior of high-concentration products upon administration could be a useful part of a product development process.

References

1. Allmendinger A, Fischer S, Huwyler B, Mahler HC, Schwarb E, Zarraga IE, Mueller R. Rheological characterization and injection forces of concentrated protein formulations: an alternative predictive model for non-Newtonian solutions. Eur J Pharm Biopharm. 2014; 87(2):318–28.
2. America, PRAMO. “Biologics 2013 Report”; 2013.
3. Amidi M, Romeijn SG, Borchard G, Junginger HE, Hennink WE, Jiskoot W. Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system. J Control Release. 2006;111(1–2):107–16.

4. Arvinte T, Palais C, Green-Trexler E, Gregory S, Mach H, Narasimhan C, Shameem M. Aggregation of biopharmaceuticals in human plasma and human serum: implications for drug research and development. MAbs. 2013;5(3):491–500.
5. Ayensu I, Mitchell JC, Boateng JS. In vitro characterisation of chitosan based xerogels for potential buccal delivery of proteins. Carbohydr Polym. 2012;89(3):935–41.
6. Bajaj H, Sharma VK, Badkar A, Zeng D, Nema S, Kalonia DS. Protein structural conformation and not second virial coefficient relates to long-term irreversible aggregation of a monoclonal antibody and ovalbumin in solution. Pharm Res. 2006;23(6):1382–94.
7. Bee JS, Stevenson JL, Mehta B, Svitel J, Pollastrini J, Platz R, Freund E, Carpenter JF, Randolph TW. Response of a concentrated monoclonal antibody formulation to high shear. Biotechnol Bioeng. 2009;103(5):936–43.
8. Bethea D, Wu SJ, Luo J, Hyun L, Lacy ER, Teplyakov A, Jacobs SA, O'Neil KT, Gilliland GL, Feng Y. Mechanisms of self-association of a human monoclonal antibody CNTO607. Protein Eng Des Sel. 2012;25(10):531–7.
9. Binabaji E, Rao S, Zydney AL. The osmotic pressure of highly concentrated monoclonal antibody solutions: effect of solution conditions. Biotechnol Bioeng. 2014;111(3):529–36.
10. Bivas-Benita M, Lin MY, Bal SM, van Meijgaarden KE, Franken KL, Friggen AH, Junginger HE, Borchard G, Klein MR, Ottenhoff TH. Pulmonary delivery of DNA encoding *Mycobacterium tuberculosis* latency antigen Rv1733c associated to PLGA-PEI nanoparticles enhances T cell responses in a DNA prime/protein boost vaccination regimen in mice. Vaccine. 2009;27(30):4010–7.
11. Bookbinder LH, Kundu A, Frost GI. (2010) Soluble hyaluronidase glycoprotein (shasegp), process for preparing the same, uses and pharmaceutical compositions comprising thereof; 2010.
12. Borwankar AU, Dinin AK, Laber JR, Twu A, Wilson BK, Maynard JA, Truskett TM, Johnston KP. Tunable equilibrium nanocluster dispersions at high protein concentrations. Soft Matter. 2013;9(6):1766–71.
13. Bowen M, Armstrong N, Maa Y-F. Investigating high-concentration monoclonal antibody powder suspension in nonaqueous suspension vehicles for subcutaneous injection. J Pharm Sci. 2012;101(12):4433–43.
14. Broersen K, Weijers M, de Groot J, Hamer RJ, de Jongh HH. Effect of protein charge on the generation of aggregation-prone conformers. Biomacromolecules. 2007;8(5):1648–56.
15. Burckbuchler V, Mekhloufi G, Giteau AP, Grossiord JL, Huille S, Agnely F. Rheological and syringeability properties of highly concentrated human polyclonal immunoglobulin solutions. Eur J Pharm Biopharm. 2010;76(3):351–6.
16. Carpenter JF, Randolph TW, Jiskoot W, Crommelin DJ, Middaugh CR, Winter G, Fan YX, Kirshner S, Verthelyi D, Kozlowski S, Clouse KA, Swann PG, Rosenberg A, Cherney B. Overlooking subvisible particles in therapeutic protein products: gaps that may compromise product quality. J Pharm Sci. 2008. https://doi.org/10.1002/jps.21530.
17. Casaz P, Boucher E, Wollacott R, Pierce BG, Rivera R, Sedic M, Ozturk S, Thomas W, Wang Y. Resolving self-association of a therapeutic antibody by formulation optimization and molecular approaches. MAbs. 2014;6:1533–9.
18. Chaudhri A, Zarraga IE, Kamerzell TJ, Brandt JP, Patapoff TW, Shire SJ, Voth GA. Coarse-grained modeling of the self-association of therapeutic monoclonal antibodies. J Phys Chem B. 2012;116(28):8045–57.
19. Chaudhri A, Zarraga IE, Yadav S, Patapoff TW, Shire SJ, Voth GA. The role of amino acid sequence in the self-association of therapeutic monoclonal antibodies: insights from coarse-grained modeling. J Phys Chem B. 2013;117(5):1269–79.
20. Cheng W, Joshi SB, Jain NK, He F, Kerwin BA, Volkin DB, Middaugh CR. Linking the solution viscosity of an IgG2 monoclonal antibody to its structure as a function of pH and temperature. J Pharm Sci. 2013;102(12):4291–304.
21. Chi EY, Krishnan S, Kendrick BS, Chang BS, Carpenter JF, Randolph TW. Roles of conformational stability and colloidal stability in the aggregation of recombinant human granulocyte colony-stimulating factor. Protein Sci. 2003;12(5):903–13.

22. Connolly BD, Petry C, Yadav S, Demeule B, Ciaccio N, Moore JM, Shire SJ, Gokarn YR. Weak interactions govern the viscosity of concentrated antibody solutions: high-throughput analysis using the diffusion interaction parameter. Biophys J. 2012;103(1):69–78.
23. Daugherty AL, Mrsny RJ. Formulation and delivery issues for monoclonal antibody therapeutics. Adv Drug Deliv Rev. 2006;58(5–6):686–706.
24. DeLano WL, Ultsch MH, de Vos AM, Wells JA. Convergent solutions to binding at a protein-protein interface. Science. 2000;287(5456):1279–83.
25. des Rieux A, Fievez V, Garinot M, Schneider YJ, Preat V. Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach. J Control Release. 2006;116(1):1–27.
26. Rickwood S, DiBiase S. Searching for terra firma in biosimilars and non-original biologics market: insight for the coming decade of change. 2013. http://www.imshealth.com/deployedfiles/imshealth/Global/Content/Healthcare/Life%20Sciences%20Solutions/Generics/IMSH_Biosimilars_WP.pdf, IMS, p. 1–24.
27. du Plessis LH, Kotze AF, Junginger HE. Nasal and rectal delivery of insulin with chitosan and N-trimethyl chitosan chloride. Drug Deliv. 2010;17(6):399–407.
28. Du W, Klibanov AM. Hydrophobic salts markedly diminish viscosity of concentrated protein solutions. Biotechnol Bioeng. 2011;108(3):632–6.
29. Elcock AH, McCammon JA. Calculation of weak protein-protein interactions: the pH dependence of the second virial coefficient. Biophys J. 2001;80(2):613–25.
30. Esfandiary R, Hayes DB, Parupudi A, Casas-Finet J, Bai S, Samra HS, Shah AU, Satish HA. A systematic multitechnique approach for detection and characterization of reversible self-association during formulation development of therapeutic antibodies. J Pharm Sci. 2013;102(1):62–72.
31. Esfandiary R, Parupudi A, Casas-Finet J, Gadre D, Sathish H. (2014) Mechanism of reversible self-association of a monoclonal antibody: role of electrostatic and hydrophobic interactions. J Pharm Sci; 2014.
32. Fathallah AM, Balu-Iyer SV. Anatomical, physiological, and experimental factors affecting the bioavailability of sc-administered large biotherapeutics. J Pharm Sci. 2015;104(2):301–6.
33. Filipe V, Que I, Carpenter JF, Lowik C, Jiskoot W. In vivo fluorescence imaging of IgG1 aggregates after subcutaneous and intravenous injection in mice. Pharm Res. 2014;31(1):216–27.
34. Fischer H, Polikarpov I, Craievich AF. Average protein density is a molecular-weight-dependent function. Protein Sci. 2004;13(10):2825–8.
35. Galush WJ, Le LN, Moore JMR. Viscosity behavior of high-concentration protein mixtures. J Pharm Sci. 2012;101(3):1012–20.
36. Geng SB, Cheung JK, Narasimhan C, Shameem M, Tessier PM. Improving monoclonal antibody selection and engineering using measurements of colloidal protein interactions. J Pharm Sci. 2014;103(11):3356–63.
37. Glazer WM, Maynard C, Berkman CS. Injection site leakage of depot neuroleptics: intramuscular versus subcutaneous injection. J Clin Psychiatry. 1987;48(6):237–9.
38. Godfrin PD, Castaneda-Priego R, Liu Y, Wagner NJ. Intermediate range order and structure in colloidal dispersions with competing interactions. J Chem Phys. 2013;139(15):154904.
39. Grenha A, Remunan-Lopez C, Carvalho EL, Seijo B. Microspheres containing lipid/chitosan nanoparticles complexes for pulmonary delivery of therapeutic proteins. Eur J Pharm Biopharm. 2008;69(1):83–93.
40. Guo J, Harn N, Robbins A, Dougherty R, Middaugh CR. Stability of helix-rich proteins at high concentrations. Biochemistry. 2006;45(28):8686–96.
41. Guo Z, Chen A, Nassar R, Helk B, Mueller C, Tang Y, Gupta K, Klibanov A. Structure-activity relationship for hydrophobic salts as viscosity-lowering excipients for concentrated solutions of monoclonal antibodies. Pharm Res. 2012;29(11):3102–9.
42. Harn N, Allan C, Oliver C, Middaugh CR. Highly concentrated monoclonal antibody solutions: direct analysis of physical structure and thermal stability. J Pharm Sci. 2007;96(3):532–46.

43. He F, Woods CE, Litowski JR, Roschen LA, Gadgil HS, Razinkov VI, Kerwin BA. Effect of sugar molecules on the viscosity of high concentration monoclonal antibody solutions. Pharm Res. 2011;28(7):1552–60.
44. Holm LS, McUmber A, Rasmussen JE, Obiols-Rabasa M, Thulstrup PW, Kasimova MR, Randolph TW, van de Weert M. The effect of protein PEGylation on physical stability in liquid formulation. J Pharm Sci. 2014;103(10):3043–54.
45. Hou YW, Chan MH, Hsu HR, Liu BR, Chen CP, Chen HH, Lee HJ. Transdermal delivery of proteins mediated by non-covalently associated arginine-rich intracellular delivery peptides. Exp Dermatol. 2007;16(12):999–1006.
46. http://www.aegisthera.com/.
47. http://www.bacter.com/.
48. http://www.cpexpharm.com.com/.
49. http://www.cpexpharm.com/.
50. http://www.dorbiopharma.com/.
51. http://www.emisphere.com/.
52. http://www.generex.com/.
53. http://www.mannkindcorp.com/.
54. http://www.phosphagenics.com/.
55. http://www.thiomatrix.com/.
56. http://www.transdermaltechnologies.com/.
57. Inoue N, Takai E, Arakawa T, Shiraki K. Arginine and lysine reduce the high viscosity of serum albumin solutions, for pharmaceutical injection. J Biosci Bioeng. 2014;117(5):539–43.
58. Inoue N, Takai E, Arakawa T, Shiraki K. Specific decrease in solution viscosity of antibodies by arginine for therapeutic formulations. Mol Pharm. 2014;11(6):1889–96.
59. Iqbal M, Lin W, Jabbal-Gill I, Davis SS, Steward MW, Illum L. Nasal delivery of chitosan-DNA plasmid expressing epitopes of respiratory syncytial virus (RSV) induces protective CTL responses in BALB/c mice. Vaccine. 2003;21(13–14):1478–85.
60. Iwura T, Fukuda J, Yamazaki K, Arisaka F. Conformational stability, reversibility and heat-induced aggregation of alpha-1-acid glycoprotein. J Biochem. 2014;156(6):345–52.
61. Jimenez M, Rivas G, Minton AP. Quantitative characterization of weak self-association in concentrated solutions of immunoglobulin G via the measurement of sedimentation equilibrium and osmotic pressure. Biochemistry. 2007;46(28):8373–8.
62. Johnston KP, Maynard JA, Truskett TM, Borwankar AU, Miller MA, Wilson BK, Dinin AK, Khan TA, Kaczorowski KJ. Concentrated dispersions of equilibrium protein nanoclusters that reversibly dissociate into active monomers. ACS Nano. 2012;6(2):1357–69.
63. Jorgensen JT, Romsing J, Rasmussen M, Moller-Sonnergaard J, Vang L, Musaeus L. Pain assessment of subcutaneous injections. Ann Pharmacother. 1996;30(7–8):729–32.
64. Kanai S, Liu J, Patapoff TW, Shire SJ. Reversible self-association of a concentrated monoclonal antibody solution mediated by Fab-Fab interaction that impacts solution viscosity. J Pharm Sci. 2008;97(10):4219–27.
65. Kijanka G, Prokopowicz M, Schellekens H, Brinks V. Influence of aggregation and route of injection on the biodistribution of mouse serum albumin. PLoS ONE. 2014;9(1):e85281.
66. Kinnunen HM, Mrsny RJ. Improving the outcomes of biopharmaceutical delivery via the subcutaneous route by understanding the chemical, physical and physiological properties of the subcutaneous injection site. J Control Release. 2014;182:22–32.
67. Krulevitch P, Wilk R, O'Connor S, Zhao M, Sieh Z, Savage D. Medical device mechanical pump. Google Patents; 2009.
68. Kwaambwa HM, Goodwin JW, Hughs RW, Reynolds PA. Visocisity, molecular weight and concentration relationships at 298 K of low molecular weight cis-polyisoprene in a good solvent. Colloids Surf, A. 2007;294:14–9.
69. Laue T. Proximity energies: a framework for understanding concentrated solutions. J Mol Recognit. 2012;25(3):165–73.

70. Lee H, Kirchmeier M, Mach H. Monoclonal antibody aggregation intermediates visualized by atomic force microscopy. J Pharm Sci. 2011;100:416–23.
71. Lee WC, Balu S, Cobden D, Joshi AV, Pashos CL. (2006) Medication adherence and the associated health-economic impact among patients with type 2 diabetes mellitus converting to insulin pen therapy: an analysis of third-party managed care claims data. Clin Ther. 2006; **28**(10):1712–25; discussion 1710-1711.
72. Li L, Kumar S, Buck PM, Burns C, Lavoie J, Singh SK, Warne NW, Nichols P, Luksha N, Boardman D. Concentration dependent viscosity of monoclonal antibody solutions: explaining experimental behavior in terms of molecular properties. Pharm Res. 2014;31(11): 3161–78.
73. Lilyestrom WG, Yadav S, Shire SJ, Scherer TM. Monoclonal antibody self-association, cluster formation, and rheology at high concentrations. J Phys Chem B. 2013;117(21): 6373–84.
74. Lin YH, Liang HF, Chung CK, Chen MC, Sung HW. Physically crosslinked alginate/N, O-carboxymethyl chitosan hydrogels with calcium for oral delivery of protein drugs. Biomaterials. 2005;26(14):2105–13.
75. Liu J, Nguyen MD, Andya JD, Shire SJ. Reversible self-association increases the viscosity of a concentrated monoclonal antibody in aqueous solution. J Pharm Sci. 2005;94(9): 1928–40.
76. Mahler H-C, Friess W, Grauschopf U, Kiese S. Protein aggregation: pathways, induction factors and analysis. J Pharm Sci. 2009;98(9):2909–34.
77. McAllister DV, Wang PM, Davis SP, Park JH, Canatella PJ, Allen MG, Prausnitz MR. Microfabricated needles for transdermal delivery of macromolecules and nanoparticles: fabrication methods and transport studies. Proc Natl Acad Sci USA. 2003;100(24): 13755–60.
78. Miller MA, Engstrom JD, Ludher BS, Johnston KP. Low viscosity highly concentrated injectable nonaqueous suspensions of lysozyme microparticles. Langmuir. 2010;26(2): 1067–74.
79. Minton AP. Macromolecular crowding. Curr Biol. 2006;16(8):R269–71.
80. Moeller EH, Jorgensen L. Alternative routes of administration for systemic delivery of protein pharmaceuticals. Drug Discov Today Technol. 2008;5(2–3):e89–94.
81. Morishita M, Barichello JM, Takayama K, Chiba Y, Tokiwa S, Nagai T. Pluronic F-127 gels incorporating highly purified unsaturated fatty acids for buccal delivery of insulin. Int J Pharm. 2001;212(2):289–93.
82. Neal BL, Asthagiri D, Lenhoff AM. Molecular origins of osmotic second virial coefficients of proteins. Biophys J. 1998;75(5):2469–77.
83. Neergaard MS, Kalonia DS, Parshad H, Nielsen AD, Moller EH, van de Weert M. Viscosity of high concentration protein formulations of monoclonal antibodies of the IgG1 and IgG4 subclass—prediction of viscosity through protein-protein interaction measurements. Eur J Pharm Sci. 2013;49(3):400–10.
84. Nezlin R. Interactions between immunoglobulin G molecules. Immunol Lett. 2010;132(1–2): 1–5.
85. Nishi H, Miyajima M, Nakagami H, Noda M, Uchiyama S, Fukui K. Phase separation of an IgG1 antibody solution under a low ionic strength condition. Pharm Res. 2010;27(7): 1348–60.
86. Nishi H, Miyajima M, Wakiyama N, Kubota K, Hasegawa J, Uchiyama S, Fukui K. Fc domain mediated self-association of an IgG1 monoclonal antibody under a low ionic strength condition. J Biosci Bioeng. 2011;112(4):326–32.
87. Pan Y, Cheng R-S. A novel interpretation of concentration dependence of viscosity of dilute polymer solution. Chin J Polym Sci. 2000;18:57–67.
88. Pathak JA, Sologuren RR, Narwal R. Do clustering monoclonal antibody solutions really have a concentration dependence of viscosity? Biophys J. 2013;104:913–23.
89. Patton JS, Foster L, Platz RM. (1999) Methods and compositions for pulmonary delivery of insulin. Google Patents; 1999.

90. Pavlou AK, Belsey MJ. The therapeutic antibodies market to 2008. Eur J Pharm Biopharm. 2005;59(3):389–96.
91. Perchiacca JM, Lee CC, Tessier PM. Optimal charged mutations in the complementarity-determining regions that prevent domain antibody aggregation are dependent on the antibody scaffold. Protein Eng Des Sel. 2014;27(2):29–39.
92. Pharma, E. World Preview 2013, Outlook to 2018; 2013.
93. Rao SV, Shao J. Self-nanoemulsifying drug delivery systems (SNEDDS) for oral delivery of protein drugs: I. Formulation development. Int J Pharm. 2008;362(1–2):2–9.
94. Rathore N, Pranay P, Bernacki J, Eu B, Ji W, Walls E. Characterization of protein rheology and delivery forces for combination products. J Pharm Sci. 2012;101(12):4472–80.
95. Rosenberg AS. Effects of protein aggregates: an immunologic perspective. AAPS J. 2006;8 (3):E501–7.
96. Roux KH, Tankersley DL. A view of the human idiotypic repertoire. Electron microscopic and immunologic analyses of spontaneous idiotype-anti-idiotype dimers in pooled human IgG. J Immunol. 1990;144(4):1387–95.
97. Rubio-Hernandez FJ, Carrique F, Ruiz-Reina E. The primary electroviscous effect in colloidal suspensions. Adv Colloid Interface Sci. 2004;107(1):51–60.
98. Saito S, Hasegawa J, Kobayashi N, Kishi N, Uchiyama S, Fukui K. Behavior of monoclonal antibodies: relation between the second virial coefficient (B (2)) at low concentrations and aggregation propensity and viscosity at high concentrations. Pharm Res. 2012;29(2): 397–410.
99. Salinas BA, Sathish HA, Bishop SM, Harn N, Carpenter JF, Randolph TW. Understanding and modulating opalescence and viscosity in a monoclonal antibody formulation. J Pharm Sci. 2010;99(1):82–93.
100. Saluja A, Fesinmeyer RM, Hogan S, Brems DN, Gokarn YR. Diffusion and sedimentation interaction parameters for measuring the second virial coefficient and their utility as predictors of protein aggregation. Biophys J. 2010;99(8):2657–65.
101. Saluja A, Kalonia DS. Nature and consequences of protein-protein interactions in high protein concentration solutions. Int J Pharm. 2008;358(1–2):1–15.
102. Schaepelynck P, Darmon P, Molines L, Jannot-Lamotte MF, Treglia C, Raccah D. Advances in pump technology: insulin patch pumps, combined pumps and glucose sensors, and implanted pumps. Diabetes Metab. 2011;37(Suppl 4):S85–93.
103. Scherer TM, Liu J, Shire SJ, Minton AP. Intermolecular interactions of IgG1 monoclonal antibodies at high concentrations characterized by light scattering. J Phys Chem B. 2010;114 (40):12948–57.
104. Shieu W, Torhan SA, Chan E, Hubbard A, Gikanga B, Stauch OB, Maa YF. Filling of high-concentration monoclonal antibody formulations into pre-filled syringes: filling parameter investigation and optimization. PDA J Pharm Sci Technol. 2014;68(2):153–63.
105. Shire SJ, Shahrokh Z, Liu J. Challenges in the development of high protein concentration formulations. J Pharm Sci. 2004;93(6):1390–402.
106. Singh SN, Yadav S, Shire SJ, Kalonia DS. Dipole-dipole interaction in antibody solutions: correlation with viscosity behavior at high concentration. Pharm Res. 2014;31:2549–58.
107. Song C, Wang P, Makse HA. A phase diagram for jammed matter. Nature. 2008;453 (7195):629–32.
108. Srinivasan C, Weight AK, Bussemer T, Klibanov AM. Non-aqueous suspensions of antibodies are much less viscous than equally concentrated aqueous solutions. Pharm Res. 2013;30(7):1749–57.
109. Stradner A, Sedgwick H, Cardinaux F, Poon WC, Egelhaaf SU, Schurtenberger P. Equilibrium cluster formation in concentrated protein solutions and colloids. Nature. 2004;432(7016):492–5.
110. Sule SV, Cheung JK, Antochshuk V, Bhalla AS, Narasimhan C, Blaisdell S, Shameem M, Tessier PM. Solution pH that minimizes self-association of three monoclonal antibodies is strongly dependent on ionic strength. Mol Pharm. 2012;9(4):744–51.

111. Sule SV, Sukumar M, Weiss WF, Marcelino-Cruz AM, Sample T, Tessier PM. High-throughput analysis of concentration-dependent antibody self-association. Biophys J. 2011;101(7):1749–57.
112. Tice TR, Gilley RM, Eldridge JH, Staas JK. (1998) Method for oral or rectal delivery of microencapsulated vaccines and compositions therefor. Google Patents; 1998.
113. Uchiyama S. Liquid formulation for antibody drugs. Biochim Biophys Acta. 2014;1844 (11):2041–52.
114. Valente JJ, Fryksdale BG, Dale DA, Gaertner AL, Henry CS. Screening for physical stability of a Pseudomonas amylase using self-interaction chromatography. Anal Biochem. 2006;357 (1):35–42.
115. Valente JJ, Payne RW, Manning MC, Wilson WW, Henry CS. Colloidal behavior of proteins: effects of the second virial coefficient on solubility, crystallization and aggregation of proteins in aqueous solution. Curr Pharm Biotechnol. 2005;6(6):427–36.
116. Valente JJ, Verma KS, Manning MC, Wilson WW, Henry CS. Second virial coefficient studies of cosolvent-induced protein self-interaction. Biophys J. 2005;89(6):4211–8.
117. Wang N, Hu B, Ionescu R, Mach H, Sweeney J, Hamm C, Kirchmeier MJ, Meyer BK. Opalescence of an IgG1 monoclonal antibody formulation is mediated by ionic strength and excipients. BioPharm Int. 2009;22(4):36–47.
118. Wang W, Nema S, Teagarden D. Protein aggregation–pathways and influencing factors. Int J Pharm. 2010;390(2):89–99.
119. Wang W, Singh SK, Li N, Toler MR, King KR, Nema S. Immunogenicity of protein aggregates–concerns and realities. Int J Pharm. 2012;431(1–2):1–11.
120. Warne NW. Development of high concentration protein biopharmaceuticals: The use of platform approaches in formulation development. Eur J Pharm Biopharm. 2011;78:208–12.
121. Williams AC, Barry BW. Penetration enhancers. Adv Drug Deliv Rev. 2004;56(5):603–18.
122. Woods JM, Nesta D. (2010) Formulation effects on opalescence of a high-concentration MAb. BioProcess Int. 2010; 48–59.
123. Wu SJ, Luo J, O'Neil KT, Kang J, Lacy ER, Canziani G, Baker A, Huang M, Tang QM, Raju TS, Jacobs SA, Teplyakov A, Gilliland GL, Feng Y. Structure-based engineering of a monoclonal antibody for improved solubility. Protein Eng Des Sel. 2010;23(8):643–51.
124. Yadav S, Shire SJ, Kalonia DS. Factors affecting the viscosity in high concentration solutions of different monoclonal antibodies. J Pharm Sci. 2010;99(12):4812–29.
125. Yadav S, Shire SJ, Kalonia DS. Viscosity behavior of high-concentration monoclonal antibody solutions: correlation with interaction parameter and electroviscous effects. J Pharm Sci. 2012;101(3):998–1011.
126. Yang MX, Shenoy B, Disttler M, Patel R, McGrath M, Pechenov S, Margolin AL. Crystalline monoclonal antibodies for subcutaneous delivery. Proc Natl Acad Sci USA. 2003;100(12):6934–9.
127. Yearley EJ, Godfrin PD, Perevozchikova T, Zhang H, Falus P, Porcar L, Nagao M, Curtis JE, Gawande P, Taing R, Zarraga IE, Wagner NJ, Liu Y. Observation of small cluster formation in concentrated monoclonal antibody solutions and its implications to solution viscosity. Biophys J. 2014;106:1763–70.
128. Yearley EJ, Zarraga IE, Shire SJ, Scherer TM, Gokarn Y, Wagner NJ, Liu Y. Small-angle neutron scattering characterization of monoclonal antibody conformations and interactions at high concentrations. Biophys J. 2013;105(3):720–31.
129. Zarraga IE, Taing R, Zarzar J, Luoma J, Hsiung J, Patel A, Lim FJ. High shear rheology and anisotropy in concentrated solutions of monoclonal antibodies. J Pharm Sci. 2013;102(8): 2538–49.

Chapter 5
Solubility, Opalescence, and Particulate Matter

Hanns-Christian Mahler and Anja Matter

Abstract "Appearance" is an analytical test often found in specifications of protein drug products. However, appearance can mean many different things, including dosage form (liquid, dried), color (coloration of solution, color of cake), cake appearance (in case of a lyophilisate), opalescence, or turbidity, and—in some cases—even visible particles. "Maximum solubility" is a parameter that has been previously evaluated for proteins. But given the nature of biologics and the analytical methods used for assessing solubility often do not yield any practically relevant information. Opalescence, respectively, solution clarity can be measured as defined by the European Pharmacopeia. It is a helpful parameter that can relate to aggregation, but also other solution phenomena like liquid phase separation. The coloration of solution is also defined in the European Pharmacopeia. Coloration in protein products can relate to concentration, type of formulation, but also process- or product-related degradants or contaminants. Solution viscosity is of utmost importance for manufacturing as well as administration of biologics at higher concentration. Finally, aggregates and particles can arise as a result of degradation pathways and subvisible and visible particles in parenteral preparations also have to comply with related pharmacopoeial requirements.

Keywords Solubility · Opalescence · Turbidity · Aggregates · Particles
Visible particles · Subvisible particles · Submicron particles · Viscosity
High-concentration formulations · Color · Appearance

H.-C. Mahler (✉) · A. Matter (✉)
Lonza AG, Drug Product Services, Basel, Switzerland
e-mail: hanns-christian.mahler@lonza.com

A. Matter
e-mail: anja.matter@lonza.com

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_5

5.1 Introduction

Protein formulations are colloidal suspensions and not perfect solutions. In most cases, proteins contain hydrophobic aromatic amino acids that would generally drive their adsorption properties. This is the main cause for some of the phenomena that such protein formulations show.

Given that "appearance" of a protein formulation can be influenced by many different aspects, namely its solubility, opalescence, color, viscosity, and aggregation propensity, and this chapter aims to discuss these relevant parameters in more detail in order to allow more specific and appropriate testing.

5.2 Appearance of Protein Formulations

5.2.1 Solubility

Solubility can be defined as the amount of a substance (the solute) that dissolves in a unit volume of a liquid substance (solvent) to form a saturated solution under specific conditions such as temperature and pressure (business dictionary). Such a definition is being used and is helpful for small molecule drugs, especially for poorly soluble small molecule drugs that require solubilization efforts. Solubility for a protein is, however, not an easily defined term. Solubility can also be considered as a limit, where typical endpoints such as HPLC analysis, visual inspection, subvisible particle testing, and other tests can be used to evaluate if there is yet undissolved solid matter and to determine these thresholds. This approach would be somewhat similar to small molecule solubility assessments. Such tests, however, can often be rather misleading when trying to establish "solubility" limits for proteins. Proteins, as mentioned earlier, are colloidal and not ideal solutions, and thus, their colloidal behavior in solution may drive some of the data. Also, it can be unclear on what is the "acceptable" threshold to derive that solubility limit from when looking at HPLC analysis (soluble aggregates), visual inspection, subvisible particle testing, or other endpoints.

Some authors tried to evaluate "protein solubility" by artificially lowering protein stability and provoking aggregation and particle formation, such as by using polyethylene glycol (PEG) as precipitant [1, 18]. However, the obtained "maximum solubility" (in mg/mL concentrations of protein) did not match other actual experiments [17].

In the author's perspective, the term "solubility" is thus misleading when being used for proteins and formulations and we recommend not using it.

5.2.2 Opalescence

Opalescence can be defined as a type of dichroism in highly dispersed systems with little opacity. The phenomenon is an example of the Tyndall effect and is named

after the appearance of opals (Wikipedia). Opalescence refers to a solutions optical characteristic caused by scattering of light, which can be explained by different scattering events like Rayleigh or Mie scattering [19]. In practical terms, opalescence is often interchangeably called turbidity.

The European Pharmacopeia has a monograph for the measurement of "Clarity and degree of opalescence of liquids" (Ph.Eur. 2.2.1, version 5). The degree of opalescence can be visually detected or alternatively determined by an instrumental method (nephelometry, turbidimetry, or ratio turbidimetry). During operator-based testing, the operators compare the solution in diffused daylight against reference standards. The solutions opalescence is usually reported in Formazin Turbidity Units (FTU). Turbidity reference standards are prepared using formazin and are diluted to the respective target opalescence. When formazin was initially adopted as the primary reference standard for turbidity, FTU or Formazin Turbidity Units were used. These units, however, do not specify how the instrument measures the sample. NTU stands for Nephelometric Turbidity Unit and signifies that the instrument is measuring scattered light from the sample at a 90° angle from the incident light.

Ph.Eur. currently defines the following categories, which relate to following NTU, as calculated for the respective dilution:

- Clear (up to 3 NTU, <Ref I);
- Slightly Opalescent (3–6 NTU, <Ref II);
- Opalescent (6–18 NTU, <Ref III);
- Strongly Opalescent (18–30 NTU, <Ref IV).

When assessed instrumentally, the test result is more accurate and independent of the analyst's vision. The disadvantage of the method becomes apparent if the degree of turbidity increases and the amount of particles is too high resulting in multiple scattering and the incident light is hindered in reaching the detector. Nephelometric measurements are therefore more reliable in low turbidity ranges, where there is a linear relationship between Nephelometric Turbidity Unit (NTU) values and relative detector signals.

Many drug products, especially for European Health Authority submissions, measure opalescence manually or instrumentally and drug product specifications often report either above FTU units (e.g., not more than 18 FTU), Ph.Eur. Reference Standards (e.g., NMT Ref III) or a descriptor as used in the Ph.Eur. (e.g., max. or not more than strongly opalescent).

The Ph.Eur. does not include any specific requirement for parenteral products in general, with regard to opalescence. In fact, there are commercial parenteral products available that are strongly opalescent (significantly above 18 FTU), such as emulsions and suspensions, for example, adjuvanted vaccines. In protein formulations, the concentration of protein, but also the type of formulation, would typically impact the degree of opalescence. The more concentrated a protein solution, the higher opalescence may be expected, as the scattering of light would increase with increasing concentration of undissolved proteinaceous spheres in the colloidal suspension. For high-concentration formulations (e.g., above 150 mg/mL

of protein), high values of opalescence can be expected and are often not a general sign of concern per se, but maybe—a bit oversimplified—considered as a cosmetic defect.

As shown in Fig. 5.1, the primary factor for solution opalescence is the protein itself. pH, ionic strength, and some specific excipients, respectively, ions, can also significantly change turbidity [32].

To note, in the Ph.Eur. monograph for "Monoclonal Antibodies for Human Use," these antibody products are described as "slightly opalescent" in the monograph text and requirements. While this expectation can be realistic for very diluted antibody formulations, it is quite unrealistic for an antibody formulation with a protein concentration well above 50 mg/mL.

As an example for formulation-dependent opalescence, citrate-containing protein formulations often have higher degrees of opalescence than comparable histidine-containing formulations of the same protein and with otherwise identical excipients [32]. The absolute level of opalescence is, however, not a sign of quality per se, yet a characteristic of a given solution.

Opalescence testing is often used during formulation development of protein formulations. In some cases, opalescence can correlate to protein aggregation [15]. It is rational that an increase in protein aggregation would change the size of colloidal spheres in solution, namely the aggregates or particles, which then would likely change the degree of opalescence. Also, the tendency of an increase in intramolecular interactions of proteins (repulsion or attraction) would be considered to impact opalescence. This is often assessed as second virial coefficient (A2 or B22).

Interestingly, depending on the aggregates or particles formed, it should be noted that opalescence values may also not show any change, despite significant protein precipitation and instability. As an example, if a protein would readily form

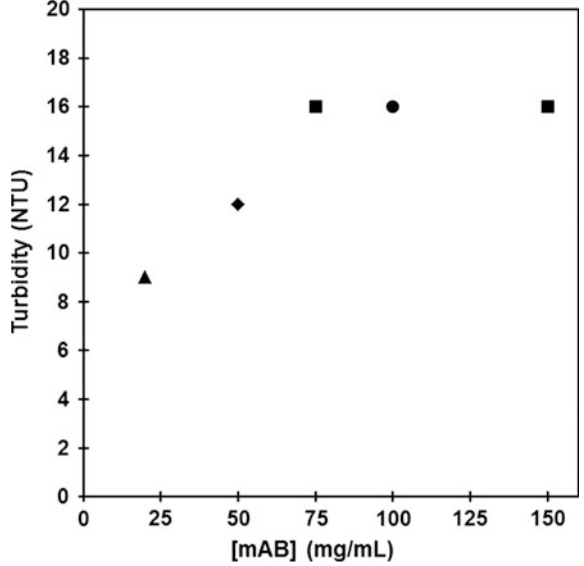


Fig. 5.1 Turbidity increase as a function of protein concentration. Four different monoclonal antibodies are depicted with different concentrations in similar formulations

significantly large particles (e.g., >100 μm in size), solution opalescence would not change. This is because only spherical aggregates that are small enough to impact the scatter of light would drive any change in opalescence. Opalescence can thus not be used as the sole method to assess protein aggregation and is incapable of replacing most valuable methods like size-exclusion chromatography (SE-HPLC), subvisible or visible particle testing.

Opalescence can also relate to other solution phenomena. This includes being a precursor of liquid phase–phase–separation [9, 16]. In other cases, opalescence has been found when protein formulations showed tendencies toward formation of gels (own unpublished data).

In summary, opalescence is a complex thermodynamic phenomenon that relates to solution properties and may depend on:

- Type of protein and its concentration;
- Buffer type and ionic strength;
- Protein aggregates and their shape;
- Phase separation phenomena.

While the absolute value of opalescence is usually not a relevant parameter, the level of change in opalescence may be important. This can be of significant interest during storage (tracked in stability studies) or for the evaluation of process changes. Opalescence testing is recommended for parenteral product development and Quality Control (QC) purposes such as release and stability testing, but cannot replace methods like SE-HPLC, subvisible or visible particle testing.

5.2.3 Coloration of Solution

Coloration of solution is defined as the degree of coloration of liquids in the range brown–yellow–red (Ph Eur 2.2.2, version 5). It refers to a solutions property and appearance caused by adsorption of specific wavelengths of light.

The European Pharmacopeia has a monograph on the degree of coloration of liquids (Ph Eur 2.2.2, version 5). This test can either be performed visually by an operator or by instrumental testing. During operator-based testing, the operators compare the solutions appearance or color to the given reference solutions, viewing horizontally against a white background. For instrumental testing, the color is measured using the reference color standards B, BY, Y, GY, and R. The degree of coloration is reflected by the respective numerical value in connection with the color scale; e.g., Y7 would be less yellow-colored than Y5.

- B (brown), intensity B1–B9;
- BY (brownish-yellow), BY1–BY7;
- Y (yellow), Y1–Y7;
- GY (greenish-yellow), GY1–GY7;
- R (red), R1–R7.

Protein formulations often show some level of solution color. In many cases, the color of protein solutions is on the "Yellow" or "Brown" scale. Given that colors on the "Yellow" and "Brown" scale may be close to each other, it is recommended to set a given color scale, once defined.

The Ph.Eur. does not include any specific requirement for parenteral products in general with regard to color. To note, in the Ph.Eur. monograph for "Monoclonal Antibodies for Human Use," these antibody products are described as "slightly yellowish" in the monograph text and requirements. While this expectation can be realistic for some very diluted antibody formulations, it is quite unrealistic for antibody formulations with a protein concentration well above 50 mg/mL, since coloration is likely stronger with high-concentration formulations. As mentioned, also other color scales than "Yellow" may often be required for protein formulations, including the "Brown" scale. For high-concentration formulations (e.g., above 150 mg/mL of protein) increased values of coloration can mostly be expected and are often not a general sign of concern. However, discoloration of protein drug products could give rise to concerns about potential quality impact. On the other hand, given the root causes of solution coloration of protein formulation, it is obvious that accelerated and stress testing, including high temperatures as well as light exposure, would lead to changes in color [21].

Recent studies on the topic have evaluated changes in color caused by oxidation [14, 27]. They identified tryptophan oxidation products as the main driver for color change to yellow in stressed monoclonal antibody drug product. Apart from the oxidation, other post-translational modifications on the antibody such as advanced glycation of end products can give rise to color [6]. Not only the molecule itself, but also various media components can contribute to drug substance color for a specific monoclonal antibody including carry over of media components [29]. Reducing the concentration of B vitamins has an effect on the intensity of the color. Derfus et al. also assessed the effect of processing conditions (medium composition and harvest conditions) on final bulk drug substance color and found that Vitamin B12 can cause a slightly red–pink color [11, 20]. Brown discoloration was also observed as a result of mixing effect of yellow and pink colors [33]. It was demonstrated that the brown color was caused by the chemically defined basal medium containing high levels of iron and vitamin B12. Iron caused tryptophan oxidation in the protein, which likely contributed to a yellow color and the pink color was caused by the residual B12. It can therefore be very cumbersome and labor-intensive to identify individual molecules that would drive coloration, since coloration may arise from multiple sources. In addition, these species are typically quite low in abundance and concentration levels.

In summary, coloration of solution relates to the solutions properties and appearance and may occur due to:

- Type of protein and concentration;
- Aggregate formation;
- Process residuals;
- Physical and chemical reactions caused by stress.

Proteins are expected to exhibit some color, especially in highly concentrated solution. While the absolute value of color is usually not a relevant parameter, the level of change in coloration, e.g., during storage or accelerated stability studies, can be of significant interest for the evaluation of process changes. The testing of the color of solution is recommended for parenteral product development and Quality Control (QC) purposes such as release and stability testing.

5.2.4 Viscosity

Viscosity of a solution can be defined as a measure of the resistance of a fluid to deformation under shear stress. It describes a fluid's internal resistance to flow and may be thought of as a measure of fluid friction (https://www.sciencedaily.com/terms/viscosity.html). Protein formulations show relevant changes in solution viscosity, as a function of:

– The active ingredient (protein) and related characteristics (e.g., size, electrostatics, dipole moment, presence/absence, and type of conjugation)
– Protein concentration
– Formulation properties (e.g., pH value, type of buffer, and excipients used with possible relevance for which counterions are used)
– Solution temperature
– Eventually shear rate. Protein formulations may or may not show shear-thinning behavior [2]. Of note is that the shear forces that may be typically occurring during manufacturing operations or injections (administration) are generally acknowledged to not lead to protein instability [3, 28].

Solution viscosity of protein formulations can be measured via different analytical methods. Most commonly used, and most applicable, is the plate/cone apparatus. This method can also vary shear rates and thus supports the evaluation if a given formulation shows Newtonian or Non-Newtonian behavior. Some plate/cone methods also can support the assessment of temperature of a solution viscosity with solvent trap and heating/cooling options. More traditionally used methods for viscosity assessments, like capillary Ubbelohde and other methods are often poorly applicable for proteins. These tests are quite crude and require significant sample volumes resulting in substantial cost per measurement due to the typically high cost of goods of therapeutic proteins. There are some recent advancements for an automated technology that requires only very little sample volume. This microfluidic Viscometer-Rheometer-On-a-Chip (mVROC) is used for the assessment of protein formulation viscosity. To note—these methods often do not correlate well to other viscosity methods given that measurements are poorly comparable related to expected shear rates.

Viscosity often becomes a limiting parameter when developing high-concentration formulations (see Fig. 5.2). A fantastic review on the topic is provided by Shire et al. [25].

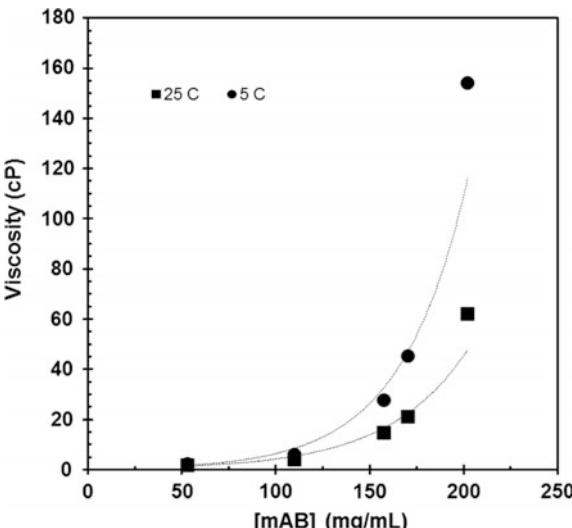


Fig. 5.2 Viscosity of an example monoclonal antibody as a function of concentration and at two different temperatures (5 °C vs. 25 °C)

It can have the following impact:

– Limiting ultrafiltration–diafiltration (UF-DF, tangential flow filtration/TFF) unit operations and leading to higher transmembrane pressures and at the same time lower flow rates.
– Limiting filtration unit operations (lower flow rates).
– Limiting filling operations, e.g., drying on/in the filling needle [24], for example, related to type and material of needle chosen, and or impacting filling precision.
– Impacting injectability [17]. More viscous solutions often lead to longer injection times when applying the same injection forces, or can increase the required injection forces. Given the applicability of Hagen–Poiseuille's Law, this issue is having increasing relevance (r to the power of 4!) with smaller inner diameters of injection needles, such as being used for some specific routes of administration (e.g., 30G needles often used for intravitreal injections).

Viscosity testing or ranges are not governed specifically by any regulatory guidance. However, given the importance of being able to manufacture and general Quality-by-Design principles, and considering the importance and regulations around patient usability, an adequate assessment of viscosity early in development is of utmost importance.

5.2.5 Aggregation and Particles

Proteins can undergo chemical or physical degradation pathways. Chemical degradation processes include the breakage of peptide bonds or amino acid

modifications such as oxidation and deamidation. On the other hand, physical degradation includes changes in the three-dimensional structure of the molecule and non-covalent interactions such as unfolding/denaturation, adsorption, aggregation, and precipitation. Aggregation and precipitation that lead to proteinaceous particle formation have received significant attention in the past years, given the concern about these being possibly connected to altered immunogenic reactions in patients, although this was discussed quite controversially [7, 26]. While there was no clinical evidence, in vitro work and animal studies suggested that artificially generated proteinaceous particles stimulated immune responses [12]. Recent studies suggest that aggregates or particles per se would, however, not be a significant immunogenicity risk unless significantly (almost quantitatively) oxidized [4, 5] to levels beyond typically observed in actual products. Yet, aggregation and particles typically remain a critical quality attribute to date. Of principal concern is also the product efficacy due to differences in biological activity of the aggregates [10].

Chapter 6 specifically discusses protein aggregates and particles and related analytical methods in more detail.

In brief, size-exclusion high-performance liquid chromatography (SE-HPLC) remains the workhorse of industry for the assessment of soluble aggregates. **Soluble aggregates** are typically oligomers like dimers, trimers, and tetramers that usually pass through a 0.22-μm filter [30]. In many cases, asymmetric flow field flow fractions (AF4) or analytical ultracentrifugation (AUC) can be used as orthogonal methods to confirm the validity of the SE-HPLC results. Soluble aggregates are required to be monitored and are specifically mentioned in relevant guidance such as ICH Q3B(R2). Acceptance criteria for soluble aggregates are per se not predefined and depend on product, process and related risk assessment. The higher the protein concentration, the more likely is the risk for aggregation to occur and the accumulation of aggregates over time.

Subvisible particles (≥10 and ≥25 μm) are required to be measured and specified, in compliance with the pharmacopeias like Ph.Eur., JP, USP, and other guidances. Light obscuration testing remains the method of choice for protein formulations. While the pharmacopeias also allow a "microscope method," the application of latter is often limited when trying to also measure proteinaceous particles in the subvisible particle range. USP recently introduced an additional monograph for biologics drug products, suggesting that flow imaging techniques would also be considerable as a relevant, orthogonal method. This method, however, is not found in Ph.Eur. or JP and the authors also believe that the value of flow imaging methods is primarily on characterization of the particles morphology based on images, rather than for quantitative counting. If used for latter, it may prove difficult to come up with relevant acceptance criteria for flow imaging methods that are meaningful and relevant for clinical safety as well as insurance of product quality and consistency. There is no common agreement on the maximum allowable aggregate levels in protein-based pharmaceutical products, because of the different behavior of proteins. Some may be largely stable and safe despite certain levels of aggregates, while for other proteins very small changes in aggregate levels may significantly affect protein stability and even safety [8]. Acceptance criteria for

light obscuration of subvisible particles are defined in the pharmacopeias and depend on product configuration: Small volume parenterals (SVPs; up to 100 mL container size) versus large volume parenterals (LVPs; >100 mL container size). When particles may be present in the drug product, it is mandatory to use of an in-line filter prior to parenteral administration. Products may be exempt from the subvisible particle requirements, if used with a final filter, but this can also be dependent on the route of administration and scope of application. While for parenteral preparations in general, the limits are (for SVPs) "NMT 6000 particles ≥10 µm and NMT 600 particles ≥25 µm"; the Ph.Eur. states that different values may be appropriate for subcutaneous or intramuscular administration. The USP <789> has established a monograph for ocular products with significantly tighter limits of "NMT 50 particles ≥10 µm and NMT 5 particles ≥25µm." Latter limits are not specifically required in the Ph.Eur., yet tighter limits than the ones listed in Ph. Eur. 2.9.19 may be appropriate in some cases, where product and process consistency would allow tighter limits, and where the disease state may warrant tighter acceptance criteria, such as when sterile products are used during surgery or for the injured eye.

The higher the protein concentration, the higher is the risk and likelihood for particles to occur. Of note, air bubbles also impact the results of subvisible particle counts, since they significantly contribute to particle counts. This is a common analytical artifact of a light obscuration method. Air bubbles are also often stabilized by surfactants, which are the most commonly used excipients in protein formulations. The impact of other parameters including color and opalescence on particle testing results was also studied. However, solution viscosity and refractive index showed a more pronounced effect on the analytical results, especially with more translucent particles [31].

There is a trend that **submicron particles** (≤1 µm) are being requested by some Health Authorities during product submissions for clinical studies (IND) as well as for marketing authorization approvals (MAA). There are various emerging methods in evaluation for that purposes, including Nanoparticle Tracking Analysis (NTA) and Resonance Mass Measurements (RMM). The available methods, however, do have significant downsides, for example, related to precision and accuracy that hinder their use for Quality Control purposes and significantly limit their applications to guide development [22, 23]. To date, these methods best suit their use for extended characterization or specific research purposes.

Visible particles should be minimized and avoided in parenteral products. The USP and Ph.Eur. require parenteral products to be "essentially/practically free of visible particles." The monograph "Monoclonal Antibodies for Human Use" requires latter products to be "without visible particles unless otherwise authorized or justified." This clearly signals the desire for parenteral products to do anything possible to avoid the presence of particles. There is no clear numerical threshold of visible particles in relation to clinical safety. This is both due to absence of relevant clinical data, as well as the variety of particle types that may be found, and due to the fact that dosing, disease state and concomitant treatments may alter the safety impact as well. Visible particle testing per Ph.Eur. 2.9.20 and USP uses a black and

white background panel with observation times of at least 5 s against each background. Testing would be performed by trained and qualified human operators. Any unit containing visible particulates would be removed from the rest of the batch during 100% inspection operations. Given that protein formulations may generate proteinaceous particulates, it is most relevant to also use best efforts during formulation, process, and packaging development, i.e., holistic drug product development toward minimizing particulates. Formulations containing proteinaceous particulates are suggested to be improved in the related drug product design. If—for whatever reason—product optimization cannot be performed in due course, a final remediation and derisking option is often the use of in-line filters during administration to patients (and animals). Such filters would need to be assessed for compatibility and ability to actually filter out the related particulates, to ensure that the administered solution would comply with expected pharmacopoeial requirements.

Formulations that have significant opalescence or coloration may adversely impact the ability to visually inspect these products for visible particles. Ph.Eur. thus specifically mentions that visible particle inspection also allows inspections using increased light intensity or longer inspection times. For further reading, we would like to refer to a review paper on the topic [15].

5.3 Conclusions

"Appearance" is a test often found in specifications of protein drug products. However, appearance can mean many different things, including dosage form (liquid, dried), color (coloration of solution, color of cake), cake appearance (in case of a lyophilisate), opalescence, or turbidity, and—in some cases—even visible particles. The European Pharmacopeia is a helpful source to establish more specific and appropriate testing parameters for development and/or quality control purposes, and this chapter aims to discuss various relevant, but often overlooked and underappreciated, parameters for protein drug products.

References

1. Ahern TJ. Chemical and physical pathways of protein degradation. Plenum; 1992.
2. Allmendinger A, Fischer S, Huwyler J, Mahler H-C, Schwarb E, Zarraga IE, Mueller R. Rheological characterization and injection forces of concentrated protein formulations: an alternative predictive model for non-Newtonian solutions. Eur J Pharm Biopharm. 2014; 87(2):318–28.
3. Bee JS, Stevenson JL, Mehta B, Svitel J, Pollastrini J, Platz R, Randolph TW. Response of a concentrated monoclonal antibody formulation to high shear. Biotechnol Bioeng. 2009; 103(5):936–43.

4. Bessa J, Boeckle S, Beck H, Buckel T, Schlicht S, Ebeling M, Weiser T. The immunogenicity of antibody aggregates in a novel transgenic mouse model. Pharm Res. 2015;32(7):2344–59.
5. Boll B, Bessa J, Folzer E, Rios Quiroz A, Schmidt R, Bulau P, Koulov AV. Extensive chemical modifications in the primary protein structure of IgG1 subvisible particles are necessary for breaking immune tolerance. Mol Pharm. 2017. https://doi.org/10.1021/acs.molpharmaceut.6b00816.
6. Butko M, Pallat H, Cordoba A, Yu XC. Recombinant antibody color resulting from advanced glycation end product modifications. Anal Chem. 2014;86(19):9816–23.
7. Carpenter JF, Randolph TW, Jiskoot W, Crommelin DJ, Middaugh CR, Winter G, Kozlowski S. Overlooking subvisible particles in therapeutic protein products: gaps that may compromise product quality. J Pharm Sci. 2009;98(4):1201–5.
8. Cordoba-Rodriguez RV. Aggregates in MAbs and recombinant therapeutic proteins: a regulatory perspective. BioPharm Int. 2008; 21(11).
9. Cromwell M, Carpenter J, Scherer T, Randolph T. Opalescence in antibody formulations is a solution critical phenomenon. Paper presented at the Papers, 236th ACS National Meeting, Philadelphia, PA; 2008.
10. Cromwell ME, Hilario E, Jacobson F. Protein aggregation and bioprocessing. AAPS J. 2006; 8(3):E572–9.
11. Derfus GE, Dizon-Maspat J, Broddrick JT, Velayo AC, Toschi JD, Santuray RT … Amanullah A. Red colored IgG4 caused by vitamin B12 from cell culture media combined with disulfide reduction at harvest. Paper presented at the MAbs; 2014.
12. Filipe V, Jiskoot W, Basmeleh AH, Halim A, Schellekens H, Brinks V. Immunogenicity of different stressed IgG monoclonal antibody formulations in immune tolerant transgenic mice. Paper presented at the MAbs; 2012.
13. Gonçalves AD, Alexander C, Roberts CJ, Spain SG, Uddin S, Allen S. The effect of protein concentration on the viscosity of a recombinant albumin solution formulation. RSC Adv. 2016;6(18):15143–54.
14. Li Y, Polozova A, Gruia F, Feng J. Characterization of the degradation products of a color-changed monoclonal antibody: tryptophan-derived chromophores. Anal Chem. 2014; 86(14):6850–7.
15. Mahler HC, Friess W, Grauschopf U, Kiese S. Protein aggregation: pathways, induction factors and analysis. J Pharm Sci. 2009;98(9):2909–34.
16. Mason BD, Zhang L, Remmele RL, Zhang J. Opalescence of an IgG2 monoclonal antibody solution as it relates to liquid–liquid phase separation. J Pharm Sci. 2011;100(11):4587–96.
17. Matheus S. Development of high concentration cetuximab formulations using ultrafiltration and precipitation techniques. Ludwig-Maximilians-Universitat; 2006.
18. Middaugh CR, Tisel WA, Haire RN, Rosenberg A. Determination of the apparent thermodynamic activities of saturated protein solutions. J Biol Chem. 1979;254(2):367–70.
19. Oster G. The scattering of light and its applications to chemistry. Chem Rev. 1948;43(2): 319–65.
20. Prentice KM, Gillespie R, Lewis N, Fujimori K, McCoy R, Bach J… Eakin CM. Hydroxocobalamin association during cell culture results in pink therapeutic proteins. Paper presented at the MAbs; 2013.
21. Qi P, Volkin DB, Zhao H, Nedved ML, Hughes R, Bass R, DalMonte P. Characterization of the photodegradation of a human IgG1 monoclonal antibody formulated as a high-concentration liquid dosage form. J Pharm Sci. 2009;98(9):3117–30.
22. Quiroz AR, Finkler C, Huwyler J, Mahler H-C, Schmidt R, Koulov AV. Factors governing the accuracy of subvisible particle counting methods. J Pharm Sci. 2016;105(7):2042–52.
23. Quiroz AR, Lamerz J, Da Cunha T, Boillon A, Adler M, Finkler C, Koulov AV. Factors governing the precision of subvisible particle measurement methods–a case study with a low-concentration therapeutic protein product in a prefilled syringe. Pharm Res. 2016; 33(2):450–61.

24. Shieu W, Stauch OB, Maa Y-F. Formulation-Nozzle Interactions To Minimize Nozzle Clogging Formulations into Pre-filled Syringes: Investigating Filling of High-Concentration Monoclonal Antibody. PDA J Pharm Sci and Tech. 2015;69:417–426.
25. Shire SJ, Shahrokh Z, Liu J. Challenges in the development of high protein concentration formulations. J Pharm Sci. 2004;93(6):1390–402.
26. Singh SK, Afonina N, Awwad M, Bechtold-Peters K, Blue JT, Chou D, Meyer BK. An industry perspective on the monitoring of subvisible particles as a quality attribute for protein therapeutics. J Pharm Sci. 2010;99(8):3302–21.
27. Song H, Xu J, Jin M, Huang C, Bongers J, Bai H, Tao L. Investigation of color in a fusion protein using advanced analytical techniques: delineating contributions from oxidation products and process related impurities. Pharm Res. 2016;33(4):932–41.
28. Thomas C, Geer D. Effects of shear on proteins in solution. Biotechnol Lett. 2011;33(3): 443–56.
29. Vijayasankaran N, Varma S, Yang Y, Mun M, Arevalo S, Gawlitzek M, Zhang B. Effect of cell culture medium components on color of formulated monoclonal antibody drug substance. Biotechnol Prog. 2013;29(5):1270–7.
30. Wang W, Roberts CJ. Aggregation of therapeutic proteins. Wiley; 2010.
31. Werk T, Volkin DB, Mahler H-C. Effect of solution properties on the counting and sizing of subvisible particle standards as measured by light obscuration and digital imaging methods. Eur J Pharm Sci. 2014;53:95–108.
32. Woods JM, Nesta D. Formulation effects on opalescence of a high-concentration MAb. BioProcess Int. 2010;8(9):48–59.
33. Xu J, Jin M, Song H, Huang C, Xu X, Tian J, Tao L. Brown drug substance color investigation in cell culture manufacturing using chemically defined media: a case study. Process Biochem. 2014;49(1):130–9.
34. European Pharmacopeia, current version, Monograph 2.2.1.
35. ICH Q3B(R2) IMPURITIES IN NEW DRUG PRODUCTS (Current Step 4 version 2 June 2006).
36. Internet: http://www.businessdictionary.com/definition/solubility.html.
37. Internet: https://en.wikipedia.org/wiki/Opalescence.
38. Internet: https://www.sciencedaily.com/terms/viscosity.html.

Chapter 6
Analytical Characterization and Predictive Tools for Highly Concentrated Protein Formulations

Andrea Allmendinger, Stefan Fischer and Robert Mueller

Abstract This chapter focuses on different aspects of development and characterization of highly concentrated protein formulations. This includes the introduction of predictive tools used during early development, specific challenges for analytical methods, and extended characterization of rheological and structural properties. The application of predictive tools during clinical lead selection and early-stage development helps to assess whether a selected clinical candidate may successfully pass the challenges of a high-concentration product in later development. In particular, parameters like B_{22} (second virial coefficient) and k_D (diffusional interaction parameter) are described and underlying principles, technologies, limitations, and applicability to highly concentrated formulations are elaborated. The specific challenges of high-concentration protein formulations with respect to analytical characterization are discussed in detail and practical recommendation is given. Different common techniques to measure viscosity are described including dependencies on protein concentration, temperature, and shear stress. Structural characterization of highly concentrated protein formulations requires analytical tools capable to measure in the non-diluted regime. Only a limited number of analytical techniques are available for this challenging task like small-angle scattering techniques (SAS) and quartz crystal impedance analysis (QCIA)/microbalance with dissipation monitoring (QCM-D). These techniques are described, and measuring principle, applications in literature as well as limitations are outlined.

Keywords High-concentration protein formulation · Protein–protein interaction
Analytical characterization · Non-ideality · Second virial coefficient

---

A. Allmendinger (✉) · S. Fischer · R. Mueller
Pharmaceutical Development & Supplies, PTD Biologics Europe,
F. Hoffmann-La Roche Ltd., Grenzacherstr. 124, 4070 Basel, Switzerland
e-mail: andrea.allmendinger.aa1@roche.com

S. Fischer
e-mail: stefan.fischer.sf2@roche.com

R. Mueller
e-mail: robert.mueller.rm1@roche.com

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_6

Diffusion coefficient · Rheology · Viscosity · Protein aggregation
Opalescence · Small-Angle scattering techniques

6.1 Predictive Tools for Clinical Lead Selection and Early-Stage Development

The desired outcome of clinical candidate selection and early-stage biopharmaceutical development is to bring an investigational new drug (IND) into clinical trials (Entry into Human (EIH)) and assess and test it as potential therapeutic protein. There is significant interest in the likelihood of the selected clinical candidate to successfully pass later development (beyond EIH) and eventually enter commercialization. As per today, novel therapeutic protein entities are emerging while still mostly therapeutic antibodies and related entities cover the majority of biotherapeutic drugs [1, 2]. The use of platform approaches and routine methods and their steady refinement often rather results in a panel of several potential candidate molecules with necessary functional properties than in "the one single candidate" [1]. This contrasts the resource- and cost-intense stages of development (bioprocessing development, pharmaceutical development and possibly device development) which need to be dedicated to the top candidate molecule [1]. Consequently, the decision about "the one candidate" is fundamental and needs to be gated prior subsequent development activities. In this context, molecular assessment is conducted; it assesses the likelihood of each candidate molecule to ultimately become a stable, efficacious and safe drug. A major component to molecule selection is the question whether the selected molecule is presumably suitable for large-scale manufacturing, the respective dosage form with the anticipated shelf-life (e.g. liquid dosage form with >24 M shelf-life at 2–8 °C intended storage) and—specific to highly concentrated formulations—has acceptable aggregation and viscosity behaviour. Such assessment is common to biotherapeutics and the approaches are often called "developability assessment" or similar. General developability approaches are beyond the scope of this chapter and the interested reader may refer to respective review articles [1].

In the context of highly concentrated formulations, a special interest lies in assessment of aggregation propensity and viscosity. Literature often suggests that these attributes are mostly governed by protein–protein interactions (PPI). In addition, literature suggests that PPI largely govern opalescence and general phase behaviour (such as seen by phase separation) [3]. Consequently, there is large interest in the direction and magnitude of intermolecular interactions and underlying principles for their assessment.

In this context, parameters such as the second virial coefficient (B22) and the diffusional interaction parameter (k_D) have been extensively studied. The following sections will elaborate on the underlying principles, limitations and—finally—on the applicability for highly concentrated formulations.

6.1.1 The Second Virial Coefficient (B22)

The second virial coefficient B22 describes non-ideal behaviour in solution and quantifies intermolecular forces between two protein molecules [4] while negative B22 values denote attractive behaviour, and positive B22 values describe repulsive behaviour. As described earlier on, attractive interactions between proteins are often related to aggregation, high viscosity, opalescence and liquid–liquid phase separation [3, 5–8].

Traditionally, the B22 has been assessed by static light scattering (SLS) [9–11] and membrane osmometry. In addition, analytical ultracentrifugation has been widely applied using the principle of sedimentation equilibrium.

SLS measures the time-averaged scattering intensity (Rayleigh scattering) that can be related to B22 by constructing the so-called Debye plot in a dilute solution regime. The methodology requires a concentration series, which can be obtained by (a) serial dilutions to be prepared and (b) a concentration detector, e.g. refractive index detector. The excess Rayleigh scattering is detected at 90° scattering angle. The classical approach is rather time—and material intense (about 50 mg of protein as assessed in a dilute solution regime and a few hours for the individual concentrations assessed with stable scattering intensities). Recently, automated approaches were developed, such as composition-gradient MALS.

Membrane osmometry is based on an osmotic pressure system consisting of two compartments and semi-permeable membrane. In the inner compartment, the buffer solution containing the protein is placed (sample solution), and in the outer compartment, only the buffer solution is placed (reference solution). An important attribute to the membrane is its impermeability to protein while being permeable to water and salt/buffer components [12]. In an equilibrium state, the chemical potential on both sides of the membrane is equal. The presence of protein (which in this case is a non-diffusible solute) reduces the chemical potential in the sample compartment and triggers solvent flow from reference to solvent compartment [12]. Applying pressure can stop solvent flow and establish equilibrium. This additional pressure applied is the osmotic pressure. Based on the McMillan–Mayer osmotic virial equation, the osmotic pressure can be linked to the osmotic second virial coefficient. The analysis is usually done by using the so-called membrane osmometers. As described for SLS, membrane osmometry requires dilution series. The analysis is work intense and does usually not allow for high-throughput analysis. This raises the question for alternative methods which allow for autosampler and automation. In this context, self-interaction chromatography (SIC) was developed. In 1996, Patro et al. reported on SIC as a means to study PPIs in bioprocessing environments [13]. SIC requires a protein immobilized solid phase chromatographic stationary phase [14], the protein being present in the mobile phase and finally injection of the solution of the protein to be studied. The elution volume is typically assessed by a liquid chromatography detector such as UV. From the classical measurement alone, there is no link to B22 yet until a model developed by Tessier et al. is used to relate osmotic second virial coefficient to the elution

behaviour in SIC [15]. SIC may come into play if the Rayleigh scattering of a molecule is not strong enough to apply SLS; this can be the case for species of lower molecular weight such as peptides [16]. In the case described by Payne et al. [16], a 36-amino acid therapeutic peptide was studied, and salt concentration, salt type, and pH were varied and related to solubility and apparent molecular weight. This specific study showcases the applicability and screening options of SIC in general. Limitations of SIC arise from the general need for column preparation, i.e. protein immobilization. Moreover, the accurate measurement of dead volumes is crucial for accurate retention time data which is—as described earlier on—the base for calculation of the B22.

Among these techniques described, SLS has been most widely used. Membrane osmometry and sedimentation equilibrium analysis (using analytical ultracentrifuge) require long measurement time, i.e. are not high-throughput methods. In addition, the material amounts required are often not yet available in early discovery, lead candidate selection.

B22 in general and its predictive power for concentrated solutions are being discussed ambiguously. As outlined earlier on, the B22 is primarily applied in a dilute solution regime. Nevertheless, good qualitative correlations could be found between the B22 as assessed in low concentration and viscosity and aggregation propensity at concentrations higher than 100 mg/mL [17].

6.1.2 The Diffusional Interaction Parameter k_D

A methodology which recently gained much interest is the assessment of the diffusional interaction parameter k_{D}. Measurements of k_{D} are usually conducted at protein concentrations of 1–20 mg/mL using dynamic light scattering (DLS) where mutual diffusion coefficients are measured as a function of solute concentration (c) and allow for the calculation of the diffusional interaction parameter (k_{D}) (with D_0 is the self-diffusion coefficient).

$$\mathrm{D} = \mathrm{D}_0(1 + k_{\mathrm{D}}c) \tag{6.1}$$

In a simplified way, a positive k_{D} signifies more repulsive interactions while less positive k_{D} implies more attractive intermolecular interactions. A major advantage of the DLS instrumentation is its applicability to a micro-well format which allows for automated assessment of k_{D} while keeping API consumption very low [17–19]. Various studies have been using dynamic light scattering, mostly due to the fact that k_{D} is often reasonably correlated to B22 [19–21]. Lehermayr et al. assessed k_{D} (via DLS) and the second virial coefficient (via SLS) for a set of monoclonal antibodies in typical pharmaceutical buffer systems and derived an empirical relationship between k_{D} and B22 applicable to standard antibodies. This data has been generated in a dilute concentration regime (as described above). The methodology allows for larger screenings based on the well-plate format and significant less protein required

for the studies. For a classical B22 measurement by SLS in dilution solution, 50 mg of protein is a good estimate. For the k_D approach by DLS applying micro-well plate format, k_D can be obtained with about 1.5 mg of protein. Several studies have demonstrated that k_D can be a fast tool to study protein aggregation propensity and solubility [22]. A comprehensive study conducted by Connolly et al. [18] correlated k_D values of 29 monoclonal antibodies (obtained at 1–20 mg/mL) to dynamic viscosity data at the high-concentration regime (≤175 mg/mL) within four different solution conditions. It should be noted that the correlation was not equally well established between the four buffer systems tested, however the study demonstrates that k_D can be a powerful parameter to assess high-concentration behaviour in the dilute solution regime, especially if only low amounts of protein are available.

In terms of predictive power, the tools described so far need to be taken with care. It should be noted that the tools based on B22 and k_D may provide sufficiently good relative ranking of different proteins, however full correlation with aggregation or viscosity may not be found. This may especially become relevant if attempts are undertaken to predict high-concentration properties, as the methods—in their classical application—are methods describing solution behaviour in dilute solution.

6.2 Analytical Characterization of Highly Concentrated Protein Formulations

6.2.1 Analytical Challenges for Characterization of Concentrated Protein Formulations

Concentrated pharmaceutical protein formulations have a protein concentration from 50 mg/mL to more than 200 mg/mL [23]. Thereby, they show significantly different physical properties, such as increased viscosity and elevated opalescence, in comparison to diluted formulations. These properties have an immediate influence on the sample preparation and method performance of analytical assays used for the characterization of concentrated protein formulations.

High viscosity poses several challenges for handling of concentrated protein formulations. Sample preparation for analytical assays using only small loading amounts requires accurate pipetting of small volumes of viscous sample and high dilution factors. In order to achieve the requested analytical precision and accuracy, comprehensive method development, operator training and use of special equipment (e.g. positive displacement pipettes) are required. The use of optimized automated liquid handling systems can further reduce variability during the sample preparation and dilution process of concentrated protein solutions.

Concentrated protein formulations may appear more opalescent than diluted solutions due to the intensified protein–protein interactions [3, 24]. The higher turbidity may handicap the visual inspection for particulate contaminations. This may complicate the qualification of automated inspection procedures or require lengthening of inspection time per sample for visual inspection operators.

Sub-visible particle measurements using the light obscuration method can be impacted by the occurrence of flow marks when a concentrated protein sample gets in contact with an aqueous rinsing solution. In order to avoid falsified results due to the formation of flow marks in the detector cell, optimization of pre-rinse volume is required.

The concentration of sub-visible particles can also be underestimated in viscous solutions due to intake of air into the pumping system of the light obscuration instrument and a resulting reduced measurement volume. Weinbuch et al. suggested sample pressurization to overcome this problem [25].

Werk et al. reported that the parameters viscosity and refractive index have a pronounced effect on sizing and counting accuracy and precision, which results in detection of smaller sizes and lower counts of sub-visible particles in concentrated protein formulations [26].

USP <787> emphasizes specific challenges of high-concentration and or high viscosity products including small differences in refractive index between particles and solution or problems with fully soakage of viscous sample solutions [27].

Furthermore, concentrated protein formulations are more prone to drying when exposed to environmental conditions. Partially drying of a protein sample may lead to sample inhomogeneity, potential physical degradation, and clogging of capillaries or needles. Hence, the sample needs to be quickly processed after the primary container has been opened and the sample solution is exposed to ambient temperature and lower relative humidity. The occurrence of needle clogging of staked-in needle syringes pre-filled with concentrated protein solutions, which have been exposed in an uncontrolled way to environmental conditions, may interfere with injection force test results of syringe-based medical devices. Controlling environmental laboratory conditions and the exposure time of opened samples may help to avoid complications during sample treatment and testing.

6.2.2 Aggregation (Soluble and Insoluble Aggregates)

Physical instability refers to changes in the protein folding or higher-order structure which include denaturation, adsorption to surfaces, aggregation, and precipitation [28, 29]. Aggregation requires bi-molecular collisions and a strong concentration dependency is expected to be dominant in high-concentration protein formulations [30]. Protein aggregation may result in covalent (e.g. disulphide-linked) or non-covalent association. Irreversible aggregation by non-covalent association typically occurs via hydrophobic regions exposed by thermal, mechanical, or chemical stresses, that alter the protein's native conformation. Protein aggregation may impact protein activity, pharmacokinetics and/or is considered relevant for changes in immunosafety [29, 31]. It occurs in most biopharmaceutical processes, especially during fermentation, refolding, purification, formulation and storage [29, 32–34].

Reversible protein association may also have a significant influence on protein activity, in vivo clearance or safety, if dissociation of associated protein molecules

is rather slow, or the equilibrium at high protein concentration is shifted to the associated state.

Analytical technologies used for characterization of proteins involve usually dilution to lower concentrations or exposure of the protein to solvent conditions that differ from the initial formulation composition. This may have considerable impact on the results of the assay since a change in solvent composition or concentration may alter a protein's physical state in a way that is not representative to the initial condition in the formulation [31]. This problem is especially important in the analysis of molecular weight and size distributions of proteins. SDS-PAGE, non-gel sieving SDS-capillary electrophoresis, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) are often used to obtain molecular weight information. These techniques are also useful for detecting covalently linked aggregates, or SDS non-dissociable aggregates, but cannot be used to determine non-covalent protein association states. The often-used gel permeation or size exclusion chromatographic method (SEC) provides such information but has several challenges. The protein's interaction with the chromatographic resin and its hydrodynamic volume may alter the elution time; thus, the use of globular protein standards to estimate the molecular weight may be erroneous. When protein–resin interactions are ionic in nature, the addition of salts will decrease such interactions, but control experiments are required to demonstrate that the increased ionic strength of the mobile phase does not perturb the size distribution of the protein. The impact of hydrodynamic volume on elution time, leading to the over-estimation of protein size, is especially noted for highly glycosylated proteins that have shapes different from the typical globular protein standards. The use of static light scattering detectors coupled with sizing chromatography (LC/LS) allows for the absolute determination of molecular mass of a protein and its higher-order aggregates and fragments during separation by gel sieving. Since elution times are not used to estimate molecular size, the problems of protein–resin interaction and hydrodynamic volume are no longer relevant [29].

The most commonly used analytical technique for quantifying aggregates in pharmaceutical formulations is SEC coupled with UV detection. In high-concentration formulations where reversible protein self-association predominates, the determination of aggregate levels by the SEC method may be inconsistent and inaccurate [35]. The SEC method involves dilution of the protein sample during injection onto the column which may lead to dissociation of aggregates with rapid dissociation rate constants. Moreover, to prevent detector saturation, high-concentration protein solutions are generally diluted even prior to injection onto the SEC column. The rapid dissociation of a monoclonal antibody upon dilution could result in varying aggregate levels, depending on the time and temperature of analysis after sample dilution. However, the use of SEC as an analytical tool for quantification of aggregates and estimation of shelf-life of protein drug products requires a high analytical precision [29, 36].

Technologies such as dynamic light scattering and analytical ultracentrifugation may provide more representative information about protein self-association states at high concentrations, because they maintain the sample composition during analysis

and can also be performed at high protein concentration [37]. Limitations of the light scattering methods are: they are not quantitative and are subject to multiple scattering artefacts at high protein concentrations. Analytical ultracentrifugation (AUC) performed as sedimentation equilibrium centrifugal analysis can be used to characterize protein aggregation. The analysis requires fitting of data to several exponentials and is model dependent. Sedimentation velocity analysis has been greatly improved and algorithms are now available to determine small amounts of protein aggregates and sedimentation coefficients. The determination of the sedimentation coefficient can provide valuable information regarding overall shape of the protein when coupled with computations of hydrodynamic bead models. However, correction of apparent molecular weights and sedimentation coefficients due to non-ideality at high protein concentrations is difficult to achieve [31, 38]. Another technique uses preparative centrifuges coupled with a specially designed micro-fractionator to obtain sedimentation equilibrium measurements of molecular weight in concentrated as well as multi-component protein systems [39].

Field-flow fractionation (FFF) methods have recently come into focus as being highly attractive for protein analysis [40]. In FFF, particles and molecules are separated according to their diffusion coefficients. Separation is performed in an open channel by the combination of a (Poiseuille) flow in the axial direction and a cross-flow through the porous wall of the channel [41]. Litzen et al. [42] compared separation and quantitation of monoclonal antibody aggregates by asymmetrical flow field-flow fractionation and gel permeation chromatography. FFF demonstrated following advantages compared to SEC: it can cover a broader size range in one single run and the size range to be analyzed can be adjusted by the flow rates using one single separation channel. It gives the same resolution but with a faster separation. Furthermore, FFF offers much faster and flexible analysis of the influence of formulation buffer on the aggregation of proteins. FFF instrumentation can be coupled to MALS-RI/UV detector, which permits direct determination of the molecular weight of the separated species.

6.2.3 Viscosity of Protein Solutions

A major challenge for the development of high-concentration protein formulations is the increase of viscosity with increasing protein concentration. The higher viscosity has a direct impact on the performance of manufacturing processes such as concentration and buffer exchange by ultrafiltration, sterile filtration and filling operations [31, 43]. Furthermore, a high viscosity may limit the ability to administer the protein drug by injection [44].

Tanford describes a viscous fluid as a fluid in which there are attractive forces between neighbouring portions of the fluid [45]. Applying an external force on the viscous solution creates flow which is opposed by a frictional force. This definition leads to the original concept from Isaac Newton on the dynamic (shear) viscosity η (Pa s) as the ratio of shear stress σ (Pa) and shear rate $\dot{\gamma}$ (s^{-1}).

$$\eta = \frac{\sigma}{\dot{\gamma}} \tag{6.2}$$

An alternative viscosity parameter is the kinematic viscosity ν ($m^2\ s^{-1}$) which is defined as the ratio of dynamic viscosity and the solution density ρ ($kg\ m^{-3}$).

$$\nu = \frac{\eta}{\rho} \tag{6.3}$$

The viscosity of a protein solution is mainly influenced by protein concentration, shear stress and temperature. Other factors include formulation properties such as pH and ionic strength or the nature of salts [39, 46].

The next sections provide a selection of commonly used models to describe the relationship between viscosity and the parameters protein concentration, shear stress and temperature.

6.2.3.1 The Impact of Shear on the Viscosity of Concentrated Protein Solutions

A Newtonian solution is defined by a constant viscosity independent of the applied shear rate. However, many fluids and solutions deviate from the Newtonian behaviour and show either shear-thinning or shear-thickening behaviour [47]. Recently, concentrated antibody formulations have been characterized for shear-thinning behaviour which was observed during filtration and injection processes [44, 48–51]. The shear-thinning effect is defined as the reduction in viscosity at high rates of deformation [52]. Depending on the nature of the system, shear-thinning can be explained by the changes of the structure of colloids as a result of deformation [53] or that polymer chains are disentangled and stretched at high rates of deformation [52]. Thereby, the viscosity curve shows three distinct sections: (1) at low shear rates an apparent Newtonian viscosity or zero shear viscosity, (2) followed by an exponential decrease of viscosity with increasing shear rate, and finally (3) an apparent Newtonian viscosity behaviour at high shear rates [54]. Allmendinger et al. [48] modelled the viscosity curve of concentrated antibody solutions with an Bird–Carreau–Yasuda model (Eq. 6.4) and observed a zero-viscosity range for shear rates up to 10,000 s^{-1} followed by an exponential decay of measured viscosity. However, a second Newtonian range could not be measured due to limitation in measuring viscosity at shear rates higher than 100,000 s^{-1} [52].

$$\eta = \eta_\infty + (\eta_0 - \eta_\infty) \cdot \left(1 + \Gamma^2 \dot{\gamma}^2\right)^{\frac{n-1}{2}} \tag{6.4}$$

The model developed by Bird, Carreau [55] and Yasuda [56] accounts for the observed Newtonian plateaus and is able to fit a wide range of shear rates. It includes five parameters, where η_0 is the zero shear rate viscosity, η_∞ is an infinite shear rate viscosity of the second Newtonian plateau, and Γ is a time constant

reflecting the critical shear rate $\dot{\gamma}_{crit} = \Gamma^{-1}$. The parameter n represents the Power Law index, which accounts for the shear-thinning behaviour and describes the width of the transition region between the zero shear viscosity and the Power Law region, which has the value 2 in the original Bird–Carreau model [52].

6.2.3.2 The Dependence of Viscosity From Protein Concentration

A major challenge for the development of concentrated protein formulations is the increase of viscosity with increasing protein concentration [31]. Thereby, the viscosity depends strongly on concentration, size and shape of the protein and the nature of protein–protein and protein–solvent interactions [57]. The concentration dependency of a protein solution can be described by a virial expansion, relating the viscosity to the solvent viscosity η_s and protein concentration c where higher-order terms of the expansion account for intrinsic viscosity [η] and the interaction parameter Huggins coefficient K_υ [58–60].

$$\frac{\eta}{\eta_s} = 1 + [\eta]c + K_v c^2 + \cdots \tag{6.5}$$

The intrinsic viscosity [η] is a quantity characteristic of a polymer and depends on the molecular weight and the conformation of the polymer. The second-order coefficient K_υ can be positive or negative and indicates attractive or repulsive forces between molecules [59, 60].

Ross and Minton [61] described an alternative concentration dependency of viscosity which was developed based on a modification of the concept by Mooney [62].

$$\eta = \eta_s \cdot \exp\left(\frac{[\eta]c}{1 - (k/\upsilon)[\eta]c}\right) \tag{6.6}$$

The factor (k/υ) is the ratio of the dimensionless self-crowding factor k and the dimensionless Simha form factor υ [61, 63].

6.2.3.3 The Influence of Temperature on the Viscosity of Concentrated Protein Solutions

The viscosity of a fluid sensitively depends on the temperature [59] and in most cases will decrease with increasing temperature. Due to the strong temperature dependence of the viscosity, it is required to report the viscosity result always together with the testing temperature and to tightly control the sample temperature during the measurement [64].

The Arrhenius relationship (Eq. 6.7) is widely used to describe the temperature dependence of viscosity [58, 64].

$$\eta(T) = \eta_0 \cdot \exp(-E_0/RT) \tag{6.7}$$

The parameter η_0 is a matter constant (Pas), E_0 is the flow activation energy (J), R is the universal gas constant 8314 J/(mol K), and T is the temperature in K.

6.2.4 *Analytical Methods for Measuring Viscosity of Protein Solutions*

There is a wide variety of analytical methods available to measure viscosity or other rheological properties of concentrated protein solutions. Frequently applied instruments in the field of protein formulation development include rotational rheometers, falling ball and capillary viscometers. In the recent years, new instrument and methods became available with advances in reducing sample volume and/or increasing sample throughput. The selection of the viscosity test method should be based on the nature of the sample, required rheological information and availability of sample volume, but should also consider the process or unit operation which is in scope of the rheological characterization.

6.2.4.1 Falling Ball and Capillary Viscometers

The use of a falling ball is one of the oldest methods in rheometry and the principle was already used by G. Stokes in 1851. F. Höppler designed in 1933 a falling ball viscometer which is meanwhile standardized (DIN 53015) and commercially available. The methodology is described in several standards such as USP <913> [65]. The instrument measures the time which a metal or glass ball takes to sink through a glass tube filled with a liquid sample. The viscosity η is calculated from the measured descent rate U_∞ according to Eq. 6.8 [65, 66].

$$\eta = \frac{C_0 \cdot (\rho_S - \rho_l) \cdot g \cdot \cos\varphi}{U_\infty} \tag{6.8}$$

C_0 is the instrument constant, which is found during calibration of the measurement tube and each sphere, g is the gravitational acceleration, ρ_S and ρ_l are the densities of the sphere and the test sample, and φ is the inclination angle of the measurement tube.

Falling ball viscometers have the advantages of simple operation, as well as the prevention of evaporation and skin formation at the air–liquid interface, a large viscosity measurement range from 0.3 m Pas to more than 10 Pas, a good repeatability (without changing the ball) and a good temperature control. The classical Hoeppler viscometer requires a large sample volume of approximately 40 mL, which limits its application for characterization of expensive concentrated protein solutions. Many companies offer variants of the classical Hoeppler

viscometer with significantly reduced sample volumes (e.g. rolling ball micro-viscometer with sample volumes less than 1 mL). Limitations of falling ball viscometers are the low sample throughput due to long measurement times, laborious cleaning of the tube, and that accurate measurements without complex corrections are only possible for Newtonian samples [64].

Another well-established principle is the measurement of kinematic viscosity with a glass capillary viscometer. The principle of the method is to measure the running time of a viscous sample, driven by gravity, which flows through a glass capillary of defined inner diameter and length [58]. Capillary viscometers are available with different designs such as Ostwald (DIN 51561), Ubbelohde (DIN 51562) and Cannon-Fenske (DIN 51336) viscometer. The different designs vary in operability, flow profiles and possibility to correct flow disturbances [64]. USP 39 provides a methods description for Ubbelohde- and Ostwald-type viscometers [67]. The required sample amount is in the range of a few mL and the method is limited in sample throughput due to laborious cleaning of the glass capillary and long measurement times. Like the falling ball principle, the capillary viscometer determines only accurate viscosity results for Newtonian samples without extensive application of corrective calculations as shear rates are not controlled and will change during the measurement [58].

6.2.4.2 Rotational Rheometers

Rotational rheometers are, in comparison with falling ball or capillary viscometers, more flexible instruments that can be used to measure the rheological behaviour over a wide range of test conditions [58]. In a rotational rheometer, a viscous sample is sheared between a static and moving surface and the resulting shear stress is determined via measurement of the generated torque. Typical test applications for rotational rheometers are measurement of shear viscosity (flow and viscosity curves), creep recovery tests, deformation and relaxation tests, and oscillation experiments [64].

Typical test geometries for rotational rheometers are cone and plate, parallel plate and concentric cylinders [64, 68]. Thereby, cone and plate measurement devices have been most frequently used for the characterization of concentrated protein solutions [46, 48, 69]. In a cone and plate device, the sample is introduced into the fixed gap between a flat plate and a truncated cone. The defined angle of the cone ensures a constant shear rate in the entire sample. The resistance caused by the viscosity of the sample is recorded as function of rotational speed of the cone. As described in Eq. 6.2, the viscosity η is the defined as the ratio of shear stress τ (Pa) and shear rate τ (pa) (s^{-1}). For a plate and cone device, the shear stress τ can be calculated from the measured torque M according to Eq. 6.9 with the cone diameter R and the shear rate τ from the rotational speed n according to Eq. 6.10 with α the cone angle in [°].

$$\tau = \frac{3 \cdot M}{2 \cdot \pi \cdot R^3} \tag{6.9}$$

$$\dot{\gamma} = \frac{6 \cdot n}{\alpha} \tag{6.10}$$

Thereby, the sensitivity to measure lower shear stresses can be extended by using cones with larger diameter, whereas the maximum applied shear rate can be increased by utilizing cones with smaller angles.

A rotational rheometer in combination with the plate and cone device has several advantages when measuring concentrated protein solutions. The design of the plate and cone device provides homogeneous shear condition within the sample, which allows the characterization of shear viscosity for Newtonian and non-Newtonian protein samples over a wide range up to shear rates of more than 10,000 s^{-1} [48]. Furthermore, the measurement requires only small sample volumes (e.g. less than 100 µl for a 0.5° cone with 25 mm diameter [48]), and the measurement device is simple to clean. However, the method is sensitive to perturbations due to sample inhomogeneity at the edges caused by solvent evaporation, skinning at the air interface, surface activity of protein molecules [69], or sample leakage and partial gap draining at high rotational speeds [64]. Temperature control only via the bottom plate can lead to a temperature gradient within the sample. Both temperature and sample inhomogeneity can be reduced by using a cover hood with inside space temperature control and a solvent trap [64]. Although the sample throughput is higher than with capillary or falling ball viscometers due to the easier cleaning of the measurement device, rotational rheometry is not a high-throughput analytical method.

6.2.4.3 Alternative Viscosity Methods

In recent years, new viscosity methods have been used for rheological characterization and formulation development of concentrated protein solutions. Capillary extrusion or micro-channel rheometry was applied to characterize Non-Newtonian protein solutions at shear rates higher than 10,000 s^{-1}, where the rotational rheometry reaches the limits of its applicability [48, 70, 71].

Nanoparticle tracking velocimetry by dynamic light scattering has been used to minimize sample amount and to increase sample throughput. He et al. [72] used 150 nm polystyrene particles as tracer particles and determined the viscosity of concentrated protein solutions using the Stokes–Einstein equation (Eq. 6.11) with D the measured diffusion coefficient, k represents the Boltzmann's constant, T the temperature in K, η the viscosity of the diffusion medium, and R is the radius of tracer particle.

$$D = \frac{k \cdot T}{6 \cdot \pi \cdot \eta \cdot R} \tag{6.11}$$

Pamar et al. [73] describe a similar approach using the small protein lyosozyme as tracer particle. The principle is easily compatible with high-throughput instrumentation using multi-well plates and is therefore a valuable tool for formulation screening of concentrated protein solutions. However, the technique will not provide details on the shear viscosity [58].

Allmendinger et al. [74] described an automated, high-throughput method to determine viscosity of protein solutions using standard capillary electrophoresis (CE) equipment. Riboflavin was used as a tracer dye to monitor movement of protein samples. The viscosity of the protein sample was calculated from the migration time of the riboflavin peak moving through the filled capillary by applying the Hagen–Poiseuille's law. The suitability of the method was demonstrated for protein formulations with viscosities in the range of 5–40 m Pas. Advantages of the CE instrumentation-based method included commercially available and established automation, short measurement times (1–15 min) and small sample volumes (few microlitres), which makes the technique applicable for high-throughput measurements.

6.3 Extended Characterization

Analytical characterization of highly concentrated protein solutions can be challenging as outlined in the previous section. In particular, the requirement to dilute the analytical sample to lower protein concentrations like for the quantification and characterization of protein aggregates for various chromatographic methods results in a limited number of analytical techniques being available for direct characterization of highly concentrated protein solutions on a molecular level. However, characterization at the molecular level is crucial to truly understand physical macroscopic properties at higher protein concentrations like solution viscosity.

Macroscopic behaviour at protein concentrations exceeding the 100–150 mg/mL is defined by several aspects. First, these are among others the intrinsic properties of the molecule (e.g. amino acid sequence/molecular weight, size/confirmation/shape). Second, the number and nature of molecules in solution leading to crowding of the molecules with increasing concentrations (volume exclusion) adds to the macroscopic behaviour. Finally, solution behaviour at high protein concentrations is governed by intermolecular interactions described by the potential of mean force between two molecules, which is a function of intermolecular distance [21, 57]. Depending on the nature of these interactions (e.g. electrostatic, hydrophobic, van der Waals forces) being repulsive or attractive in nature, and dependent on other solutes around the molecule (formulation composition), they can lead to non-ideal behaviour of the protein solution [75]. Since predictions of macroscopic behaviour

are based on analytical techniques measuring at low protein concentrations, the non-ideality can only be accounted for to some parts and thus limits the outcome of the prediction [76]. Formation of transient higher-order structures in solution as a result of protein–protein interactions has been reported to be the underlying reason for increased viscosities for some monoclonal antibody formulations [77]. These structures are reversible and consist of loosely bound clusters of various sizes depending on protein concentration and formulation composition [78]. The cluster size has been reported to be directly linked to macroscopic behaviour, specifically to viscosity, thus driving the need for analytical techniques measuring at high protein concentrations.

Characterization of these structural components on a molecular level can be performed by use of small-angle scattering techniques (SAS) measuring in the non-diluted sample regime as well as characterization by use of quartz crystal impedance analysis (QCIA) and quartz crystal microbalance with dissipation monitoring (QCM-D). The structural information can also elucidate information on protein–protein interaction at high protein concentrations. These methods, which are used for extended characterization at high protein concentrations, are described in detail in the following paragraph including the measuring principle, applications in literature as well as limitations.

6.3.1 Storage/Loss Modulus G′/G″ Determined by Quartz Crystal Impedance Analysis and Quartz Crystal Microbalance with Dissipation Monitoring

Quartz crystal impedance analysis (QCIA) and Quartz crystal microbalance with dissipation monitoring (QCM-D) are non-destructive test methods to characterize viscoelastic properties of a liquid. The quartz crystal is resonating at high frequencies in the megahertz range (5–10 MHz) applying an oscillating strain onto the sample [79, 80]. The underlying principle is the electromechanical coupling, which means the interplay of the electric properties of the piezoelectric quartz crystal and the mechanical properties of the load. Deformation of the quartz crystal in both directions resulting in mechanical vibrations leads to separation of charges in the piezo crystal and thus build-up of an electrical potential proportional to the applied stress. The resonance frequency is determined by the mass of the crystal as well as by the mass of the load. Thus, loading of a sample results in a frequency shift. To solely determine the mechanical properties of the sample, the effect of mass needs to be differentiated from the density–viscosity effect by either impedance monitoring (QCIA) [81] or by determination of the dissipation factor (QCM-D) [82, 83]. These parameters can be converted to the storage modulus G′, which is a parameter normally used for quantification of viscoelastic material under application of an oscillating strain. G′ is frequency-dependent and a measure of energy stored in the system. The loss modulus G″ in contrast is proportional to the loss of energy during

oscillation and characterizes the viscous, plastic part of a sample. The solution appears as a liquid as long as G′ is below G″ and vice versa as a gel-like structure if G″ is higher than G′. Both parameters are governed by the molecular interaction within the material. An increase in intermolecular interactions may result in (higher-order) structural changes leading to an increase in energy stored in the system upon application of a strain. The relaxation time increases as the molecules are no longer able to re-orient in the timescale of a single oscillation. As an example, this results in a smaller shift in impedance for QCIA compared to an ideal solution, which show a linear correlation of impedance shift and viscosity/G″. For an ideal fluid, molecular relaxation times are small enough at high frequencies and molecules re-orient themselves. Therefore, G′ and G″ measured in the megahertz frequency range can be used as a parameter to characterize viscoelasticity directly translating into a measure for protein–protein interactions.

Application of QCIA to aqueous solutions in the pharmaceutical relevant viscosity range for parenteral injections was first shown by Saluja and colleagues in 2004 for ideal formulations like sucrose, urea, PEG-400, glucose, and ethylene glycol solutions as well as for a non-ideal PEG 800 formulation [81]. In 2005, they studied highly concentrated BSA formulations up to a protein concentration of 200 mg/mL at various pH (2–9) and ionic strengths (25 and 150 mM sodium chloride). They reported a decrease in storage moduli with higher ionic strength due to shielding of electrostatic interactions showing the potential to characterize and link the rheological properties of the formulations with protein–protein interactions [84]. In 2006, Saluja and colleagues studied monoclonal antibody formulations (IgG_2) at protein concentrations up to 120 mg/mL at a pH between 4 and 9 and an ionic strength up to 300 mM. Consistent with the change in ionic strength and in pH, they reported a direct relation between the storage moduli and protein–protein interactions [85]. In 2007, they established a qualitative relation between the second virial coefficient from light scattering measurements performed at low protein concentrations and the storage modulus as a novel measure for protein–protein interactions at higher protein concentrations. They studied IgG_2 formulations for a pH between pH 3.0 and 9.0, however only up to a protein concentration of 120 mg/mL [86]. The QCM-D method was applied in 2009 by Patel and colleges to various IgG_2 solutions (70 mg/mL, pH 4–6.2) for analysis of the storage modulus G′ characterizing the rheological properties as well as characterizing protein–protein interactions [87]. In general, determination of the storage and loss moduli by QCIA and QCM-D requires a low sample consumption in the microlitre range of only 10–20 μL. Further advantages of these methods are the short measurement time of 2–3 min, fast stabilization after loading, and overall easy handling. However, accurate and uniform drop placement as well as complete coverage of the electrodes is challenging. Importantly, the QCM-D instrument used in the mentioned publications is commercially available.

6.3.2 Small-Angle Scattering Techniques

Small-angle scattering (SAS) techniques are powerful methods to investigate nanostructures of colloidal macromolecular systems like protein solutions at high protein concentrations. These techniques use elastic scattering characterized by zero energy transfer of either an X-ray (Small-angle X-ray scattering, SAXS) or neutron beam (Small-angle neutron scattering, SANS) when directed at the sample. The intensity of scattered photons or neutrons $I(q)$ is detected at very small scattering angles (typically 0.1–10°) as a function of the scattering vector q to resolve structures at a length scale of about 1–100 nm (Fig. 6.1). The scattering data give information about the fluctuations of electronic or nuclear densities in the matter, which can be related to the size (distribution), shape and orientation of nanostructures within the sample. They specifically account for random arrangements of density inhomogeneity like higher-order arrangements in protein solutions at elevated concentrations. The scattering data also elucidate inter-particle interactions and can thus be used to study molecule interactions in solution, which will be outlined below.

SAXS and SANS are two similar methods which provide complementary information. X-rays interact primarily with the electron cloud surrounding each atom. The higher the atomic number, the higher the scattering intensity. Neutrons interact with the nuclei of atoms or by interaction with the magnetic momentum of unpaired electrons. Therefore, the contribution to the diffracted intensity depends on each isotope. To increase resolution, hydrogen is exchanged by deuterium during sample preparation for SANS measurements. This is the major advantage of neutron diffraction over X-ray diffraction that neutron scattering is sensitive to the presences of hydrogen/deuterium with the latter scattering neutrons to a stronger extent. The deflection of the neutrons is dependent on the size of the nanostructures in solution in which smaller structures yield higher scattering density.

The application of SAS techniques is a unique, non-destructive way to obtain direct structural information on colloidal systems with only little sample preparation. The X-ray source can be a laboratory source or a synchrotron. Neutrons are

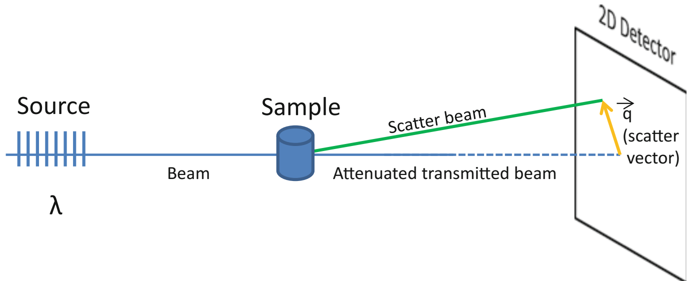


Fig. 6.1 Measuring principle of small-angle scattering techniques

usually generated in a nuclear reactor or spallation source. However, beam time in one of the few globally distributed synchrotrons is scarce and costly.

The scattering intensity of colloidal protein solutions $I(q)$ is given by the product of the structure factor $S(q)$, the form factor $P(q)$, and the molecular protein concentration ρ as a function of q [88–90]:

$$I(q) = \rho P(q) S(q) \tag{6.12}$$

q is the scattering vector, which is a function of the scattering angle θ and the wavelength λ:

$$|q| = \frac{4\pi}{\lambda} \sin\frac{\theta}{2} \tag{6.13}$$

The form factor is a function of the size and shape of the macromolecular structures in solution and an average overall orientations. It is characteristic for repeated distances of partially ordered materials and capable of delivering structural information of up to 150 nm. The structure factor is the average distribution of intermolecular distances and can thus be related to the potential of mean force.

Application of SAXS to protein solutions to study their interaction potential in solution was demonstrated in 2003 by Liu and co-workers. They studied lysozyme solutions correlating X-ray scattering data with the second osmotic virial coefficient at low salt concentrations. With increasing ionic strength, however, the attractive potential was overestimated not accounting for short-ranged interactions [91]. Zhang and colleagues have studied the effect of ionic strength on protein–protein interaction with BSA as a model system for a large range of salt (up to 2 M) and protein concentrations (2–500 mg/mL) using SAXS. The data revealed that repulsive Coulomb interactions dominate the overall interaction potential at high protein concentrations and at low ionic strength. Addition of salt changed the nature of the interaction potential smoothly from repulsive to attractive and revealed the presence of an increasing attractive potential at high salt concentration attributed to van der Waals forces [92]. The influence of the type of the salt being chaotrope or kosmotrope in nature and the dependence of their position in the Hofmeister series as well as their influence on the molecular structure was studied in 2012 by Scherer and co-workers for IgG_1 formulations [93]. They also reported in 2013 that this IgG_1 formed reversible dimers in dilute solutions and protein cluster at higher protein concentrations studied up to 175 mg/mL. The cluster formation was dependent on the ionic strength of the formulation. The cluster size correlated with the viscosity of the IgG_1 solutions [77]. Also in 2012, Mosbaek et al. applied SAXS to IgG_2 formulations up to protein concentrations of 122 mg/mL. They demonstrated that SAXS can measure short- and long-ranged interactions at high protein concentrations simultaneously being strongly affected by the added excipients in the formulation [94].

SANS was applied in a number of studies in the recent years focusing on proteinaceous solutions. Velev and co-workers have studied protein interaction by

neutron scattering for liquid lysozyme and chymotrypsinogen formulations dependent on various formulation parameters (pH 3–11; addition of 5–300 mM NaCl). They demonstrated agreement between the second virial coefficient determined by static light scattering and by neutron scattering determined at a protein concentration of 10 mg/mL, as well as agreement to the theoretically calculated second virial coefficient according to the DLVO theory. The data revealed that measurement at low protein concentration can account for repulsive and attractive electrostatic interaction, long-ranged van der Waals forces, and the excluded volume of the protein molecules equivalent to colloidal spheres. However, the measurement was unable to resolve complex short-ranged interactions [95]. An increase in the intensity distribution when q is approaching zero was also demonstrated for other proteins at very low scattering angles due to the formation of ordered clusters. This was shown in 2005 by Liu and colleagues for Cytochrome C and lysozyme solutions and interpreted as the presence of weak long-ranged attractive interaction between the molecules dependent on the formulation composition [96]. Application of SANS to monoclonal antibody formulations was reported in 2013 by Liu et al. They studied the intermolecular potential at high protein concentrations of 150 mg/mL revealing highly anisotropic attractive potentials for one of the antibodies dependent on formulation composition [97]. These attractive intermolecular forces led to the formation of reversible dynamic clusters, which were shown in 2015 to be the underlying reason for unusally high solution viscosity [78]. They also reported the formation of reversible dimer formation at dilute protein concentration, which interact with each other forming loosely bound transient clusters at increasing protein concentrations. These data were in line with neutron spin echo measurements determining antibody diffusion at short-time diffusion coefficients.

The published literature data show that small-angle scattering techniques are useful methods to characterize the overall nature of intermolecular interactions at low and especially high protein concentrations dependent on the composition of the formulation. At increasing protein concentration, these techniques enable the characterization of higher-order arrangements in the protein solution at the nanoscale level being either sensitive to inhomogeneities in electron densities (SAXS) or to fluctuations in the density of nuclei (SANS).

References

1. Jarasch A, et al. Developability assessment during the selection of novel therapeutic antibodies. J Pharm Sci. 2015;104(6):1885–98.
2. Ecker DM, Jones SD, Levine HL. The therapeutic monoclonal antibody market. MAbs. 2015;7(1):9–14.
3. Salinas BA, et al. Understanding and modulating opalescence and viscosity in a monoclonal antibody formulation. J Pharm Sci. 2010;99(1):82–93.
4. Curtis RA, Prausnitz JM, Blanch HW. Protein-protein and protein-salt interactions in aqueous protein solutions containing concentrated electrolytes. Biotechnol Bioeng. 1998;57(1):11–21.

5. Perchiacca JM, Tessier PM. Engineering aggregation-resistant antibodies. Annu Rev Chem Biomol Eng. 2012;3:263–86.
6. Vazquez-Rey M, Lang DA. Aggregates in monoclonal antibody manufacturing processes. Biotechnol Bioeng. 2011;108(7):1494–508.
7. Nishi H, et al. Phase separation of an IgG1 antibody solution under a low ionic strength condition. Pharm Res. 2010;27(7):1348–60.
8. Cromwell ME, Hilario E, Jacobson F. Protein aggregation and bioprocessing. AAPS J. 2006;8 (3):E572–9.
9. Scherer TM, et al. Intermolecular interactions of IgG1 monoclonal antibodies at high concentrations characterized by light scattering. J Phys Chem B. 2010;114(40):12948–57.
10. Some D. Light-scattering-based analysis of biomolecular interactions. Biophys Rev. 2013; 5(2):147–58.
11. Arzensek D, Kuzman D, Podgornik R. Colloidal interactions between monoclonal antibodies in aqueous solutions. J Colloid Interface Sci. 2012;384(1):207–16.
12. Moon YU, et al. Osmotic pressures and second virial coefficients for aqueous saline solutions of lysozyme. Fluid Phase Equilib. 2000;168(2):229–39.
13. Patro SY, Przybycien TM. Self-interaction chromatography: a tool for the study of protein-protein interactions in bioprocessing environments. Biotechnol Bioeng. 1996;52(2): 193–203.
14. Hedberg SH, et al. Self-interaction chromatography of mAbs: accurate measurement of dead volumes. Pharm Res. 2015;32(12):3975–85.
15. Tessier PM, Lenhoff AM, Sandler SI. Rapid measurement of protein osmotic second virial coefficients by self-interaction chromatography. Biophys J. 2002;82(3):1620–31.
16. Payne RW, et al. Second virial coefficient determination of a therapeutic peptide by self-interaction chromatography. Biopolymers. 2006;84(5):527–33.
17. Saito S, et al. Behavior of monoclonal antibodies: relation between the second virial coefficient (B (2)) at low concentrations and aggregation propensity and viscosity at high concentrations. Pharm Res. 2012;29(2):397–410.
18. Connolly BD, et al. Weak interactions govern the viscosity of concentrated antibody solutions: high-throughput analysis using the diffusion interaction parameter. Biophys J. 2012;103(1):69–78.
19. Lehermayr C, et al. Assessment of net charge and protein-protein interactions of different monoclonal antibodies. J Pharm Sci. 2011;100(7):2551–62.
20. Yadav S, et al. The influence of charge distribution on self-association and viscosity behavior of monoclonal antibody solutions. Mol Pharm. 2012;9(4):791–802.
21. Yadav S, Shire SJ, Kalonia DS. Factors affecting the viscosity in high concentration solutions of different monoclonal antibodies. J Pharm Sci. 2010;99(12):4812–29.
22. He F, et al. High-throughput assessment of thermal and colloidal stability parameters for monoclonal antibody formulations. J Pharm Sci. 2011;100(12):5126–41.
23. Warne NW. Development of high concentration protein biopharmaceuticals: the use of platform approaches in formulation development. European Journal of Pharmaceutics and Biopharmaceutics: Official Journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V. 2011;78(2):208–12.
24. Raut AS, Kalonia DS. Opalescence in monoclonal antibody solutions and its correlation with intermolecular interactions in dilute and concentrated solutions. J Pharm Sci. 2015;104(4): 1263–74.
25. Weinbuch D, Jiskoot W, Hawe A. Light obscuration measurements of highly viscous solutions: sample pressurization overcomes underestimation of subvisible particle counts. AAPS J. 2014;16(5):1128–31.
26. Werk T, Volkin DB, Mahler HC. Effect of solution properties on the counting and sizing of subvisible particle standards as measured by light obscuration and digital imaging methods. European J Pharm Sci Official J Eur Fed Pharm Sci. 2014;53:95–108.
27. USP, General Chapters: <787> Subvisible particulate matter in therapeutic protein injections, in USP 392016.

28. Manning MC, Patel K, Borchardt RT. Stability of protein pharmaceuticals. Pharm Res. 1989;6(11):903–18.
29. Mahler HC, et al. Protein aggregation: pathways, induction factors and analysis. J Pharm Sci. 2009;98(9):2909–34.
30. Morris AM, Watzky MA, Finke RG. Protein aggregation kinetics, mechanism, and curve-fitting: a review of the literature. Biochim Biophys Acta. 2009;1794(3):375–97.
31. Shire SJ, Shahrokh Z, Liu J. Challenges in the development of high protein concentration formulations. J Pharm Sci. 2004;93(6):1390–402.
32. Wang W. Protein aggregation and its inhibition in biopharmaceutics. Int J Pharm. 2005; 289(1–2):1–30.
33. Manning MC, et al. Stability of protein pharmaceuticals: an update. Pharm Res. 2010;27(4): 544–75.
34. Cromwell ME, Hilario E, Jacobson F. Protein aggregation and bioprocessing. AAPS J. 2006;08(03):E572–9.
35. Gabrielson JP, et al. Quantitation of aggregate levels in a recombinant humanized monoclonal antibody formulation by size-exclusion chromatography, asymmetrical flow field flow fractionation, and sedimentation velocity. J Pharm Sci. 2007;96(2):268–79.
36. Philo JS. A critical review of methods for size characterization of non-particulate protein aggregates. Curr Pharm Biotechnol. 2009;10(4):359–72.
37. Li YI, Weiss WF, Roberts CJ. Characterization of high-molecular-weight nonnative aggregates and aggregation kinetics by size exclusion chromatography with inline multi-angle laser light scattering. J Pharm Sci. 2009;98(11):3997–4016.
38. Liu J, Shire SJ. Analytical ultracentrifugation in the pharmaceutical industry. J Pharm Sci. 1999;88(12):1237–41.
39. Liu J, et al. Reversible self-association increases the viscosity of a concentrated monoclonal antibody in aqueous solution. J Pharm Sci. 2005;94(9):1928–40.
40. Caldwell KD, Wahlund K-G. Field-flow fractionation. In: Jiskoot W, Crommelin FJA, editors. Methods for structural analysis of protein pharmaceuticals. AAPS; 2005. p. 379–412.
41. Zhu R, et al. Studying protein aggregation by programmed flow field-flow fractionation using ceramic hollow fibers. Anal Chem. 2005;77(14):4581–6.
42. Litzen A, et al. Separation and quantitation of monoclonal antibody aggregates by asymmetrical flow field-flow fractionation and comparison to gel permeation chromatography. Anal Biochem. 1993;212(2):469–80.
43. Shire SJ. Formulation and manufacturability of biologics. Curr Opin Biotechnol. 2009;20(6): 708–14.
44. Burckbuchler V, et al. Rheological and syringeability properties of highly concentrated human polyclonal immunoglobulin solutions. Eur J Pharm Biopharm. 2010;76(3):351–6.
45. Tanford C, editor. Physical chemistry of macromolecules. New York: Wiley; 1961.
46. Kanai S, et al. Reversible self-association of a concentrated monoclonal antibody solution mediated by Fab-Fab interaction that impacts solution viscosity. J Pharm Sci. 2008.
47. Chhabra RP, Richardson JF. Non-newtonian flow and applied rheology—engineering applications. 2nd ed. Elsevier.
48. Allmendinger A, et al. Rheological characterization and injection forces of concentrated protein formulations: an alternative predictive model for non-newtonian solutions. European Journal of Pharmaceutics and Biopharmaceutics: Official Journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V. 2014; 87(2):318–28.
49. Allmendinger A, et al. Sterile filtration of highly concentrated protein formulations: impact of protein concentration, formulation composition, and filter material. J Pharm Sci. 2015; 104(10):3319–29.
50. Rathore N, et al. Characterization of protein rheology and delivery forces for combination products. J Pharm Sci. 2012;101(12):4472–80.
51. Fischer I, et al. Calculation of injection forces for highly concentrated protein solutions. Int J Pharm. 2015;493(1–2):70–4.

52. Osswald T, Rudolph N. Polymer rheology—fundamentals and applications. Hanser Publishers.
53. Ostwald W. Ueber die Geschwindigkeitsfunktion der Viskositaet disperser Systeme. Kolloid-Z. 1925;36(99).
54. Malkin AY, editor. Rheology fundamentals. ChemTec Publishing; 1994.
55. Bird RB, Carreau PJ. A nonlinear viscoelastic model for polymer solutions and melts. Chem Eng Sci. 1968;23:427–34.
56. Yasuda K, Armstrong RC, Cohen RE. Shear flow properties of concentrated solutions of linear and star branched polystyrenes. Rheol Acta. 1981;20:163–78.
57. Saluja A, Kalonia DS. Nature and consequences of protein-protein interactions in high protein concentration solutions. Int J Pharm. 2008;358(1–2):1–15.
58. Jezek J, et al. Viscosity of concentrated therapeutic protein compositions. Adv Drug Deliv Rev. 2011;63(13):1107–17.
59. Teraoka I, editor. Polymer solutions: an introduction to physical properties. New York: Wiley; 2002.
60. Goodwin JW, Hughes RW, editors. Rheology for chemists—an introduction. Cambridge: Royal Society of Chemistry; 2000.
61. Ross PD, Minton AP. Hard quasispherical model for the viscosity of hemoglobin solutions. Biochem Biophys Res Commun. 1977;76(4):971–6.
62. Mooney M. The viscosity of a concentrated suspension of spherical particles. J Colloid Sci. 1951;6(2):162–70.
63. Mehl JW, Oncley JL, Simha R. Viscosity and the shape of protein molecules. Science. 1940;92(2380):132–3.
64. Metzger T. The rheology handbook. 4th ed. Hanover: Vincentz Network; 2014.
65. USP, General Chapters: <913> Viscosity—rolling ball method, in USP 392016.
66. Malkin AY, Isayev AI. Rheology—concepts, methods, and applications. 2nd ed. ChemTec Publishing.
67. USP, General Chapter <911> Viscosity—capillary methods, in USP 392016.
68. USP, General Chapter <912> Viscosity—rotational methods, in USP 392016.
69. Patapoff TW, Esue O. Polysorbate 20 prevents the precipitation of a monoclonal antibody during shear. Pharm Dev Technol. 2009;14(6):659–64.
70. Bee JS, et al. Response of a concentrated monoclonal antibody formulation to high shear. Biotechnol Bioeng. 2009;103(5):936–43.
71. Dharmaraj VL, et al. Rheology of clustering protein solutions. Biomicrofluidics. 2016;10(4):043509.
72. He F, et al. High-throughput dynamic light scattering method for measuring viscosity of concentrated protein solutions. Anal Biochem. 2010;399(1):141–3.
73. Parmar AS, Muschol M. Lysozyme as diffusion tracer for measuring aqueous solution viscosity. J Colloid Interface Sci. 2009;339(1):243–8.
74. Allmendinger A, et al. High-throughput viscosity measurement using capillary electrophoresis instrumentation and its application to protein formulation. J Pharm Biomed Anal. 2014;99:51–8.
75. Chari R, et al. Long- and short-range electrostatic interactions affect the rheology of highly concentrated antibody solutions. Pharm Res. 2009;26(12):2607–18.
76. Yadav S, Shire SJ, Kalonia DS. Viscosity behavior of high-concentration monoclonal antibody solutions: correlation with interaction parameter and electroviscous effects. J Pharm Sci. 2012;101(3):998–1011.
77. Lilyestrom WG, et al. Monoclonal antibody self-association, cluster formation, and rheology at high concentrations. J Phys Chem B. 2013;117(21):6373–84.
78. Godfrin PD, et al. Effect of hierarchical cluster formation on the viscosity of concentrated monoclonal antibody formulations studied by neutron scattering. J Phys Chem B. 2016;120(2):278–91.
79. Mason WP, et al. Measurement of shear elasticity and viscosity of liquids at ultrasonic frequencies. Phys Rev. 1949;75:936–46.

80. Mason WP. Measurement of the viscosity and shear elasticity of liquids by means of a torsially vibrating crystal. Trans Am Soc Mech Eng. 1947;68:359–70.
81. Saluja A, Kalonia DS. Measurement of fluid viscosity at microliter volumes using quartz impedance analysis. AAPS PharmSciTech. 2004;5(3):68–81.
82. Rodahl M, et al. Quartz-crystal microbalance setup for frequency and Q-factor measurements in gaseous and liquid environments. Rev Sci Instrum. 1995;66(7):3924–30.
83. Rodahl M, Kasemo B. On the measurement of thin liquid overlayers with the quartz-crystal microbalance. Sens Actuators a-Phys. 1996;54(1–3):448–56.
84. Saluja A, Kalonia DS. Application of ultrasonic shear rheometer to characterize rheological properties of high protein concentration solutions at microliter volume. J Pharm Sci. 2005; 94(6):1161–8.
85. Saluja A, et al. Application of high-frequency rheology measurements for analyzing protein-protein interactions in high protein concentration solutions using a model monoclonal antibody (IgG2). J Pharm Sci. 2006;95(9):1967–83.
86. Saluja A, et al. Ultrasonic storage modulus as a novel parameter for analyzing protein-protein interactions in high protein concentration solutions: correlation with static and dynamic light scattering measurements. Biophys J. 2007;92(1):234–44.
87. Patel AR, Kerwin BA, Kanapuram SR. Viscoelastic characterization of high concentration antibody formulations using quartz crystal microbalance with dissipation monitoring. J Pharm Sci. 2009;98(9):3108–16.
88. Hunter RJ. Foundation of colloid science. Vol II. Oxford: Calderin Press; 1989, p. 675–873.
89. Belloni L. Interacting monodisperse and polydisperse spheres. In: Lindner P, Temb T, editors. Neutron, X-ray and light scattering. Amsterdam: Elsevier Science; 1991, p. 135–55.
90. Kaler E. Small-angle scattering from complex fluids. In: Brumberger H, editor. Modern aspects of small-angle scattering. Dordecht: Kluwer Academic Publishers; 1995, p. 239–353.
91. Narayanan J, Liu XY. Protein interactions in undersaturated and supersaturated solutions: a study using light and X-ray scattering. Biophys J. 2003;84(1):523–32.
92. Zhang F, et al. Protein interactions studied by SAXS: effect of ionic strength and protein concentration for BSA in aqueous solutions. J Phys Chem B. 2007;111(1):251–9.
93. Lilyestrom WG, Shire SJ, Scherer TM. Influence of the cosolute environment on IgG solution structure analyzed by small-angle X-ray scattering. J Phys Chem B. 2012;116(32):9611–8.
94. Mosbaek CR, et al. High concentration formulation studies of an IgG2 antibody using small angle X-ray scattering. Pharm Res. 2012;29(8):2225–35.
95. Velev OD, Kaler EW, Lenhoff AM. Protein interactions in solution characterized by light and neutron scattering: comparison of lysozyme and chymotrypsinogen. Biophys J. 1998;75(6): 2682–97.
96. Liu Y, et al. Effective long-range attraction between protein molecules in solutions studied by small angle neutron scattering. Phys Rev Lett. 2005;95(11):118102.
97. Yearley EJ, et al. Small-angle neutron scattering characterization of monoclonal antibody conformations and interactions at high concentrations. Biophys J. 2013;105(3):720–31.

Chapter 7
Practical Considerations for High Concentration Protein Formulations

Deirdre Murphy Piedmonte, Jian Hua Gu, Stephen R. Brych and Monica M. Goss

Abstract Practical issues that arise for high concentration protein formulations can complicate manufacturing and affect injectability/device compatibility. High concentration protein formulations have an increased tendency for high solution viscosity, physical stability sensitivities (aggregation/particulation), and non-Newtonian solution behavior (shear thinning) due to high shear rates. Process unit operations can be negatively impacted by these factors, and it is critical to understand how they influence process performance. Device compatibility can be affected by changes in protein concentration and temperature that will impact product viscosity and injectability. Complete characterization of the solution physical properties (viscosity and shear thinning profile) as well as the stability profile must be understood to ensure efficient processing, delivery, and efficacy of the therapeutic product. If potential candidates with impeding viscosity values are not identified early in development, subsequent mitigation efforts to reduce viscosity likely pivot from a protein engineering approach to changes in formulation.

Keywords High protein concentration formulation · Viscosity
Shear thinning · Device compatibility · Injectability · Syringeability

---

D. M. Piedmonte · J. H. Gu · S. R. Brych · M. M. Goss (✉)
Amgen, Inc., Process Development, One Amgen Center Drive,
Thousand Oaks, CA 91320-1799, USA
e-mail: monicap@amgen.com

D. M. Piedmonte
e-mail: deirdrem@amgen.com

J. H. Gu
e-mail: jiang@amgen.com

S. R. Brych
e-mail: sbrych@amgen.com

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_7

7.1 Introduction

Many of the practical challenges associated with manufacturing and administering high concentration protein formulations are related to the high viscosity of the solution because viscosity can increase as a function of protein concentration [1–5]. Increases in viscosity (i.e., resistance to flow) are typically due to protein intermolecular interactions that can be hydrophobic, polar, or ionic in origin and may even occur at interfaces [4, 6–8]. Regardless of the precise mechanism of intermolecular interaction, self-association of high concentration protein formulations typically manifests as high viscosity. Although the range of 15–30 cP is considered higher viscosity [9], 'high' viscosity is less of an absolute number and more situational, meaning when the viscosity of the product necessitates adaptations of manufacturing processes and administration. In addition to being highly dependent on protein concentration, viscosity is influenced by solution temperature. This is of practical significance because the viscosity of a product can change within the fairly narrow and controlled temperature range experienced during manufacturing unit operations, storage, and patient administration. Viscosity dictates solution behavior (i.e., fluid dynamics) and can therefore impact 'processability' during certain unit operations in manufacturing. Moreover, the viscosity of a drug product solution drives critical device design choices and functionality, which are often termed 'syringeability/injectability.' Solutions with increased viscosities may lead to increased injection times, which can be correlated with the perception of a painful injection.

High concentration protein formulations enable the administration of smaller volumes for subcutaneous injection. Whereas the delivery volume for subcutaneous injection was once thought to be limited to 1 mL due to concerns of injection pain from volume, viscosity, and formulation characteristics, larger volumes are now successfully being delivered [10, 11]. Berteau et al. reported that abdominal injections of up to 3 mL and 20 cP were tolerated without perceived pain [12]. Interestingly, the authors reported that the high viscosity (15–20 cP) injections were the most tolerated (with respect to pain) compared to injections with lower (1 cP) and medium (8–10 cP) viscosities. Moreover, within the conditions assessed, neither injection volume nor flow rate impacted perceived pain [12]. Ease of administration is complex and is influenced by many factors, including injection volume, needle gauge, time of injection, composition of the formulation, site of injection, and the viscosity of the solution.

Regarding product quality of protein therapeutic formulations, degradation reactions can be broadly categorized into those affecting chemical and physical/colloidal stability. Chemical modifications (e.g., oxidation, deamidation, isomerization, fragmentation, glycation) typically do not pose an added concern for higher protein concentrations. However, high concentration protein formulations can have an increased propensity to aggregate and/or particulate (subvisible or visible particles) and can even undergo gelation. Moreover, formulation has been shown to have an impact on aggregation/particulation and high concentration protein

formulations, in particular, can show increased kinetics of aggregate/particle formation [13, 14, 16]. While formulation parameters (e.g., pH, ionic strength, buffer, surfactant type, and concentration) are typically balanced to optimize chemical and physical stability, particular effort at high protein concentrations may be required to reduce potential physical stability and viscosity challenges.

This chapter will focus on the practical aspects of developing, manufacturing, and administering high concentration, viscous protein therapeutic formulations.

7.1.1 Overview of Viscosity Measurement Techniques

For high concentration protein solutions, accurate viscosity measurements generated under relevant conditions aid in the rational design of the manufacturing process as well as the selection of an appropriate delivery device and delivery parameters (e.g., force profile and injection time). The complex rheological behaviors of high concentration protein formulations make both the prediction of viscosity measurements and the measurements themselves challenging.

7.1.2 Complex Behavior Under Shear: The Practical Significance of Shear Thinning

For classical 'Newtonian' solutions, fluid dynamics is mainly determined by viscosity and is independent of the rate of shear. Practically, this means that the viscosity of a Newtonian fluid remains constant regardless of how fast it is forced to flow through an orifice (e.g., filling needle during manufacturing or a syringe during administration). More complex fluid behavior is deemed 'non-Newtonian' behavior, where the flow varies with time, shear rate (γ), and shear stress (τ). The effective viscosity is dependent on the shear rate. The simplest model for non-Newtonian fluid dynamics is the power-law fluid model/Ostwald–de Waele model, which describes that the shear stress (τ) is proportional to the shear rate (γ) to the nth power [15].

$$\tau = K\gamma^n$$

Shear thinning is a phenomenon used to describe non-Newtonian behavior of fluids whose viscosity decreases under increasing shear strain, meaning less resistance to flow at higher shear rates than at lower shear rates. High concentration protein solutions with high intrinsic viscosities often exhibit shear thinning. Moreover, proteins differ in their susceptibility to shear thinning. Even the same protein may exhibit different shear-thinning behaviors as a function of protein concentration, when measured at the same shear rate [16, 17]. Therefore, a viscosity measurement executed at a single shear rate may not represent the actual viscosity

throughout processing/manufacturing unit operations and patient administration because a drug product solution can experience a wide range of shear stress throughout its lifecycle.

Various techniques can be used to measure solution viscosity, and their advantages and disadvantages have been reviewed thoroughly elsewhere [18–20].

To select the most appropriate instrument to measure viscosity, it is key to remember that the experimental data should be generated at a shear rate that is within the range that will be encountered during the unit operation or administration step of interest. In brief, rotational viscometers commonly use a cone and plate or parallel plate geometry to measure the solution rheology. A constant shear rate across the geometry, such as with the cone/plate, enables precisely defined viscosity measurements for non-Newtonian liquids at that shear rate. Slit viscometers feature a wide shear rate range, while both rotational and glass capillary rheometers operate at lower shear rates.

Figure 7.1 illustrates relative shear rate ranges for protein drug product unit operations during manufacturing (e.g., mixing, bulk transfer, tangential flow filtration (TFF), automated visual inspection, filtration, and filling) and administration overlaid with the capability of viscosity-measuring techniques (slit, rotational, and glass capillary viscometers) [21–24]. The shear rates listed in Fig. 7.1 are relative ranges and not absolute values because shear rates are dependent on the specific operating conditions under which the shear was generated. Note that the shear rate experienced upon extrusion through a needle is orders of magnitude greater than shear rates experienced during most other unit operations (Fig. 7.1).

Rotational and glass capillary rheometers operate at lower shear rates (on the order of 100–1000 s^{-1}) than the shear rate range typically encountered during extrusion through a narrow bore needle (typically on the order of 100,000–1,000,000 s^{-1})

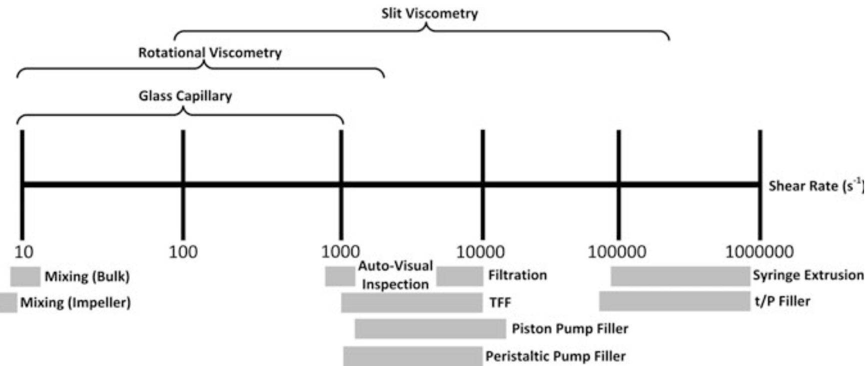


Fig. 7.1 Illustrative, relative shear rate ranges for manufacturing unit operations [e.g., mixing, bulk transfer, tangential flow filtration (TFF), automated visual inspection, filtration, filling (piston, peristaltic, and time-over-pressure (t/P))] as well as extrusion from syringe overlaid with the capability of viscosity-measuring techniques (slit, rotational, and glass capillary) with respect to typical shear rate range [21, 23, 24]

(Fig. 7.1). The apparent viscosity of a solution prone to shear thinning during extrusion could be significantly less than the viscosity measured during characterization of the product. The practical result is that highly concentrated protein therapeutics may require significantly reduced injection forces because of shear-thinning (i.e., non-Newtonian) behavior than would be anticipated for a Newtonian fluid [17].

A viscosity–shear rate profile of the protein therapeutic solution can be generated over a range of shear rates that are relevant to both drug product manufacturing unit operations and administration by combining viscosity data generated at high shear rates with data obtained using different viscosity methods.

Because protein therapeutic formulations can experience a broad range of shear rates spanning several orders of magnitude, a viscosity measurement generated at a single shear rate likely will not be representative of the various shear rates experienced throughout manufacturing and administration. Viscosity should be measured under appropriate conditions (meaning at a relevant shear rate), particularly for high protein concentration therapeutic solutions that exhibit prominent shear-thinning behavior. Representative viscosity measurements take on an added importance as a program matures through development and can improve technical transfers (e.g., when switching filling technologies between piston, peristaltic, or time-over-pressure fillers) as well as help identify appropriate injection forces for administration and device design.

7.1.3 Technical Considerations to Accurately Measure Viscosity

Beyond choosing the proper viscometer that operates in the shear rate range that will be experienced during the unit operation(s) of interest, there are additional practical considerations for accurately and reproducibly measuring viscosity given the complexity of measuring the rheological properties of high protein concentration solutions. In the following section, we discuss technical considerations to enable the generation of accurate and reliable viscosity measurements of high protein concentration solutions.

Obtaining accurate viscosity measurements using rotational rheology is complicated by the potential for protein film formation at the air/sample liquid interface, particularly for high concentration protein formulations. The presence of an elastic protein film at the air–water interface of the periphery of the cone and plate or parallel plate geometry can cause unusual behavior in viscosity measurement of protein solutions, such as a shear thinning behavior for solutions whose behavior is expected to be Newtonian [25]. This specific challenge of film formation at the air–liquid interface can be mitigated by adding a small amount of surface active excipient (such as a surfactant) into the solution, which serves to prevent irreversible adsorption of protein molecules at the interface. The challenge can be also addressed by using a different technique to measure viscosity such as capillary or

slit viscometry (such as the microfluidics viscometer–rheometer-on-a-chip (m-VROC)), which does not have a liquid/air interface.

In addition to protein concentration, another solution property well known to affect viscosity is temperature [16]. To reliably and accurately measure viscosity, the accurate and precise determination of protein concentration of samples of interest as well as stringent temperature control during viscosity measurements are essential.

Sample preparation, loading, the use of carefully chosen standards, and even cleaning between samples are all particularly important for highly concentrated, viscous protein samples. Care should be taken during sample preparation and analysis not to induce product quality changes such as aggregation or particulation. Careful sample loading is required for the slit and capillary viscometers because the introduction of bubbles into the systems during sample loading can significantly influence the viscosity measurement. Cleaning protocols should be executed consistently to prevent the sample carryover, which will ensure accurate measurements. Moreover, instrument performance should be routinely assessed using reference fluids traceable to national standards and/or water [26, 27]. Especially for high protein concentration solutions, appropriate aqueous solutions with viscosities known to be similar to the samples of interest can be used to check the performance of the instrument. Instrument manufacturers sometimes use cellulose solution standards because cellulose solutions present Newtonian behavior under low shear, but convert to non-Newtonian at higher shear rates. Due to the complex behavior of proteins under shear, viscosity measurements of high protein concentration solutions require a judicious choice of instrumentation, testing at different and relevant shear rates, and meticulous execution.

7.2 Manufacturing Unit Operations

To minimize potential impact to product quality, protein therapeutics may be processed at refrigerated temperatures, where solution viscosity is increased. The solution characteristics of high protein concentration drug products such as high viscosity can impact the performance of manufacturing unit operations, specifically tangential flow filtration (TFF), filtration, filling, the effectiveness of automated inspections, and even the cleanability of manufacturing equipment. In the following sections, we will discuss each of the aforementioned unit operations in detail, as they pertain to high protein concentration, viscous therapeutic formulations.

Tangential Flow Filtration (TFF)

The final stages of processing protein therapeutics typically requires a solution exchange and concentration step into the final formulation composition at the final target concentration. Other manufacturing process techniques to deliver high concentration drug products by drying such as lyophilization and spray drying are discussed elsewhere [28, 29]. Here, we focus our discussion on tangential flow

filtration (TFF), which is commonly used to formulate and concentrate liquid drug product formulations to generate a high enough concentration to deliver in an injectable volume in the range of 1–1.5 mL [28]. TFF is a pressure-driven technique that uses a membrane to separate large protein molecules and water/solutes where the protein is retained based on its size. When performing TFF to achieve high final protein concentrations (>100 mg/mL), several challenges can be encountered. Particularly for high protein concentration therapeutics that have low solubility, TFF can result in protein precipitation, consequent membrane fouling, and reduced recovery [1, 30, 31]. To avoid TFF issues related to protein precipitation caused by low solubility, the protein can be diluted back into its 'solubility zone' during the diafiltration step. This step requires that the facility can accommodate larger volumes of diluted protein (i.e., appropriate tank sizes are available). If accommodating facility fit infrastructure is unavailable, a variable volume diafiltration step can be used where the protein is concentrated during buffer exchange to skirt areas of low solubility [31, 32].

During TFF, the maximum achievable protein concentration allowed by the system above which no further increase in protein concentrations is feasible is referred to as C_{wall} [30, 33]. Approaching C_{wall}, solution viscosity can increase, and there is an increase in system transmembrane pressure due to the formation of a polarized layer of highly concentrated protein on the retentate side of the membrane. This can cause increased system back pressure and processing time.

A cumulative or 'stacking' phenomenon can occur, for example, at antibody concentrations at the membrane of 150–200 mg/mL and corresponds to a 55–70 mg/mL retentate antibody concentration [34]. Increasing the pressure generally does not assist in increasing flux across the membrane since the gel layer only grows thicker [35]. If issues arise due to decreased flux at the required processing protein concentrations, alternate formulations to minimize protein–protein interactions or increased shear stress to sweep protein incorporated in the gel layer back into solution should be assessed [1, 36, 37]. Naturally, an evaluation will be required to determine if alternate formulations provide adequate shelf life stability or if increased shear during processing is tolerated by the protein since proteins can become destabilized in high shear environments [38].

It is well known that viscosity decreases with increasing temperature. Therefore, an obvious practical solution to overcome processing challenges due to increased viscosity (and related issues due to increased pressure, the C_{wall} concentration, and recovery) is to operate at elevated temperatures. A thorough assessment of drug product quality at the higher operating temperature is necessary to ensure quality is maintained. Moreover, forward processing into drug product (DP) and subsequent monitoring on stability is important to ensure that no impact to product quality was incurred due to higher temperature processing that will manifest during DP shelf life at the recommended storage condition. The development of an appropriate formulation to increase protein solubility and counter high viscosity effects has shown to be an effective mitigation approach to ease issues encountered during the TFF processing step [28].

Moreover, accurately targeting the final formulation pH and final excipient concentrations can be challenging when performing TFF because of intermolecular interactions between the protein and excipients. At high protein concentrations, the pH and excipient levels in the starting diafilitration buffer may increase in the final drug product due to intermolecular interactions with the protein. This phenomenon is termed the Donnan effect and is excipient-specific [39]. The Donnan effect can lead to significant differences in filtrate and retentate excipient concentrations during TFF [39]. Depending on the pI of the protein and its charge at the formulation pH, the conjugate charged buffer species can cooperatively interact with the protein due to electrostatic interactions, potentially leading to unequal partitioning of that particular charged solutes across the membrane. This will lead to increased levels of the conjugate acid or base of the buffer on the retentate side of the membrane, which can either lower or raise the pH (based on whether the conjugate acid or base, respectively, of the buffer is retained) in addition to lowering the level of non-protein interacting isotonicity agents (e.g., sugars and amino acids), due to volume exclusion effects [39, 40]. For instance, after the TFF of a concentrated monoclonal antibody formulation, the pH increased 0.8 units (from 4.0 to 4.8) and the buffer concentration increased more than 40 mM (from 10 to 51 mM) [39].

The latest models for molecular interactions/volume exclusion effects during TFF processes can be used to predict final pH and excipient concentrations post-TFF [39, 41, 42]. In addition, informative experiments should be conducted throughout development to characterize and understand the effects that the TFF processes can have on highly concentrated protein solutions.

Bulk Drug Substance Storage

Protein drug substance (DS) is often stored for extended periods of time and is often stored frozen to minimize degradation during its relatively long shelf life [43]. Commonly used containers for frozen bulk drug substance include stainless steel cryovessels, carboys, and disposable 'single-use' systems [43]. Cryoconcentration is a phenomenon that can lead to differences in protein and excipient concentrations within a DS container. Cryoconcentration results in formation of concentration gradients of the protein and excipients and has been mapped in carboys [44] and cryowedges [45, 46]. Due to their dimensions, 'single-use' bulk drug substance bags have the advantage of reduced cryoconcentration compared to what has been observed in traditional containers, but compatibility testing with single-use bags is warranted to test for leachates and any potential, consequent product quality impact [47, 48]. Freezing rate is known to impact cryoconcentration, and the effect was most noticeable in the osmolality distribution after a slow freezing process [49]. The impact of cryoconcentration may be more pronounced at higher protein concentrations, potentially resulting in increased aggregation propensity and higher viscosity, which could make subsequent mixing and filtering unit operations more complex.

When formulations are stored frozen, changes to the physical state (such as crystallization) of the stabilizing excipients that may occur in the frozen state should be assessed. For frozen formulations containing stabilizing excipients, such as

sorbitol (an isomer of mannitol), crystallization can result in protein instability (i.e., aggregation) due to the removal of the stabilizing excipient from the protein in the amorphous phase [50]. Similarly, the crystallization of trehalose in the frozen state was reported [51] and characterized [52], and its effect on aggregation was later assessed [53]. High protein concentration monoclonal antibody formulations showed evidence of delayed excipient crystallization as well as decreased aggregation rates after crystallization, presumably due to the self-stabilization afforded by higher protein concentration formulations [54].

An understanding of the potential degradations in the frozen state is important to ensure DS stability over its intended shelf life, particularly for high concentration protein formulations that can be aggregation- and particulation-prone.

DS thawing times and temperatures are dependent on practical constraints like the DS volume and container (carboy, 'single-use' bag, or cryovessel) as well as facility infrastructure. Thaws can be static or dynamic at either refrigerated temperature or room temperature, and mixing after thawing can be used to generate a homogenous solution for forward processing.

7.2.1 Drug Product Fill/Finish Unit Operations

Mixing

Mixing is a unit operation designed to achieve a homogenous solution and is of particular importance for high concentration protein therapeutics. The choice of mixing tanks is often dictated by practical constraints such as batch size and existing facility infrastructure. Mixing operations should be performed such that product quality is not adversely impacted.

Mixing operations can be more challenging for high protein concentration therapeutic solutions, particularly if the solution is viscous. Technical considerations including the mechanism of mixing, the geometry of the mixing tank as well as the mixing speed time and temperature of operation should be assessed. Regarding tank geometry, the ratios of impeller to tank diameter and impeller diameter to impeller distance from tank base as well as mixing speed and time should be carefully considered [55–57]. Bottom-mounted mixers are common due to their ease of use and low tank fill volume requirement but in some cases have been reported to induce protein aggregation and particulates during processing [58]. Subvisible particles have been observed with bottom-mounted mixers due to the protein passing between two surfaces (e.g., the impeller bearings and drive unit) during operation [59]. This can induce several potential stresses: mechanical stress, interfacial interactions (e.g., solid–liquid and/or air–liquid), cavitation, and even local thermal stress [58]. In contrast, top-mounted impellers do not have the close contact stress with tank and mixer components, but these types of mixers have volume constraints. The batch volume must be large enough for the impeller to be sufficiently covered with solution in order to avoid foaming and vortexing during mixing [56]. Foaming during mixing can induce aggregation. In contrast,

top-mounted impellers can induce shear, particularly at the impeller tip. Two studies that assessed top-mounted impellers reported no changes in product quality [55, 59]. Although there may not be product quality impact as a result of mixing, characterization under relevant processing conditions is recommended because some molecules are more sensitive to shear.

To achieve homogeneity for viscous, high concentration protein formulations, longer mixing times at lower mixing speeds may be used, where the protein therapeutic solution will experience lower shear rates. Moreover, mixing can be performed at a warmer temperature, where solution viscosity is decreased. However, it is important to verify that the protein therapeutic solution is robust to mixing at the chosen operating temperature, particularly if mixing occurs at an elevated temperature to mitigate viscosity [55, 60, 61]. As shown in Fig. 7.1, the shear rate of mixing is fairly low compared to other unit operations, but prolonged exposure to even low shear rates has been reported to induce protein degradation [59, 61, 62]. Assessing homogeneity by osmolality and/or protein concentration measurements taken from different depths in the mixing tank ensures that the product is being adequately mixed with the selected choices of impeller, mixing speed time and temperature.

Filtration

Aseptic filtering of protein drug product (DP) can occur at two separate stages: bioburden reduction filtration immediately after compounding/mixing the DP and/or sterile filtration prior to filling. The filtration process can be affected by properties of both the therapeutic protein formulation (e.g., viscosity, formulation composition, and particle load) and filter (e.g., pore size and surface properties of the filter material) [57, 63, 64]. Incremental increases in protein concentration can lead to increased solution viscosity that requires increased force during filtration [63].

High concentration protein formulations can exhibit an increased propensity to aggregate and/or particulate [65]. Protein particulation resulting from processing stresses (especially for suboptimal formulations) can result in downstream filter plugging and fouling due to a high subvisible particle load [66]. Gradual pore plugging and fouling result from pore narrowing by the accumulation and adsorption of deformable protein aggregates/particles [57, 63]. To understand the implications of potential filter pore plugging, filter capacity tests are performed during development to assess the flux of solution through the filter at a constant filtration pressure. The outcome of this test will identify the proper filter surface area to volume ratio to avoid manufacturing slowdown or stoppages and without a negative impact to product quality.

Reversible self-association networks have a higher predominance at high protein concentration and can lead to increase in viscosity that causes difficulty during filtration [2]. Increase in the force required to filter high protein concentration solutions can increase both processing time and shear stresses. Upon filtration through narrow pores, highly concentrated, viscous protein therapeutic formulations can exhibit non-Newtonian filtration behavior associated with shear thinning. In fact, shear thinning behavior can differ based on the type of filter. Shear thinning

was observed for the same protein solution during filtration with a polyether sulfone (PES) membrane but not with a polyvinylidene difluoride (PVDF) membrane with the same pore size, but the filters were shown to have a different pore network [63]. The shear thinning was caused by the tighter network of pores associated with the PES membrane that disrupted the reversible self-association interaction network thought to increase solution viscosity [63]. The disruption of the self-associated networks can likely increase filtration rate [63, 67]. Although small pore sizes restrict flux, associated shear thinning can compensate somewhat for the loss in flux. It has been observed that the shear thinning effect did not contribute to negative product quality impact when formulated in the presence of a surfactant [68]. Choosing a differently sized filter and/or different filter material can improve processability.

Filling (Pump Considerations)

There are several filling technologies used in protein drug product manufacturing. Fillers are categorized based on their mechanism of action and typically include peristaltic, time-over-pressure (t/P), and positive displacement (piston) pumps. Choosing the proper filling technology is particularly important for high protein concentration, viscous protein therapeutics to minimize dripping and clogging during the fill as well as any potential negative impact to product quality (particularly particles) that can occur due to increased shear rate [61, 69, 70].

As depicted in Fig. 7.1, t/P fillers impart shear rates on the same order of magnitude as syringe extrusion during administration because t/P fillers typically control the flow rate out of the pressure vessel using a narrow orifice. Particularly at high shear rates, non-Newtonian shear thinning of high protein concentration viscous solutions can cause dripping during filling. Dripping can cause filler nozzle clogging due to rapid water evaporation of remnant solution left at the tip after a pump stroke or during necessary line stoppages. Dripping/clogging can result in fill weight inaccuracies that lead to unexpected line stoppages and impact manufacturing efficiency and can potentially lead to batch failures. Factors for nozzle clogging include protein concentration, solution viscosity, air flow around the nozzle tip, and nozzle size [69]. Suck-back (or drawback), which is a slight reversal of fluid flow that brings the solution back into the nozzle, has been shown to minimize or eliminate nozzle clogging by minimizing dripping at the nozzle tip [69]. Furthermore, mitigating any variation in the 'suck-back' height was shown to be key to improved fill weight precision [70].

Dripping during filling may require greater optimization of the filling process [69, 71]. If the shear rate is imposing complications during filling (i.e., inducing shear thinning), then peristaltic or piston-driven pumps have been shown to apply lower shear rates (Fig. 7.1). Moreover, high shear can potentially impact product quality and has been reported to induce the formation of protein particles [72]. Optimization of the fill speed and nozzle size should be considered if a negative impact to product quality is observed.

In addition, understanding the effect of environmental factors including temperature and humidity on rheological properties and filling operations of high

concentration protein formulations is key, as is choosing appropriate filling needles, filling speed, and verifying the robustness of line stoppages during filling operations as part of process qualification.

Automated Visual Inspection (AVI)

Visual inspection is a required release assay that can be manual or automated. Here, we focus on automated visual inspection (AVI), because it is known to be sensitive to solution properties such as density, viscosity, and surface tension. This is because AVI systems detect particles in solution after spinning the primary container (at a fixed rate) and solution properties affect the movement of a particle in solution. Ji et al. showed decreased performance of an AVI system as a function of DP formulation viscosity [73]. Moreover, the authors reported that differences in surface tension and density can affect the detection rates of an AVI system, even between formulations with the same measured viscosity. Increasing the spin speed improved the overall performance, particularly for viscous samples [73]. Therefore, the selection of a spin rate for an AVI system may need to be altered for a highly viscous drug product solution. This finding reinforces the idea that physical properties should be characterized during development and taken into consideration even when developing settings for an AVI system.

Cleanability

Solution behavior such as surface tension and viscosity can affect cleanability [74]. High concentration protein solutions may have a stronger propensity to form a soilant layer on surfaces and as a result may be more difficult to clean. This is of practical significance both during analytical testing and manufacturing. While cleanability is formally assessed prior to manufacturing to validate that cleaning and sterilization practices are sufficient, caution should be taken well before cleanability protocols have been tested in a manufacturing setting. Cleaning characterization can be rapidly performed using UV-Vis spectroscopy [75]. If a drug product solution poses cleanability issues in a manufacturing setting, this finding could translate back to the analytical testing environment, where instruments should be cleaned between samples to eliminate carryover between testing highly viscous samples. Known solution behavior can be somewhat predictive of cleanability issues.

7.2.2 Mimic solutions

Beyond specific unit operations, there are other process considerations where solution properties of the drug product formulation are relevant. One example is mimic solutions, which can be used when drug product is unavailable for process development. Mimic solutions can be used throughout development: during process development for fill/finish, syringe/device functionality studies, combination product human factor engineering studies and even in blinded clinical studies. A single mimic solution may not be fit for use for all purposes and should be carefully

evaluated. Identifying the solution properties that need to be matched can help select an appropriate mimic solution. Particularly for viscous, high protein concentration product solutions, the rheological properties of the intended mimic solution including shear-thinning behavior and temperature dependence of solution viscosity should be carefully considered. A mimic solution that matches solution properties (over a range of temperatures) including density, viscosity, and surface tension may be sufficient to evaluate fill/finish operations for filling and syringe stoppering conditions. However, different surface interactions can be noted between a high concentration protein formulation and its mimic solution with similar solution properties, because proteins tend to adsorb to surfaces [76]. For clinical studies, any excipients added to the mimic solution to increase viscosity and hence match the drug product solution (such that the injection extrusion force or device injection time is similar for the mimic/placebo and active drug product) must be designated as 'generally regarded as safe' (GRAS). Mimic solutions should be fit for purpose and exhibit the same properties as the drug product solution.

7.3 Delivery Considerations

7.3.1 Pre-filled Syringes and Autoinjectors

To allow for a streamlined administration experience for patients, drug product is commonly provided in a pre-filled syringe (PFS) [77]. PFS are often used in pens/autoinjector devices, which provide an opportunity for home dosing and reduce the potential for needle sticks. It is worth noting that technology allowing needle-free injections has been reported to enable the administration of highly viscous gels that are not amenable to traditional administration with a syringe due to their high viscosity [78]. Currently, PFS are ubiquitous and will therefore be the focus of subsequent discussions.

7.3.2 Syringeability/Injectability

The term 'syringeability' refers to the force required to expel a solution through a needle and is typically used to describe the ease of flow through a needle and any tendency to clog or foam. The broader term 'injectability' incorporates the force required to inject, which also depends on tissue back pressure. The flow through a needle is best characterized by the Hagen–Poiseuille equation, expressed below as the pressure drop necessary to generate the hydrodynamic force to expel the fluid out of the syringe, which varies with the inverse of the inner diameter (or radius) of the needle to the fourth power (r^4) [15].

$$\Delta P = \frac{8\mu LQ}{\pi r^4}$$

Equation 1: ΔP = pressure drop; μ = fluid viscosity; L = needle length; Q = volumetric flow rate (expressed as volume/time); r = needle inner radius.

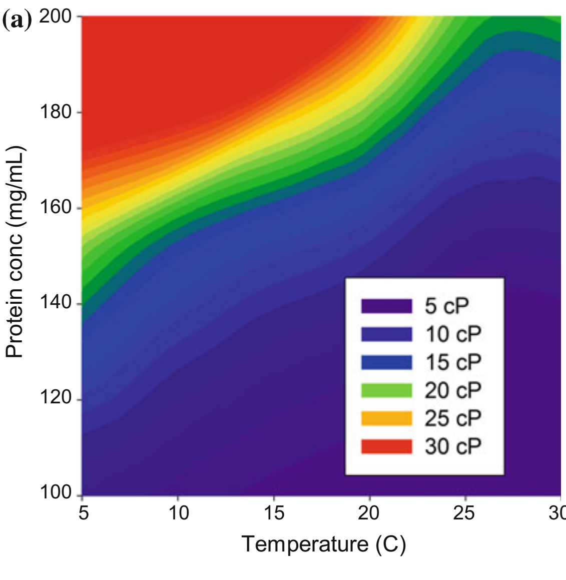
Needle gauge (specifically the inner diameter of the needle) is critical to both syringeability and injectability. Needle gauge also possesses an inherent duality in relation to patient comfort and ease of injection. A narrow bore needle may be more comfortable to insert but requires a greater force to inject [79]. Other geometric properties of the needle (e.g., inner diameter, length, and shape of the bevel) affect both syringeability and injectability.

Characterization of the injection time and delivery force required for a manual injection or with a delivery device such as an autoinjector is essential. Because the viscosity of a solution dictates the fluid dynamics of injection, solution viscosity has a dominant effect on injectability and therefore device performance. At high protein concentrations (>100 mg/mL), changes in viscosity tend to be nonlinear. Even relatively small differences in protein concentration within a typical specification range can lead to fairly large increases in solution viscosity that could potentially impact processability and/or device function.

Given the impact of both protein concentration and temperature on viscosity, it is important to assess their combined impact on solution viscosity. A traditional viscosity curve for a monoclonal antibody as a function of protein concentration and temperature is shown in Fig. 7.2a. These data were generated using a cone and plate viscometer, at a relatively low shear rate of 750 s^{-1}. In Fig. 7.2a, the effect of protein concentration on viscosity is most pronounced at the lowest temperature assessed.

Although the temperature range shown in Fig. 7.2a is relevant to manufacturing operations, long-term refrigerated storage, and patient administration, the relatively low shear rate at which the data was generated (750 s^{-1}) is not representative of typical filling unit operations or administration (refer to Fig. 7.1). Therefore, in Fig. 7.2b, we show theoretical values estimated at a higher shear rate ($\sim$100,000 s^{-1}) to illustrate how effective viscosity can decrease as a result of shear thinning. The decrease in effective viscosity due to shear thinning is most pronounced at low temperature and high protein concentration.

A holistic understanding of the impact changes in protein concentration and temperature will have on viscosity and consequently on injectability and device functionality hinges on mapping viscosity data over a relevant range of both protein concentrations and temperatures. As discussed earlier, and highlighted in Fig. 7.2a and b, during development the apparent viscosity should be assessed at shear rates consistent with shear rates experienced upon injection. Differences in viscosity as a function of temperature (even at the same protein concentration) can potentially result in requiring a high extrusion force during the device design. It is particularly important to assess the potential for shear-thinning behavior during administration to avoid an overpowered device design. The failure to recognize the potential to



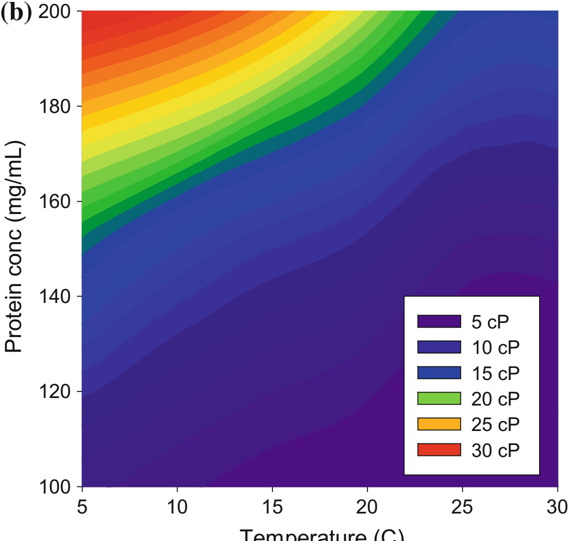


Fig. 7.2 **a** (Top panel): Viscosity (reported in cP, measured at 750 s^{-1}) of a monoclonal antibody as a function of both protein concentration (mg/mL) and temperature (degrees Celsius). **b** (Bottom panel): Theoretical values estimated at a higher shear rate of 100,000 s^{-1}

undergo shear thinning during administration can lead to the recommendation of an overestimated injection force that could potentially affect patient comfort (high delivery forces resulting in fast, potentially painful flow rates) and device functionality [80]. The force required to expel high concentration protein therapeutic solutions from a syringe can be significant. Acceptable variability in the protein concentration and or temperature at which the device is used to administer the product can lead to a wide range of viscosity conditions under which the device must be able to reliably function.

Typically, protein therapeutics are processed and stored long term at refrigerated temperatures, where solution viscosity is often the highest. A protein therapeutic solution is often equilibrated to room temperature before injection to improve a patient's experience by potentially reducing the injection time and the extrusion force required for injection.

Potential Effect of Syringe Siliconization

Although PFS can be either glass (usually Type 1 borosilicate) or plastic, glass syringes currently dominate the PFS market [81]. Glass PFSs are siliconized to allow the plunger to move along the barrel. Silicone oil can be baked or sprayed on. The distribution of silicone oil can impact the force required to extrude the contents because uneven silicone oil distribution, particularly an insufficient amount of silicone oil near the needle, may impede the injection [80, 82]. This may impact both a manual injection from a pre-filled syringe or with an injection device. If an injection device uses a compressed spring to expel the contents, this issue can be exacerbated because the force also weakens near the end of the injection, which could potentially lead to a stalled injection.

Injection time is an outcome that is highly relevant to the patient. Rathore et al. reported model-based quantification that identified key sources of injection time variability: product viscosity, needle diameter, and the spring constant [83]. Despite the small variability within GMP-caliber autoinjector components themselves, injection time can be variable due to its dependence on several other system parameters [83]. It is understood that models cannot capture all potential sources of variability, such as the homogeneity of syringe siliconization, potential (albeit minor) performance degradation due to component age, protein drying in the needle, and the precise temperature at injection [9, 83]. Careful and methodical testing, modeling and assessment for potential modes of failure are necessary to choose appropriate conditions for delivery.

7.3.3 Impact of Subcutaneous Tissue Back Pressure on Injectability

In silico models can predict the performance parameters of injection devices such as injection force and time [17, 83]. However, these models are based on fluid dynamics principles and therefore omit the potential impact of the tissue back pressure during subcutaneous (SC) injection. Similarly, analytical testing to assess injection forces and syringe functionality testing (i.e., break loose and extrusion force) are typically performed into air and may not be representative of an in vivo injection where the skin and subcutaneous milieu also play a role. In silico modeling and analytical testing can assess syringeability and identify potential modes of failure, but to assess injectability, the role of tissue back pressure must also be

considered. Despite growing interest in this topic, limited data exist for assessing the contribution of the tissue back pressure during SC injection [79]. Allmendinger et al. reported that the contribution of the subcutaneous back pressure to the injection forces increased linearly with viscosity and reported that when estimating injection forces, the contribution of the tissue as well as the conditions of injection should be considered [17, 84].

An appropriate animal model should be selected to predict tissue back pressure in humans. Due to the morphological and physiological similarities between minipig and human skin, the minipig has been successfully used as a model for studies of drugs administered subcutaneously [84, 85]. Variations in tissue back pressure measurements can be observed, particularly for high viscous solutions. Patte et al. examined tissue resistance pressure in patients at different infusion rates and found that stepwise increases in the infusion rate were associated with significant rises in median tissue resistance pressure [86]. Several factors can contribute to the variability in the tissue back pressure measurements (which can in turn affect the injection forces), including needle geometry (e.g., inner diameter, length) and even the surface finish of the syringes [84]. The natural variability of skin tissues and injection site location can also introduce variability in the injection force measurements into skin tissue. Thus, these factors should be carefully taken into consideration while measuring injection forces representative for in vivo conditions.

The development of robust combination products requires a better understanding of injection forces dependent on various parameters including tissue back pressure. The development of ex vivo models (e.g., obtaining functional skin tissue) and in vivo animal models for assessment of device functionality including injection force measurements can aid in the overall success of holistic device design, which accounts for the solution properties of the therapeutic protein formulation.

7.3.4 Potential Needle Clogging

Drying and subsequent needle clogging may occur upon storage of the device and is hypothesized to be due to evaporation of the liquid in the formulation or local precipitation of the protein. The volume of the precipitated material affects the area of the needle that may be partially blocked. This impacts the extrusion force to varying degrees depending upon the extent of the clog. Increased protein concentrations have seemingly increased probability of these types of blockage events. The extent of blockage is also dependent upon storage conditions such as temperature, time, orientation, relative humidity, and device parameters including needle gauge and length, and needle shield rubber formulation. The rubber formulation can theoretically affect the permeability of the needle shield and/or potentially introduce leachables that initiate protein precipitation [87, 88]. This drying phenomenon is

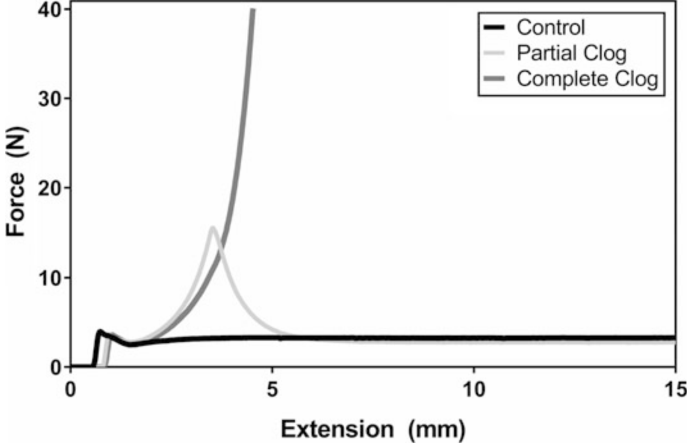


Fig. 7.3 Extrusion force diagram of a high concentration antibody (100 mg/mL) stored under various environmental conditions to induce clogging. The extrusion forces of a control, a partially clogged syringe as well as a completely clogged PFS (force > 40 N) are shown

readily observed when the needle shield is removed from the syringe but the contents of the syringe are not immediately injected, similar to issues observed with filling needles [69]. The extent of the blockage is dependent upon the protein concentration, syringe orientation, temperature, relative humidity, and time of exposure without the needle shield. This phenomenon can be detected by monitoring the extrusion force as shown in Fig. 7.3, which shows extrusion force diagram of a high concentration antibody formulation that was stored under various environmental conditions to induce clogging.

In a typical load–force diagram, the breakloose force (N) is a maximum, which is readily observed as the plunger begins to move. The force then decreases as plunger traverses the air gap. The maximum force to expel the syringe contents is termed the extrusion force. If protein dries in the needle and partially blocks the fluid path, a new peak may be observed after the breakloose peak. The extent to which the dried protein clogs the needle may translate into a greater force required to expel the blockage or for complete blockages, potentially result in cessation of extrusion. Partially clogged syringes may not result in a noticeable change in injection forces during manual or device-driven expulsions, but may be predictors of increased risks of future clogging events.

Due to the myriad potential causes of needle clogging, understanding the mechanism is critical. To that end, neutron scattering has been recently used to image liquid in the needle and monitor the injection process [89]. This work highlights the potential for in situ imaging techniques to aid in the investigation and prevention of device malfunction.

7.4 Potential Mitigation

It is worthwhile to briefly mention the causes of high viscosity because mitigation efforts may vary based on the mechanism of interaction. Protein self-association can be due to myriad interactions: non-specific molecular crowding, specific interactions, weak associations, and/or electrostatic interactions [90–92]. To mitigate viscosity driven by intermolecular interactions, understanding the type of interaction (at a molecular level) is important. If the underlying mechanism is unknown, then screening of formulation excipients that can disrupt the protein–protein interactions can be informative. The underlying mechanism driving high solution viscosity can sometimes be inferred based on the excipient class that is effective in mediating it. Practically speaking, mitigation options depend on the phase of development. An unusual and problematic tendency to self-associate can be identified by measuring viscosity as a function of protein concentration and temperature during early screening studies to influence the choice of one molecule over another in the pre-discovery phase. Judicious point mutations have been shown to decrease viscosity [93]. However, a protein engineering approach hinges on a nuanced understanding of the molecular mechanism driving increased viscosity.

For molecules further along in development, and depending on the underlying mechanism, self-association (and consequent high viscosity) can sometimes be mitigated by prudent choices of excipients to modulate attractive and/or repulsive interactions. Certain excipients, specific cations/anions, or amino acids have been shown to help reduce solution viscosity, decrease aggregation, and increase solubility [94–96]. Multiple self-association mechanisms can be at work in high protein concentration solutions, in which case the dominant mechanism affecting viscosity should be identified. For example, Nichols et al. identified that neutralizing negatively charged surface patches was more effective in reducing solution viscosity of a highly concentrated antibody solution than disruption of an aggregation-prone region of the protein [93]. Although it is best to choose a molecule during development that does not have intrinsic high viscosity, this approach can be impractical because efficacy is often the dominant molecular attribute.

7.5 Summary

Practical issues that arise for high concentration protein formulations are typically due to high viscosity, which can complicate manufacturing unit operations and affect injectability/device compatibility. The viscosity of high protein concentration drug products can impact the performance of manufacturing unit operations, specifically tangential flow filtration (TFF), mixing, filtration, filling, the effectiveness of automated inspections, and even the cleanability of manufacturing equipment. Each potentially affected unit operation must be carefully considered to

determine the effects, if any, that high viscosity, particulation, or shear sensitivity could have on process performance. Potential mitigations (like processing at higher temperatures where viscosity is lower) must be balanced with any impact to product quality.

Solution properties that affect viscosity and downstream operations such as device compatibility include slight variations in protein concentration and the temperature of the product at administration. Variability in the homogeneity and level of siliconization of the PFS, primary container dimensions, the characteristics of the autoinjector spring, and tissue back pressure can potentially affect injectability. Due to the potential for non-Newtonian behavior (shear thinning), viscosity–shear rate profiles of high concentration protein formulations should be generated over a range of shear rates relevant to both drug product manufacturing unit operations and administration. Early screening of potential candidates can identify highly viscous candidates and perhaps eliminate such molecules from consideration. If such issues are not identified until later in development, perhaps concurrent with an increase in dosing regimen that necessitates a higher protein concentration, then mitigation efforts to reduce viscosity likely pivot from a protein engineering approach to focus on formulation changes.

Acknowledgements The authors wish to acknowledge the work of Drs. Nitin Rathore and Joseph Bernacki, particularly their data visualization, as Fig. 7.1 was adapted from their work. Thanks to Dr. Nicholas Clark for generating breakloose extrusion data. We would also like to thank Dr. Sugunakar Patro for his critical review and support.

References

1. Shire SJ, Shahrokh Z, Liu J. Challenges in the development of high protein concentration formulations. J Pharm Sci. 2004;93(6):1390–402.
2. Liu J, Nguyen MD, Andya JD, Shire SJ. Reversible self-association increases the viscosity of a concentrated monoclonal antibody in aqueous solution. J Pharm Sci. 2005;94(9):1928–40.
3. Jezek J, Rides M, Derham B, Moore J, Cerasoli E, Simler R, Perez-Ramirez B. Viscosity of concentrated therapeutic protein compositions. Adv Drug Deliv Rev. 2011;63(13):1107–17.
4. Tomar DS, Kumar S, Singh SK, Goswami S, Li L. Molecular basis of high viscosity in concentrated antibody solutions: Strategies for high concentration drug product development. mAbs. 2016;8(2):216–28.
5. Yadav S, Shire SJ, Kalonia DS. Viscosity behavior of high-concentration monoclonal antibody solutions: correlation with interaction parameter and electroviscous effects. J Pharm Sci. 2012;101(3):998–1011.
6. Kanai S, Liu J, Patapoff TW, Shire SJ. Reversible self-association of a concentrated monoclonal antibody solution mediated by Fab-Fab interaction that impacts solution viscosity. J Pharm Sci. 2008;97(10):4219–27.
7. Berg JC. An introduction to interfaces and colloids: the bridge to nanoscience. Singapore: World Scientific; 2009.
8. Pathak JA, Sologuren RR, Narwal R. Do clustering monoclonal antibody solutions really have a concentration dependence of viscosity? Biophys J. 2013;104(4):913–23.

9. French DL, Collins JJ. Advances in parenteral injection devices and aids. In Sandeep Nema JDL, editors. Pharmaceutical dosage forms. London: Informa Healthcare; 2010. pp.71–85.
10. Dias C, Abosaleem B, Crispino C, Gao B, Shaywitz A. Tolerability of high-volume subcutaneous injections of a viscous placebo buffer: a randomized, crossover study in healthy subjects. AAPS PharmSciTech. 2015;16(5):1101–7.
11. Doughty DV, Clawson CZ, Lambert W, Subramony JA. Understanding subcutaneous tissue pressure for engineering injection devices for large-volume protein delivery. J Pharm Sci. 2016;105(7):2105–13.
12. Berteau C, Filipe-Santos O, Wang T, Rojas HE, Granger C, Schwarzenbach F. Evaluation of the impact of viscosity, injection volume, and injection flow rate on subcutaneous injection tolerance. Medical devices. 2015;8:473–84.
13. Mahler HC, Friess W, Grauschopf U, Kiese S. Protein aggregation: pathways, induction factors and analysis. J Pharm Sci. 2009;98(9):2909–34.
14. Wang W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. Int J Pharm. 1999;185(2):129–88.
15. Bird B, Stewart W, Lightfoot E. Transport phenomena. New York: John Wiley and Sons; 1960. pp. 1–780.
16. Zarraga IE, Taing R, Zarzar J, Luoma J, Hsiung J, Patel A, Lim F. High shear rheology and anisotropy in concentrated solutions of monoclonal antibodies. J Pharm Sci. 2013;102 (8):2538–49.
17. Allmendinger A, Fischer S, Huwyler J, Mahler H-C, Schwarb E, Zarraga IE, Mueller R. Rheological characterization and injection forces of concentrated protein formulations: an alternative predictive model for non-Newtonian solutions. Eur J Pharm Biopharm. 2014;87 (2):318–28.
18. Viswanath DS, Ghosh T, Prasad DHL, Dutt NVK, Rani KY. Viscosity of liquid—theory, estimation, experiment, and data. Berlin: Springer; 2010.
19. Anderson AM, Bruno BA, Smith LS. Viscosity Measurment part IV. Handbook of measurment in science and engineering. Published Online. Hoboken: John Wiley & Sins, Inc.;2013.
20. Jezek J, Rides M, Derham B, Moore J, Cerasoli E, Simler R, Perez-Ramirez B. Viscosity of concentrated therapeutic protein compositions. Adv Drug Delivery Rev. 2011;63(13):1107–17.
21. Bee JS, Stevenson JL, Mehta B, Svitel J, Pollastrini J, Platz R, Freund E, Carpenter JF, Randolph TW. Response of a concentrated monoclonal antibody formulation to high shear. Biotechnol Bioeng. 2009;103(5):936–43.
22. Mahler H-C, Fischer S, Randolph TW, Carpenter JF. Protein aggregation and particle formation: effects of formulation, interfaces, and drug product manufacturing operations. In: Wei Wang CJR, editors. Aggregation of therapeutic proteins; 2010. pp. 301–5.
23. Neumaier R. Hermetic pumps: the latest innovations and industrial applications of sealless pumps. Houston, TX: Gulf Publishing Company; 1997.
24. Bechtold-Peters K. Development challenges and validation of fill and finish processes for biotherapeutics in Pharmaceutical Dosage forms-parenteral medications. In: Nema S, Ludwig JD, editors. Pharmaceutical dosage forms—parenteral medications, third edition: volume 3: regulations, validation and the future. Boca Raton: CRC Press; 2010. pp. 25–51.
25. Grigoriev DO, Derkatch S, Kraegel J, Miller R. Relationship between structure and rheological properties of mixed BSA/Tween 80 adsorption layers at the air/water interface. Food Hydrocolloids. 2007;21(5–6):823–30.
26. Swindellsr JF, Coe RJ, Godfrey TB. Absolute viscosity of water at 20 °C. J Res Nat Bur Stand 1952; 48(1):2279.
27. Kestin J, Sokolov M, Wakeham WA. Viscosity of liquid water in the range −8° to 150 °C. J Phys Chem Ref Data. 1978;7(3):941–8.

28. Shire SJ, Liu J, Friess W, Jorg S, Mahler H-C. High-concentration antibody formulations. In: Feroz Jameel SH, editors. Formulation and process development strategies for manufacturing biopharmaceuticals. Hoboken, NJ: Wiley; 2010. pp. 349–81.
29. Dani B, Platz R, Tzannis ST. High concentration formulation feasibility of human immunoglubulin G for subcutaneous administration. J Pharm Sci. 2007;96(6):1504–17.
30. Rao S, Gefroh E, Kaltenbrunner O. Recovery modeling of tangential flow systems. Biotechnol Bioeng. 2012;109(12):3084–92.
31. Gefroh E, Lutz H. An alternate diafiltration strategy to mitigate protein precipitation for low solubility proteins. Biotechnol Prog. 2014;30(3):646–55.
32. Jaffrin M, Charrier J. Optimization of ultrafiltration and diafiltration processes for albumin production. J Membr Sci. 1994;97:71–81.
33. Zeman LJ ZA. Microfiltration and ultrafiltration—principles and applications. New York: Marcel Dekker, Inc.; 1996. pp. 618.
34. Mitra GL, J. Ultrafiltration of immune serum globulin and human serum albumin: regression analysis studies. Sep Sci Technol. 1978; 13:89–94.
35. Porter MC. Concentration polarization with membrane ultrafiltration. Ind Eng Chem Prod Res Dev. 1972;11(3):234–48.
36. van Reis R, Zydney A. Bioprocess membrane technology. J Membr Sci. 2007;297(1–2):16–50.
37. Hung JJ, Borwankar AU, Dear BJ, Truskett TM, Johnston KP. High concentration tangential flow ultrafiltration of stable monoclonal antibody solutions with low viscosities. J Membr Sci. 2016;508:113–26.
38. Jaspe J, Hagen SJ. Do protein molecules unfold in a simple shear flow? Biophys J. 2006;91(9):3415–24.
39. Stoner MR, Fischer N, Nixon L, Buckel S, Benke M, Austin F, Randolph TW, Kendrick BS. Protein-solute interactions affect the outcome of ultrafiltration/diafiltration operations. J Pharm Sci. 2004;93(9):2332–42.
40. Arakawa T, Timasheff SN. Preferential interactions of proteins with solvent components in aqueous amino-acid solutions. Arch Biochem Biophys. 1983;224(1):169–77.
41. Miao F, Velayudhan A, DiBella E, Shervin J, Felo M, Teeters M, Alred P. Theoretical analysis of excipient concentrations during the final ultrafiltration/diafiltration step of therapeutic antibody. Biotechnol Prog. 2009;25(4):964–72.
42. Teeters M, Bezila D, Benner T, Alfonso P, Alred P. Predicting diafiltration solution compositions for final ultrafiltration/diafiltration steps of monoclonal antibodies. Biotechnol Bioeng. 2011;108(6):1338–46.
43. Singh S, Nema S. Freezing and thawing of protein solultions. In: Hershenson FJaS, editors. Formulation and process development strategies for manufacturing biopharmaceuticals. Hoboken, NJ: John Wiley & Sons, Inc.; 2010. pp. 624–75. http://www.biopharminternational.com/large-scale-freezing-biologics-understandingprotein-and-solute-concentration-changes-cryovessel-p-0?id=&sk=&date=&pageID=7.
44. Kolhe P, Badkar A. Protein and solute distribution in drug substance containers during frozen storage and post-thawing: a tool to understand and define freezing-thawing parameters in biotechnology process development. Biotechnol Prog. 2011;27(2):494–504.
45. Kolhe P, Holding E, Lary A, Chico S, Singh S. Large-scale freezing of biologics: understanding protein and solute concentration changes in a cryovessel–part I. BioPharm Internat. 2010;23(6):53–60.
46. Singh S, Chico S, Lary A, Kolhe P, Holding E. Large-scale freezing of biologics: understanding protein and solute concentration changes in a cryovessel—Part 2. BioPharm Internat. 2010; 23(7).
47. Shire SJ. Formulation and manufacturability of biologics. Curr Opin Biotechnol. 2009;20(6):708–14.
48. Xiao NJ, Medley CD, Shieh IC, Downing G, Pizarro S, Liu J, Patel AR. A small-scale model to assess the risk of leachables from single-use bioprocess containers through protein quality characterization. PDA J Pharm Sci Technol. 2016;70(6):533–46.

49. Kolhe P, Amend E, Singh SK. Impact of freezing on pH of buffered solutions and consequences for monoclonal antibody aggregation. Biotechnol Prog. 2010;26(3):727–33.
50. Piedmonte DM, Summers C, McAuley A, Karamujic L, Ratnaswamy G. Sorbitol crystallization can lead to protein aggregation in frozen protein formulations. Pharm Res. 2007;24(1):136–46.
51. Sundaramurthi P, Patapoff TW, Suryanarayanan R. Crystallization of trehalose in frozen solutions and its phase behavior during drying. Pharm Res. 2010;27(11):2374–83.
52. Connolly B, Patapoff TW, Wang YJ, Moore JM, Kamerzell TJ. Vibrational spectroscopy and chemometrics to characterize and quantitate trehalose crystallization. Anal Biochem. 2010;399(1):48–57.
53. Connolly BD, Le L, Patapoff TW, Cromwell ME, Moore JM, Lam P. Protein aggregation in frozen trehalose formulations: effects of composition, cooling rate, and storage temperature. J Pharm Sci. 2015;104(12):4170–84.
54. Piedmonte DM, Hair A, Baker P, Brych L, Nagapudi K, Lin H, Cao W, Hershenson S, Ratnaswamy G. Sorbitol crystallization-induced aggregation in frozen mAb formulations. J Pharm Sci. 2015;104(2):686–97.
55. Martin-Moe S, Lim FJ, Wong RL, Sreedhara A, Sundaram J, Sane SU. A new roadmap for biopharmaceutical drug product development: integrating development, validation, and quality by design. J Pharm Sci. 2011;100(8):3031–43.
56. Nail S, Akers M. Pharmaceutical biotechnology. In: Hershenson FJaS, editors. Development and manufacture of protein pharmaceuticals. Hoboken: Wiley; 2010.
57. Jiang G, Thummala A, Wadhwa MV. Applications of statistical regression and modeling in fill-finish process development of structurally related proteins. J Pharm Sci. 2011;100(2): 464–81.
58. Gikanga B, Roshan-Eisner D, Ovadia R, Day ES, Stauch OB, Maa YF. Processing impact on monoclonal antibody drug products: protein subvisible particulate formation induced by grinding stress. PDA J Pharm Sci Technol 2016.
59. Gikanga B, Eisner DR, Ovadia R, Day ES, Stauch OB, Maa YF. Processing impact on monoclonal antibody drug products: protein subvisible particulate formation induced by grinding stress. PDA J Pharm Sci Technol. 2017;71(3):172–88.
60. Brose D. Membrane filtration. In: Nail SL, Akers, MJ, editors. Development and manufacture of protein pharmaceuticals. Berlin, US: Springer; 2002. pp. 213–79.
61. Sethuraman APX, Mehta B, Radhakrishnan V. High-concentration antibody formulations. In: Jameel F HS, editors. Formulation and process development strategies for manufacturing biopharmaceuticals. Hoboken: John Wiley & Sons; 2010. pp. 839–56.
62. Cornell Manning M, Evans G, Payne R. Protein stability during bioprocessing. In: Jameel F, Hershenson S, editors. Formulation and process development strategies for manufacturing biopharmaceuticals. Hoboken, NJ: John Wiley & Sons Inc.; 2010. pp. 605–23.
63. Allmendinger A, Mueller R, Huwyler J, Mahler HC, Fischer S. Sterile filtration of highly concentrated protein formulations: impact of protein concentration, formulation composition, and filter material. J Pharm Sci. 2015;104(10):3319–29.
64. Mahler HC, Huber F, Kishore RS, Reindl J, Ruckert P, Muller R. Adsorption behavior of a surfactant and a monoclonal antibody to sterilizing-grade filters. J Pharm Sci. 2010;99 (6):2620–7.
65. Fink A. Protein aggregation: folding aggregates, inclusion bodies and amyloid. Fold Des. 1998;3(1):R9–23.
66. Maa YF, Hsu CC. Investigation on fouling mechanisms for recombinant human growth hormone sterile filtration. J Pharm Sci. 1998;87(7):808–12.
67. Lilyestrom WG, Yadav S, Shire SJ, Scherer TM. Monoclonal antibody self-association, cluster formation, and rheology at high concentrations. J Phys Chem B. 2013;117(21): 6373–84.
68. Liu L, Qi W, Schwartz DK, Randolph TW, Carpenter JF. The effects of excipients on protein aggregation during agitation: an interfacial shear rheology study. J Pharm Sci. 2013;102 (8):2460–70.

69. Shieu W, Torhan SA, Chan E, Hubbard A, Gikanga B, Stauch OB, Maa YF. Filling of high-concentration monoclonal antibody formulations into pre-filled syringes: filling parameter investigation and optimization. PDA J Pharm Sci Technol. 2014;68(2):153–63.
70. Shieu W, Lamar D, Stauch OB, Maa YF. Filling of high-concentration monoclonal antibody formulations: investigating underlying mechanisms that affect precision of low-volume fill by peristaltic pump. PDA J Pharm Sci Technol. 2016;70(2):143–56.
71. Shieu W, Stauch OB, Maa YF. Filling of high-concentration monoclonal antibody formulations into pre-filled syringes: investigating formulation-nozzle interactions to minimize nozzle clogging. PDA J Pharm Sci Technol. 2015;69(3):417–26.
72. Kiese S, Papppenberger A, Friess W, Mahler HC. Shaken, not stirred: mechanical stress testing of an IgG1 antibody. J Pharm Sci. 2008;97(10):4347–66.
73. Rathore N, Chen C, Gonzalez O, Ji W. Challenges and strategies for implementing automated visual inspection for biopharmaceuticals. Pharm Technol. 2009;2009(6).
74. Sharnez R, Lathia J, Kahlenberg D, Prabhu S, Dekleva M. In situ monitoring of soil dissolution dynamics: a rapid and simple method for determining worst-case soils for cleaning validation. PDA J Pharm Sci Technol. 2004;58(4):203–14.
75. Rathore N, Qi W, Ji WC. Cleaning characterization of protein drug products using UV-vis spectroscopy. Biotechnol Prog. 2008;24(3):684–90.
76. Eu B, Cairns A, Ding G, Cao X, Wen ZQ. Direct visualization of protein adsorption to primary containers by gold nanoparticles. J Pharm Sci. 2011;100(5):1663–70.
77. Makwana S, Basu B, Makasana Y, Dharamsi A. Prefilled syringes: an innovation in parenteral packaging. Int J Pharm Invest. 2011;1(4):200–6.
78. Breitsamer M, Winter G. Needle-free injection of vesicular phospholipid gels—a novel approach to overcome an administration hurdle for semisolid depot systems. J Pharm Sci. 2016;106(4):968–72.
79. Cilurzo F, Selmin F, Minghetti P, Adami M, Bertoni E, Lauria S, Montanari L. Injectability evaluation: an open issue. AAPS PharmSciTech. 2011;12(2):604–9.
80. Rathore N, Pranay P, Bernacki J, Eu B, Ji W, Walls E. Characterization of protein rheology and delivery forces for combination products. J Pharm Sci. 2012;101(12):4472–80.
81. Jenke DR. Extractables and leachables considerations for prefilled syringes. Expert Opin Drug Deliv. 2014;11(10):1591–600.
82. Wen ZQ, Vance A, Vega F, Cao X, Eu B, Schulthesis R. Distribution of silicone oil in prefilled glass syringes probed with optical and spectroscopic methods. PDA J Pharm Sci Technol. 2009;63(2):149–58.
83. Rathore N, Pranay P, Eu B, Ji W, Walls E. Variability in syringe components and its impact on functionality of delivery systems. PDA J Pharm Sci Technol. 2011;65(5):468–80.
84. Allmendinger A, Mueller R, Schwarb E, Chipperfield M, Huwyler J, Mahler HC, Fischer S. Measuring tissue back-pressure–in vivo injection forces during subcutaneous injection. Pharm Res. 2015;32(7):2229–40.
85. Kang DW, Oh DA, Fu GY, Anderson JM, Zepeda ML. Porcine model to evaluate local tissue tolerability associated with subcutaneous delivery of protein. J Pharmacol Toxicol Method. 2013;67(3):140–7.
86. Patte C, Pleus S, Wiegel C, Schiltges G, Jendrike N, Haug C, Freckmann G. Effect of infusion rate and indwelling time on tissue resistance pressure in small-volume subcutaneous infusion like in continuous subcutaneous insulin infusion. Diabetes Technol Therapeutics. 2013;15 (4):289–94.
87. Thyagarajapuram N. Presented at the PDA Europe The universe of pre-filled syringes and injection devices, Basel, Switzerland, November 7–11 2011.
88. Adler M. Challenges in the development of pre-filled syringes for biologics for a formulation scientist's point of view. Am Pharm Rev. 2012;2/1/2012 ed. p 9.
89. Kaestner A, Roth J, Grunzweig C. Real-time neutron imaging to detect origin of blocking in drug injection devices. PDA J Pharm Sci Technol. 2016;70(4):353–60.

90. Yearley EJ, Zarraga IE, Shire SJ, Scherer TM, Gokarn Y, Wagner NJ, Liu Y. Small-angle neutron scattering characterization of monoclonal antibody conformations and interactions at high concentrations. Biophys J. 2013;105(3):720–31.
91. Connolly BD, Petry C, Yadav S, Demeule B, Ciaccio N, Moore JM, Shire SJ, Gokarn YR. Weak interactions govern the viscosity of concentrated antibody solutions: high-throughput analysis using the diffusion interaction parameter. Biophys J. 2012;103(1):69–78.
92. Li L, Kumar S, Buck PM, Burns C, Lavoie J, Singh SK, Warne NW, Nichols P, Luksha N, Boardman D. Concentration dependent viscosity of monoclonal antibody solutions: explaining experimental behavior in terms of molecular properties. Pharm Res. 2014;31 (11):3161–78.
93. Nichols P, Li L, Kumar S, Buck PM, Singh SK, Goswami S, Balthazor B, Conley TR, Sek D, Allen MJ. Rational design of viscosity reducing mutants of a monoclonal antibody: hydrophobic versus electrostatic inter-molecular interactions. mAbs. 2015; 7(1):212–30.
94. Shiraki K, Kudou M, Fujiwara S, Imanaka T, Takagi M. Biophysical effect of amino acids on the prevention of protein aggregation. J Biochem. 2002;132(4):591–5.
95. Tsumoto K, Umetsu M, Kumagai I, Ejima D, Philo JS, Arakawa T. Role of arginine in protein refolding, solubilization, and purification. Biotechnol Prog. 2004;20(5):1301–8.
96. Arakawa T, Ejima D, Tsumoto K, Obeyama N, Tanaka Y, Kita Y, Timasheff SN. Suppression of protein interactions by arginine: a proposed mechanism of the arginine effects. Biophys Chem. 2007;127(1–2):1–8.

Part IV
Container-Closure Systems

Chapter 8
Parenteral Container Closure Systems

Roman Mathaes and Alexander Streubel

Abstract Parenteral container closure systems are in direct contact with the drug product formulation and can significantly impact the product quality. The interaction between the drug product formulation and the primary packaging components may also impact adequate delivery of the product to the patient. Therefore, primary packaging development should be fully integrated into parenteral drug product development. This book chapter describes the basic function and requirements of a parenteral container closure system, advantages, and challenges with commonly used container closure system materials and components and the key benefits of combination products compared to traditional vial systems.

Keywords Parenteral container closure systems · Primary packaging
Glass · Combination products

8.1 Introduction

Recombinant proteins comprise the largest segment of the parenteral pharmaceutical market and continue to outpace overall pharma spending growth. Following the approval of insulin in 1982, the total annual revenue of biologics increased to 140 billion dollars in 2013. Yet, biologics are very sensitive and delicate systems with complex upstream and downstream processes as well as an aseptic fill and finish process, where the drug product is transferred into its container closure system (CCS). The selection and validation of an adequate CCS is a key process during the product development. Different container closure systems for parenteral pharmaceuticals are available, which need to be carefully designed and chosen to provide several requirements.

R. Mathaes (✉) · A. Streubel
Hochbergerstrasse 60 a, 4057 Basel, Switzerland
e-mail: roman.mathaes@lonza.com

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_8

8.2 Requirements of a Parenteral CCS

Several requirements of a parenteral CCS are mandatory (Table 8.1).

1. Containment

The basic and fundamental function of a CCS is the containment of the DP. This includes no leakage, diffusion, or permeation of the DP during manufacturing, distribution, and normal handling. In addition, the CCS must be inert to alteration by the DP [1, 2].

2. Protection and Compatibility

The CCS must protect the DP from all external influences, in the case of therapeutic proteins from, e.g., oxygen by allowing a nitrogen protective gas headspace or in the case of freeze dried DPs from moisture.

In addition, the compatibility of the primary packaging with the final DP plays an important role in the design and selection of the CCS. Several possible interactions between the primary packaging and the parenteral DP can be critical for the DP stability over shelf life. Ensuring the compatibility of the final primary packaging and the DP is an essential part during the product development (Table 8.2).

The different primary packaging components are the source of specific extractables and leachables, which possibly lead to physical or chemical DP degradation. Extractables are substances that can be extracted from the primary packaging under harsh conditions like aggressive solvents or elevated temperatures. Leachables are substances that leach into the DP solution under normal conditions. For example, leaching metal ions from glass vials can lead to protein precipitation. The leachable profile is dependent on the glass type and quality as well as the DP formulation (mainly pH), (Table 8.2). Other case studies of CCS–DP interactions are discussed in detail later in this chapter.

3. Sterility

Parenteral DPs are sterile products, as per pharmacopeial requirements and are manufactured by terminal sterilization, if possible. However, the terminal sterilization of therapeutic protein adversely impacts product quality. Therefore, therapeutic proteins are manufactured aseptically in combination with sterile filtration. Parenteral DPs are protected by appropriate CCS. A CCS needs to be designed,

Table 8.1 Overview of requirements of a parenteral CCS

Containment: no leak, diffusion, or permeation of the DP
Protection: protect the DP from all external influences
Sterility: ensure container closure integrity (CCI) over shelf life
Presentation and identification: source of information, e.g., labels
Convenient application: comfortable and easy administration
Anti-counterfeiting: unique packaging schemes

Table 8.2 Overview of possible interactions of the parenteral DP and the primary packaging

Example of source	Example of interaction, degradation pathway	Ref.
Glass: vials, syringes	Leachables of particles and metal ions, adsorption, precipitation	[3–7]
Plastics: vials, syringes	Leachables, poor gas barrier: oxidation	[8]
Elastomers: rubber stoppers, plunger	Leachables, adsorption, permeation	[9–11]
Metals: syringe	Leachables of tungsten: oxidation, precipitation	[12–15]
Adhesive: PFS stake-in needles	Leachables of adhesive components: oxidation	[16]
Coatings (e.g., silicone oil): syringes	Leachables of silicon oil: adsorption, aggregation	[17–22]

chosen, and validated to ensure protection from any possible microbial contaminations during manufacturing (intrinsic contaminations) or storage (CCI). Obviously, any microbiological contamination of a sterile product can cause serious harm in patients [23]. Sterility assurance of parenteral DPs during manufacture or storage is clearly mandated by health authorities globally [24, 25]. Ensuring CCI over the product shelf life (storage, shipment, intended use) is one of the most critical aspects in the development and commercialization of a parenteral pharmaceutical product.

As a result, a variety of leak test methods have been developed to assess CCI and have different advantages and challenges: (1) The microbial immersion challenge test (mCCI) is a traditional CCI test method, where filled and sealed drug product units are immersed in a defined challenge organism solutions under specific conditions and possible contaminants are assessed. (2) Physical CCI (pCCI) tests exist, where any kind of surrogate is being used to assess possible leaks and thus CCI. The most commonly used CCI tests includes the blue dye test, the electrical field test, or the helium leak test, with the latter being the currently most sensitive pCCI test [26–28].

4. Presentation and Identification

The label on the CCS is an important source of information of pharmaceutical products. The pharmaceutical company must deliver a product within label claims. Label information usually includes the identification of the active pharmaceutical ingredient (API), the dosage form, the trade name, storage condition, and the batch number. The provided information on the label must follow national legislation [1, 29].

5. Convenient application

The global healthcare environment undergoes a paradigm shift from hospital care to home—self-administration. As a result, the possibility of a convenient self-administration of a pharmaceutical has received increasing attention. A common trend in the competitive parenteral biopharmaceutical market is the use of combination products like pre-filled syringes (PFSs) or other drug delivery

devices instead of the conventional vial and disposable syringe. Combination products offer numerous benefits for the patient like safety and dosing accuracy (discussed below) [2, 30].

6. Anti-counterfeiting

Counterfeiting has become an extremely serious problem in the pharmaceutical industry. Biopharmaceuticals can have single treatment cost of 1000 dollars and are an attractive product for counterfeits. Obviously, counterfeit parenteral pharmaceuticals which are possibly non-sterile can cause serious harm to patients.

Therefore, several solutions have been introduced for anti-counterfeiting. For example, visual features include breakable temper evident caps, or covered features, which are hidden from public [2, 29].

8.3 Container Closure Systems for Parenteral Pharmaceuticals

Several CCSs for parenteral pharmaceuticals are available: vials with rubber stopper and a crimp cap, pre-filled syringes, cartridges, ampoules. The prevalent CCS configuration for parenteral pharmaceuticals is the glass vial sealed with a rubber stopper and a crimp cap (around 50–55% of injectables [10]), followed by pre-filled syringes (around 25–30% of injectables [10]) (Fig. 8.1).

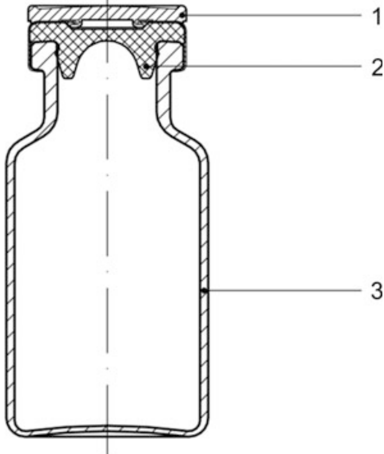


Fig. 8.1 **a** Pharmaceutical vial sealed with a rubber stopper and an aluminum flip-off cap: (1) crimp cap with plastic flip-off button, (2) rubber stopper, (3) vial

8.4 Vials

The glass vial offers several advantages over the plastic vial. For instance, it provides compression strength, considerable chemical inertness, impermeability to gas, resistance to heat, and transparency for product visibility. In contrast, the plastic vial lacks all these benefits of the glass vial. However, they are not fragile, light, cheap, and can be manufactured in precise dimensions and in a variety of shapes [31].

The USP pharmacopeia addresses glass quality in chapter <660> "Containers-Glass," which classifies glass into three types. First, the type I borosilicate glass is considerable chemical inert and has a low thermal coefficient of expansion. Second, the type II surface-treated soda lime glass is chemically treated to reduce alkali leachables (e.g., by ammonium sulfate), and third the type III standard soda lime glass (not used for parenteral drug products). In addition to the classification of the glass types, the USP chapter <660> provides three tests to determine the glass type and the inner surface hydrolytic resistance. For example, the "Powdered Glass Test" challenges small pieces of glass in an autoclave cycle.

For the production of glass, the raw materials are heated up until they are homogenously molten. After homogenization of the melt, vials can be produced in several ways: by a blow or pressing process ("molded" vials) or via tubes which are subsequently cut and formed ("tubular" vials). Today, glass vials from glass tubings are preferred over glass vials from molding as they have less cosmetic defects, are lighter, show better dimensional consistency, and display a better heat transfer during lyophilization [10].

During the hot forming process, the tubes are heated up again partially by gas flames to form the bottom and the neck/opening region of the vial using forming tools made out of steel. Sticking of the liquid glass to these forming tools is avoided by a constant rotation of the tubes with the forming tools which are lubricated by food grade oil. Such vials reveal high internal stresses from the high temperatures differences during forming process. These stresses are released by heating up the vials close to their transformation temperatures T_g (approximately 580 °C) in an annealing or cooling oven. At the end of the manufacturing process, the vials are usually packed in polymer boxes and are being shipped to the customer.

Fused silica or fused quartz is a glass made out of pure SiO_2. It has outstanding properties like a low thermal expansion coefficient, high temperature resistance, and high chemical resistance. It is inert against acids except hydrofluoric and hot phosphoric acid. But it has a main disadvantage which excludes it from being used in the above-described vial (or syringe) forming process: Transformation and softening and processing temperatures are all significantly higher compared to other glass types used for pharmaceutical applications. As a consequence, additional raw materials are added to lower the processing temperature, mainly alkali oxide (Na_2O or K_2O, so-called network modifiers). The modifiers are mobile ions within the glass backbone formed by SiO_2 and other network formers and lead to a disruption of the network. This results in a lower processing temperature. The addition of network formers like B_2O_3 or Al_2O_3 helps to improve the chemical durability (borosilicate

glasses). Depending on the desired chemical and physical properties, several more substances (metal oxides) are added, e.g., coloring agents like iron or titanium in case of brown glass. It is the aim to keep the elemental composition as tight as possible. Therefore, the raw materials need to have a constant and high quality.

8.5 Rubber Stopper

The elastomeric rubber stoppers are an important primary packaging component of several CCS. For example, they are used for closures of vials and syringes and as plungers tip (pistons) for syringes or cartridges. The elastomeric rubber stopper is a complex chemical part of the primary packaging [32]. Closures and pistons are available in a variety of sizes and shapes. In case of the pharmaceutical vial, two important designs are the serum rubber stopper and lyophilization rubber stopper. The lyophilization rubber stoppers are manufactured with protruding nods to keep the rubber stopper in the correct position with open vents during the lyophilization process (Fig. 8.2) [33].

The USP covers elastomeric rubber stoppers with several chapters. USP chapter <381> "Elastomeric Closures For Injections" [34] provides physicochemical and functional tests for elastomeric rubber stoppers. The different rubber stopper types (silicon coated, lubricious coated, barrier coating) require individual test setups. In addition to USP chapter <381>, USP chapter <87> [35] and <88> [36] provide in vitro and in vivo "Biological Reactivity Tests."

The rubber formulation usually consists of an elastomer, a filler, an activation agent, a vulcanization agent, and color pigments [37–39]. In contrast to the complex rubber formulations before the 1980s with over 10 components, the rubber formulations are cleaner today [40]. The most common elastomer for stoppers is halobuty, which shows a superior gas and moisture barrier [37, 41]. The vulcanization agent (usually sulfur) provides the rubber shape and elasticity [42]. The process of vulcanization is controlled by an activator (metal oxides) [39, 42]. In addition, fillers reduce tack and modify the rubber stopper hardness.

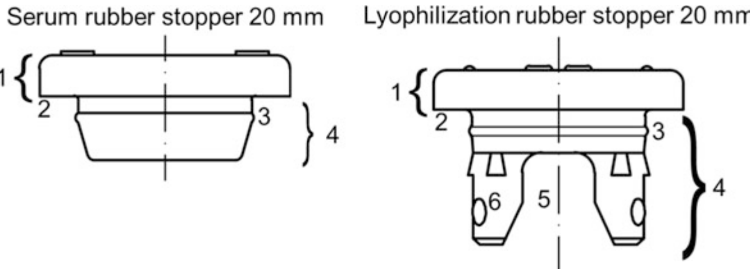


Fig. 8.2 Serum and lyo rubber stoppers for pharmaceutical vials. (1) Rubber stopper flange height, (2) vial sealing area, (3) no pop ring, (4) rubber stopper plug, (5) vent, (6) nods

The final elastomer composition as well as the size and shape of the rubber stopper determine the physical and chemical properties like hardness, modulus, compression, permeability to gas [37, 43, 44], and the individual profile of extractables/leachables [9, 45, 46].

In addition to the complex chemical rubber formulation, rubber stoppers are usually coated to reduce the leaching of rubber components into the drug product [45]. Moreover, it can replace silicone on the top of the stopper, which is required for a convenient process and handling. For, example, the proprietary West Pharmaceuticals/Daikyo Flurotec® coating contains ethylene and tetrafluoroethylene (EFTE) and protects the drug product from the rubber leachables [47]. Furthermore, it can be applied to the top of lyophilization rubber stoppers to prevent the sticking of the rubber stoppers to the freeze drying shelf.

The ability of a rubber stopper to close a vial is a function of the rubber's mechanical properties. Mortan et al. described the viscoelastic properties of elastomeric stoppers [43]. The intermediate response (combination of viscous and elastic) of a stopper during the capping process ensures a sufficient seal. First, the viscous behavior allows the rubber stopper to flow into imperfections of the vial sealing area. Second, the elastic behavior of the stopper generates a pressure of the stopper against the vial sealing area, the residual seal force.

The viscoelastic properties of a rubber stopper are defined by the rubber stopper hardness, which is usually measured by a shore durometer and elasticity [48]. Rubber hardness can be measured with a shore A durometer. Today, stoppers are available from shore A hardness 40 up to shore A hardness 80. Another important parameter of a rubber is the compression set. Compression set test measures the elastic properties of a rubber after prolonged compression stress. Therefore, a high compression set stopper applies less force to a closure after storage [49].

In addition to the stopper dimensions, the hardness of the rubber formulation impacts CCI. Soft stoppers fill vial imperfections more efficient. However, a soft stopper increases the risk of stopper dimpling into the vial neck. In addition, soft stoppers are susceptible to ruptures.

Rubber stopper and vial manufacturers do not collaborate to match their product configurations. Especially, the no pop ring of the rubber stopper should match the blowback on the vial. Lam et al. addressed this problem and described a method to visualize the fit of rubber stoppers and vials [50].

Moreover, several environmental stress factors influence the stopper performance. For example, heat during washing and autoclaving accelerates stress relaxation and crosslink reversion, which increases the gas permeability of a rubber stoppers [51]. In addition, Chan et al. described cracked pre-filled syringe rubbers upon ozone exposure [52]. Most common rubber formulations display a Tg between −50 and −70 °C. Therefore, stoppers stored on dry ice or −80 °C lose their elasticity, which can possibly impact CCI [53].

In conclusion, the stopper is a complex component of the primary packaging. Several interactions with the DP are possible or can influence CCI. The process of the stopper selection should always stay in context with the vial selection and includes several physicochemical and dimensional aspects.

8.6 Crimp Caps

During the capping process, the rubber stopper is compressed and kept in its compressed position by the crimp cap. The compressed rubber stopper applies a force against the vial sealing area, which seals the CCS.

Different crimp cap designs are commercially available. The majority of crimp caps consist of an aluminum part and feature an injection site or are completely removed (tear-off design) before usage. Plastic buttons (flip-off buttons) can further protect the injection site. A variety of different designs of flip-off buttons are available, e.g., with the same diameter than the aluminum part or with a larger diameter than the aluminum part, which eases the removal of the flip-off button.

8.7 Combination Products

The global healthcare environment undergoes a paradigm shift from hospital care to home—self-administration. Parenteral drugs for long-term treatments including rheumatoid arthritis or metabolic disease are candidates for self-administration. The increasing competition of several products of different pharma companies in certain disease areas like the above-mentioned rheumatoid arthritis led to the development of a variety of combination products for self-administration [54]. A combination product is defined as: "A product comprised of two or more regulated components, i.e., drug/device, biologic/device,…" [55] and eliminates the filling step from a second container like a vial. Pre-filled syringes comprise the largest segment of all combination products with a predicted growth to 6.83 billion units in 2025 [30]. However, injection pens or other more complex auto-injection devices receive increasing attention lately [54]. The most prominent examples for pen devices are the insulin pens in the European market.

PFS shows several advantages over conventional glass vials. However, some challenges remain (Table 8.3).

PFS can be classified into two different groups: luer lock luer tip PFSs where the barrel is locked to the needle with a mechanism similar to a screw and staked-in PFSs where the needle is fixed to the barrel with an adhesive [30]. The barrel of the luer lock luer tip PFSs can get siliconized in a bake-in process, and no adhesive is needed (low leachable levels). The staked-in PFSs are siliconized in a spray-on process and use an adhesive (possible leachable source). However, they are convenient to use [54].

In addition, PFSs can feature a dual chamber design, where two liquid drugs products or a lyophilized drug product and a diluent are separated in two chambers in the same container. The two chambers are separated by an internal plunger, which is pushed over an bypass prior usage allowing the two components to be mixed [60, 61].

Table 8.3 Advantages and challenges of pre-filled syringes

Advantages	Ref.	Challenges	Ref.
Better compliance	[30, 56]	Technical: (break loose and gliding forces, plunger movement during shipping, wet plungers)	[57, 58]
Convenient administration	[54, 57]	Leachables: (tungsten, silicone oil, adhesives)	[54]
Less microbial contamination	[54, 57]	Syringeability: (viscosity)	[54]
Less over-fill needed	[30]	Lyophilization	[59]
Less dosing errors	[10, 30]		
Safety	[57]		

PFSs are available in glass and plastics. A glass barrel has the advantage of transparency for visual inspection, absolute gas tightness, and competitive overall costs. Plastic barrels were historically produced out of polymers like polypropylene and polyethylene and had the disadvantage a lower transparency and a lower gas barrier compared to glass barrels. The low transparency of conventional plastic syringes was addressed by the development of cyclic olefin copolymers (COC) and cyclic olefin polymers (COP) PFSs. COC and COP pre-filled syringes also feature a good moisture barrier and low extractable levels (no tungsten pin during manufacturing needed, no silicone oil coating needed). Today, PFS in the European and US markets is preferably made of glass, whereas PFS in the Japanese markets is preferably made of plastic.

References

1. Annex 9, WHO Technical Report Series 902, (2002).
2. Zadbuke N, Shahi S, Gulecha B, Padalkar A, Thube M. Recent trends and future of pharmaceutical packaging technology. J Pharm Bioallied Sci. 2013;5:98.
3. Jiang G, Goss M, Li G, Jing W, Shen H, Fujimori K, Le L, Wong L, Wen Z-Q, Nashed-Samuel Y. Novel mechanism of glass delamination in Type 1A borosilicate vials containing frozen protein formulations. PDA J Pharm Sci Technol. 2013;67:323–35.
4. Sloey C, Gleason C, Phillips J. Determining the delamination propensity of pharmaceutical glass vials using a direct stress method. PDA J Pharm Sci Technol. 2013;67:35–42.
5. Zhao J, Lavalley V, Mangiagalli P, Wright JM, Bankston TE. Glass delamination: a comparison of the inner surface performance of vials and pre-filled syringes. AAPS PharmSciTech. 2014;1–12.
6. Ratnaswamy G, Hair A, Li G, Thirumangalathu R, Nashed-Samuel Y, Brych L, Dharmavaram V, Wen ZQ, Fujimori K, Jing W. A case study of nondelamination glass dissolution resulting in visible particles: implications for neutral pH formulations. J Pharm Sci. 2014;103:1104–14.
7. Cao S, Jiao N, Wen Z-Q. Classification of glass particles in parenteral product vials by visual, microscopic, and spectroscopic methods. PDA J Pharm Sci Technol. 2014;68:362–72.

8. Nakamura K, Abe Y, Kiminami H, Yamashita A, Iwasaki K, Suzuki S, Yoshino K, Dierick W, Constable K. A strategy for the prevention of protein oxidation by drug product in polymer-based syringes. PDA J Pharm Sci Technol. 2015;69:88–95.
9. Bee JS, Randolph TW, Carpenter JF, Bishop SM, Dimitrova MN. Effects of surfaces and leachables on the stability of biopharmaceuticals. J Pharm Sci. 2011;100:4158–70.
10. Sacha GA, Saffell-Clemmer W, Abram K, Akers MJ. Practical fundamentals of glass, rubber, and plastic sterile packaging systems. Pharm Dev Technol. 2010;15:6–34.
11. Nassar MN, Nesarikar VV, Lozano R, Huang Y, Palaniswamy V. Degradation of a lyophilized formulation of BMS-204352: identification of degradants and role of elastomeric closures. Pharm Dev Technol. 2005;10:227–32.
12. Liu W, Swift R, Torraca G, Nashed-Samuel Y, Wen Z-Q, Jiang Y, Vance A, Mire-Sluis A, Freund E, Davis J. Root cause analysis of tungsten-induced protein aggregation in pre-filled syringes. PDA J Pharm Sci Technol. 2010;64:11–9.
13. Bee JS, Nelson SA, Freund E, Carpenter JF, Randolph TW. Precipitation of a monoclonal antibody by soluble tungsten. J Pharm Sci. 2009;98:3290–301.
14. Seidl A, Hainzl O, Richter M, Fischer R, Böhm S, Deutel B, Hartinger M, Windisch J, Casadevall N, London GM. Tungsten-induced denaturation and aggregation of epoetin alfa during primary packaging as a cause of immunogenicity. Pharm Res. 2012;29:1454–67.
15. Jiang Y, Nashed-Samuel Y, Li C, Liu W, Pollastrini J, Mallard D, Wen ZQ, Fujimori K, Pallitto M, Donahue L. Tungsten-induced protein aggregation: solution behavior. J Pharm Sci. 2009;98:4695–710.
16. Markovic I. Considerations for extractables and leachables in single use systems: a risk-based perspective. In: PDA Single use systems workshop; 2011. pp. 22–3.
17. Demeule B, Messick S, Shire SJ, Liu J. Characterization of particles in protein solutions: reaching the limits of current technologies. AAPS J. 2010;12:708–15.
18. Thirumangalathu R, Krishnan S, Ricci MS, Brems DN, Randolph TW, Carpenter JF. Silicone oil-and agitation-induced aggregation of a monoclonal antibody in aqueous solution. J Pharm Sci. 2009;98:3167–81.
19. Ludwig DB, Carpenter JF, Hamel JB, Randolph TW. Protein adsorption and excipient effects on kinetic stability of silicone oil emulsions. J Pharm Sci. 2010;99:1721–33.
20. Ludwig DB, Trotter JT, Gabrielson JP, Carpenter JF, Randolph TW. Flow cytometry: a promising technique for the study of silicone oil-induced particulate formation in protein formulations. Anal Biochem. 2011;410:191–9.
21. Britt KA, Schwartz DK, Wurth C, Mahler HC, Carpenter JF, Randolph TW. Excipient effects on humanized monoclonal antibody interactions with silicone oil emulsions. J Pharm Sci. 2012;101:4419–32.
22. Gerhardt A, Bonam K, Bee JS, Carpenter JF, Randolph TW. Ionic strength affects tertiary structure and aggregation propensity of a monoclonal antibody adsorbed to silicone oil–water interfaces. J Pharm Sci. 2013;102:429–40.
23. Macías AE, Muñoz JM, Bruckner DA, Galván A, Rodríguez AB, Guerrero FJ, Medina H, Gallaga JC, Cortés G. Parenteral infusions bacterial contamination in a multi-institutional survey in Mexico: considerations for nosocomial mortality. Am J Infect Control. 1999;27:285–90.
24. Sterile Product Packaging—Integrity Evaluation, The U.S. Pharmacopeia 37 General Chapter 1207.
25. DesRoches CM, Blendon RJ, Benson JM. Americans' responses to the 2004 influenza vaccine shortage. Health Aff. 2005;24:822–31.
26. Kirsch LE, Nguyen L, Moeckly CS. Pharmaceutical container/closure integrity I: mass spectrometry-based helium leak rate detection for rubber-stoppered glass vials. PDA J Pharm Sci Technol. 1997;51:187–94.
27. Kirsch LE, Nguyen L, Moeckly CS, Gerth R. Pharmaceutical container/closure integrity II: The relationship between microbial ingress and helium leak rates in rubber-stoppered glass vials. PDA J Pharm Sci Technol. 1997;51:195–202.

28. Kirsch LE, Nguyen L, Gerth R. Pharmaceutical container/closure integrity III: validation of the helium leak rate method for rigid pharmaceutical containers. PDA J Pharm Sci Technol. 1997;51:203–7.
29. Bauer E. Pharmaceutical packaging handbook. New York: Informa Healthcare USA Inc; 2009.
30. Ingle RG, Agarwal AS. Pre-filled syringe-a ready-to-use drug delivery system: a review. Expert Opin Drug Deliv. 2014;11:1391–9.
31. Bauer E. Pharmaceutical packaging handbook. Informa Health Care; 2009.
32. Edward JS. Elastomeric components for the pharmaceutical industry. In: Encyclopedia of pharmaceutical technology, 3rd ed. UK: Taylor & Francis; 2013. pp. 1466–81.
33. Mungikar A, Ludzinski M, Kamat M. Effect of the design of the stopper including dimension, type, and vent area on lyophilization process. PDA J Pharm Sci Technol. 2010;64:507–16.
34. Elastomeric Closures For Injections, The U.S. Pharmacopeia 37 General Chapter 381.
35. Biological Reactivity Test, In-vitro, The U.S. Pharmacopeia 37 General Chapter 87.
36. Biological Reactivity Test, In-vivo, The U.S. Pharmacopeia 37 General Chapter 88.
37. Wood RT. Validation of elastomeric closures for parenteral use: an overview. PDA J Pharm Sci Technol. 1980;34:286–94.
38. Datta RN, Flexsys B. Rubber curing systems. Rapra Technology Limited; 2002.
39. Coran A. Chemistry of the vulcanization and protection of elastomers: a review of the achievements. J Appl Polym Sci. 2003;87:24–30.
40. Mahler H-C, Borchard G, Luessen HL. Protein pharmaceuticals: formulation, analytics and delivery. Germany: ECV-Editio Cantor-Verlag; 2010.
41. Fusco J, Hous P. Butyl and halobutyl rubbers. In: Rubber technology. Berlin: Springer; 1999. pp. 284–321.
42. Kuntz I, Zapp RL, Pancirov RJ. The chemistry of the zinc oxide cure of halobutyl. Rubber Chem Technol. 1984;57:813–25.
43. Morton DK. Container/closure integrity of parenteral vials. PDA J Pharm Sci Technol. 1987;41:145–58.
44. ASTM D2240: Rubber property - Durometer hardness, American Society for Testing and Materials.
45. Corredor CC, Haby TA, Young JD, Shah PA, Varia SA. Comprehensive determination of extractables from five different brands of stoppers used for injectable products. PDA J Pharm Sci Technol. 2009;63:527–36.
46. Mueller R, Karle A, Vogt A, Kropshofer H, Ross A, Maeder K, Mahler H-C. Evaluation of the immuno-stimulatory potential of stopper extractables and leachables by using dendritic cells as readout. J Pharm Sci. 2009;98:3548–61.
47. Sharma B. Immunogenicity of therapeutic proteins. Part 2: Impact of container closures. Biotechnol Adv. 2007;25:318–24.
48. Hopkins GH. Elastomeric closures for pharmaceutical packaging. J Pharm Sci. 1965;54:138–43.
49. ASTM D395-14: Standard Test Methods for Rubber Property-Compression Set, American Society for Testing and Materials.
50. Lam P, Stern A. Visualization techniques for assessing design factors that affect the interaction between pharmaceutical vials and stoppers. PDA J Pharm Sci Technol. 2010;64:182–7.
51. Shelton JR. Aging and oxidation of elastomers. Rubber Chem Technol. 1957;30:1251–90.
52. Chan EK, Hubbard A, Hsu CC, Vedrine L, Maa Y-F. Root cause investigation of rubber seal cracking in pre-filled cartridges: ozone and packaging effects. PDA J Pharm Sci Technol. 2011;65:445–56.
53. Zuleger B, Werner U, Kort A, Glowienka R, Wehnes E, Duncan D. Container/closure integrity testing and the identification of a suitable vial/stopper combination for low-temperature storage at −80 °C. PDA J Pharm Sci Technol. 2012;66:453–65.
54. Jezek J, Darton NJ, Derham BK, Royle N, Simpson I. Biopharmaceutical formulations for pre-filled delivery devices. Expert Opin Drug Deliv. 2013;10:811–28.
55. US Food and Drug Administration: CFR Code of Federal Regulations Title 21.

56. Buysman E, Conner C, Aagren M, Bouchard J, Liu F. Adherence and persistence to a regimen of basal insulin in a pre-filled pen compared to vial/syringe in insulin-naïve patients with type 2 diabetes. Curr Med Res Opin. 2011;27:1709–17.
57. Badkar A, Wolf A, Bohack L, Kolhe P. Development of biotechnology products in pre-filled syringes: technical considerations and approaches. AAPS PharmSciTech. 2011;12:564–72.
58. Rathore N, Pranay P, Eu B, Ji W, Walls E. Variability in syringe components and its impact on functionality of delivery systems. PDA J Pharm Sci Technol. 2011;65:468–80.
59. Korpus C, Haase T, Sönnichsen C, Friess W. Energy transfer during freeze-drying in dual-chamber cartridges. J Pharm Sci. 2015;104:1750–8.
60. Teagarden DL, Speaker SM, Martin SW, Österberg T. Practical considerations for freeze-drying in dual chamber package systems, Freeze drying/lyophilization of pharmaceutical and biological products. Boca Raton: CRC Press Inc; 2010. pp. 494–526.
61. Michaels TM Jr. Dual chamber internal by-pass syringe assembly. In: Google patents; 1997.

Chapter 9
Development of Prefilled Syringe Combination Products for Biologics

Mariana N. Dimitrova, Jared S. Bee, Ling Lu and Jason E. Fernandez

Abstract Biologics for treatment of a growing number acute and chronic conditions are often developed as parenteral injectables. Prefilled syringes (PFS) and other combination products offer patients the convenience of at-home self-administration. This can result in considerable cost savings compared to dosing in a clinical setting. PFS systems improve patient compliance and dosing accuracy, reduce overfill cost and waste, and can reduce sharps injuries ('needle sticks') with the inclusion of a safety system. With the advantages of PFS also come potential challenges that require early development taking into account the drug product and formulation requirements; drug compatibility and stability, solution properties such as viscosity, good understanding of the syringe functionality, user experience and requirements, comprehensive risk assessment to assess and prevent failures of syringes at home or in emergency settings. This chapter describes design controls, human factors, scientific, engineering, and regulatory aspects of development of PFS as combination products. Finally, advanced PFS offerings and emerging syringe technologies with improved material, mechanical, dimensional, and compatibility properties are discussed.

Keywords Prefillable syringes · Drug/device combination products
Syringe functionality · Protein/device compatibility · PFS design controls
and risk management · Human factor studies

M. N. Dimitrova (✉)
Technical Operations, Jazz Pharmaceuticals, Philadelphia, PA, USA
e-mail: Mariana.Dimitrova@jazzpharma.com

J. S. Bee
Analytical Sciences, MacroGenics Inc, Rockville, MD, USA

L. Lu
BioTherapuetics Pharmaceutical Sciences, Pharmaceutical R&D,
Pfizer, Andover, MA, USA

J. E. Fernandez
Technical Development, Biogen, Cambridge, MA, USA

© American Association of Pharmaceutical Scientists 2018
N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_9

9.1 Introduction: Why Prefilled Syringe Injectables?

Although oral delivery is highly convenient, biologics are subject to a significant first-pass effect, greatly reducing oral bioavailability [1]. As a result protein therapeutics and large molecular weight biologics are typically delivered parenterally. Subcutaneous delivery is an attractive route of administration as it offers both acceptable bioavailability and a convenient route of administration for patients associated with reduced pain upon injection and greater tolerability. The combination of convenience and bioavailability is a large driver in the development of prefilled syringes (PFS) for use with biologics. Figure 9.1 shows a typical relative bioavailability for different routes of administration [1–3].

Outpatient dosing and self-administration of biologics are desirable for patients with chronic conditions where frequent long-term dosing is required with minimum impact on patient ambulation and quality of life. Prefilled syringes, autoinjector devices, and large volume subcutaneous wearable combination products represent a rapidly growing segment of the biologics market because they are convenient and cost-effective offerings that can meet this need. A growing number of commercial biologics on the market are available as PFS or other SC combination products. Humira® (adalimumab), Enbrel® (etanercept), and Avonex® (interferon γ-1a) PFS presentations have been used by patients for many years. Figure 9.2 shows PFS offerings for different types of molecular entities over the past 20 years. Prefilled syringes can be a step toward development of an autoinjector presentation as part of product lifecycle management. Insulin is one of the best-known biopharmaceutical products and is currently available in various pen injectors from multiple companies including NovoPen® 4® (Novo Nordisk), Lantus® SoloSTAR pen (Aventis), and Humalog® KwikPen® (Eli Lilly) (Fig. 9.3).

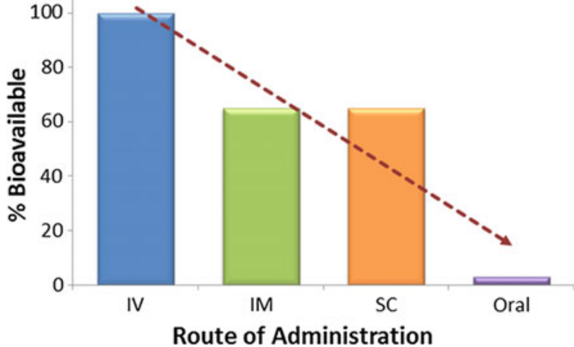


Fig. 9.1 General bioavailability trend of intravenous (IV), intramuscular (IM), subcutaneous (SC), and oral delivery of 145 kDa molecular weight biologics (e.g., monoclonal antibodies)

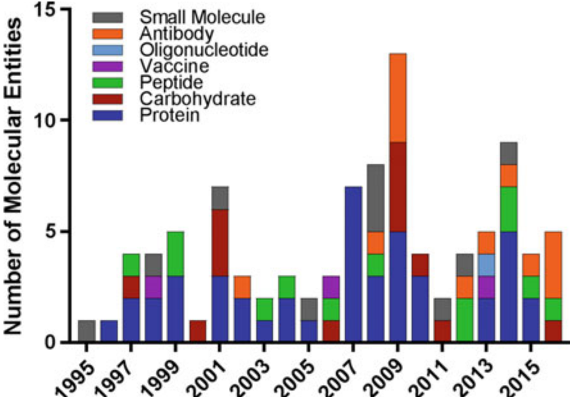


Fig. 9.2 Prefilled syringe product approvals since 1995 by molecular class. The approved products include diverse classes of biologics including proteins (e.g., etanercept), monoclonal antibodies (e.g., Amjevita, adalimumab), vaccines (e.g., Fluvax), oligonucleotides (e.g., mipomersen sodium), peptides (e.g., Copaxone, glatiramer acetate), carbohydrates (e.g., Thorinane, enoxaparin sodium), etc. *Source* PharmaCircle prepared 06/2017

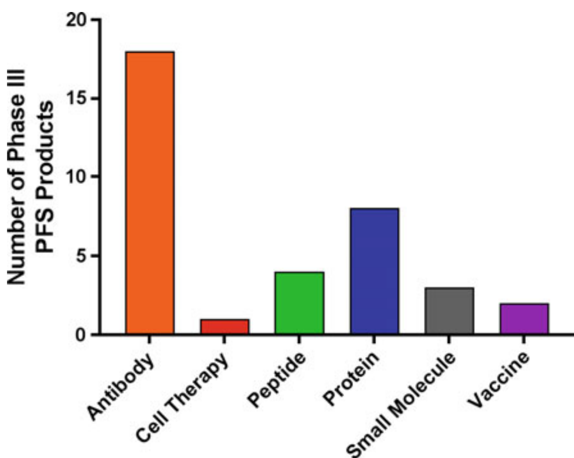


Fig. 9.3 Number of PFS products currently in Phase 3 trials by molecular class. The graph illustrates that biologics continue to drive the PFS market, which underscores the criticality of PFS delivery systems to protein-based therapeutics. *Source* PharmaCircle prepared 06/2017

9.2 Advantages of PFS Presentations for Biologics

Prefilled syringe product presentations offer many advantages over vials for injectable biologics. The ability to safely self-administer and avoid treatment in a clinical setting is one of the many advantages prefilled syringes offers in terms of patient convenience. When combined with a safety system, prefilled syringes reduce the risk of accidental sharps injuries (‘needle sticks’). This feature is also a compliance requirement for prefilled syringes in the USA, European Union, and increasingly the rest of the world. Prefilled syringes reduce the risk of

contamination, needle reuse and improve dosing accuracy. By dosing the complete contents of the PFS, there is a higher level of confidence in achieving the correct dose, relative to withdrawing a dose from a vial and measuring the dose with a disposable syringe. This is especially desirable in the case of unit-based treatment independent of the body weight of the patients (Fig. 9.4).

In terms of overall manufacturing cost, PFS may offer surprising benefits over vial presentations. The lower overfill of PFS compared to vials due to lower holdup volume often reduces unnecessary wastage and overfill of life-saving valuable biologics, thus favorably impacting the overall cost of PFS compared to vials. Cost reductions could lead to notable price reduction, thus better coverage by healthcare payer organizations and therefore greater potential access to patients. Figure 9.5 shows an illustrative example relative cost of goods manufactured (COGM) comparison for an accessorized PFS compared to a vial. This calculation does not include other drug development costs incurred in discovery, translational science, clinical development, marketing, logistics, human factor studies for combination product, and other business overheads. The cost of antibody manufacturing is on the order of $100–$1000 per gram, depending on the expression system, the titer, the manufacturing process, and scale. The vial components and filling costs are estimated to be on the order of $10 per dose [4]. Syringe container components typically cost slightly more than vials and for this estimate, we assumed $15 per dose. A dose of 1 mL of 150 mg/mL antibody (0.15 g dose for 70 kg patient = 2 mg/kg) at $200 per gram is $30. Based on this, we assume typical relative costs of 33% for the vial and 50% for the PFS relative to the dose. The overfill cost is directly related to the dose cost, and we assumed a larger overfill of 30% is needed for a viscous drug product in a vial and only 5% for a PFS. The key driver for the vial cost is the overfill needed due to the holdup volume. This is illustrated in Fig. 9.5.

The higher overfill cost for a vial compared to a PFS presentation has a greater impact for lower titer or yield processes but is less impactful for lower dose or for

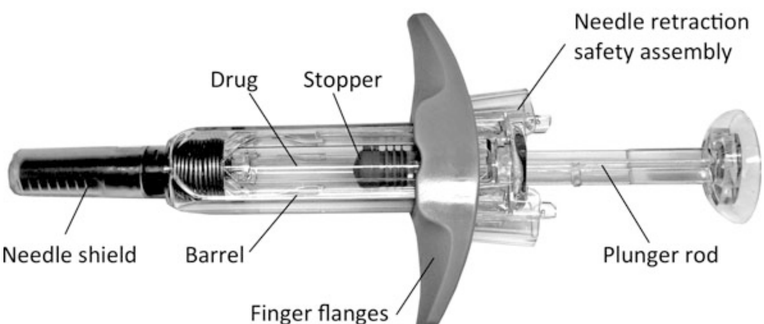


Fig. 9.4 Example of an accessorized prefilled syringe configuration showing the various components

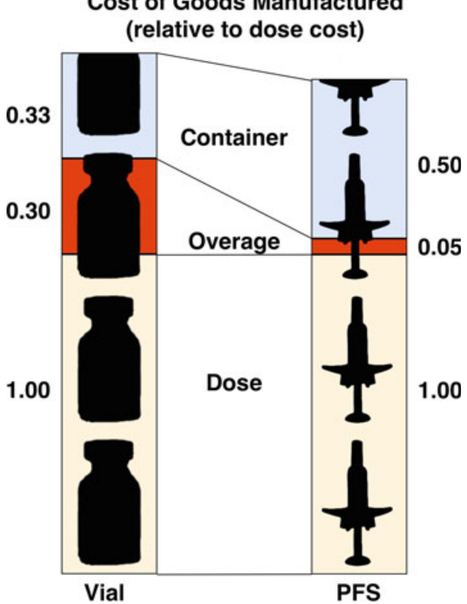


Fig. 9.5 Illustrative example comparing cost of goods manufactured (COGM) for a vial compared to an accessorized prefilled syringe presentation at commercial stage. In this scenario, the higher cost of the PFS container closure components compared to the vial cost (50% vs. 33%) is more than compensated by the higher cost of the extra biologic in the overfill for a vial (30% vs. 5%)

lower cost products. Another substantial total cost of a drug is the healthcare provider facility and staff time and resources when dosing is performed in a clinical setting. This is not part of the COGM analysis. If a prefilled syringe can be used for self-administration at home instead of hospital, then additional cost savings (e.g., $2,000/day) could also add to the advantages PFS presentations offer for parenteral injectables.

9.3 Development of PFS Presentations for Biologics

Combination products development incorporates a range of considerations associated with the therapeutic formulation, the syringe injection device, the fill–finish process and packaging operations, user/patient requirements, etc. Some of the critical considerations and their interdependencies are illustrated in Fig. 9.6 and summarized succinctly in Table 9.1.

When considering a transformation of a biologic therapeutic from intravenous into patient-convenient subcutaneous route for administration, it is critical to consider all factors that may potentially impact the product immunogenicity and address them in the PFS development process. A recent review [12] of clinical studies found that four out of six products with clinical data for both SC and IV administration routes had slightly higher patient anti-drug antibodies (ADA) rates for SC compared to IV route [12]. For example, Herceptin™ had the highest ADA rates of 14.9% by SC compared to 8.1% by IV. However, the SC administration

Fig. 9.6 Critical areas of considerations in the development of PFS presentation

Table 9.1 Considerations in the early development of PFS presentations for biologics

Patient/user requirements	
Requirements	*Example considerations and possible studies*
Target product profile and human factors	User needs could require that the drug is easy to self-administer in the home setting using a PFS with a low perception of pain on injection. Some considerations for design inputs could be the needle gauge, dose volume, injection force/time, excipients, competitive landscape, patient training, and dexterity. An informative study may lead to design inputs such as needle gauge >27, dose volume <1 mL, injection time <10 s, and injection force <25 N. Engineering studies can then be performed with configurations that could meet these design control inputs
Formulation suitability	
Excipients	A thorough compatibility assessment of the therapeutic formulation with the injection syringe device should be performed. Evaluate the solution properties of the formulation to fit the device requirements, e.g., viscosity which correlates with the glide and break loose forces and functionality of the syringe. Weak interactions and therefore viscosity may be reduced by addition of excipients to the formulation [5, 6] Assess if a freeze-dried compound can be reformulated and developed as a stable liquid form Assess if different levels of key stabilizing excipients are needed (e.g., surfactant)
Volume	Substantial progress has been made in recent years expanding the volumes delivered subcutaneously by injection devices, e.g., Hizentra® of CSL Berings. For PFS presentations, typical ranges

(continued)

Table 9.1 (continued)

Patient/user requirements	
Requirements	*Example considerations and possible studies*
	of volumes include ≤ 1 mL in the past and ≤ 2.25 mL in recent years for biologics. The 1 mL has been a historic benchmark for SC injection volume based on backpressure, pain, local adverse events, and leakage; however in recent years, it has been proposed that these factors might be re-examined and further explored for volumes of up to 3.5 mL [7] for PFS. It is noted, large volume subcutaneous delivery devices have greatly expanded the delivery volumes into the subcutaneous interstitial space to tens and hundred milliliters per administration. To ensure efficacious dosing, often these volumes are also associated with higher concentration formulations for biologics such as monoclonal antibodies. Typical volumes for vaccines are much smaller
Concentration	Assess the ability to formulate the protein at a suitably high concentration to achieve the dose in the desired volume. Assess impacts of concentration on phase separation, viscosity, osmolality, long-term stability, and syringe functionality
Viscosity	Measure viscosity versus concentration profile for potential formulations Perform evaluation of expected forces needed to inject for different needle gauges Assess impact of concentration variation on viscosity (e.g., at $\pm 10\%$ concentration) Assess impact of refrigerated versus room temperature on viscosity Perform glide force functionality assessments on stability
Stability	Assess impact of high concentration and viscosity-modifying excipients on product stability
Robustness	Assess impact of variability in levels of key excipients on formulation properties and stability such as bracketing of the surfactant level on stability to assess impact on subvisible particles, or impact of key excipient on viscosity Perform the general suite of formulation and stability studies as required such as freeze/thaw studies. For example, stopper movement during freezing and subsequent thawing of a PFS may result in loss of sterility which needs to be assessed thoroughly in early development
Redispersion of adjuvant	For vaccine-based products, which contain an adjuvant, redispersion of the adjuvant is required prior to administration to the subject. PFS containing adjuvanted vaccines should be routinely assessed for redispersion when stored and shipped in a variety of configurations (e.g., tip down, tip up, sideways, etc.)
Compatibility	
Sensitivity to silicone oil	Perform agitation studies with spiked silicone oil emulsion Perform stress, accelerated and long-term storage stability studies in syringes with different silicone oil levels

(continued)

Table 9.1 (continued)

Patient/user requirements	
Requirements	*Example considerations and possible studies*
Sensitivity to tungsten	Assess low-tungsten syringe offerings and perform tungsten pin-extract spiking for glass staked needle syringes. Evaluate key degradation pathways (e.g., soluble aggregates, particles, potency) under excess stressed conditions and over the course of shelf life at intended storage conditions
Overall stability in a prefilled syringe	Perform studies in different prefilled syringe options (e.g., glass vs. plastic, staked vs. luer lock) and vendors Perform stress, accelerated and long-term storage stability studies
Shipping and handling	Add simulated or real transportation stress arms to stability studies and assess product quality
Stopper movement	Stoppers may move due to changes in pressure during shipping or by freeze-thaw. Products should be evaluated to determine whether stopper movement could result in a loss of sterility and, if so, whether restraining the stopper with active secondary packaging is required
Filling	Perform a scale-down pumping stress study. Fill development stability lots with a commercial scale filler pump or scale-down model Assess impact of filling settings on the final quality (e.g., drip retraction, fill temperature) Assess the impact of sterilization on stability (e.g., assess potential impact of residual vaporized hydrogen peroxide, gamma irradiation, or e-beam) Several case studies have been published demonstrating that there are additional engineering challenges relating to practical aspects of the PFS fill–finish processing operations such as dissolved gas and liquid leaks upon removal of tip-cap [8], sterilization and packaging impacts on components [9], and filling nozzle and process optimization [10, 11]
Functionality	
Injection force profile	Measure injection force profile to determine break loose force, glide force, and smoothness of gliding for different lots of syringes on stability. Assess forces and smoothness of the injection force profile within the context of the desired profile. The syringe functionality should be closely aligned with the patient population requirements and dexterity. Typical glide force requirement might be <25 N glide force for a smooth injection force profile Perform stress, accelerated and/or long-term storage stability studies in syringes with different silicone oil levels and/or in aged syringes
Container closure integrity (CCI)	Container closure integrity should be established upon manufacturing (filling) of the drug product and demonstrated to be maintained through subsequent operations such as primary container shipment and device assembly using an appropriate integrity test method. Review Chap. 12, for further information

route for Herceptin™ was concluded to be noninferior to IV administration and to have a similar overall safety profile. In another example, Orencia™ had low ADA rates of 1.1 and 2.3% for SC and IV respectively. For all six products, the overall assessment was that ADA rates were low and without impact on safety or efficacy [12]. Factors proposed to potentially contribute to differences in ADA rates for SC compared to IV include injection site damage, mechanism of action, disease state, protein aggregate levels, formulation excipients [12] and need to be evaluated in the formulation development of PFS presentations.

Examples of compatibility and functionality considerations for PFS combination product presentations of biologics are illustrated in Sects. 9.4 and 9.5.

9.4 Compatibility of Biologics with PFS

Contact with surfaces and leachables has been shown to impact biologics during manufacturing and during storage [13]. The change in product contact surfaces and leachables in PFS configurations compared to vials requires additional compatibility and stability assessment. Silicone oil is the most commonly used lubricant in glass syringe configurations which is required for a patient's convenient and compliant delivery of parenteral injectables. Some proteins are sensitive to silicone oil, forming aggregates or particles while others may be less sensitive with no adverse impact on the protein detected [14]. Thus, the sensitivity to silicone oil needs to be thoroughly assessed early in PFS development, a suitable syringe configuration selected for further development, and the formulation tailored to the needs of the PFS combination product.

Clouding of insulin caused by excess silicone oil on syringes was reported in some historical (1980s) cases [15, 16]. More recently, the Fc-fusion protein abatacept has been shown to form particles consisting of droplets entangled in a fibrous structure upon exposure to siliconized syringes [17]. Agglomeration of emulsified silicone oil droplets from syringes can be accelerated by adsorption of protein, resulting in larger particle-like silicone droplet 'grape clusters' in the solution [18, 19]. In contrast, there was no impact on protein purity, higher order structure, stability, subvisible particle counts, or biological activity of interferon alfa-2b fusion protein in siliconized (at various target levels) glass cartridges [20]. There was an increase in turbidity, for both the placebo and active drug, in the siliconized glass cartridges that correlated with the silicone oil level and was attributed to the presence of silicone droplets [20]. Similarly, the only detectable impact for moving from a vial to a PFS configuration for two different mAbs was a small increase in subvisible particles that could have potentially been related to emulsified silicone droplets released from the barrel [21]. No differences in safety, efficacy, or immunogenicity were reported for patients receiving either the vial or the PFS presentation [21]. Several publications have indicated that formulation can substantially impact the adsorption, and adsorbed structure, of proteins to silicone oil and that forced degradation studies with spiked silicone emulsions combined with

vigorous agitation can enhance aggregation [17, 18, 22–26]. These examples explain why it is very important to determine the optimal level of silicone oil lubrication of prefilled syringes that will ensure smooth functionality over the course of shelf life without resulting in instability of the biopharmaceutical formulation contents. For example, Fig. 9.7 illustrates soluble aggregation formation as a function of the amount of silicone oil present.

Tungsten is a leachable derived from the glass staked PFS needle hole forming process. Similar to silicone oil, some proteins have been found to be sensitive to tungsten leachates present in staked needle syringes—forming aggregates or particles, while many proteins show no sensitivity. Tungsten residue has been associated with immunogenicity resulting from tungsten-induced denaturation and aggregation of epoetin alfa [27]. Earlier investigative studies into initial observations of aggregation in PFS demonstrated that tungsten residues were the root cause of the aggregation [28]. Further studies demonstrated that the impact of tungsten on protein precipitation and aggregation was greatest at lower pHs [29] and largely mediated by soluble forms of tungsten polyanions [30]. The discovery of protein–tungsten interactions has led PFS vendors to take steps to control and monitor tungsten residues in PFSs and develops low tungsten or tungsten-free product line offerings intended for biologics that may require them.

Screening studies can be performed to identify potential sensitivities to silicone oil and tungsten. Performing these studies in the development phase allows product development teams to take appropriate steps to mitigate any risk identified through continued formulation or container development. The most common type of screening studies involves spiking extracted tungsten or silicone oil into formulated proteins and subsequently exposing the spiked solutions to stressed conditions such as shaking or high-temperature storage. Samples are then evaluated by appropriate analytical tests, such as particle analysis and compared to control containers. These studies are intended to exaggerate the effects that may be encountered in a PFS

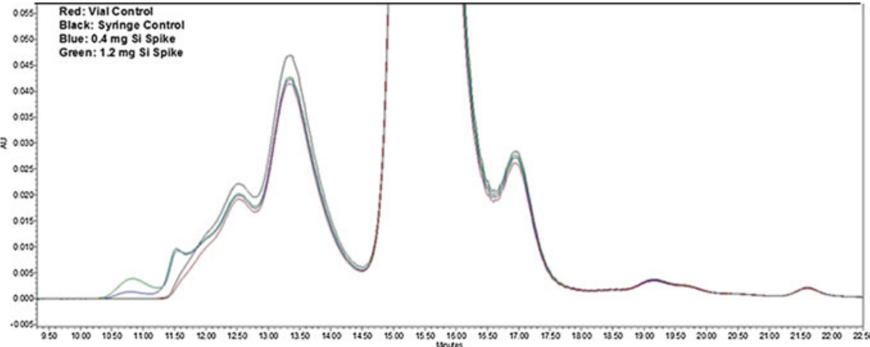


Fig. 9.7 Example of silicone oil induced aggregation and soluble high molecular weight species (HMWS) formation in a protein therapeutic. Increasing the silicone oil results in a small increase in aggregation and formation of additional aggregate species as shown by the appearance of new peaks in the chromatogram

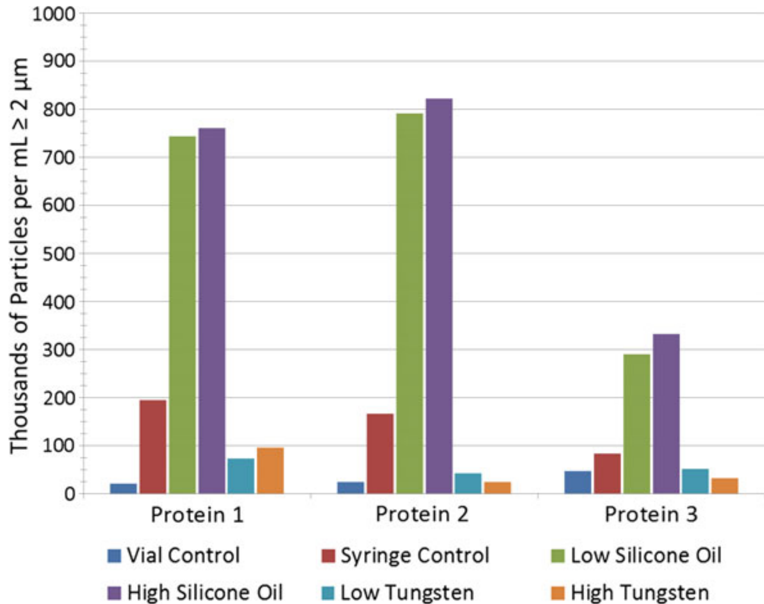


Fig. 9.8 Example of a screening study investigating the sensitivity of various biologic therapeutics to free silicone oil and tungsten. The microflow imaging (MFI) analyses have been utilized to detect changes in subvisible particles under stressed conditions

presentation and proactively identify areas for continued development. An example of data that may be obtained from this type of screening study is provided in Fig. 9.8. Data presented in Fig. 9.8 shows that impact of silicone oil and tungsten, assessed by subvisible particle count, varies from protein to protein.

9.5 Syringe Functionality Considerations

The injection force profile and smoothness are important aspects of the functionality of a prefilled syringe presentation. Patients, especially those with limited dexterity or strength, may experience difficulties in completing a manual injection with an irregular and/or high injection force profile. To a first estimate, the force required to expel the formulation through the needle can be assessed using the Hagen–Poiseuille fluid flow equation with an extra term to account for friction of the stopper against the barrel. This approach has been used to model the impact of component variability on the expected functional performance variation of delivery devices [31].

Currently, glass prefilled syringes are the leading syringe configuration for PFS product presentations of biologics. Silicone oil is coated onto the inner barrel of glass syringes to lower, and smooth, the frictional forces upon injection.

Glass barrels with non-uniform silicone oil distributions, especially bare patches near the needle end, were found to have large undesirable fluctuations in the measured gliding force profile [32]. This can have an impact on the patient experience during self-administration and can result in 'stalling' if the PFS is packaged within an autoinjector. The use of 'diving nozzle' siliconization in the manufacturing process of glass staked needle syringes is a mitigation that has enabled a more uniform distribution while minimizing application of excess silicone to the barrel. Frictional contributions are notably lower for siliconized glass barrels than for plastic barrels which do not typically have silicone lubrication applied.

The needle gauge and the viscosity of the formulation are the factors that have the greatest impact on the force needed to inject, whereas friction contributes to a lesser extent. In a feasibility assessment, it is useful to calculate the expected impact of needle gauge, formulation viscosity, and injection speed on the force to expel the formulation from the syringe. In Fig. 9.9, we show the application of the Hagen–Poiseuille equation to calculate the force required to expel 1 mL from a prefilled syringe in 7.7 s. Results are shown as predicted upper value for prefilled syringes with different gauge needles as defined by ISO 9626. Backpressure depends upon the injection rate and the viscosity and has been reported to be in the order of 2.4 ± 1.9–4.7 ± 3.3 N for 10 and 20 mPas solutions injected at 0.2 mL/s in subcutaneous tissue and could be as high as potentially 12 N in certain cases [33]. It is noted the measured and perceived user forces arising from tissue backpressure may vary between different syringe designs and depend on factors such as the needle diameter, the barrel bore diameter, the design of finger flanges, convenient grip of the syringe barrel, drug formulation. In this calculation, we used a value of 5 N as an average estimate for backpressure. The calculation results shown in Fig. 9.9 may be used to assess feasibility of the injection force for a PFS

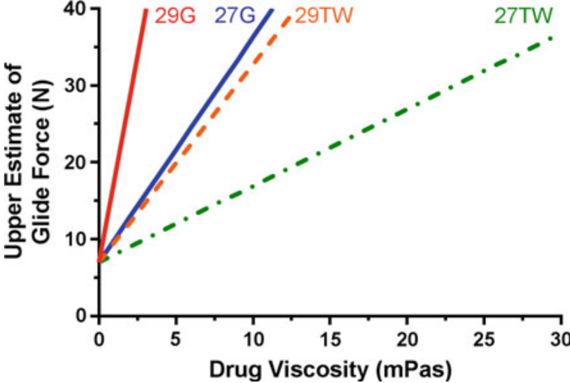


Fig. 9.9 Calculated upper glide force estimate as a function of the drug viscosity for different needle gauges. Parameters used in the calculation are needle length = 20 mm, barrel diameter = 6.35 mm, frictional force = 2 N, volumetric flow rate = 0.13 mL/min (1 mL in 7.7 s), and issue backpressure estimate (see text) of 5 N. EN ISO 9626 minimum needle IDs: 29G = 133 μm, 29GTW = 190 μm; 27G = 184 μm, 27GTW = 241 μm

presentation. For example, if it is known that the formulation viscosity is 10 mPas at room temperature, then a prefilled syringe with a 27TW (Thin Wall) needle may be preferred over a 29TW (Thin Wall) needle. A formulation with a viscosity of 3 mPas might be considered acceptable whereas more research studies may be recommended if the current formulation has a viscosity of ≥ 20 mPas at the target concentration. The assumptions used in calculated injection force estimates should be evaluated for the actual design space situation. For example, PFS vendors offer needle designs with inner diameters larger than the ISO limit and with more defined specifications which can therefore reduce glide forces substantially from the upper estimate based on the ISO specifications.

The formulation viscosity can increase on refrigerated storage which should also be considered in the design of a robust prefilled syringe offering. Figure 9.10 shows a typical example of how viscosity versus temperature data trends for high-concentration biologics. The impact of temperature fluctuation on viscosity profile is important to consider within the context of PFS functionality and user force requirements (recall Hagen–Poiseuille equation). Room temperature equilibration of PFS presentations prior to injection is a common practice used to reduce viscosity, glide, and break loose forces as well as reduce pain upon injection.

The choice of suitable syringe configurations offering improved compatibility with biologics while also offering smooth functionality is greatly improved in recent years as discussed in Sect. 9.7.

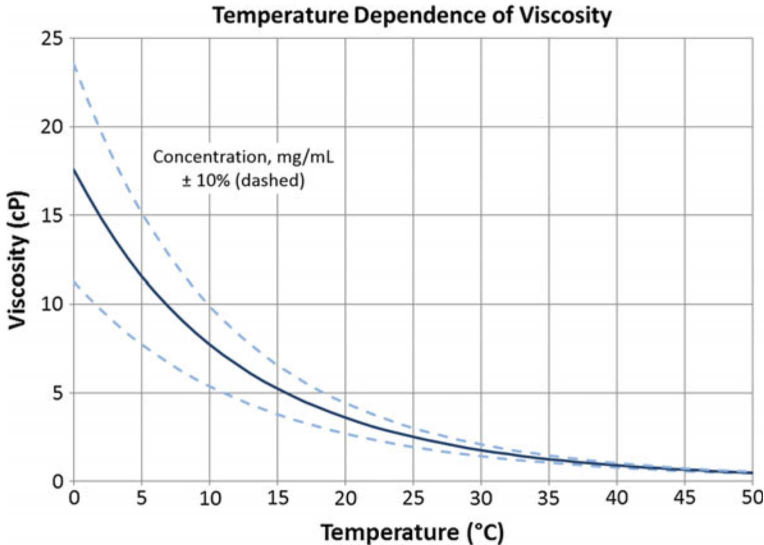


Fig. 9.10 Viscosity as a function of temperature for an example biologic. Concentration and temperature are critical parameters to consider in the selection of PFS needle configurations as normal variability in these parameters can greatly impact the viscosity and resulting functionality performance of the PFS system

9.6 Regulatory Considerations in PFS Development—Design Controls

Between 2010 and 2013, a number of PFS failures in emergency rooms and intensive critical units' settings resulted in serious adverse events. After learning the failure was linked to the design of the luer connectors on syringes, the regulatory agencies determined that these syringes were not designed, reviewed, and approved for these specific intended uses and deemed design controls necessary for PFS design. In April 2013, the FDA issued draft guidance on glass syringes to supplement the ISO 11040-4:2007 glass syringe standard, which lacked connectivity performance requirements and had limited dimension specifications. In June 2013, the FDA also finalized its guidance for marketing application owners to consider the technical requirements in the regulatory submissions of the injectors used to deliver drugs and biologics. The guidance recommended that the combination product development and manufacturing should consider the combination product as a whole, including the interfaces between drug and device, device and device, device and users.

Thus, PFS presentations which were originally regulated as a drug product based on the primary mode of action became a drug/device combination product in 2013 [21 CFR Part 4 'Current Good Manufacturing Practice (CGMP) Requirements for Combination Products' (Final Rule)]. According to the regulation, PFS combination product manufacturers need to meet the requirements in either of two ways. One way is to meet both the drug cGMPs (21 CFR 210/211) and medical device Quality System Regulations (QSR) (21 CFR 820). The second is a streamlined approach that uses drug cGMP as a base and adds on relevant provisions from device QSR. In the PFS combination product, the syringe is a device constituent part and the drug formulation is a drug constituent part. Accordingly, the PFS development process needs to apply design controls as part of device QSR requirements (FDA Guidance CGMP for Combination Product 2015 [34]), in addition to the drug cGMP (Fig. 9.11).

Design controls are based upon quality assurance and engineering principles, interrelated practices and procedures that are incorporated into the design and

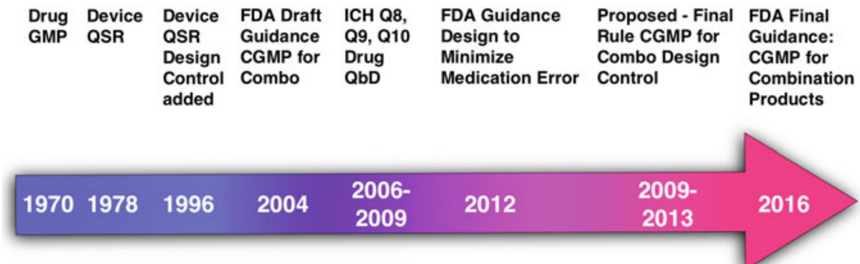


Fig. 9.11 PFS development—regulation history

Table 9.2 Drug versus device process development

Drug development	Device development
Regulation and quality system	
PFS (syringe = drug container (CCS))	Syringe = device, PFS = combination product
21 CFR 210/211 cGMP for manufacturing	21 CFR 820 QSR(GMP) for development and manufacturing
Guidance: Q8, Q9, Q10 for development [36–38]	21 CFR 820.30 for development—auditable
Technical	
CCS requirement for drug protection	ISO standards for delivery performance and safety
Prescriptive per USP, FDA guidance, Test-in approach	Systematic approach, add device–users' interfaces design-in, design requirements
FDA guidance: reduce medication error	FDA guidance: human factors-reduce use error
SME knowledge, experience	Quality system, documented objective evidence, traceability

development process, forming a system of checks and balances (FDA Design Control Guidance 1997 [35]). Typical drug development focuses more on the manufacturing process risks and their impact on drug quality. Design controls also address additional interface risks from the ground up, including not only device design or selection, but also manufacturing and uses. Table 9.2 describes a PFS development process, differentiating between drug and device development.

9.6.1 PFS Design Controls

Design controls are useful tools in PFS combination product development including risk management and human factors. Effective use of these tools can improve the quality of the product, add value and increase efficiency in the development process. Moreover, design controls create a blueprint for the development process from start to end, synthesizing multiple sources to fill any knowledge gaps in the development process while ensuring compliance and adherence to applicable regulations.

Design controls consider the PFS combination product as a whole, including the interfaces, such as drug and device, device and device, device and manufacturing process, and device and user. Design controls start from a documented and approved plan (Design and Development Plan (DDP)) (FDA Design Control Guidance [35]). The DDP describes the design and development process and activities including risk management and human factor activities.

The outcome of the design controls is documented, approved, and compiled in a design history file (DHF). Design input starts by identifying: (a) user needs,

(b) regulatory requirements, (c) device industry standards, (d) risks from similar product experience, and (e) other stakeholder needs, such as manufacturing machine compatibility and marketing presentation.

The design input requirements are generated by transforming all of the listed requirements into a list of engineering parameters, which include functional, performance, usability, safety, and interface requirements.

For the selected off-the-shelf (OTS) syringe components with same intended use, the development program can start from the risk management activities. The risk management tools can be used to document known risks and controls with documented evidence for safety and effectiveness while identifying new risks and new controls. In this case, design controls may focus on reviewing the existing specifications and data including existing manufacturing process or setting up new design requirements due to the new risks. The development responsibility may focus on supplier management, such as verifying supplier data for design controls and in regulatory submissions, quality assurance of syringe components, change notification, and controls through purchasing controls.

For early clinical phases using OTS syringe, the Design & Development Plan (DDP) may define the scope of the project as focusing on the clinical study only. The objective of design controls is to ensure safe and effective use of the device constituent part and combination product in the clinical trials and collect user data to inform or improve the design. The DDP can provide the overarching strategy and enable combination of the activities and documents from risk management, design verification, and design validation. The level of documentation required may be further reduced by including one design review before the clinical study starts.

Syringe development in the device industry follows device development regulatory guidance and ISO standards regulations. In April 2013, the Food and Drug Administration issued a draft guidance for Glass Syringes, a Supplement to ISO 11040-4:2007 to help the pharmaceutical industry understand and prevent luer connectivity issues when using glass syringes. In June 2013, the FDA issued a Final guidance—Technical Consideration on Injectors. It explained what technical data to submit in license application filings in regards to interface compatibility, e.g., syringe and autoinjector, drug and injector, user and injector. In 2015, the third edition of ISO 11040-4:2015 [39] was published to provide technical guidance and facilitate discussion between pharma PFS manufacturers and syringe suppliers to determine appropriate product specifications. If certain sections are not applicable or not required, pharma companies may ask syringe suppliers to explain or provide the scientific justification. This ISO standard also called out other standards such as (a) ISO 7886-1 for syringe function and performance, (b) ISO 7864 [40] for needle dimension and performance, (c) ISO 9626 for needle material and dimension, and (d) ISO 594-1 [41–43], -2 or ISO 80369-7 for Luer connector dimensions and performance.

The pharmaceutical companies may reference ISO 11040-4:2015 [39] standard to identify gaps in the supplier specifications and in the verification data regarding

device safety, function, and performance. Additionally, ISO 11040-5 can aid with filling gaps in function and performance requirements for plunger stopper, which are not included in USP <381>.

9.6.2 Risk Management in the Design Control Process

Risk management approach is based on objective evidence, e.g., scientific literature, supplier data, manufacturing data, clinical evaluation, or complaint data. As a part of the combination product market application, the current regulatory requirements include provision of objective evidence or justification to prove safety (e.g., biocompatibility) and effectiveness (e.g., syringe force, luer connectivity) of the product for the intended use. Thus in addition to risk assessment of the drug Critical Quality Attributes (CQA), the pharma industry now needs to reference ISO 14971 for the device constituent parts of a combination product to conduct a Hazard Analysis, use risk analysis (e.g., uFMEA), product risk analysis (e.g., components and/or system level FMEA), risk–benefit analysis, production and postproduction review to meet additional requirements for the device. For example, a Hazard Analysis for a PFS system may summarize the hazards from therapeutic (e.g., dose accuracy, overdose, underdose), chemical (e.g., leachable, drug-related impurities, other contaminants), biological (e.g., sterility, biocompatibility, cross-contamination), and mechanical causes (e.g., sharp effect). These hazards may be caused by improper selection of components or the design of a combination product as a whole, as well as incompatibility with the manufacturing process or the use of the combination product.

Historically pharmaceutical companies relied on suppliers to provide product data, but now pharmaceutical developers need to review the objective evidence from suppliers, assess the data, and qualify (verify and validate) the components for safe and effective use with the drug formulation, with the manufacturing process, and with the intended user population. If there is a gap in supplier documentation, a required risk control may involve a new verification or validation or a new requirement.

9.6.3 Design Validation and Human Factors

Why perform human factors studies for PFS? Even though an off-the-shelf syringe has been used for many years and for various applications, if it is used in a new therapy and/or a combination product, clinical evaluation will be required. As part of design validation per regulation, the syringe should be tested in the actual and/or simulated use environment. Human variability, different user groups, and different use environments may introduce use errors not previously seen or envisaged.

User preference may also become a source of use errors. User interfaces include user interaction with the syringe, packaging, and labeling (e.g., instructions for use). Patients may not always use the product as directed—for example, some may prefer to inject on thigh not abdomen area, may like to inject perpendicularly instead of at an angle, plunge intermittently rather than continuously to reduce the pain, or inject more quickly, wear gloves or not; in a family setting, there may be pets or children running around to interfere with injection.

Human factors formative testing is exploratory and serves to inform and optimize device design. Cognitive and ergonomic interactions with the operation of the device are assessed with different user populations in a simulated setting that mimics actual use environment (e.g., light, noise) to identify and mitigate use errors in the design and instructions for use (IFU). Finally, design validation (human factor summative testing) confirms that the design meets user needs, the user can operate the devices safely and effectively, can clearly understand the IFU, and risks are mitigated to an acceptable level. User FMEA is a common tool to leverage existing scientific data, complaint data, or clinical evaluation with objective evidence, to document or justify risk controls, or identify needs for additional human factor studies. A well-designed IFU may help users to more easily understand and utilize the product. Nevertheless, some users may not understand the IFU and may require training by professionals. Therefore, the FDA recommended that pharma companies should ensure the HCP labeling includes training for the patient by the HCP prior to use of the device.

9.7 Emerging Technologies for Prefilled Syringe Presentations

Prefillable syringe systems offer a convenient platform for the subcutaneous and intramuscular delivery of biologics. As the use of PFS has grown in the development of therapeutic biologics, challenges associated with the integration of PFS systems have been encountered. Tungsten and silicone oil, already discussed, are known to be directly related to the presence of subvisible particulate (SVP) matter in prefilled systems and may contribute to the aggregation of some therapeutic proteins [22, 23]. The theoretical role that aggregates and SVP matter play in eliciting immunogenic responses is of further concern. These factors have led to a more thorough characterization of tungsten and silicone present in PFS systems and have in turn driven the development of prefillable syringe product lines offering enhanced control of these parameters: reducing tungsten and free silicone oil. Polymeric syringe systems have also emerged to provide a solution for products that require an absence of silicone oil and tungsten [44]. The evolution of polymeric syringes has introduced additional challenges to users of these components, including increased oxygen permeation across the polymer barrel, label adhesive and ink permeation, and susceptibility to cosmetic defects. The use of radiation

sterilization in polymeric syringes resulted in radical formation that caused oxidation of the protein, whereas steam sterilization did not have a negative impact on compatibility [45].

Advanced technologies focused on glass PFS lubrication enhancements leading to improved compatibilities with sensitive biologics have also emerged. These technologies offer a reduction or even elimination of the silicone oil lubricant from the system in order to mitigate potential challenges with SVP and aggregation, effectively coupling the barrier properties of glass with the lubricant free features of polymeric syringes. A novel fluoropolymer stopper that eliminates the need for silicone oil lubrication has been developed [46], while systems that seek to modify and immobilize the silicone oil lubricant layer [47] or replace the silicone oil [48] have also emerged. In parallel, advances in polymeric syringe technologies are beginning to offer barrier systems [49], providing polymeric syringe barrels with some of the desirable properties of glass systems.

The integration of PFS into autoinjector systems provides an additional level of patient convenience. While this convenience is highly desirable, it has shed light on an additional set of challenges related to the dimensional fit, functionality, and strength performance of PFS systems. This has led to more insightful studies of the distribution of lubricating silicone oil in PFS systems and the introduction of siliconization process controls in PFS product lines. It has additionally spurred the development of analytical tools [50] to aide in the characterization of siliconization. These advancements are geared toward the goal of optimization of the functionality of the combination PFS-device product. The mechanical strength of glass PFS has also emerged as an important attribute in the development of combination products. Glass breakage presents both a safety concern and a dose delivery concern. To address glass breakage, glass strengthened offerings have been developed with superior mechanical performance compared to standard Type I borosilicate glass barrels. Noted previously, polymeric syringes have also been positioned as an enhancement to mitigate glass breakage.

Considering the growing integration of biologics into PFS-based subcutaneous delivery systems, the development of technologies to mitigate challenges encountered in the development of combination products is expected to continue to grow in parallel. The end result is a range of technologies available for the development of convenient, safe, and effective PFS-based biologic therapeutics.

References

1. Bruno BJ, Miller GD, Lim CS. Basics and recent advances in peptide and protein drug delivery. Ther Deliv. 2013;4:1443–67.
2. Plavina T, Fox EJ, Lucas N, Muralidharan KK, Mikol D. A randomized trial evaluating various administration routes of natalizumab in multiple sclerosis. J Clin Pharmacol. 2016;56:1254–62.
3. Zhao L, Ji P, Li Z, Roy P, Sahajwalla CG. The antibody drug absorption following subcutaneous or intramuscular administration and its mathematical description by coupling

physiologically based absorption process with the conventional compartment pharmacokinetic model. J Clin Pharmacol. 2013;53:314–25.
4. Kelley B. Industrialization of mAb production technology: the bioprocessing industry at a crossroads. MAbs. 2009;1:443–52.
5. Connolly BD, Petry C, Yadav S, Demeule B, Ciaccio N, Moore JM, Shire SJ, Gokarn YR. Weak interactions govern the viscosity of concentrated antibody solutions: high-throughput analysis using the diffusion interaction parameter. Biophys J. 2012;103:69–78.
6. Saito S, Hasegawa J, Kobayashi N, Kishi N, Uchiyama S, Fukui K. Behavior of monoclonal antibodies: relation between the second virial coefficient (B (2)) at low concentrations and aggregation propensity and viscosity at high concentrations. Pharm Res. 2012;29:397–410.
7. Mathaes R, Koulov A, Joerg S, Mahler HC. Subcutaneous injection volume of biopharmaceuticals-pushing the boundaries. J Pharm Sci. 2016;105:2255–9.
8. Chan E, Maa YF, Overcashier D, Hsu CC. Investigating liquid leak from pre-filled syringes upon needle shield removal: effect of air bubble pressure. PDA J Pharm Sci Technol. 2011;65:363–71.
9. Chan EK, Hubbard A, Hsu CC, Vedrine L, Maa YF. Root cause investigation of rubber seal cracking in pre-filled cartridges: ozone and packaging effects. PDA J Pharm Sci Technol. 2011;65:445–56.
10. Shieu W, Stauch OB, Maa YF. Filling of high-concentration monoclonal antibody formulations into pre-filled syringes: investigating formulation-nozzle interactions to minimize nozzle clogging. PDA J Pharm Sci Technol. 2015;69:417–26.
11. Shieu W, Torhan SA, Chan E, Hubbard A, Gikanga B, Stauch OB, Maa YF. Filling of high-concentration monoclonal antibody formulations into pre-filled syringes: filling parameter investigation and optimization. PDA J Pharm Sci Technol. 2014;68:153–63.
12. Hamuro L, Kijanka G, Kinderman F, Kropshofer H, Bu DX, Zepeda M, Jawa V. Perspectives on subcutaneous route of administration as an immunogenicity risk factor for therapeutic proteins. J Pharm Sci. 2017;106(10):2946–54. https://doi.org/10.1016/j.xphs.
13. Bee JS, Randolph TW, Carpenter JF, Bishop SM, Dimitrova MN. Effects of surfaces and leachables on the stability of biopharmaceuticals. J Pharm Sci. 2011;100:4158–70.
14. Bai S, Landsman P, Spencer A, DeCollibus D, Vega F, Temel DB, Houde D, Henderson O, Brader ML. Evaluation of incremental siliconization levels on soluble aggregates, submicron and subvisible particles in a prefilled syringe product. J Pharm Sci. 2016;105:50–63.
15. Bernstein RK. Clouding and deactivation of clear (regular) human insulin: association with silicone oil from disposable syringes? Diabetes Care. 1987;10:786–7.
16. Chantelau E, Berger M, Bohlken B. Silicone oil released from disposable insulin syringes. Diabetes Care. 1986;9:672–3.
17. Majumdar S, Ford BM, Mar KD, Sullivan VJ, Ulrich RG, D'Souza A J. Evaluation of the effect of syringe surfaces on protein formulations. J Pharm Sci. 2011;100:2563–73.
18. Ludwig DB, Carpenter JF, Hamel JB, Randolph TW. Protein adsorption and excipient effects on kinetic stability of silicone oil emulsions. J Pharm Sci. 2010;99:1721–33.
19. Britt KA, Schwartz DK, Wurth C, Mahler HC, Carpenter JF, Randolph TW. Excipient effects on humanized monoclonal antibody interactions with silicone oil emulsions. J Pharm Sci. 2012;101:4419–32.
20. Auge KB, Blake-Haskins AW, Devine S, Rizvi S, Li YM, Hesselberg M, Orvisky E, Affleck RP, Spitznagel TM, Perkins MD. Demonstrating the stability of albinterferon alfa-2b in the presence of silicone oil. J Pharm Sci. 2011;100:5100–14.
21. Lubiniecki A, Volkin DB, Federici M, Bond MD, Nedved ML, Hendricks L, Mehndiratta P, Bruner M, Burman S, Dalmonte P, et al. Comparability assessments of process and product changes made during development of two different monoclonal antibodies. Biologicals. 2011;39:9–22.
22. Thirumangalathu R, Krishnan S, Ricci MS, Brems DN, Randolph TW, Carpenter JF. Silicone oil- and agitation-induced aggregation of a monoclonal antibody in aqueous solution. J Pharm Sci. 2009;98:3167–81.

23. Jones LS, Kaufmann A, Middaugh CR. Silicone oil induced aggregation of proteins. J Pharm Sci. 2005;94:918–27.
24. Gerhardt A, Bonam K, Bee JS, Carpenter JF, Randolph TW. Ionic strength affects tertiary structure and aggregation propensity of a monoclonal antibody adsorbed to silicone oil-water interfaces. J Pharm Sci. 2013;102:429–40.
25. Gerhardt A, McGraw NR, Schwartz DK, Bee JS, Carpenter JF, Randolph TW. Protein aggregation and particle formation in prefilled glass syringes. J Pharm Sci. 2014;103: 1601–12.
26. Mehta SB, Lewus R, Bee JS, Randolph TW, Carpenter JF. Gelation of a monoclonal antibody at the silicone oil-water interface and subsequent rupture of the interfacial gel results in aggregation and particle formation. J Pharm Sci. 2015;104:1282–90.
27. Seidl A, Hainzl O, Richter M, Fischer R, Bohm S, Deutel B, Hartinger M, Windisch J, Casadevall N, London GM, et al. Tungsten-induced denaturation and aggregation of epoetin alfa during primary packaging as a cause of immunogenicity. Pharm Res. 2012;29:1454–67.
28. Liu W, Swift R, Torraca G, Nashed-Samuel Y, Wen ZQ, Jiang Y, Vance A, Mire-Sluis A, Freund E, Davis J, et al. Root cause analysis of tungsten-induced protein aggregation in pre-filled syringes. PDA J Pharm Sci Technol. 2010;64:11–9.
29. Jiang Y, Nashed-Samuel Y, Li C, Liu W, Pollastrini J, Mallard D, Wen ZQ, Fujimori K, Pallitto M, Donahue L, et al. Tungsten-induced protein aggregation: solution behavior. J Pharm Sci. 2009;98:4695–710.
30. Bee JS, Nelson SA, Freund E, Carpenter JF, Randolph TW. Precipitation of a monoclonal antibody by soluble tungsten. J Pharm Sci. 2009;98:3290–301.
31. Rathore N, Pranay P, Eu B, Ji W, Walls E. Variability in syringe components and its impact on functionality of delivery systems. PDA J Pharm Sci Technol. 2011;65:468–80.
32. Eu B, Cairns A, Ding G, Cao X, Wen ZQ. Direct visualization of protein adsorption to primary containers by gold nanoparticles. J Pharm Sci. 2011;100:1663–70.
33. Allmendinger A, Mueller R, Schwarb E, Chipperfield M, Huwyler J, Mahler HC, Fischer S. Measuring tissue back-pressure–in vivo injection forces during subcutaneous injection. Pharm Res. 2015;32:2229–40.
34. Guidance for Industry and FDA Staff: Current Good Manufacturing Practice Requirements for Combination Products, 2017.
35. Design control guidance for medical device manufacturers 1997.
36. ICH harmonised tripartite guideline: Pharmaceutical development Q8(R2) 2009.
37. Guidance for Industry: Q9 Quality Risk Management 2006.
38. Guidance for Industry: Q10 Pharmaceutical Quality System 2009.
39. ISO 11040-4:2015: Prefilled syringes—Part 4: Glass barrels for injectables and sterilized subassembled syringes ready for filling 2015.
40. ISO 7864: 1993: Sterile hypodermic needles for single use 1993.
41. ISO 80369-7:2016: Small-bore connectors for liquids and gases in healthcare applications—Part 7: Connectors for intravascular or hypodermic applications 2016.
42. ISO 594-1:1986: Conical fittings with a 6% (Luer) taper for syringes, needles and certain other medical equipment – Part 1: General requirements 1986.
43. ISO 594-2:1998: Conical fittings with 6% (Luer) taper for syringes, needles and certain other medical equipment – Part 2: Lock fittings 1998.
44. Bose SC, Constable K. Design & evaluation of a polymer-based prefillable syringe for biopharmaceuticals with improved functionality and performance. Drug Dev Deliv. 2015;15:24–9.
45. Kiminami H, Krueger AB, Abe Y, Yoshino K, Carpenter JF. Impact of sterilization method on protein aggregation and particle formation in polymer-based syringes. J Pharm Sci. 2017;106:1001–7.
46. Teska BM, Brake JM, Tronto GS, Carpenter JF. Aggregation and particle formation of therapeutic proteins in contact with a novel fluoropolymer surface versus siliconized surfaces: effects of agitation in vials and in prefilled syringes. J Pharm Sci. 2016;105:2053–65.

47. Depaz RA, Chevolleau T, Jouffray S, Narwal R, Dimitrova MN. Cross-linked silicone coating: a novel prefilled syringe technology that reduces subvisible particles and maintains compatibility with biologics. J Pharm Sci. 2014;103:1384–93.
48. Thorton JDJ, DE, Sakhrani V. Next generation lubrication solutions for pharmaceutical packaging. ONdrugDelivery. 2015:10–15.
49. Weikart CM, Pantano CG, Shallenberger JR. Performance stability of silicone oxide-coated plastic parenteral vials. PDA J Pharm Sci Technol. 2017;71:317–27.
50. Wen ZQ, Vance A, Vega F, Cao X, Eu B, Schulthesis R. Distribution of silicone oil in prefilled glass syringes probed with optical and spectroscopic methods. PDA J Pharm Sci Technol. 2009;63:149–58.

Chapter 10
Special Topics in Analytics of Pre-filled Syringes

Atanas Koulov

Abstract Pre-filled syringes (PFSs) are extensively used as a container closure system offering convenience of administration. PFS features special and unique characteristics, enabling their proper functionality, but which, in some cases, may result in novel and unanticipated challenges. Such challenges are, for example, the potential interaction of the drug product with trace amounts of tungsten, originating from the pins used to form the syringe, or the presence of large concentrations of silicone oil droplets, potentially masking changes in proteinaceous particle load in a PFS product. This chapter discusses some of the analytical approaches used to tackle such challenges in the development of PFS drug products.

Keywords Pre-filled syringe · Silicone oil · Particles · Tungsten
Leachables and extractables · Container closure

10.1 Introduction

The pre-filled syringe (PFS) is a particular container closure system (CCS), presenting specific and often unique physical and functional features (see Fig. 10.1). For example, in contrast to liquid-filled vials, PFSs typically contain very little headspace and have unique product contact surfaces and materials (steel needle, silicone oil covered barrel). PFSs require careful design and development to ensure proper functionality. The latter typically depends on the combination and interplay of various characteristics such as product solution viscosity, needle and syringe dimensions, fill volume, siliconization technology/level. Thus, the development of PFS products requires additional—and often highly specialized—analytical characterization and testing. In this chapter, some of these special analytical topics and requirements will be reviewed.

A. Koulov (✉)
Drug Product Services, Analytical Development and Quality Control,
Lonza AG, Basel, Switzerland
e-mail: atanas.koulov@lonza.com

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_10

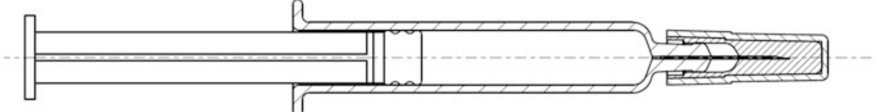


Fig. 10.1 A schematic representation of a staked-in-needle pre-filled syringe, showing the various components: (1) plunger, (2) syringe barrel, (3) needle and (4) rigid needle shield

10.2 Protein Aggregation and Sub-visible Particles

One of the main differences between PFS and vials most commonly used in development of biotech products is the presence of silicone oil (SO; polydimethylsiloxane)[1]. Silicone oil is used as a lubricant of the glass barrel of PFS in order to reduce the frictional force between the rubber stopper/piston and the syringe barrel and in turn ensure proper syringe functionality (break lose and gliding forces allowing unobstructed administration over the shelf-life of the product).

The silicone oil is applied to the barrel of the PFS using different technologies. For example, the silicone oil can be sprayed onto the barrel directly, resulting in a layer of "free" (or loosely bound) silicone oil. Alternatively, the silicone oil can be baked onto the glass surface of the barrel, resulting in a much more tightly bound silicone oil layer. Typically, spray-on processes also result in larger amounts of silicone oil delivered to the PFS barrel surface: typically approximately 1 mg SO/barrel or less versus approximately 0.1 mg SO/barrel or less for baked-on SO processes. Clearly, such apparent differences in both the amount of silicone oil and the mobility of the silicone oil layer on the barrel of the PFS are very important, particularly in the context of the frequently asked question about potential interactions between SO and the protein API in biotech products [1]. Such potential interactions have been discussed in recent studies [2], which have raised the possibility for serious adverse effects of SO on proteins, such as induced protein aggregation, presumably caused due to the very hydrophobic nature of SO. Interestingly, such studies have so far demonstrated measurable effects only in highly exaggerated and unlikely conditions (e.g. at very large SO/protein ratios, far exceeding the ones typically used in biotech products). Furthermore, it has been demonstrated that the presence of a surfactant (a nearly mandatory pharmaceutical excipient for protein therapeutics) obliterates the induction of protein aggregation by SO in such experiments [3, 4]. Nevertheless, testing the compatibility of protein therapeutics with SO remains a very important endpoint of PFS development.

The presence of SO in PFSs contributes to another important facet of the PFS target product profile (TPP), namely the sub-visible particle load of the product. Interestingly, SO present on the barrel surface may migrate to the bulk of the product in the form of microscopic SO droplets, thus contributing to the overall

[1]With some exceptions, liquid-filled vials typically do not feature siliconized glass walls.

particle load of the product. Here it is important to emphasize that the mechanism of migration of SO to the aqueous protein solution is still not well understood. In fact, it is likely that the spontaneous migration of SO to the bulk aqueous solution is a misconception. Clearly, in static conditions and in the absence of external energy added to the system, such migration constituting essentially the creation of a SO micro-suspension would not be favoured thermodynamically due to the hydrophobic effect. Naturally, in cases where PFSs are subjected to external forces, such as agitation for example, one could envision how such external energy may lead to partitioning of SO micro-droplets into the aqueous solution of the product. However, it is arguable to what extent do such effects contribute to the presence of large amounts of SO droplets in the PFS products. The fact is that during the testing of PFSs, large amounts of SO micro-droplets are commonly observed. It should be pointed out that sample preparation practices likely vary widely between different laboratories and testing sites. Nevertheless, in the context of the discussion above, one of the more drastic examples of exertion of force onto PFS (and the barrel-bound silicone oil layer in particular) is the ejection of the PFS content prior to testing. This is a typical and most common practice in order to ensure that the analytical tests are performed on the product solution as administered to the patient (ejected through the needle or at the very least through the tip of the syringe in cases of luer lock configurations). It is easy to imagine the action of the syringe piston moving down the barrel of the syringe and essentially stripping the silicone oil layer. In these circumstances, a relatively large volume of SO likely is mixed with the product solution in the form of micro-suspension during ejection of the syringe contents. This line of thought poses an interesting possibility, i.e. the possibility that the majority of silicone oil droplets observed in PFS product solutions is generated during the sample preparation. This idea is supported by the observation that the faster the contents of PFS is expelled, the larger amounts of SO droplets are observed in the test solution (*unpublished data*). Nevertheless, the amount of SO droplets in solution may increase over the shelf-life of a syringe, owing to various factors, such as the syringe manufacturing process, storage and transportation.

With the caveats mentioned above, the SO droplets still constitute a large (in most cases the dominant) portion of the sub-visible particles in the PFS solutions. Whereas proteinaceous sub-visible particles have been and continue to be a topic of high scientific interest within the biopharmaceutical community, particularly in the context of concerns about potential adverse effects [5, 6], SO droplets are generally thought to be relatively inert and safe if administered to patients parenterally, particularly in the low-microgram quantities contained in solutions expelled from PFS units. Thus, the presence of different types of particles (proteinaceous particles, SO droplets and other particles) effectively poses a challenge to sub-visible particle testing.

The light obscuration sub-visible particle test universally used for quality control of biotech therapeutic products in accordance with the major pharmacopoeias is (in its current technical implementation) unable to differentiate between a SO droplet and a non-SO (e.g. proteinaceous) particle. Thus, SO droplets may effectively "mask" the particle load in a product. This may become particularly

important if the proteinaceous particles in a given product exhibit a tendency to increase over the product's shelf-life—a tendency which may not be readily detectable due to the masking effect of the often more numerous SO droplets. Thus, during the development of a PFS program, it is very important to characterize the sub-visible particle profile of the product and understand the relative contribution of the SO droplets and non-SO particles in the product. Within the last several years, the development of three technologies in particular made possible the relatively detailed characterization of SO/non-SO sub-visible particles.

The first technology which enabled characterization of the SO droplets is the technique known as flow imaging microscopy (aka dynamic imaging microscopy) [7, 8]. Flow imaging microscopy features a flow-cell set-up somewhat similar to the one of light obscuration, but in contrast to the latter, which simply enumerates the particles passing through the flow cell, flow imaging microscopy features imaging capabilities, i.e. provides images of the individual particles collected by a high-speed camera. The ability of this technique to produce the images of individual particles has proven very useful for characterization of SO droplets in PFS. Due to their hydrophobic nature, the SO droplets have a very regular circular appearance, regardless of their size [7, 9]. Furthermore, the refractive properties of SO lead to the particular appearance of larger SO droplets in flow microscopy images (the exact size thresholds depend on the specific instrument set-up)—i.e. darker outer border and lighter interior. This characteristic appearance of SO droplets allows for differentiating them from other microscopic particles, which are commonly present in PFS test solutions, e.g. air bubbles, proteinaceous particles (see Fig. 10.2).

The second advance that we have witnessed within the last decade is the development of algorithms for automatic, machine differentiation of SO and non-SO particles. This operation until recently was performed manually, with operators sorting sometimes through thousands of particles and deciding how to categorize

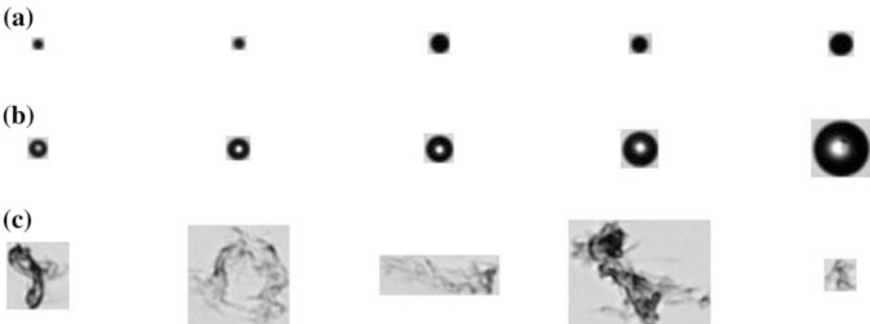


Fig. 10.2 Examples of different types of sub-visible particles commonly present in PFS: **a** air bubbles, **b** silicone oil droplets, **c** proteinaceous particles

them based on their personal experience. Clearly, such manual procedure is extremely cumbersome and prone to error. Thus, the recent invention of several approaches for automatic categorization of particle images ([7, 9, 10, 11], Schnaible and Koulov unpublished) allowed for more efficient accurate and precise differentiation of particles. Further, the latest approaches allow for differentiation of particles of sizes which were previously inaccessible via manual categorization (<5 um).

It needs to be mentioned that in general, due to the different detection principles of flow imaging microscopy and light obscuration, these techniques produce different results (in terms of particle enumeration) when measuring identical samples. Whereas this has been the subject to extensive research and debate in the community and the physical causes have been largely understood ([12, 13], Koulov et al. unpublished), the fact of the matter is that this fact is not that relevant to the characterization of sub-visible particles in PFS. More specifically, LO is likely to remain the tool of choice for quality control and flow imaging microscopy—the method of choice for "extended/additional characterization"—e.g. SO/non-SO particle profile characterization in PFS.

Paradoxically, the presence of different commercial flow imaging microscopy systems which use different optical systems and non-harmonized particle morphology descriptors has effectively stymied the efforts on development of common techniques for data analyses. Recent efforts to develop algorithms for data analysis have overcome these differences and have made major progress in direction of harmonization of these data and in understanding these differences [14].

In practical terms, during the development of PFS biotech therapeutic products, various studies to understand the sub-visible particle profile of the product need to be carried out. Typically, these encompass the analyses of stability samples (long-term storage, accelerated and stressed conditions) from various product lots accompanying the PFS development. Such extended characterization efforts are particularly important to understand the proportion of SO particles in the product as well as the kinetics of the different categories of particles over the shelf-life of the product. This knowledge may prove essential when defining product-specific limits for sub-visible particles (typically required for licensure in the USA) for PFS products and in general—in order to ensure the definition of a sound control system.

As a final note, it may be mentioned that the current practices with regard to sub-visible particle characterization of PFS vary significantly from company to company (and even sometimes—from site to site). Different approaches are applied to not only sample preparation, measurement and instrument settings, but also to the characterization "philosophy" applied during the duration of the lifecycle of a product. Whereas regulators provide certain latitude to sponsors in justifying individual control strategies (based on the specifics of each product), such broad variance in practices and approaches may contribute to larger discrepancies in the characterization of different products than often appreciated.

10.3 Container Closure Integrity

The guarantee of sterility of parenteral dosage forms is a critical facet in their development. In this aspect, PFS presents a more complex challenge simply due to the larger number of closure interfaces (see Fig. 10.3). In addition, some of these interfaces are formed with moveable parts—e.g. the syringe plunger, the syringe needle shield; thus, factors present during storage and shipping (e.g. air pressure differences) may affect the container closure integrity (CCI) of PFS.

It is critical that during the development of PFS products, special attention is paid to the characterization and qualification of the CCS, particularly with regard to the aspects mentioned above. However, such careful characterization requires the availability of analytical tools and approaches to measure the relative contributions of the different container closure interfaces (sealing areas). For example, it is important to carefully select the method for measuring container closure integrity in order to distinguish small differences in tightness of the various sealing areas. Typically, only very sensitive deterministic methods (e.g. the helium leak CCI test method) may be suitable for this purpose. In addition, the methodology for CCS characterization should be developed so the individual contributions of the various sealing areas can be revealed (Pelaez et al., in preparation) and characterized. Also, the CCI testing methodology needs to allow for accurate and precise measurement of the contributions of the movements of the various moveable PFS components mentioned above. For example, one such critical question is the robustness of the tip cap seal. It should be noted that such methodologies to date are not commercially available, but need to be designed for purpose and custom-built (Pelaez et al., in preparation).

10.4 Extractables and Leachables

Similarly to any other parenteral CCS, PFS requires leachable and extractable evaluations. However, this is also an area where PFS presents additional analytical challenges as compared to other CCSs (e.g. vials). In general, the presence of additional (and unique) product contact surfaces in PFS requires that the potential

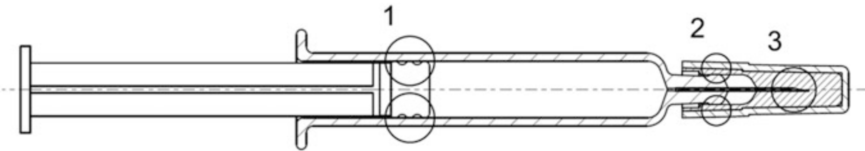


Fig. 10.3 A schematic overview of a rigid needle shield (RNS) staked-in-needle pre-filled syringe showing the main sealing interfaces: (1) ribs of syringe plunger, (2) RNS/tip cone and (3) RNS/needle tip

contributions of these contact surfaces are specifically addressed. An example of such distinct contribution may be the interface of the stainless steel needle and the tip cone of the glass barrel of the syringe. Typically, the syringe needle is fixed to the tip cone using an adhesive (see Fig. 10.1). In most syringe designs, the adhesive comes into direct contact with the product and therefore has the potential of affecting the product stability. Some cases of such occurrences have been reported, where either the incomplete curing or incomplete drying of the needle adhesive has resulted in leaching of compounds from the adhesive into the product with adverse consequences, such as protein oxidation [15, 16].

Another example of unique product contact surface in syringes, which may potentially contribute to the elemental impurity profile of the product, is the metal needle itself. Of course, the elemental impurity profiles of PFS are typically assessed according to ICH Q3D, similarly to other dosage forms. However, it is worth mentioning one interesting aspect of the elemental impurity profile of PFS, namely the potential influence of the syringe manufacturing process. It has been widely reported [17, 18, 19] that the manufacturing process of syringes (formation of the glass tip cone orifice using a hot tungsten pin) may result in the presence of tungsten and a variety of tungsten-compound residues (such as oxides and salts), which may have adverse effects on the stability (and potentially on the safety) of the product. More specifically, it has been demonstrated that such tungsten compounds may induce protein aggregation. Thus, the potential sensitivity of a given product to the presence of tungsten compounds is important to assess during the development of pre-filled syringes. Typically, this is achieved by means of tungsten spiking studies using either pure model tungsten compounds or preferably tungsten pin extracts.

10.5 Closing Remarks

There are several unique aspects in the development of PFS, resulting in distinct quality attributes, which need to be specifically addressed during the development process. The characterization of these special quality attributes in turn often requires the use of specialized analytical approaches. Whereas some of these aspects (e.g. the presence of adhesive in staked-in-needle syringes as a potential source of leachables) are unique in origin, they can be characterized, monitored and controlled using analytical tools that are readily available and applied to other parenteral formats. Others, however, (e.g. assessing the robustness of the tip cap seal or the differentiation of SO and non-SO particles), present unique analytical challenges and require the development of new, custom-built technical solutions, which are not readily available.

Although many of the analytical challenges encountered during product development can be anticipated by following the general logic and rules of product development, there have been cases of surprising challenges, which were difficult to anticipate. One such example is the clogging of syringes due to evaporation of

product in the syringe needle. As the number of market approvals of PFS products is continuously increasing, it is likely that such surprises will decrease in the future. However, the complexities of PFS development will likely still pose analytical challenges for the foreseeable future.

Finally, it is important to remember that the different quality attributes of PFS are often interlinked and interdependent (e.g. the viscosity profile of the drug product as a function of temperature, which determines the syringe functionality parameters, needs to be taken into consideration in developing the protein content specifications for the product). Thus, the development of PFS products in the future may benefit from development approaches such as quality by design (QbD). However, regardless of the development strategy of a given program, proper PFS drug product development will always surely require a holistic analytical approach integrating state-of-the-art analytical tools and advanced scientific knowledge.

References

1. Funke S, Matilainen J, Nalenz H, Bechtold-Peters K, Mahler HC, Friess W. Silicone migration from baked-on silicone layers. particle characterization in placebo and protein solutions. J Pharm Sci. 2016;105(12):3520–31.
2. Gerhardt A, Mcgraw NR, Schwartz DK, Bee JS, Carpenter JF, Randolph TW. Protein aggregation and particle formation in prefilled glass syringes. J Pharm Sci. 2014;103 (6):1601–12.
3. Basu P, Blake-Haskins AW, O'Berry KB, Randolph TW, Carpenter JF. Albinterferon α2b adsorption to silicone oil-water interfaces: effects on protein conformation, aggregation, and subvisible particle formation. J Pharm Sci. 2014;103(2):427–36.
4. Gerhardt A, Mcumber AC, Nguyen BH, Lewus R, Schwartz DK, Carpenter JF, Randolph TW. Surfactant effects on particle generation in antibody formulations in pre-filled syringes. J Pharm Sci. 2015;104(12):4056–64.
5. Carpenter JF, Randolph TW, Jiskoot W, Crommelin DJ, Middaugh CR, Winter G, Fan YX, Kirshner S, Verthelyi D, Kozlowski S, Clouse KA, Swann PG, Rosenberg A, Cherney B. Overlooking subvisible particles in therapeutic protein products: gaps that may compromise product quality. J Pharm Sci. 2009;98(4):1201–5.
6. Singh SK, Afonina N, Awwad M, Bechtold-Peters K, Blue JT, Chou D, Cromwell M, Krause HJ, Mahler HC, Meyer BK, Narhi L, Nesta DP, Spitznagel T. An industry perspective on the monitoring of subvisible particles as a quality attribute for protein therapeutics. J Pharm Sci. 2010;99(8):3302–21.
7. Sharma DK, Oma P, Pollo MJ, Sukumar M. Quantification and characterization of subvisible proteinaceous particles in opalescent mAb formulations using micro-flow imaging. J Pharm Sci. 2010;99(6):2628–42.
8. Wuchner K, Büchler J, Spycher R, Dalmonte P, Volkin DB. Development of a microflow digital imaging assay to characterize protein particulates during storage of a high concentration IgG1 monoclonal antibody formulation. J Pharm Sci. 2010;99(8):3343–61.
9. Strehl R, Rombach-Riegraf V, Diez M, Egodage K, Bluemel M, Jeschke M, Koulov AV. Discrimination between silicone oil droplets and protein aggregates in biopharmaceuticals: a novel multiparametric image filter for sub-visible particles in microflow imaging analysis. Pharm Res. 2012;29(2):594–602.

10. Saggu M, Patel AR, Koulis T. A random forest approach for counting silicone oil droplets and protein particles in antibody formulations using flow microscopy. Pharm Res. 2017;34 (2):479–91.
11. Weinbuch D, Zölls S, Wiggenhorn M, Friess W, Winter G, Jiskoot W, Hawe A. Micro-flow imaging and resonant mass measurement (Archimedes)—complementary methods to quantitatively differentiate protein particles and silicone oil droplets. J Pharm Sci. 2013;102 (7):2152–65.
12. Ripple DC, Hu Z. Correcting the relative bias of light obscuration and flow imaging particle counters. Pharm Res. 2016;33(3):653–72.
13. Zhao H, Diez M, Koulov A, Bozova M, Bluemel M, Forrer K. Characterization of aggregates and particles using emerging techniques. In: Mahler H-C, Jiskoot W, editors. Analysis of aggregates and particles in protein pharmaceuticals. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2012.
14. Cavicchi RE, Collett C, Telikepalli S, Hu Z, Carrier M, Ripple DC. Variable threshold method for determining the boundaries of imaged subvisible particles. J Pharm Sci. 2017;106 (6):1499–507.
15. Adler M. Challenges in development of pre-filled syringes for biologics—a formulation scientist's point of view. Presented at the 2011 PDA Europe The Universe of Pre-filled Syringes and Injection Devices. Basel, Switzerland, 7–11 Nov 2011.
16. Markovic, I. Considerations for extractables and leachables in single use systems: a risk-based perspective. Presented at the PDA Single Use Systems Workshop, Bethesda, USA, 22–23 June 2011.
17. Jiang Y, Nashed-Samuel Y, Li C, Liu W, Pollastrini J, Mallard D, Wen ZQ, Fujimori K, Pallitto M, Donahue L, Chu G, Torraca G, Vance A, Mire-Sluis T, Freund E, Davis J, Narhi L. Tungsten-induced protein aggregation: solution behavior. J Pharm Sci. 2009;98 (12):4695–710.
18. Liu W, Swift R, Torraca G, Nashed-Samuel Y, Wen ZQ, Jiang Y, Vance A, Mire-Sluis A, Freund E, Davis J, Narhi L. Root cause analysis of tungsten-induced protein aggregation in pre-filled syringes. PDA J Pharm Sci Technol. 2010;64(1):11–9.
19. Seidl A, Hainzl O, Richter M, Fischer R, Böhm S, Deutel B, Hartinger M, Windisch J, Casadevall N, London GM, Macdougall I. Tungsten-induced denaturation and aggregation of epoetin alfa during primary packaging as a cause of immunogenicity. Pharm Res. 2012;29 (6):1454–67.

Chapter 11
C Mini-pumps

Dirk Gläser, Jürg Liniger and Daniel Peter

Abstract Mini-pumps for bolus injection contribute to keep pace with the requirements for drug delivery devices driven by specific properties of protein therapeutics in combination with particular usability needs. Introductory Sect. 11.1 reflects on the growing requirements on drug delivery devices for biologics in connection with new indications and user groups. Section 11.2 discusses the boundary conditions, physical basics and technical requirements. Section 11.3 deals with the different types of pumps and their classification. In Sect. 11.4, the different drive concepts and their application areas are discussed and Sect. 11.5 looks at new trends and related requirements for mini-pumps. Finally, Sect. 11.6 makes considerations on USA and EU regulations applicable for those devices.

Keywords Drug delivery device · Mini-pump · Bolus injector
Pen injector · Patch injector · Micro-dosing pump

11.1 Introduction

Beside intravenous infusion and hand-held syringes, Mini-pumps play an important role for the bolus administration of protein therapeutics. Pumps used for bolus injection facilitate the process of drug delivery by improved usability and more precise control of the administration process, for example by means of mechanical or electronic control of the delivery process. These factors represent essential prerequisites for potential self-administration, even for special target user groups, with consequently positive impact on patient convenience and reduced cost for

D. Gläser (✉) · J. Liniger · D. Peter
Device Development, F. Hoffmann-La Roche Ltd, Basel, Switzerland
e-mail: dirk.glaeser@roche.com

J. Liniger
e-mail: juerg.liniger@roche.com

D. Peter
e-mail: daniel.peter.dp1@gmail.com

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_11

health care systems. The market of protein therapeutics is further growing [1, 2], and hence, adequate delivery systems are needed to cope with the expectations of patients, healthcare professionals, and authorities. Consequently, drug delivery systems like Mini-pumps are gaining importance. Mini-pumps generally can be single- or multiuse drug delivery devices with replaceable or non-replaceable drug containers. They can be hand-held (e.g. pen injectors) or, especially when longer administration times are needed, body-worn (e.g. patch injectors), which requires different considerations in terms of usability. Protein therapeutics such as monoclonal antibodies (mAb) creates new requirements for drug delivery, due to partially relatively high volumes (>2 ml) and high viscosities in combination with specific administration routes. Additionally, there is a growing awareness of user needs and usability aspects including the potential for self-administration. Traditional devices such as prefilled syringes in combination with auto-injectors, so-called pen injectors, are not always capable of providing the required features. Patch injectors represent a suitable alternative which address these specific needs. Body-worn patch pumps are typically needle-based injection devices delivering drug volumes from 1 ml up to several 10 ml, at viscosities up to 30 cP and higher, often associated with longer injection times than traditional pen injectors (Fig. 11.1).

In addition to individual volume and viscosity ranges, the consideration of user needs and convenience aspects is essential when designing such devices. First of all, ease of use is a central requirement in order to fulfil user needs and to prevent from use errors under real-use environment conditions. This can be achieved for

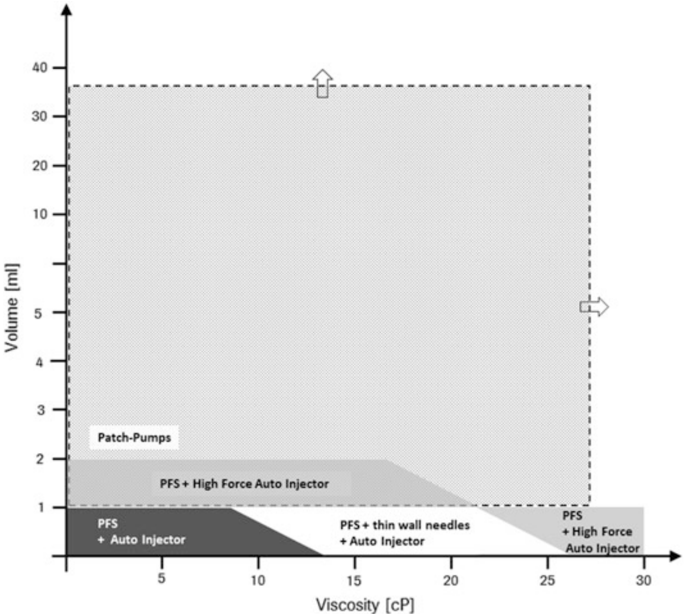


Fig. 11.1 Application areas of bolus injectors in comparison

example by means of prefilled, ready to use devices, featuring as less handling steps as possible and an intuitive and easy to understand user interfaces. Another aspect is convenience. It may be appropriate to choose a smaller needle size and to hide the needle in order to reduce the pain sensation perceived by the patient and to manage 'needle phobia'. Depending on the specific target user group, there might be additional usability requirements like adequate user interfaces to be considered, for example in connection with visual impairment or disabilities, especially when considering self-administration. Such Mini-pump devices may be intended for different routes of administration, like transdermal, subcutaneous, intravenous, intramuscular, intrathecal, ocular, intraperitoneal and others. For protein products, the main focus is on both, the intravenous and subcutaneous routes, whereas the subcutaneous injection is becoming more important [3]. However, due to the growing number of protein therapeutics for an increasing variety of indications, alternative routes of administration like the ones mentioned above might become more important for certain disease areas. Another relevant aspect is the integration with the drug packaging. Typically, well-known primary packaging systems like vials are preferred which create specific requirements for the device, e.g. integration of the primary container in the device. Alternative concepts suggest the filling of the device from a drug reservoir or the insertion of a separate drug container right before administration to the patient, but typically require additional handling steps.

Special requirements associated with protein therapeutics which need to be taken into account include cold storage from filling until the time of use.

In summary, protein therapeutics in combination with today's usability requirements create new and very specific requirements for drug delivery devices, depending on the indication, target user group and way of using the device, e.g. self-administration.

11.2 Boundary Conditions of Mini-pumps for Biologics

11.2.1 Physical Basics

In Mini-pumps, a fluid is normally transported through a fluidic path typically comprised of hoses and needles forming a tube system. In the simplest case, the injection needle is the only tube in the entire system.

If a fluid is to be transported through a straight, circular tube, a pressure difference must exist, which can be calculated according to the Hagen–Poiseuille law [4] as follows (Fig. 11.2):

$$p_1 - p_2 = \frac{128 \cdot L \cdot \eta \cdot Q}{d^4 \cdot \pi}$$

In simplified terms, this law applies to laminar flow (Reynolds number Re < 2300, [5, 6]) and Newtonian fluids. This assumption does not hold true for

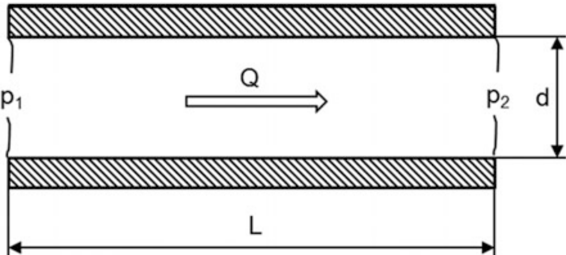


Fig. 11.2 Laminar flow through a circular tube, where L is the length of the tube, η is the dynamic viscosity, Q is the flow rate, d is the inner diameter of the tube, and p_1 and p_2 are the pressure values at the corresponding positions

turbulent flow, where the pressure difference is greater because of additional flow losses. The assumption likewise does not apply to fluids that display non-Newtonian behaviour.

As a simplification, the viscosity of Newtonian fluids such as water depends solely on temperature. The viscosity of protein therapeutics varies with temperature, but also with protein concentration. Protein therapeutics may display non-Newtonian behaviour. In this case, viscosity depends non-proportionally on the shear forces acting on the fluid. There are known shear-thinning effects that lead to a decrease in viscosity with increasing shear forces [7].

Given the conditions noted above, it is necessary to determine for every real situation whether the Hagen–Poiseuille law is applicable or whether the calculation model must be adapted [8, 9].

11.2.2 Basic Requirements

The basic task of a Mini-pump is the transport of the liquid drug from the drug container into the patient's body. This can be either a controlled dosing of the drug or the delivery of the entire content of the drug reservoir at acceptable flow rate.

For a Mini-pump according to Fig. 11.3, where the drug container is a syringe and the fluidic path is the injection needle, the required driving force can be calculated as follows (assuming Hagen–Poiseuille law is applicable):

$$F_{\text{Drive}} = \frac{32 \cdot D^2 \cdot L \cdot \eta \cdot Q}{d^4} + F_{\text{Fr}}$$

With syringe inner diameter D, length of the injection needle L, dynamic viscosity η, flow rate Q and inner diameter of the injection needle d. The friction force between the syringe barrel and plunger is considered with F_{Fr}.

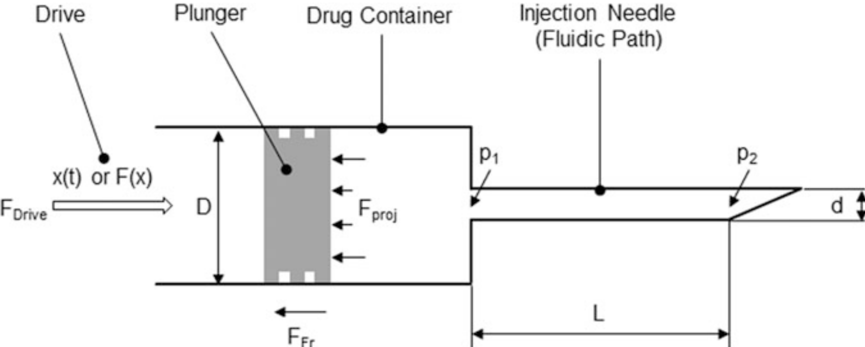


Fig. 11.3 Symbolic view of Mini-pump fluidic path, where L is the length of the injection needle (fluidic path), d is the inner diameter of the injection needle, p_1 and p_2 are the pressure values at the corresponding positions, F_{proj} is the force resulting by the pressure projected to the plunger, and F_{Fr} is the friction force of the plunger. F_{Drive} represents the force to move the plunger to achieve the desired flow rate. In more detail, $F(x)$ represents a force which depends on the plunger position (spring-driven) or $x(t)$ represents a motor drive where the position and/or the speed of the plunger is controlled

Figure 11.3 shows the schematic structure of the fluidic path of a Mini-pump. Its central component is the drug container. The fluidic path is understood here as the fluid-conducting link from the drug container until discharge into the patient's body. Normally, a combination of needles and hose sections is used. In the simplest case, the transfer system consists merely of the injection needle connected directly to the drug container.

Besides the rheological and volume requirements imposed by the fluid to be transported, the Mini-pump design must consider numerous technical boundary conditions that depend on the treatment given. Some boundary conditions may be of secondary importance and overridden by other boundary conditions, depending on the treatment. In designing a Mini-pump, it is often necessary to reach a compromise between competing requirements and boundary conditions. However, it is important to understand that the Mini-pump is developed around the drug container. The drug container is the heart of the pump, and its form and properties are not only governed by the properties of the fluid to be transported, but are also key determinants of the pump design.

In the following, important boundary conditions are detailed.

Reconstitution

When reconstituting a freeze-dried powder, a key consideration is whether reconstitution with a suitable fluid is to take place inside or outside the Mini-pump. Reconstitution in the Mini-pump is technically demanding. External reconstitution results in additional handling steps for the user.

Volume to Inject

Normally, the amount of drug to be administered is at least the fill volume of the drug container. Where the drug container is a cylindrical cartridge, the design of the Mini-pump must find a balance between cartridge diameter and length. From the drug standpoint, there may be an optimum in terms of minimizing the contact surface between drug and cartridge. From the standpoint of Mini-pump size, for a given injection volume, a large cartridge diameter results in a short length, and this may appear advantageous at first sight. However, a large cartridge diameter also requires a correspondingly strong pump drive, which is often technically challenging.

Viscosity/Pressure in the Fluidic Path Depending on the viscosity of the drug, the desired flow rate and the geometry of the fluidic path through which the drug is transported, it is possible to determine the required driving force of the Mini-pump and the pressures produced in the fluidic path and cartridge.

Injection Time and Tolerance

The injection time—the time until the drug has been completely injected—depends on the total injection volume and flow rate. A change in flow rate or its tolerance is accompanied by a change in the injection time, which is potentially noticed by the user and may be undesirable.

Electronically controlled pump drives tend to be non-critical with regard to flow rate tolerance. Spring-based drives, in which a spring exerts force on the plunger, and the fluidic path acts as a flow restrictor determining drug flow, are usually inaccurate because the flow rate is largely determined by injection needle diameter tolerances and the viscosity of the drug. Flow rate and injection time tolerance specifications may preclude certain drive designs.

Flow Rate and Viscosity Tolerability

Whereas injection volume and viscosity of the drug are determined by therapeutic and formulation requirements, the flow rate during subcutaneous delivery is often the subject to debate during the development phase of the device because of conflicting requirements, such as injection time and physiological tolerability. Physiological acceptance is most commonly equated with the pain sensation of the patient. From a safety and convenience perspective, the shortest possible injection time is desired, whereas the intention to reduce the pain experience leads to the desire for lower flow rates which consequently results in longer injection times.

Generally, it is assumed that smaller drug volumes can be injected faster, whereas larger volumes require slower delivery. Auto-injectors typically deliver approximately 1 ml within less than 10 s which corresponds to a flow rate of approximately 6 ml/min. High-volume injectors currently target for significantly lower flow rates typically in the range of 1–3 ml/min. Recent data suggest the physiologic tolerability of larger SC dose volumes and high viscosities in combination with higher flow rates, showing that faster delivery can be associated with even less pain sensation for a certain delivered volume [10, 11].

Dose Accuracy
In designing a Mini-pump, it is important to consider how the pump design affects dose accuracy. In the simplest case, the entire drug container is emptied in a defined time (bolus injector). Here, dose accuracy is largely influenced by the fill volume of the drug container and the ability of the Mini-pump to empty the entire container. Within certain limits, flow rate accuracy is normally of secondary importance.

In the case of an insulin pump, high demands are placed on dose accuracy, so-called micro-dosing, because a few millilitres of insulin must be released over several days according to a precise administration profile. Under- or overdosing would have bad consequences for the patient.

Storage
The Mini-pump is required to work always safely and reliably. Therefore, storage times and conditions, depending on the drug, have to be considered when designing such devices. In case of protein therapeutics, cool storage of the drug together or without the device for a certain time is to be expected. The maximum storage time is typically defined by the stability of the drug.

11.2.3 Additional Requirements

As well as the basic requirement of controlled delivery of the drug product at a defined flow rate, Mini-pumps must satisfy a large number of additional requirements.

Many of these requirements aim at simplifying Mini-pump use or treatment as a whole and/or making them safer.

Control, High Dose Accuracy and Communication
The use of electronics and software results in new possibilities for controlling, monitoring and displaying Mini-pump functions that are not feasible with purely mechanical systems.

Electronic controlled pump drives allow for high-precision dose accuracy pump drives. Logging of user operations or communication via the Internet or with other devices such as cell phones or blood glucose meters provides new opportunities for treatment monitoring or aids fault-finding during servicing.

Ergonomics and Design
In terms of ergonomics and industrial design, customer requirements and expectations have changed in recent years, driven by numerous examples from the consumer goods industry and mobile consumer electronics. Manufacturers are called upon to develop ergonomic devices that are suitable for the target groups and also have an appealing design.

Mini-pumps where the injection needle is never visible to the user shall help to reduce the pain sensation perceived by the patient and manage ‘needle phobia’.

Simple and Safe Handling

To reduce handling steps and to simplify the use of a Mini-pump, one option is to integrate a prefilled drug cartridge into the Mini-pump. Various handling steps—that are necessary if the drug is filled by the user/patient into the Mini-pump—are no longer necessary what may be an important benefit for the patient/user.

An other option to increase the ease of use (and also the safe handling) is the integration of automated functions into the Mini-pump. Automated functions, such as the automated connection of the injection needle to the drug container, the automated insertion/retraction of the injection needle, the automated needle shielding before and after use and the automated needle stick injury protection after use, could be requirements to increase the easy and safe handling of the Mini-pump.

Reusability

Mini-pumps can be constructed as single-use or reuseable devices. Single-use pumps are disposed of after a single use. Reuseable pumps are often designed for the controller, drive and possibly the power source to be reused, while the drug container and all other components that are in contact with the drug and/or must be sterile are used once only.

Reusable pumps seem at a first glance to better cope with sustainability expectations, however, might require a more complex logistics and drive additional requirements. These factors must be carefully weighed in order to come to a realistic assessment regarding sustainability.

11.3 Types of Mini-pumps

Among the Mini-pumps commonly available on the market, a fundamental distinction is made between bolus injectors for short-term administration and micro-dosing pumps for long-term administration. Bolus injectors are further differentiated into pen injectors, which are hand-held during drug administration, and patch injectors, which are adhered to the body at the administration sites. Widely used micro-dosing pumps are ones worn close to the body with a transfer system for the drug fluid (infusion set comprising a flexible tube with an injection needle and a coupling for connection to the pump). For some years, micro-dosing pumps have also been available that can be adhered directly to the user's body (Fig. 11.4).

All types are familiar both as single-use articles, which are disposed of after complete or partial administration of the contents of the drug container, and as multiuse articles, which are fitted with a new drug container after complete or partial administration and can be reused.

Single-use articles are generally characterized by less complex operation steps and are thus less error-prone, especially if the devices are provided to the user already fitted with the filled drug container. A further advantage is lower cost due to lower requirements for robustness and long-term reliability.

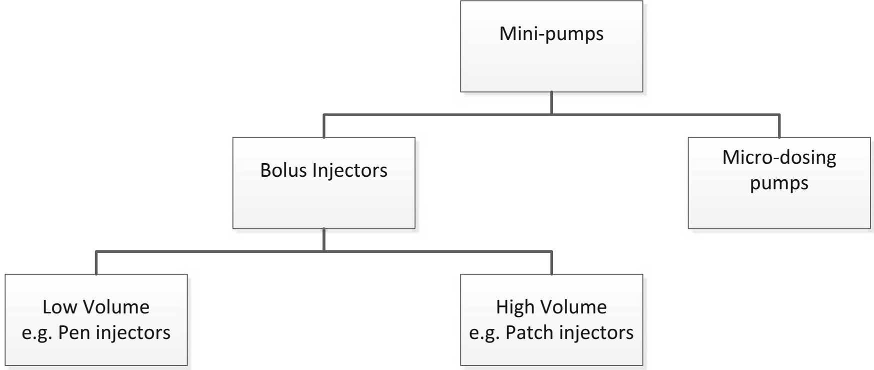


Fig. 11.4 Types of mini-pumps

The main advantages of multiuse devices are in the area of sustainability, but they are also capable of meeting more extensive requirements. In the area of micro-dosing, because the use of higher-quality and more accurate components makes it possible to meet considerably higher requirements not only for dose accuracy, but also for flexibility (e.g. individual programming of dosing profiles, recording of treatment history, visual display of information for the user).

11.3.1 Bolus Injectors

When using bolus injectors, a relatively large amount of drug fluid (>0.1 ml) is injected during a single administration. Either pen or patch injectors are used for administration.

11.3.1.1 Pen Injectors

Description

A pen injector is held in position by hand to administer the drug fluid. Various models possess a protection against unintended operation, which allows administration of the fluid only when the injection needle is a certain depth under the skin (Figs. 11.5 and 11.6).

With a single-dose injector, all the fluid in the drug container (usually ≤ 2 ml) is delivered at once and the injector is then discarded or fitted with a new drug container. With multidose injectors, several doses (usually ≤ 0.5 ml) of the fluid are delivered in several administrations over a certain time (e.g. several days or weeks). Multidose injectors have either a fixed or selectable dosage.

Fig. 11.5 Pen injector Pegasys ProClick (single-use, single-dose)

Fig. 11.6 Roferon or comparable (reusable, multidose)

Drug administration with a single-dose injector is often effected by a pretensioned mechanism, which is released by the patient when the injector is in the correct position. With multidose injectors, administration is generally accomplished by a user-applied force, where a small amount of fluid is to be delivered, or by a mechanical or electromechanical drive in the case of larger dose volumes.

Pen injectors are particularly suitable for administration of protein therapeutics as long as the volume is small (typically <2 ml) and the viscosity is low. For higher viscosities, pen injectors with thin wall needles and elevated force are suggested to allow acceptable injection times because the device is hand-held.

11.3.1.2 Patch Injectors

Description

A patch injector is attached to the patient's body at the administration site with an adhesive patch on the device to administer the drug fluid. The injection needle is inserted into the tissue to the defined depth, the drug delivered and the needle then retracted to avoid risk of injury. Here again, some models are equipped with a safety device which prevents actuation of the device before it is on the patient's

Fig. 11.7 Roche single-use injection device

body and/or which can interrupt drug delivery if the device is removed from the body before all the drug has been administered (Fig. 11.7).
Patch injectors are generally used with administered drug volumes exceeding 2 ml.

Drug administration from a patch injector is effected by a pretensioned mechanism or an electromechanical drive.

Patch injectors are particularly suitable for administration of protein therapeutics with larger volume (typically >2 to several 10 ml) and the high viscosities. The drive allows to apply higher forces, and the injection time is not critical because the device is body-worn.

11.3.2 Micro-dosing Pumps for Long-Term Use

When using micro-dosing pumps for long-term administration, relatively small amounts of drug fluid are administered at a so-called basal rate (amount administered per unit time, e.g. micro-litres per hour), although a bolus can often also be delivered with the same device (Fig. 11.8).

Description

To administer the drug fluid, a micro-dosing pump is fitted with a filled drug container (or the container is filled if integrated) and connected to the transfer system. For certain therapies (e.g. insulin treatment of diabetes), this transfer system must be filled with drug fluid before the injection needle is placed on the patient's body.

After placement of the injection needle, the pump is switched to operating mode, causing it to deliver the predefined amount of drug fluid. Certain therapies (e.g. diabetes treatment) make use of micro-dosing pumps that permit the delivery of freely selectable boluses (e.g. after meals) in addition to the basal rate.

Fig. 11.8 Accu-Chek spirit combo

With most micro-dosing pumps, drug administration is effected by an electromechanical drive.

Micro-dosing pumps are widely used for therapy of diabetes and less important for protein therapeutics where predominantly administration of a high-volume bolus is required.

11.4 Drive Concepts for Mini-pumps

Protein products often have high to very high viscosity because of their concentration of active substance. For most of the concepts discussed below, the principle applies that, apart from larger drug container volumes, the size of the device also increases with the viscosity of the drug fluid because more powerful and hence larger actuators have to be used and the device's supporting structures have to be built more robustly. Alternatively, this can be avoided by enlarging the flow cross sections in the fluidic path, although this means that a larger-diameter injection needle has to be used which is not desirable from a convenience point of view.

11.4.1 Driven by Mechanical Actuator

Purely mechanical pump concepts can be implemented, for example, by using a pretensioned actuator element, as illustrated here by a mechanism with a compression spring (Fig. 11.9).

Description

The drug fluid is contained in a cartridge which is sealed with a needle-pierceable septum at the end of its outlet and has a sliding plunger at its open end. The actuator element consists of a pretensioned compression spring, which is released at the start

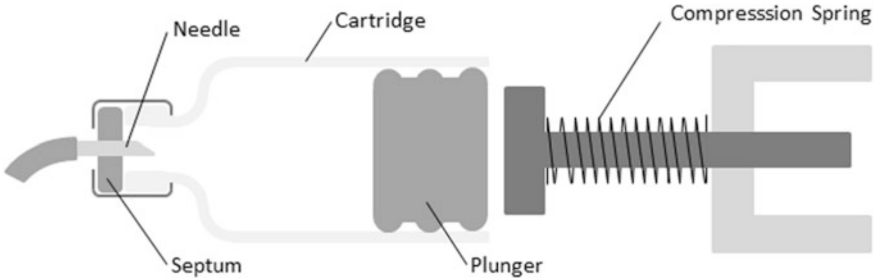


Fig. 11.9 Schematic representation of a compression spring-driven mini-pump

of drug delivery and then exerts a force on the plunger that pushes it towards the outlet, thereby expelling the drug fluid. Alternatively, a spring mechanism can act on a collapsible drug container (e.g. bag) to expel the drug fluid. This type of drive is characterized by a low level of complexity. The delivery rate, and hence the delivery time for a given amount of fluid, is determined by the spring characteristic, the viscosity of the fluid, the plunger friction and the geometry of the transfer system components, especially the injection needle, and can thus be predetermined by appropriate dimensioning of certain components. However, the tolerances of some of these parameters have a major impact on delivery time variation. The spring characteristic also makes it difficult to achieve a uniform flow rate.

With high-viscosity drug fluids, a powerful (spring) actuator must be used to deliver the dose volume within an acceptably short time. This actuator must be pre-tensioned throughout the storage period, a factor that must be taken into account in designing the components exposed to this force and which can negatively impact size.

11.4.2 Driven by Electromechanical Actuator

Electromechanical pump concepts feature an actuator element that is electrically driven and powered by a power source. They also often use an electronic control unit (hardware and software), permitting flexible programming of the pump.

High-viscosity drug fluids require a powerful actuator with corresponding power source in order to deliver the dose volume within an acceptably short time, and this can negatively impact size.

11.4.2.1 Syringe Pumps

Description

The drug fluid is contained in a cartridge which is sealed with a needle-pierceable septum at the end of its outlet and has a sliding plunger at its open end.

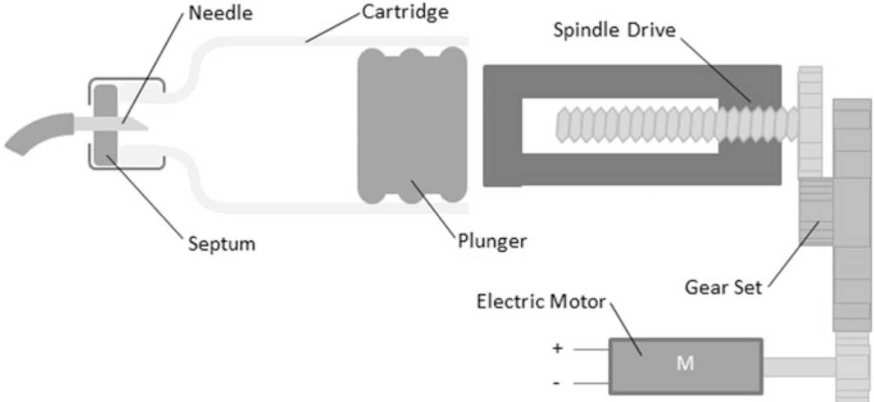


Fig. 11.10 Schematic representation of an electromechanically driven mini-pump

Alternatively, a prefilled syringe can be used as primary drug container. The actuator element consists of an electric motor whose rotational movement is stepped down via a gearbox and converted to linear movement with a spindle drive. The resultant impact force propels the plunger towards the cartridge outlet, thereby expelling the drug fluid (Fig. 11.10).
While this drive concept displays a certain level of complexity, it makes it possible to set a defined delivery rate almost regardless of fluid viscosity, plunger friction or other confounders and their tolerances, and low delivery time variation is achievable. With appropriate design, this concept also permits administration of minute quantities in the micro- to nanolitre range and is therefore suitable, for example, for use in micro-dosing pumps for the treatment of diabetes. The use of telescopic lead screws allows to limit the form factor and the overall size of device although adding complexity to the design.

11.4.2.2 Peristaltic Pumps

Description
The drug fluid is located in a collapsible drug container (e.g. bag), which is connected to the transfer system into the patient's body. Part of this transfer system consists of a relatively soft and flexible—and hence squeezable—length of tubing. Two plungers act as inlet and outlet valves. Between these valve plungers is a delivery plunger. Controlled actuation of these three plungers, e.g. with cam discs, produces drug flow in the squeeze tube, resulting in delivery of the bag contents into the patient (Figs. 11.11 and 11.12).
A variation of this principle is the carousel pump, in which the squeeze tube is arranged in a rough semicircle, along which at least three rollers attached to a disc follow a circular path. In this case, the rollers assume the function of the valve plungers and delivery plunger.

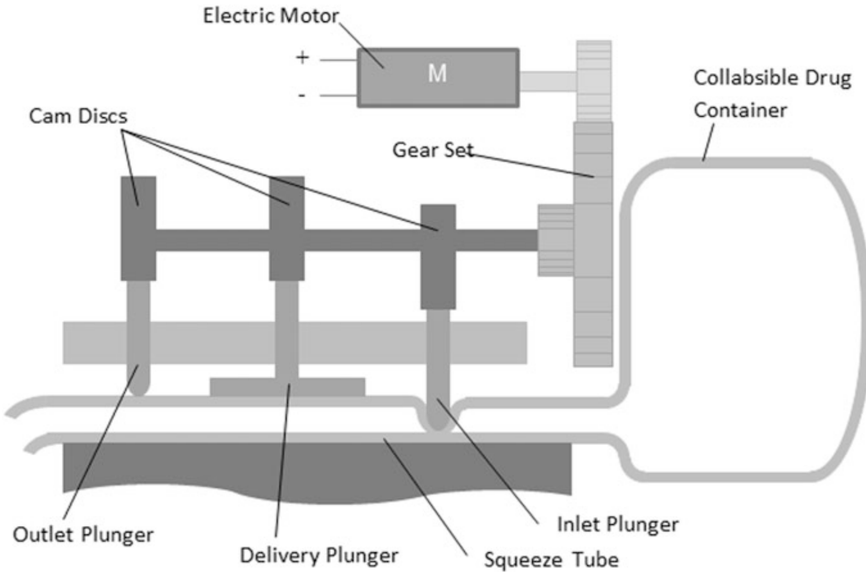


Fig. 11.11 Schematic representation of electromechanically driven linear peristaltic pump

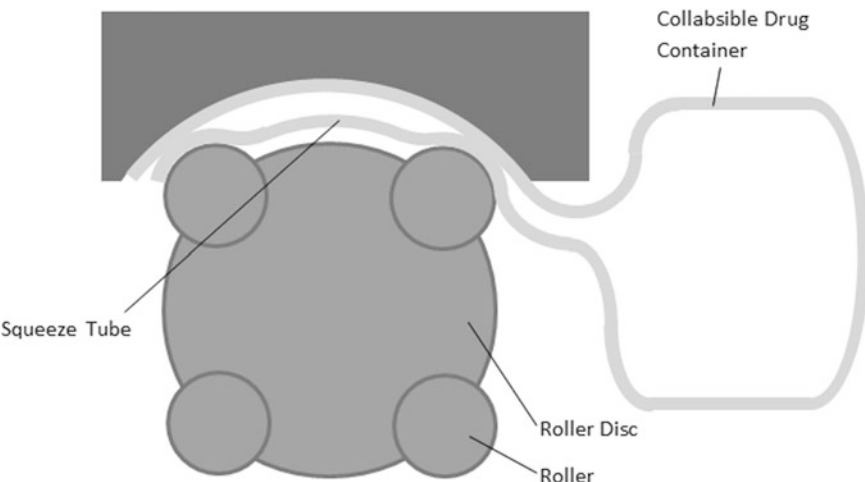


Fig. 11.12 Schematic representation of electromechanically driven carousel peristaltic pump

The delivery mechanisms explained above can be driven by an electric motor and, where necessary, an additional gearbox, which are connected to the cam disc in the case of the linear peristaltic pump and to the roller disc in the case of the carousel pump.

This drive concept is less suitable for micro-dosing than for delivering larger amounts of fluid. Examples include intravenous administration of drugs requiring delivery of amounts in excess of 50 ml within a few hours and parenteral nutrition in which several hundred millilitres must be delivered within a day and thus play not an important role for delivery of protein therapeutics.

11.4.2.3 Micro-pump Engines

The concept of a micro-pump engine represents an alternative approach which provides flexibility in the device design and has advantages for the overall size of the device due to the typically small size of the actual pump engine. Instead of compressing the primary packing or parts of the fluidic path in order to expel the drug, the fluid is delivered by means of suction. This concept enables amongst others the use of standard primary containers. Since, in contrast to the concepts where the drive is completely separated from the primary packaging and the fluidic path, the drug comes in direct contact and mechanically interacts with parts of the pump and, e.g. its lubrication, this concept has additional requirements with respect to compatibility and bears additional risks regarding impact on drug stability that must be examined thoroughly during development.

Description

Micro-pump engines typically consist of one or more movable parts that alternatively open and close a valve creating an underpressured cavity which sucks the drug from the reservoir, e.g. the primary packaging, followed by creating an overpressure in the cavity that then expels the drug. The pump itself represents an integral part of the fluidic path of the device. The drive of the pump engine is electrically powered and typically electronically controlled, which allows good control of the flow rate over a certain range.

11.4.3 Driven by User-Applied Force

In these pump concepts the force required to expel, the drug fluid is applied by the user him- or herself (Fig. 11.13).

Description

The drug fluid is contained in a cartridge which is sealed with a needle-pierceable septum at the end of its outlet and has a sliding plunger at its open end. The dosing element consists, for example, of a spindle drive in which a dosage can be manually preset by twisting a rotary knob. Manual pressure on the dosing button moves the entire dosing element linearly as far as it will go, which moves the plunger towards the cartridge outlet, thereby expelling the drug fluid.

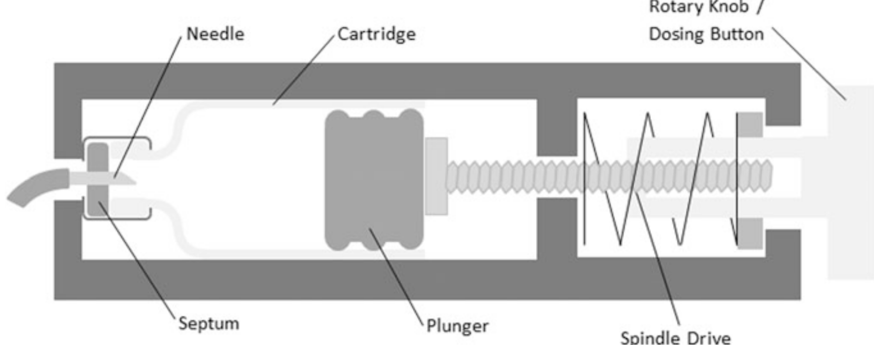


Fig. 11.13 Schematic representation of a pen drive based on a spindle drive

These and similar drive concepts are widely used in insulin therapy for diabetics, but are also familiar for additional applications in which smaller dose volumes are required. Dose volumes >0.5 ml or dosing of high-viscosity fluids is scarcely achievable with this concept because the user has to exert a force on the dosing button throughout the administration process. The force required for delivery is mainly determined by the viscosity of the fluid, plunger friction, return spring force and the geometry of the injection needle.

11.4.4 Gas-Driven

Gas-driven pump concepts use compressed gas as the actuator or possess an actuator in the form of a cell in which a gas is produced by a chemical or electrochemical reaction (Fig. 11.14).

Description

The drug fluid is contained in a cartridge which is sealed with a needle-pierceable septum at the end of its outlet and has a sliding plunger at its open end. The incoming compressed gas exerts pressure on the plunger, propelling it towards the cartridge outlet and thereby expelling the drug fluid.

Although this drive concept has a low level of complexity in terms of the principle employed, ensuring a hermetic seal poses a major challenge. The delivery rate is determined by the actuator characteristic, the viscosity of the fluid, the plunger friction and the geometry of the transfer system components, especially the injection needle.

This pump concept has not yet gained wide market acceptance.

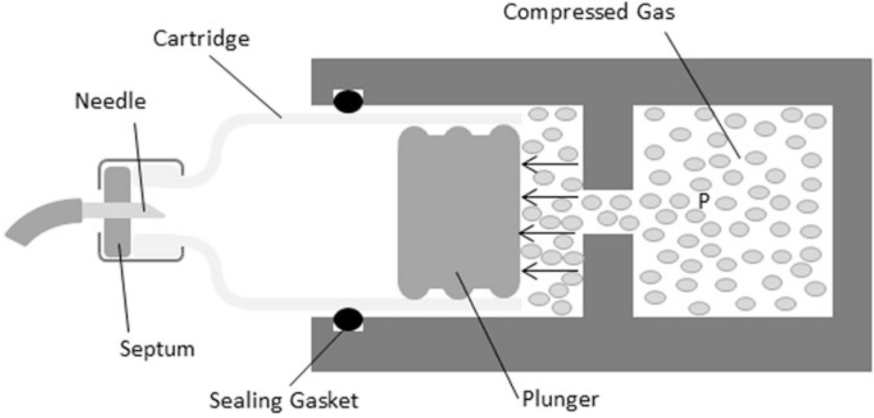


Fig. 11.14 Schematic representation of a gas-driven mini-pump

11.4.5 Other Drive Concepts

The last few years have seen intensive research and development on new pump concepts, as well as alternative actuator concepts. In some cases, the alternative actuator concepts are combined with traditional pump concepts, but there are also some revolutionary new approaches. The aim of these new concepts may be to further miniaturize the pumps or to gain a cost advantage by using cheaper components. Some of these approaches are briefly outlined below.

Shape Memory Alloys
Already on the market is a product that although using a conventional threaded spindle drive is driven by shape memory alloy wires instead of an electric motor.

Piezo Actuators
Various projects are working on the use of piezoelements as actuators. Approaches range from replacement of the conventional electric motor and possibly the spindle drive by rotary or linear piezoactuators to the so-called inchworm motor consisting of two piezoactuators for caulking the actuator at the cartridge internal diameter and an element to generate propulsion, or a so-called 'hula-hoop' threaded spindle drive.

Already in use are products with piezoactuators arranged in the form of a linear peristaltic concept, for example, in implantable micro-dosing pumps.

Balloon
Also known are concepts in which the drug fluid is pumped by an additional filling system into a balloon contained in the device either manually or automatically before use. The balloon then acts similarly to the purely mechanical drive using a compression spring described in Sect. 4.1.

11.5 New Trends in Mini-pumps

Drug Requirements
Among others, the need to support more highly viscous drugs and higher volumes is a driver for the development and industrialization of technically advanced drug delivery systems, primarily patch pumps. Latest developments suggest patch pumps which are capable of delivering several 10 ml of highly viscous fluids at considerable flow rates.

Platforms and Costs
Meanwhile, there is generally a trend towards technology and product platforms in order to achieve cost-efficient targets. Due to the nature of the targeted drugs, the administration routes and patient populations, it can be a significant challenge to meet all the individual needs at the same time. Thus, platforms are often a compromise between a bespoke solution for a specific use case (disease areas, therapeutic boundary conditions, target user groups, drug properties) and costs involved in the development, manufacturing and regulatory effort of the devices.

Device-Tailored Primary Packaging concepts
Since the integration with the primary packaging of the drug plays an essential role in the development of the devices, new drug container concepts, or even custom-tailored primary packaging design, e.g. plastic bags or tubes, might allow new and enhanced device designs.

Usability
Generally, there is a trend to make drug delivery devices more robust against use errors and consider usability aspects throughout the requirement engineering and design process. It is not sufficient that a device technically works properly; it must be easy to use for, in particular, the target user group, and the device design must contribute to prevent from unwanted use error under real-use environment conditions. This is typically achieved by usability and handling studies which generate input for the design process and therefore become increasingly important.

Industrial Design
When looking at the industrial design requirements driven by the user expectations, first of all small size and attractive design are key elements. This involves the desire for state-of-the-art user interfaces, including, for example, visual and audible feedback, which is in addition driven by usability considerations, on the one hand, and regulatory requirements on the other hand. In the next step, integration with personal electronics like mobile phones is conceivable, although this creates new hurdles on the design validation side.

Sustainability
Sustainability aspects and environmental awareness of the users are not least drivers for semi-disposable and loader concepts, which are perceived to be less harmful to

the environment. In addition, environmental considerations play an important role during material selection and design in order to allow for a viable waste management concept during the commercial phase.

11.6 Regulatory Considerations for Mini-pumps

Medical devices are, depending on type and classification, subject to a variety of regulatory requirements. Pumps for medicinal products are in general medical devices which fall under the 93/42/EEC Medical Device Directive in Europe and 21 CFR 800ff in the USA.

Many of today's Mini-pumps for biologics are drug products integrated with delivery system, e.g. prefilled single-use patch injectors. The regulation of such combinations is different, depending on the region. In Europe, if the device and the medicinal product form a single integral product which is intended exclusively for use in the given combination and which is not reusable is regulated as medicinal products according to Directive 2001/83/EC. However, relevant essential requirements of Annex I of Directive 93/42/EEC apply as far as safety- and performance-related device features are concerned [12].

In the USA, device-drug combinations are regulated as combination products according to 21 CFR Part 3.

In contrast, devices with exchangeable cartridges, for examples, reusable pumps are regulated in the EU as medical devices according to Directive 93/42/EEC, whereas in the USA as combination products according to 21 CFR Part 3.

Depending on the Primary Mode of Action (PMOA), it has to be decided which regulatory requirements need to be fulfilled in detail. Depending on the type of product, the intended use and the Primary Mode of Action, other requirements may apply, e.g. Directive 90/385/EEC Active Implantable Medical Devices for implantable pumps.

When developing, manufacturing and marketing medical devices, EN ISO 13485 in Europe and QSR/21 CFR 820 in the USA, describes the requirements for a Quality Management System which includes as well design and development control requirements.

A Risk Management (e.g. according to ISO14971) and a Usability Engineering Process (e.g. according to IEC62366) must be considered.

It is important to note that the complete regulatory requirements for each individual type of medical device must be evaluated thoroughly for all anticipated markets.

11.7 Conclusions

The specific properties of protein therapeutics, e.g. rheological properties in combination with particular usability needs, create new challenges for drug delivery devices and combination products. The major issue is to meet the technical requirements given by the drug, e.g. viscosity and volumes, and simultaneously satisfy these usability expectations. Mini-pumps for bolus injection contribute to keep pace with these growing demands. Mini-pumps are typically needle-based injection systems capable of delivering a wide range of drug volumes and viscosities, which help to cope with these increasing technical requirements. Different types of Mini-pumps are existing which differ particularly in the drive concepts. They can be hand-held or body-worn with replaceable or non-replaceable drug containers. Depending on the specific requirements of the therapy, the drug product and the user group, the most suitable type must be chosen for the individual application. For subcutaneous delivery of protein therapeutics, the following two device types are currently predominantly used: for smaller drug volumes, pen injectors, and for larger volumes, patch injectors. However, other device types and administration routes are conceivable and might be required for certain disease areas. Usability consideration plays an essential role throughout the entire development process, starting from the definition of requirements. Good knowledge about drug properties and the target user group is key in order to meet the usability needs and to prevent from use errors under real conditions.

In summary, Mini-pumps represent an effective and flexible tool in order to cope with the demands of protein therapeutics and continue to gain in importance in the future.

References

1. Elvin JG, Couston RG, van der Walle CF. Therapeutic antibodies: market considerations, disease targets and bioprocessing. Int J Pharm. 2013;440:83–98.
2. Aggarwal SR. What's fueling the biotech engine—2012 to 2013. Nat Biotechnol. 2014;32:32–9.
3. Shpilberg O, Jackisch C. Subcutaneous administration of rituximab (MabThera) and trastuzumab (Herceptin) using hyaluronidase, 2013.
4. Poiseuille JLM. Recherches expérimentales sur le mouvement des liquides dans les tubes de très petits diamètres. CR Acad Sci. 1840;11:1041–8.
5. Reynolds O. An experimental investigation of the circumstances which determine whether the motion of water shall be direct or sinuous, and of the law of resistance in parallel channels. Philos Trans. 1883;174:935–82.
6. Reynolds O. On the dynamical theory of incompressible viscous fluids and the determination of the criterion. Philos Trans Royal Soc London. A. 1895;186:123–64.
7. Zarraga E et al. High shear rheology and anisotropy in concentrated solutions of monoclonal antibodies. J Pharm Sci. Aug 2013;102(8):2538–49.
8. Rathore N et al. Pharmaceutics, preformulation and drug delivery - characterization of protein rheology and delivery forces for combination products, J Pharm Sci. Dec 2012;101(12).

9. Allmendinger A, et al. Rheological characterization and injection forces of concentrated protein formulations: an alternative predictive model for non-Newtonian solutions. Eur J Pharm Biopharm. 2014. https://doi.org/10.1016/j.ejpb.2014.01.009.
10. Dias C et al. Tolerability of high-volume subcutaneous injections of a viscous Placebo buffer: a randomized, crossover study in healthy subjects, AAPS PharmSciTech. Oct 2015;16(5).
11. Berteau C, et al. Evaluation of the impact of viscosity, injection volume, and injection flow rate on subcutaneous injection tolerance. Med Dev Evid Res. 2015;8:473–84.
12. COUNCIL DIRECTIVE 93/42/EEC of 14 June 1993 concerning medical devices, 1993L0042—EN—11.10.2007— 005.001.

Chapter 12 Container Closure Integrity Testing of Primary Containers for Parenteral Products

Shu-Chen Chen

Abstract Container closure integrity (CCI) is the ability of a container closure system—i.e., a package system—to provide containment and protection for the content inside. Ensuring the CCI of a parenteral product package is essential during the entire lifecycle of a product. The chapter first reviews the U.S. FDA regulatory requirements on CCI. Regulations for several regulatory authorities outside the USA are also presented for comparison. From these regulatory requirements, attributes of CCI test are discussed. The chapter is then devoted to two aspects of CCI tests—(1) selection criteria for the test method to use and (2) introduction of six different methods. Selection criteria cover sensitivity and reliability of the method, destructive versus non-destructive methods, inline monitoring versus off-line test as well as the material of construction of the primary container. Six methods reviewed are microbial challenge test, liquid tracer leak test, vacuum decay leak test, electrical conductivity leak test, tracer gas leak test, and headspace gas analyzer. Finally, the development, qualification, and validation of a method to be used are discussed. Each CCI test method has its advantages and disadvantages. The selection of an appropriate CCI method depends on the purposes and requirements of the test. In the Summary section, comparisons on the pros and cons for the various methods are presented.

Keywords Container closure integrity · Leak · Test method · Sensitivity Reliability · Destructive · Qualification · Validation

S.-C. Chen (✉)
Agoura Hills, USA
e-mail: sychen100@gmail.com

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*, AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_12

12.1 Introduction

12.1.1 Purpose of Container Closure Integrity Test

Container closure integrity (CCI) is the ability of a container closure system (i.e., a package system) to provide containment and protection for the content inside. It is an important quality attribute for a package to safeguard and maintain its content to the required specifications for various industries. For example, CCI of a food package can prolong food freshness by limiting water loss, CCI of a drug package can preserve content safety by preventing contaminant ingress, CCI of a nuclear waste package can protect environment by keeping toxic materials sealed, and CCI of a petroleum drum can curtail oil degradation by reducing oxygen transmission.

Container closure integrity is especially critical for parenteral product package due to the mode of administration of the parenteral product to the user. CCI can prevent microbial ingress, maintain product sterility and stability, and retain drug efficacy. Most importantly, it ensures the safety of patients who use the products. A non-efficacious and non-sterile drug not only loses medicinal and therapeutic effects but also poses potential threats to the well-being and life of patients. The serious consequence with the loss of CCI has made it a critical defect in quality controls for parenteral product release. Numerous factors, from the material selection at early stage of container design to the storage conditions at final stage of product shelf life, can affect CCI of a package system significantly. Therefore, ensuring the CCI of a parenteral product package is essential during the entire lifecycle of a product.

12.1.2 Primary Container Closure System for Parenteral Products

A parenteral package system includes various components that keep the content safe and effective until time of use. According to FDA's guidance for packaging human drugs and biologics, a *container closure system* refers to the sum of package components that together contain and protect the dosage form. ***A package system*** is another term equivalent to ***a container closure system***. A package system typically consists of primary package components (or primary package system) that are in contact with the product content and the secondary package components (or secondary package system) that are used to provide additional protection to product during transportation and distribution. This chapter will focus on the primary package components used for parenteral products in pharmaceutical and biological industries. Therefore, a package system or a package used hereafter refers to a primary package system or a primary package.

Vial, syringe, and cartridge are various primary package systems used for the finished parenteral products in pharmaceutics and biologics. The components for

vial package system normally comprise of a vial, a stopper, and a flip-off aluminum cap. The components for syringe package system include a syringe barrel, a plunger-stopper, and a luer lock or a stacked needle with shield. And the components for cartridge package system typically consist of a barrel, a plunger-stopper, and a diaphragm encased in a cap for needle penetration. In this chapter, the term ***primary container*** is also used for the three ***primary package systems vial, syringe, and cartridge***.

Borosilicate Type I glass is still the main material of construction for majority of the primary containers used in parenteral products, although plastic has gained significant recognition and usage recently due to easy breakage of glass. Plastic container also has the advantage of being free from silicone oil coating, which is needed for extruding the content from a glass syringe.

12.1.3 Container Closure Integrity Test for Parenteral Products

A container closure integrity (CCI) test for parenteral product is to detect flow in and out the container through a leak path that can lead to non-sterility. ***CCI test*** is often interchangeably used with ***leak test*** which is a method that detects leak path on a package that allows the escape of content inside a container to the environment or the entry of foreign materials from environment into the container.

A CCI test is conducted under specified test conditions using selected equipment. Test result measures the degree of leakage. For a CCI test that quantitatively measures the amount of gas flow, the degree of leakage is usually expressed as the amount of gas flow per unit time. When the amount of gas flow is expressed in volume, the conditions for gas volume need to be stated because gas volume depends on temperature and pressure. Often the detected leakage flow is converted and expressed in terms of ***hole size*** on a thin orifice plate for easy conceptualization on the degree of defect.

The CCI test of a parenteral product is to be conducted throughout its lifecycle prior to use to ensure sterility of the content inside the package. During the manufacturing process, the container integrity is typically checked by a vision inspection system, which usually limits to detectable leak size of larger defects from chips, cracks, and breakages. Some manufacturing process does employ leak detection system that is capable of detecting smaller leaks for 100% online monitoring. However, it is often limited to detection technologies that require packages with certain conditions, such as the nitrogen-blanketed package. With recent FDA guideline on CCI test in lieu of sterility test, the CCI test is now included in the stability study. The CCI test is also performed frequently in forensic investigations when concern on product sterility is raised due to exceptional conditions, such as temperature excursion during transport. In these applications, the good-quality products can often be saved by passing a non-destructive CCI test.

The CCI test provides a valuable means for monitoring product quality throughout its entire lifecycle. Therefore, selecting a proper CCI test is critical. The suitability of a CCI test depends on the use, requirement, and timeline of the parenteral product. These parameters that influence the selection of a CCI test are discussed in Sect. 12.3.

12.1.4 Background on Container Closure Integrity Test and Method

The manufacture and distributions of pharmaceutics and biologics are government-regulated industries. The regulations in the USA clearly state a container closure system that permits penetration of microorganisms is unsuitable for a sterile product. Safeguards should be implemented to strictly preclude the delivery of products that may lack CCI and lead to non-sterility. Other countries in the world also have similar regulations on sterile drugs, for example, the *Manufacture of Sterile Medicinal Products* in the European Union, the *Ministerial Ordinance on Standards for Manufacturing Control and Quality Control for Drug and Quasi-Drugs* in Japan, and the *Food and Drugs Act and Regulations* in Canada.

Historically, the sterility of drug products has long been evaluated and verified using microbial challenge with media-filled containers as part of aseptic process validation prior to product manufacturing. The sterility for product lifecycle was also evaluated and demonstrated through microbiological challenge with sterile outcome on the stored products. This traditional microbial challenge test for sterility verification, however, has scientific and practical limitations according to FDA guidance; it can only detect viable organisms present at the time of tests, the viable organisms present at the time of test can only be detected if they are capable of growth in the culture media used, and it is destructive and prone to contamination. A negative finding in sterility test does not guarantee the integrity of a container closure system throughout the product lifecycle.

In 2008, FDA issued the guidance on CCI test in lieu of sterility test for stability study. The guidance suggests the CCI be maintained and demonstrated throughout the product shelf life in addition to sterility testing. EudraLex Volume 4 (EU Guidelines to GMP Medicinal Products for Human and Veterinary Use) in 2008 also indicates container integrity should be checked with appropriate procedures. With these guidelines and the limitations of microbial challenge, physicochemical test for container closure integrity has been used by many companies to replace the microbial challenge.

A wide range of instruments for physicochemical tests are available in the market. Many companies in pharmaceutical and biological industries have used them to develop, validate, and implement the physicochemical test methods for CCI of parenteral products within the last decade. The major and frequently used physicochemical methods as well as the traditionally used microbial challenge for leak detection of primary containers in pharmaceutics and biologics industries are presented and discussed in Sect. 12.4.

12.2 Regulatory Requirements on Container Closure Integrity

12.2.1 Regulations on Sterile Drugs

12.2.1.1 US Federal Regulations on Sterile Drugs

For the protection of public health, sterile pharmaceutical and biological products are required by federal laws under ***Title 21—Food and Drugs*** and ***Title 42—The Public Health and Welfare*** of the United States Code (U.S.C.). They provide the federal statutes for the requirements of releasing human drugs and biologics to the markets. These requirements are legally enforced under the Code of Federal Regulations (CFR). Title 21 of the CFR is the portion that governs food and drugs within the USA. Three chapters are provided in Title 21 CFR with each chapter, respectively, for the Food and Drug Administration (FDA), the Drug Enforcement Administration (DEA), and the Office of National Drug Control Policy (ONDCP). Most of the regulations in Chapter I for the FDA are based on the ***Federal Food, Drug, and Cosmetic Act (FD&C Act)*** of Chapter 9 in Title 21 of the U.S.C. Since biologics is also covered under Title 42 of the U.S.C., FDA's regulatory authority for biologics in Title 21 CFR resides in the ***Public Health Service Act (PHS Act)*** under Chapter 6A of Title 42 U.S.C. In short, human drugs and biologics in the USA are regulated by Title 21 CFR under the FDA, Department of Human and Health Services. The two centers in the FDA, *Center for Drug Evaluation and Research* (CDER) and *Center for Biologics Evaluation and Research* (CBER), oversee and execute these regulations for human drugs and biologics. Another center in the FDA, *Center for Device and Radiological Health* (CDRH), will also carry out the enforcement of these regulations to pharmaceutical and biological industries if the drug or biologics is part of a combination product which includes a medical device, and the device is the primary mode of action that provides the most important therapeutic function of the combination product.

In Chapter I of Title 21 CFR for the FDA, Subchapters C (the 200 series), D (the 300 series), and F (the 600 series) are regulations pertaining to pharmaceuticals and biologics. More specifically, the regulatory controls on containers and closures are covered in Part 211—*Current Good Manufacturing Practice for Finished Pharmaceuticals* and Part 600—*Biological Products: General*. Section §211.94(b) in Subpart E states the container closure system shall provide adequate protection against foreseeable external factors in storage and use that can cause deterioration or contamination of the drug product. Section §600.11(h) in Subpart B also iterates the requirements that the containers shall be sealed to maintain the integrity of the products during the dating period. In addition, the containers and closures for products intended for use by injection shall be sterile and free from pyrogens.

The FDA often issues documents to provide guidance for industry on general principles of the regulations. While such guidance represents FDA's current thinking on the topic, it is not a legally binding document. There are several FDA

guidance documents pertaining to container closure integrity for maintaining sterile drug products. The three main ones are "*Container Closure Systems for Packaging Human Drugs and Biologics*," "*Sterile Drug Products Produced by Aseptic processing—Current Good Manufacturing Practice,*" and "*Container and Closure System Integrity Testing in Lieu of Sterility Testing as a Component of the Stability Protocol for Sterile Products.*" Again, these guidance documents affirm that a well-sealed container closure system shall provide protection from microbial contamination (loss of sterility or added bioburden) and maintain product integrity (prevent loss of product and efficacy) over its shelf life. The container closure system should be monitored, and the evaluation of the packaging system should be included in the stability protocol.

12.2.1.2 Regulations Outside the U.S. on Sterile Drugs

Other countries have similar requirements in GMP pertaining to the manufacturing and quality controls of sterile drugs. For products supplied to countries outside the USA, the CCI test shall also meet the specific rules and regulations required in the marketed countries. Table 12.1 shows the correspondent regulations and agencies to CFR and FDA for several other countries.

With the requirement on demonstrating the maintenance of drug sterility through a CCI test, a number of standards and references from various organizations (ASTM, ISO, USP, EP, etc.) are available to facilitate the implementation and execution of various CCI tests for meeting regulatory compliance. Information from these documents provides useful guidelines for evaluating, developing, and

Table 12.1 Example of regulations and regulatory authority outside the USA

Country	Regulations	Regulatory body/authority
Australia	Therapeutic Goods Regulations 1990 (Therapeutic Goods Act 1989)	Therapeutic Goods Administration (TGA) of Department of Health and Ageing
Brazil	Resolution RDC 17/2010 (Drug GMP) Market Regulations	National Health Surveillance Agency (ANVISA) of Ministry of Health
Canada	Food and Drugs Regulations (Food and Drugs Act)	Therapeutic Products Directorate and Biologics and Genetic Therapies Directorate, Health Products and Food Branch of Health Canada
European Union	EudraLex from European Commission	Respective regulatory body in each country (e.g., Federal Institute for Drugs and Medical Devices of Ministry of Health in Germany)
Japan	Ministerial Ordinances of MHLW	Pharmaceuticals and Medical Devices Agency (PMDA) of Ministry of Health, Labour and Welfare (MHLW)

validating a selected CCI method. However, each container closure system needs to select a suitable CCI method first, which depends on the requirements, specifications, and applications of its content.

12.2.2 Attributes of Container Closure Integrity Test from Regulatory Requirements

12.2.2.1 Test on Container with Product Content

FDA guidance states the CCI test shall be conducted on product-filled containers to provide the true status of container closure integrity. Depending on the material of construction of the container closure system, some unexpected interference between container package material and parenteral product could occur over the dating period, resulting in degradation of material and thus compromising the integrity of the closure system. Testing samples filled with actual product is a true assessment of the CCI as any compromise in CCI due to the interactions between product and container closure system over the shelf life can only be detected by using these samples.

The CCI test using the conventional microbial challenge requires the container filled with growth promotion media. As a result of using such media, the test is conducted on container that has no contact to the actual product. However, in some cases, a CCI test cannot be done on container that holds the actual product, for instance, the product contains a material which biases the CCI test result. If no suitable CCI test is available for container filled with actual product, then the use of media-filled containers may be acceptable providing no adverse interaction between product and container exists.

12.2.2.2 Test on Container Over Product Lifecycle

Historically, the CCI test was performed only at the beginning of product lifecycle, and sterility test was performed over the product lifecycle. Since the sterility of a product does not guarantee the integrity of a container and container integrity is a critical quality attribute for parenteral product, the CCI should be assessed, monitored, and controlled during the entire lifecycle of the product. The lifecycle starts in the design of primary container at the beginning stage of package development, continues through the routine manufacturing process, and finishes at the end of the product shelf life. In the beginning stage of package development, the requirements for product content and package's properties, function, manufacturing, storage, stability, shipment, distribution, and human factors all need to be considered to ensure the CCI of the package. Applicable test methods for the content and package shall be evaluated during design process, and a suitable method is selected,

developed, and qualified. The selected test method is then used to challenge the final design of the container under normal and potential extreme conditions for a successful pass of the CCI test.

Proper in-placed process controls for routine manufacturing can reduce the occurrence of container integrity failure. The vision inspection system commonly used for cosmetic defects can monitor and minimize the risk of accepting leak containers. However, these procedures cannot eliminate all potential CCI failures. Leak test, either 100% non-destructive online or statistically significant in-process sampling, should be conducted to supplement the process control and vision inspection for confirmation of container integrity prior to product release.

Interaction between package materials and drug content or degradation of package materials could occur over time and affect material's sealing property. This issue might subsequently impact the integrity of the package system, yet not discovered immediately post-filling of the content. A slow interaction or degradation might not even be detectable until product reaches the end of its long shelf life. Therefore, it is essential, as well as required by the regulations, that container integrity be evaluated in stability study over product shelf life to ensure no detrimental effects occur over storage period to compromise container integrity and cause harms to patients.

12.3 Selection Criteria for Leak Test Method

A particular leak test method may not be applicable to all product packaging systems. The selection of a leak test method depends on the attributes of the method, manufacturing of the product, intended use of the content, and configuration, material and design of the container closure system. FDA guidance on validation of an ***analytical*** test recommends the characterizations of a method's accuracy, specificity, detection limit, linearity, range, precision, and robustness. Some of the characteristics may not be applicable for a ***physicochemical*** test. For example, the specificity is not a relevant characteristic for a non-chemical test. Critical characteristics such as detection limit (or sensitivity) and precision (or reliability) shall be considered at the minimum for a physicochemical method. In addition, method type (destructive or non-destructive), method applicability (online or offline), product content (small or large molecules), and container's material of construction (glass or plastic) are other important factors to consider in selecting a suitable leak test method for primary containers to meet the specific application and objective. These parameters are discussed in details below.

12.3.1 Sensitivity or Detection Limit

A leak test is to measure the leak rate from the inside of a container through a leak path out. The measured leak rate is often converted into an equivalent flow rate

through an orifice on a thin plate under the same test conditions. For easy conceptualization, the *sensitivity* of a method is then commonly expressed as the *smallest size of an orifice* (*a hole size*) the method can detect. The smaller a leak size a method is capable of detecting, potentially the higher the sensitivity of the method.

The sensitivity of a method should not be confused with sensitivity of an instrument. A test method is an integral system that consists of instrument(s), test parameters, test procedures, and peripheral equipment such as test chamber, connecting tubing, flow meter and pump, etc. Sensitivity of a test method is usually lower than that of the instrument used in the integrated system which contains additional components. The difference between the two sensitivities will be further illustrated later in the sections where the methods are discussed.

Another misunderstanding on the method sensitivity is how the sensitivity of a method can be claimed. The sensitivity of a method is the lowest achievable leak detection for the *entire* primary container system. A method cannot claim a sensitivity of a certain leak size if this leak size can only be detected at an easy location on the primary container closure system (i.e., syringe barrel), but not at a difficult location on the system (i.e., plunger-stopper of a syringe).

The ability to verify the sensitivity of a method also affects its attainable sensitivity. It is necessary to demonstrate the method will consistently reject defects at the claimed sensitivity, which requires reproducible and well-defined defects at the sensitivity to be verified for testing. Since laser-drilled hole has a well-defined leak size and is the closest mimic to a realistic torturous leak path, it is often used on the barrel of a container as defect. The current laser-drilling technology is capable of creating small leaks of approximately 5 ± 1 μm with reasonable reproducibility. Therefore, the sensitivity of leak tests for parenteral containers is commonly validated as a nominal 5 μm orifice despite the higher sensitivity of some instruments.

No container can be claimed as *absolute* zero leak. The elastomer seal of a container can permeate minute gas flow and thus a tiny gas leak. The adequacy of a method's sensitivity depends on the requirements of product quality specifications and the availability of leak detection technology. For some applications, a method with lower sensitivity (higher leak rate) might be acceptable as only the sterility of the product needs to be preserved. In other applications, a higher sensitivity might be necessary to prevent adverse effect on product efficacy and stability due to leak. Once the sensitivity (i.e., allowable leak rate) for the intended use is established, method selection can be narrowed to technologies that are capable to deliver the required performance.

12.3.2 Reliability or Precision

The reliability of a test method indicates the variations of the test results under specified test conditions. The higher the reliability of a test the lower the variation in results will be. Various terms such as *repeatability*, *intermediate precision,* and

reproducibility are used to express different sources for the variation of data from a test method. In *analytical* method for laboratory measurement from the ICH Q2, repeatability accounts for the variations over a short interval of time under the same test conditions; intermediate precision evaluates the variations in data from tests by different analysts, in different days and using different instruments; and reproducibility examines the variations between laboratories.

These terms are defined differently in the Reference Manual of Measurement Systems Analysis (MSA) from the American Society for Quality Control (ASQC) and the Automotive Industry Action Group (AIAG). Here, the *repeatability* is defined as the variation in measurements obtained by an operator on one sample with the same gage several times, and the *reproducibility* is the variation in the average of the measurements made by different operators using the same gage on one sample.

Regardless of the various definitions of the terminologies, the bottom line is that the method has to be able to produce consistent results (i.e., within the allowable variations) when performed by different operators, at different time, with different machines, and at different sites.

Reliability of a method is critical to prevent false conclusion. It should be verified with statistically significant confidence using sufficient sample size, randomized sample order, and alternating operator orders. This is especially crucial for test that is highly probabilistic and provides only attribute (i.e., pass/fail) data. This will be further illustrated in the sections where the various methods are discussed.

12.3.3 Destructive Versus Non-destructive Methods

The destructive or non-destructive nature of a test method can influence its application and selection of a CCI method. It is more desirable to use non-destructive method as it offers the advantages of cost saving and preservation of samples. The destructive method causes the loss of potentially good products and packaging components, does not offer the opportunity to reexamine the samples, and does not allow the execution of other tests on the same samples. More than one test on a sample is important when link between tests on the same sample is needed as in forensic investigation. However, the destructive method may be the only choice due to certain limitations or requirements of the product.

12.3.4 Contents Inside Primary Container

Drug product typically is not the only content inside a primary container. Moisture, air, or nitrogen can be present in the headspace of a container. The type, property, and quantity of the contents inside a container can affect the function and therefore the selection of a CCI test method.

The liquid product inside a container is often substituted by water during CCI method development because the product is expensive and hard to obtain. The use of water may not represent the worse-case conditions for leak detection of the method selected. The selection of a method should be checked using the container with actual product inside to ensure the selected method is appropriate for the targeted product and container system.

12.3.5 Inline Manufacturing Monitor Versus Offline Laboratory Test

Cycle time of a test is another factor to consider when selecting a test method for different end uses. The processing speed in fill-and-finish and stoppering-and-capering for drug manufacturing is typically in the range of hundreds of containers per minute. The cycle time of a CCI test for inline application has to be comparable to this production speed. Reducing the throughput due to CCI test may require major modification of production lines thus can be costly for business.

A method that can be in sync with production speed but with slightly lower reliability is more preferable than one with a higher reliability but at a speed too low to be good for 100% inline use. Tighter acceptance criteria can be set for the faster method to compensate for the slightly less reliable result. Although this approach can potentially reject good containers, it maintains production speed while still keep container integrity in check for the passing products.

On the other hand, in a stability study a more reliable method, even slower, is more appropriate for the CCI test. A longer test cycle is less a concern for stability protocol as the study typically has a limited number of samples. Applying a less reliable method to the limited samples in a stability study can be problematic. If a non-leaker is falsely identified as a leaker in stability study due to the unreliable data or unnecessarily tight acceptance criteria, it can trigger a false non-conformance and needless investigation for the marketed products.

Therefore, depending on the purpose of the application, whether a test method can be inline 100% manufacturing monitor or offline sampling test will influence the selection of the method.

12.3.6 Primary Container Material of Construction

Traditionally glass has been used as material of construction for primary container of parenteral product. Glass is usually considered as impermeable and does not absorb tracer gas (usually helium) or liquid (such as dye) in method that uses tracer. But the elastomer material that provides sealing function for the glass containers

(such as the stopper in vial and the plunger-stopper in syringe) may absorb tracer gas and outgas during test cycle to give false result.

Plastic is now gaining recognition as the material of construction for the parenteral containers. Rigid plastic used for primary containers usually has lower permeability than the elastomer seal material. However, a primary container has a much larger surface area compared to an elastomer seal. It may absorb sufficient amount of trace gas in a test. The absorbed gas is then released during test cycle for the leak detector to register it as a leak to lead to false result. Furthermore, plastic material exhibits high propensity of absorbing color dye. As will be discussed in further details in a later section on test method, absorption of dye by the primary container could bias or impair the method to be selected. Therefore, before a test method that uses tracer is selected studies are required to ensure that the elastomer or plastic materials to be used do not influence the test result.

12.4 Container Closure Integrity Test Method for Primary Container

The test methods presented in this section include the frequently used physicochemical tests and the traditionally used microbial challenge for rigid primary containers. The general principles, applications, advantages, and challenges of these tests are discussed. Some exemplary data are provided to further illustrate different features of each method.

12.4.1 Microbial Challenge Test

Microbial test, one of the earlier and widely used methods for verifying the sterility of drugs, has two types—immersion or aerosol. In the immersion test, the containers to be tested are filled with media solution and then submerged in a vessel containing nutrient broth and the motile-indicating microorganisms. Upon completion of the exposure period, the containers are incubated and the media solution inside the containers is examined for turbidity. The turbid containers are sub-cultured on media to confirm the presence of the indicating microorganism. Sterility or CCI is demonstrated when the challenged container shows no turbidity, indicating no presence of the microorganism inside the container. The aerosol challenge is performed in a similar procedure. Instead of immersion, however, the media-filled samples are exposed to aerosolized microbial spores in a high-bioburden environment for an allotted time.

Since the microorganism used in the challenge is extremely small, a leak size of 1 μm can potentially be detected if the organisms migrate through the leak path. With this feature, the method may seem to have a high sensitivity. The success of

leak detection by microbial challenge, however, depends on several other factors; the chances for the microbes to find and traverse the leak path and the wetness of the entire leak path. Due to such unpredictable movement of microorganisms and the potential air blockage of the micro-channel, microbial method has been shown to be probabilistic. Figure 12.1 shows an example of the unrepeatable nature of the results from a microbial immersion challenge. For each leak size, up to three separate tests were conducted under the same test conditions using containers with extremely small leaks. The leak rate in the figure is presented as vacuum decay rate. The range of leak rate represents a range in hole size of approximately from 0 to 5 μm. In the figure, a generally increasing trend in the percentage of microbial ingress is observed with increasing leak size. However, the percent positive units varied significantly from test to test on leaks of similar size.

Various studies have been published in an attempt to correlate the leak size to percent positive leak detection. Unfortunately, the probability of microbe ingress, limited sample size, and types of microorganism used in the studies prevented such correlation attempts from reaching a firm and consistent conclusion, thus, making the determination of method's true sensitivity difficult.

Several other shortcomings of the method such as microorganism type, destructive and contamination prone nature tend to drive the leak detection for primary containers toward other physicochemical tests. Nevertheless, in some instances, microbial challenge may be the only viable CCI test. One such example is the case of an intricate auto-injector device. The following factors of an auto-injector—numerous movable parts, obstruction of container headspace, difficulty of preparing the positive control, and potential compromise of container integrity from device disassembling procedures—limit the use of other test methods for its CCI test.

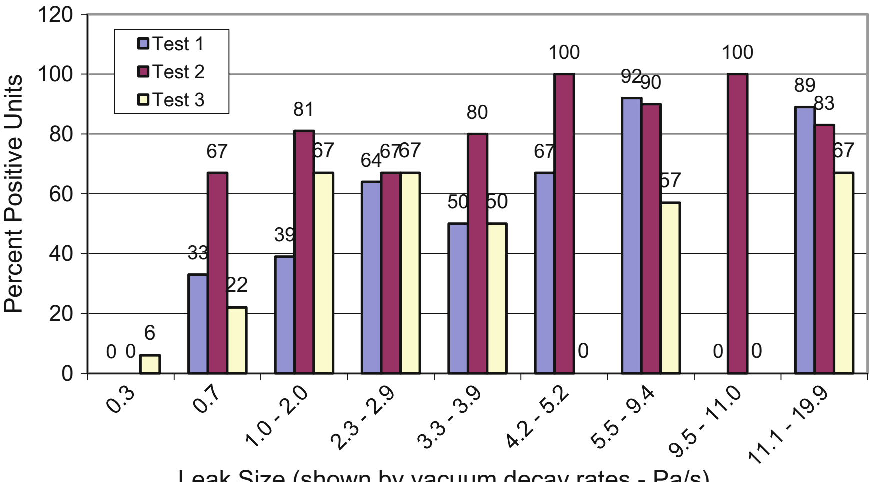


Fig. 12.1 Probability of positive microbial ingress on various leak sizes

12.4.2 Liquid Tracer Leak Test

Liquid tracer test, or commonly called dye penetration or dye ingress test, is an easy, fast, and inexpensive way for leak detection. Figure 12.2 shows the typical setup for a dye penetration test. The test starts by placing the challenged samples fully immersed in a tub holding the dye solution (or liquid tracer). The dye solution tub is placed inside a pressure chamber and subjected to a specified vacuum pressure for an allotted time. The pressure in the chamber is then restored to atmosphere for another specified interval. In some cases, the samples in dye solution tub are subjected to an additional over-pressure for an extra duration prior to being removed from the test chamber. The dye solution tub is then removed from the test chamber. The samples are then removed from the dye solution, and the external surfaces of the samples are thoroughly rinsed and dried. The samples are examined for dye penetration into the interior using visual inspection or UV/Vis spectrometer by comparing them to the negative controls.

The most commonly used liquid tracers are Methylene Blue and Rhodamine B. The USP <381>, EP 3.2.9, ISO 8362-5, and ISO 8871-5 all mention the use of methylene blue dye as a liquid tracer for closure integrity and self-sealing tests. Testing conditions in these documents were slightly different in previous releases,

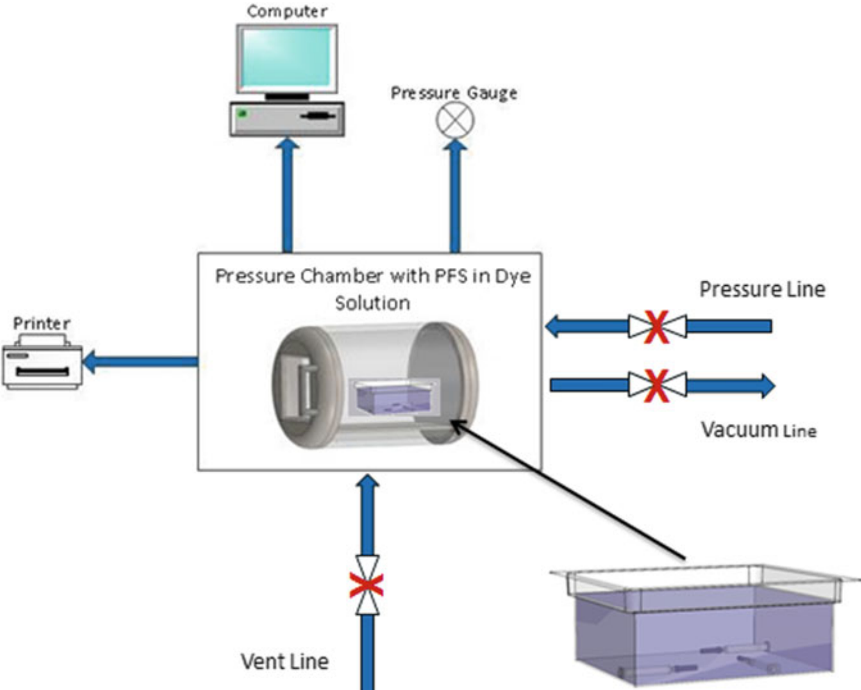


Fig. 12.2 Typical setup for dye immersion test (syringe shown as test sample)

Table 12.2 Pressure profile and test duration for methylene blue dye test

Source document	Sample size	Exposure at reduced pressure		Exposure at atmospheric pressure
		Vacuum pressure	Duration	Duration
USP<381>, EP 3.2.9, ISO 8362-5 and 8871-5	10	−27 kPa gauge	10 min	30 min

but are synchronized in the latest versions. Table 12.2 shows the test conditions and sample size from these documents. Significant modifications on these test conditions have been made by many companies to increase the sensitivity of the test method. In addition to the extra over-pressure and test duration mentioned above, one major change to improve the sensitivity of the method is the use of surfactant in the dye solution, which reduces liquid surface tension to facilitate easier ingress of dye solution.

One critical requirement missing from the documents in Table 12.2 is the positive control for the test. Negative and positive controls in an assay are essential to ensure that the testing system functions properly at the time of test. The test may be questionable if no positive control is used to demonstrate the test article is actually challenged during the test. Without the confirmation of a positive control, negative result of a test sample could be due to the insensitive or probabilistic issue of the method. The use of validated positive control can rule out these uncertainties.

Due to the poor sensitivity and low repeatability of dye penetration under the test conditions of Table 12.2, the method has been shown to require relatively large needles as positive controls to assure consistent 100% dye ingress in positive controls. Figure 12.3 shows an example of the inconsistent dye penetration result. Eighty syringes, each contained a "micro" leak of same size, were tested in a single run using test conditions of 30 min of 9″Hg vacuum and 30 min of ambient pressure. Significant variation in the amount of methylene blue dye penetrating through the leaks was indicated by the huge difference in shades of blue color.

Despite the above drawback of the liquid tracer test, the test can be a valuable tool for the detection of extremely small leak during container development process. When the pressure exposure time was increased significantly to hours or days, additional positive pressure was added to the test cycles and higher concentration of surfactant was used, the dye ingress test actually detected leaks that were considerably smaller than 5 μm. Under the test conditions of Table 12.2, liquid tracer test is a simple method for detecting larger leaks, but not a sensitive method for detecting small leaks consistently, thus not an excellent CCI test method for sterility confirmation of parenteral products. However, it is a useful tool during container development for checking container integrity. The inconsistent outcome can be compensated by using severe test conditions of high vacuum, high over-pressure, extremely long test duration, high concentration of surfactant in dye solution, and substantially large sample size.

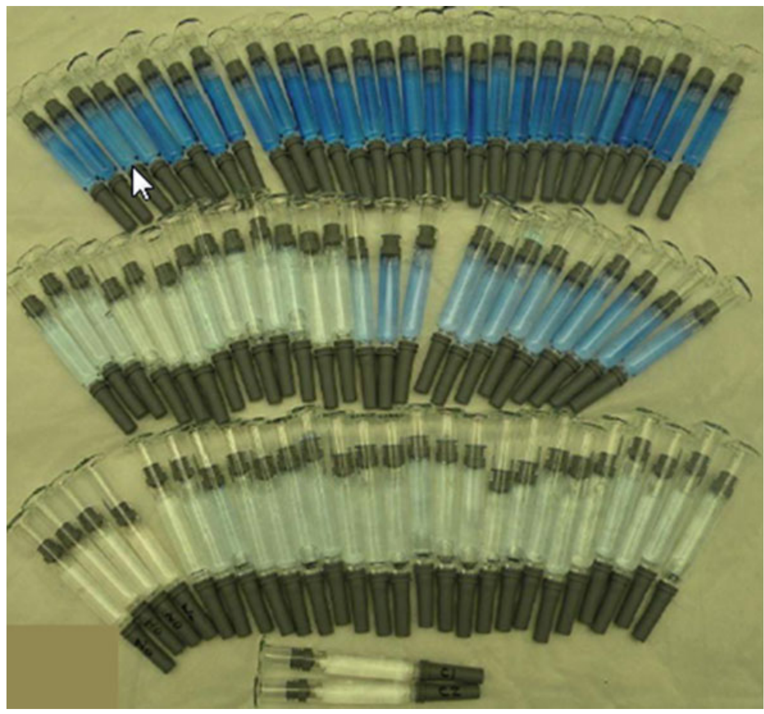


Fig. 12.3 Variation of blue color in 80 syringes with same leak size from a single methylene blue dye penetration test. Bottom two is negative control

12.4.3 Vacuum Decay Leak Test

Vacuum decay test utilizes the principle of pressure change for leak detection. Setup of the test system generally includes a pressure monitor device, a test chamber, and vacuum mechanism as illustrated in Fig. 12.4. There are three stages in the leak test; evacuation, equalization, and test cycles. The system applies vacuum inside the test chamber containing a sample during evacuation cycle. Test chamber is then isolated from the vacuum source by the normally closed fill valve. The absolute pressure transducer monitors the vacuum level in the closed system during equalization cycle. After the equalization cycle, the normally open bypass valve closes and the change of vacuum level in test chamber over the predetermined test cycle is measured by the differential pressure transducer. The vacuum decay rate is correlated to the leak rate, which in turn determines if a leakage is present in the test sample based on a preset acceptance criterion.

Vacuum decay test detects leak from any location in a container, thus the leak location cannot be isolated from the single test. But the simplicity and robustness of the test makes the method attractive. The test method is used in ASTM F2338, an FDA recognized consensus standard for non-destructive detection of leaks in

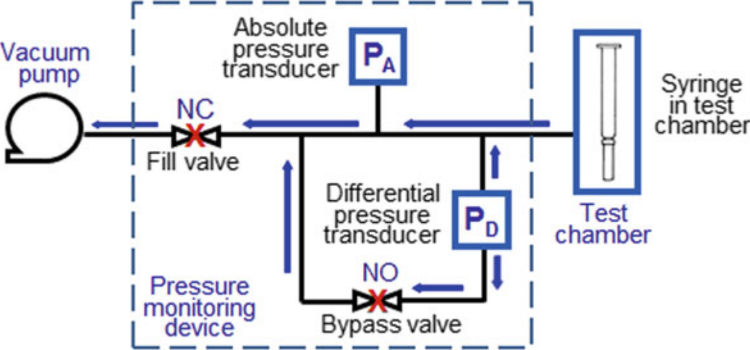


Fig. 12.4 Schematic diagram of a vacuum decay leak test system

packages. As demonstrated in ASTM F2338, the commonly recognized sensitivity of 5 μm for the primary containers can easily be validated. Another advantage of the method is the simple yet reliable positive control, which is simulated by using a precise microflow meter to represent the targeted sensitivity. The non-destructive nature of the test adds one more favorable factor to the method selection. This is especially so for products that are expensive and scarce to procure during the method development.

One drawback of the vacuum decay leak test is the occlusion of leak path when testing a package containing a product of large molecules. The high vacuum applied during the test provides a high sensitivity to the method and allows the detection of small leak in contact with liquid solution. But, it also simultaneously pulls the large molecules in the solution toward the leak. The large molecules are left in the torturous pathway after the vaporization of water and ultimately clogging the small leak. In one study of such clog phenomenon, fifty biological drug-filled syringes with laser-drilled hole size of 3.5–7 μm were tested using vacuum decay method. The samples were tested daily for 10 days. The results in Fig. 12.5 show that more than 97% of the data had pressure decay values equal to or less than the clog criterion based on the responses of no-leak samples, producing false-negative results.

Figure 12.6 further illustrates the outcome of clog phenomenon. Two groups of glass syringes with one group containing a biologics solution and the other group containing its placebo solution were repeatedly tested over 10 days. Each syringe in both groups had a laser-drilled hole size ranging from 3 to 7 μm on the glass barrel. The large variations of differential pressures in placebo group were attributed to the wide range of hole size (3–7 μm), partial impediment on some holes prior to the leak test, and baseline noise of the instrument. Nonetheless, the clog phenomenon is evident that leaks on all syringes containing biologics were completely occluded after three tests, while the ones containing placebo solution were still detectable at the tenth test.

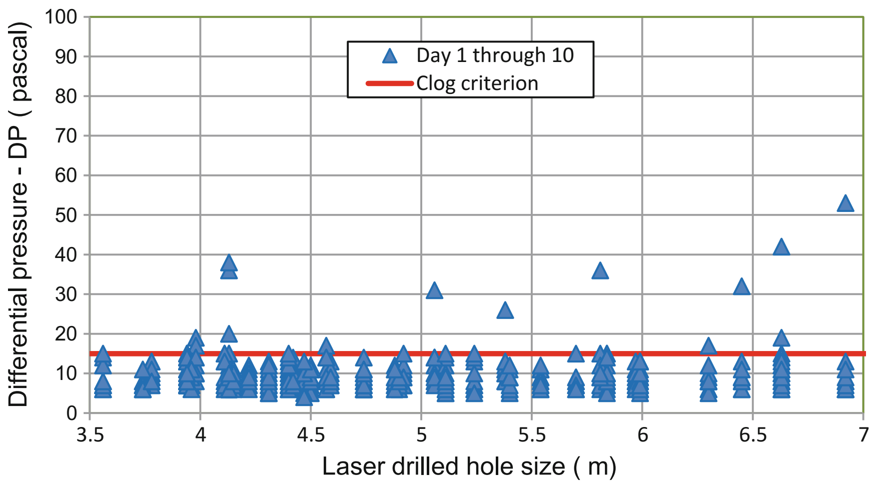


Fig. 12.5 Differential pressures from vacuum decay leak tests for laser-drilled syringes filled with biological drug

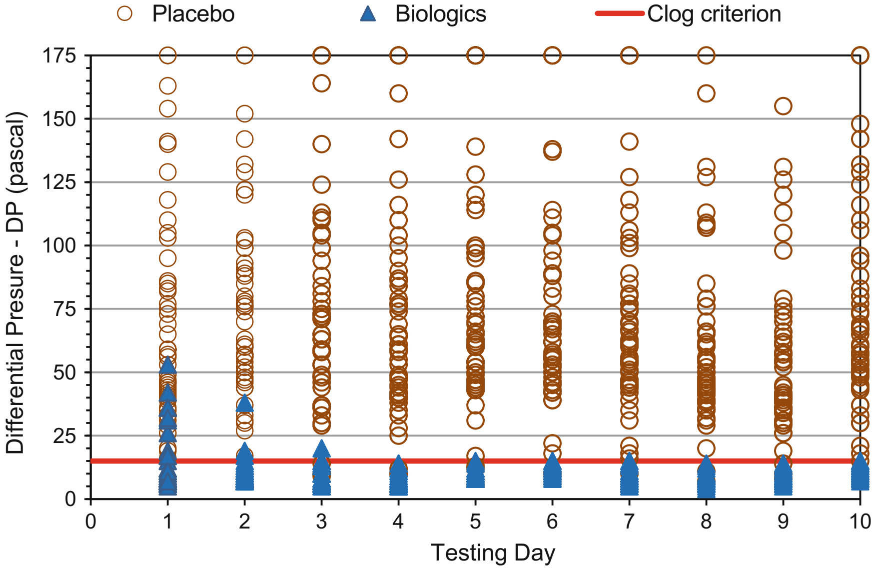


Fig. 12.6 Differential pressures from vacuum decay leak tests for laser-drilled syringes with biologics or placebo (175 used for DP > 175 in the plot)

12.4.4 Electrical Conductivity Leak Test

The electrical conductivity leak test, commonly known as spark test or High Voltage Leak Detection (HVLD) system, is based on the principle of electricity conduction through materials of different conductivities. Two fundamental requirements for the method to work are: (1) the container is made of electrically insulated material such as glass, rubber, or plastics and (2) the solution in the container is an electrically conductive material. Figure 12.7 shows the basic setup and principle of a HVLD system for leak testing a syringe.

During the test, a high voltage is applied to a specific area of the tested container through an inspection electrode. If the container has a leak at the inspected area, a discharge current will flow through the pinhole or crack into the container. The current flow through the container and conductive solution is collected at a detection electrode. A defective container has no resistance at the leak site, thus a higher current volume is collected at the detection electrode. Detecting the change in this current volume enables the presence of a leak to be identified. HVLD is a localized leak detection method as the inspection electrode needs to be placed near the defect for an effective detection. Therefore, the test sample needs to be rotated 360° circumferentially and the electrodes need to move laterally (Fig. 12.7). Alternatively multi-channel electrode stations can be installed laterally (Fig. 12.8), instead of moving electrodes laterally, to cover the entire test sample.

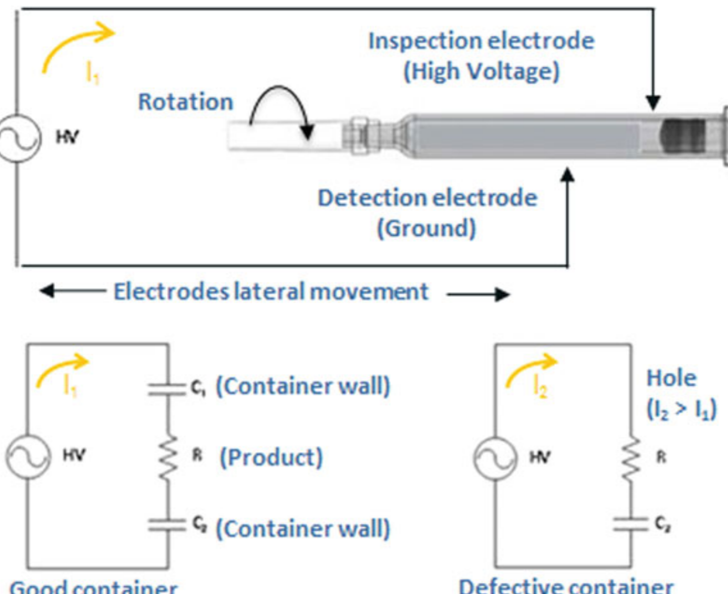


Fig. 12.7 Setup and leak detection principle for an electrical conductivity test

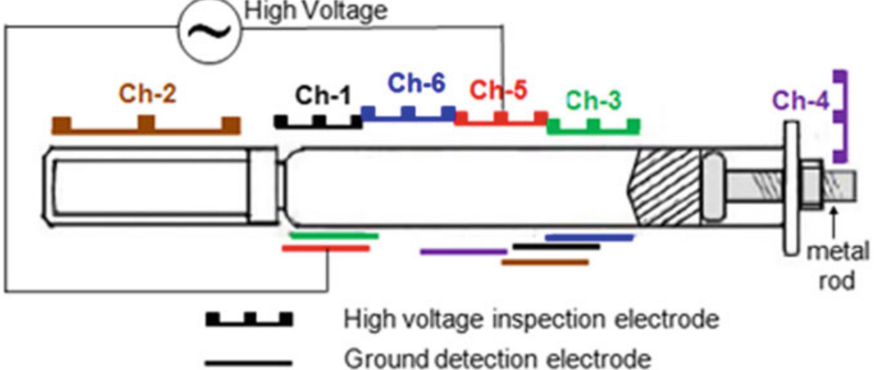


Fig. 12.8 Schematic diagram of a multi-channel HVLD system for syringe

Since the HVLD is a localized leak detection method, its method sensitivity needs to be verified at locations throughout the entire primary container closure system. The method cannot claim a sensitivity of 5 μm for a prefilled syringe if the 5 μm leak can only be detected at the syringe barrel, but not at the plunger-stopper of the syringe.

The electrical conductivity leak test method has the assurance of absolutely no external contamination to the content in the container. It is also considered as a non-destructive test if the high voltage does not impact the efficacy of the drug or interact with any of the contents. One study presented by ImClone Systems at 2010 PDA Annual Meeting on the HVLD systems showed the high voltage used in vial leak detection presented no detectable impact on the efficacy of their biological drug. However, another study showed the high voltage caused the conversion of oxygen in the headspace to ozone and could potentially impact the product. It is still unknown whether the impacts on other parenteral products would be negative or positive. Thus, it is vital that the impact of high voltage on the content be evaluated for each specific product over the entire shelf life to ensure no undesirable effects from high voltage on the efficacy and safety of the drug.

There are several advantages of using the HVLD system for leak detection. The detection principle of the system allows the test be performed at a much higher speed than the other methods. It also allows the machine design be flexible to accommodate various line speeds. The test parameters of the machine, high voltage and sensitivity factor, are easily adjustable, so a variety of products and containers can be tested on the same equipment with minimal changeover time. Therefore, the HVLD system is commonly used in manufacturing for continuous 100% online monitoring.

Since HVLD system uses liquid product in the container as a conductive path, a small leak, which might potentially be clogged by large molecule proteins in a biological product, can still be detected as long as the leak path remains wet. This benefit can be an important factor influencing the selection of leak test for container

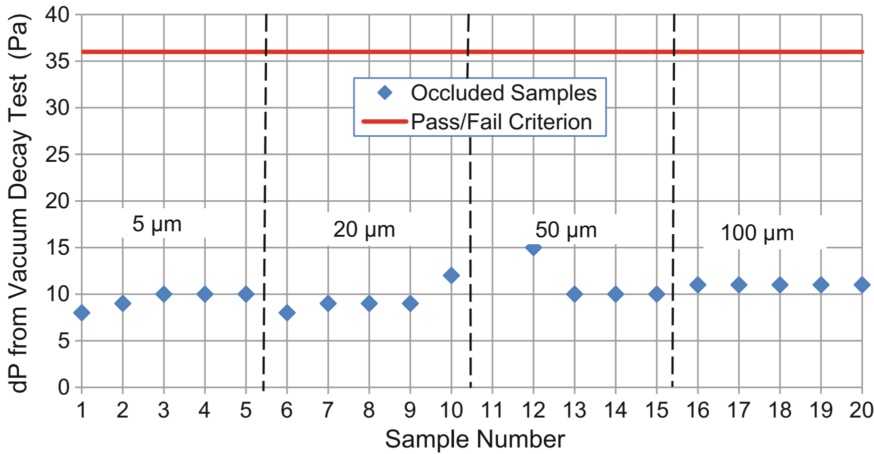


Fig. 12.9 Leak detection results from vacuum decay method for 20 laser-drilled syringes

with large molecule content that poses high likelihood of obstructed leak path. To demonstrate the advantage of HVLD method over the vacuum decay method for CCI test of biologics, 20 syringes with hole size of 5–100 µm filled with biological drug were tested initially using vacuum decay method and subsequently the HVLD method. Results in Fig. 12.9 show the leaks on all 20 syringes were not detected by vacuum decay method. Results in Fig. 12.10 show successful detection by HVLD method.

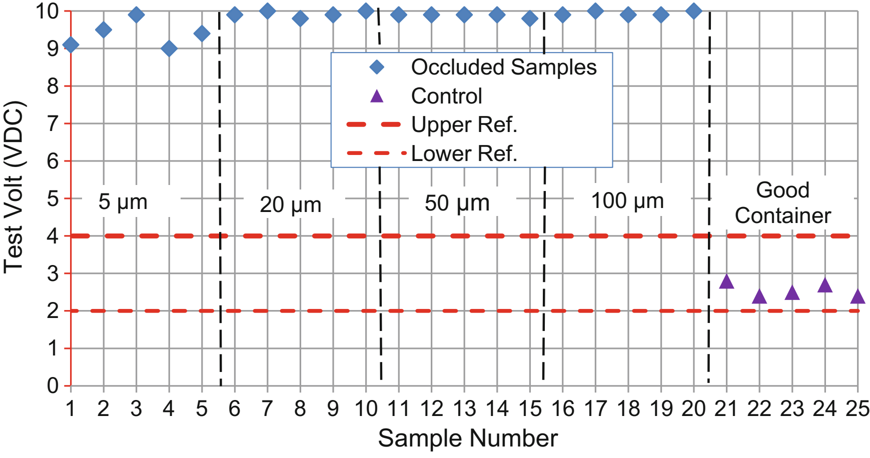


Fig. 12.10 Leak detection results from HVLD method for the same 20 laser-drilled syringes from Fig. 12.9

Several studies performed on HLVD method indicated a few downsides of the system. When a small laser-drilled leak was located in an area of thicker wall, where the leak path was not completely wet, the system could not detect the leak. However, such leak undetected through HLVD was found after the leak path became wet through a vacuum procedure. Although increasing high voltage can improve the success of HLVD in detecting leaks through a thicker wall, it also increases the occurrence of false reject due to the likelihood of a short circuit. Such short circuit was observed as occasionally inconsistent signals received at the detection electrode in repeated tests. Figure 12.11 shows the results of replicate tests for ten samples with similar leak size on a multi-channel HVLD system. The signals were fairly consistent between replicates except for Sample no. 4, where a large discrepancy in signal between replicates was observed. A short circuit between electrodes of adjacent channels was identified as the cause for the high signal in the second run. This issue was later corrected through optimization of the test parameters.

Another factor found to affect the inspection signal is the surface condition of a container due to environment. The signals varied appreciably when a cold non-defective sample was tested on days of substantially different humidity. Such variation can be minimized by controlling the humidity of the testing environment, warming up the sample and wiping the surface of the sample prior to testing. An unexpected outcome could trigger a costly false non-conformance investigation. Therefore, in-depth studies and optimizations of the test parameters are recommended during the method development to minimize the method's downsides.

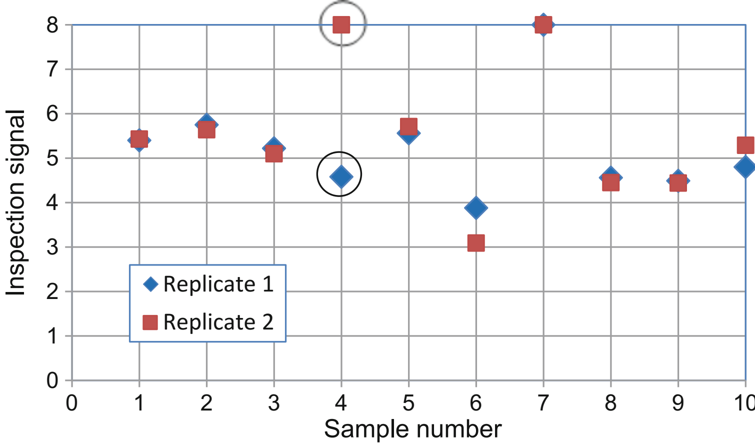


Fig. 12.11 Inspection signals of 10 samples with nominal 5 μm laser-drilled hole from 2 replicates

12.4.5 Tracer Gas Leak Test

The tracer gas leak testing is used in various industries for wide range of applications. Helium, due to its super property, was already used in the 1940s as the tracer gas for leak detection of the diffusion plant in the separation of uranium for the first atomic bomb. There are two different ways to run tracer gas leak test for detecting container leak. The test can generally be categorized as outside-in way or inside-out way. In the outside-in way, a tested part is placed in a chamber filled with pressured helium or the outer surface of a tested part is bombarded with helium whereas in the inside-out way, a tested part is filled with helium. The escaped helium from the leak in both ways is detected by an integrated detection system which is consisted of a helium mass spectrometer, a vacuum or pressure system, test chamber, helium filling equipment, etc. One of the factors in selecting between the two ways is the rigidity of the containers to be tested. Since it is slow for a rigid container with a small elastomer seal to absorb sufficient helium in bombing method or it is challenging to seal a detection probe inserted into a rigid container for the outside-in method, the inside-out method is generally used for the rigid parenteral product containers.

For the inside-out method, the principle for helium leak detector is similar to that of a vacuum decay leak detector. The tested container is placed inside a vacuum chamber in both methods. The major difference between the two methods is the need of helium filling in the tracer gas method. Another difference is that the leak is detected by a mass spectrometer with the rise of helium concentration in tracer gas method while a pressure transducer is used in the vacuum decay method.

Figure 12.12 shows an example of an integrated helium leak detection system. The system has the capability of investigating container integrity at various temperatures with the use of an environmental chamber. It also has duel modes of operation; one for measuring leak rate with a vacuum chamber and the other for pinpointing the leak location with a sniffer probe. As shown in Fig. 12.12, a vial to be tested is filled with helium from a tank via needles puncturing through its stopper seal. Adhesive is applied to the puncture site afterward to seal any potential leak from the puncture. The vial is then placed inside a vacuum test chamber (shown at the upper right in the figure) for leak rate measurement or under a cover containing a sniffer probe (shown at lower right in the figure) for leak location identification. If a leak exists, helium escaped from the leak path is detected by the mass spectrometer.

Figure 12.13 presents the helium leak test results of samples from an investigation of subzero temperature excursion during shipping. In this test, the actual filled helium concentration was not 100%. The measured leak rates shown in the figure had to be corrected. The actual leak rates were obtained from the measured leak rates corrected by actual filled helium concentrations in the container. The acceptance criterion of 6×10^{-6} std.cc/sec is based on Kresch's study results from 1999. The investigation confirmed the integrity of the containers. The high

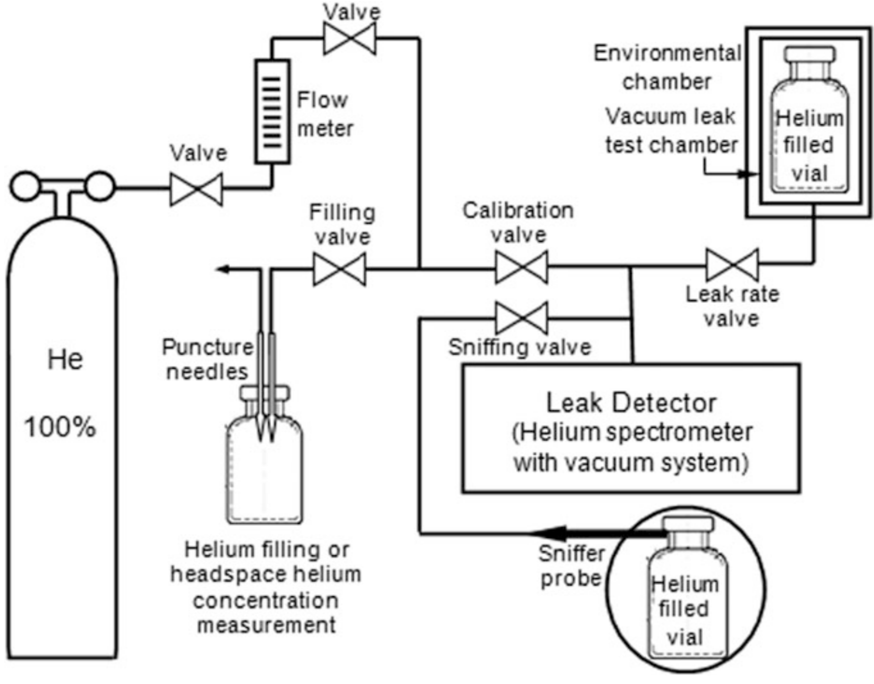


Fig. 12.12 An integrated helium leak detection system for leak rate measurement and leak location identification

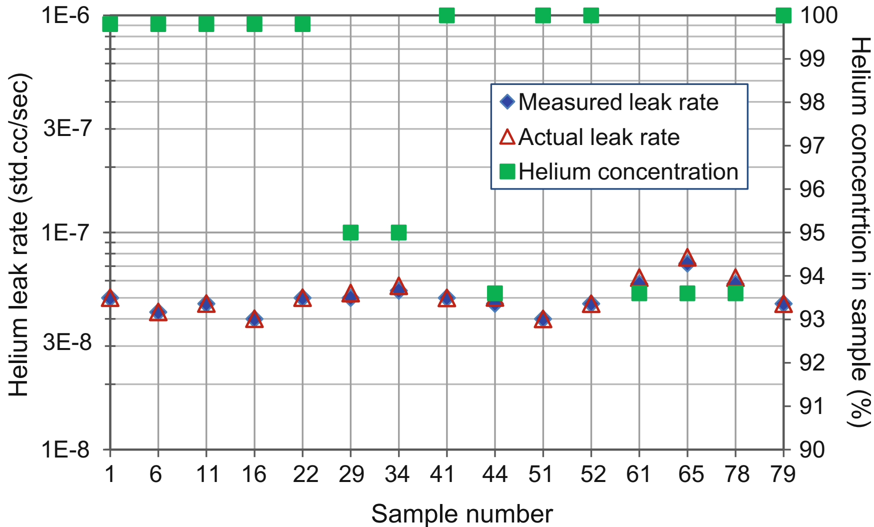


Fig. 12.13 Helium leak rates from containers exposed to temperature excursion during shipping

sensitivity of the method, as seen in the extremely low leak rates measured, makes it an excellent method for a package when a hermetic seal is required.

Helium is non-toxic, non-reactive, inexpensive, and modestly present in atmosphere. These attributes enable it to go through the smallest leak without affecting the container and be readily and precisely measured by a mass spectrometer. Therefore, helium leak detector is currently the most sensitive method for a CCI test.

Although the method has the benefit of highest sensitivity, it also has some drawbacks. The commonly used containers for parenteral products typically have seals made of rubber or other elastomeric material. This kind of materials usually has higher permeability compared to glass and can potentially absorb tracer gas to influence the leak detection outcome. To demonstrate the effect of helium permeability on leak test of plastic containers, thirty plastic vials were tested at two temperatures. Results in Fig. 12.14 show that the same good vials passed the leak test at −70 °C failed at room temperature with the same pass/fail criterion. This is due to the effect of higher permeability of helium in plastic material at warmer temperature. The results also point out that different pass/fail criteria are needed for tests conducted at different temperatures.

Finally, the use of helium as a tracer gas can be considered as disadvantage. Since the parenteral products are typically not packaged with helium, the helium filling step makes it a destructive test. In addition, the test conducted on non-product-filled container is also a disadvantage.

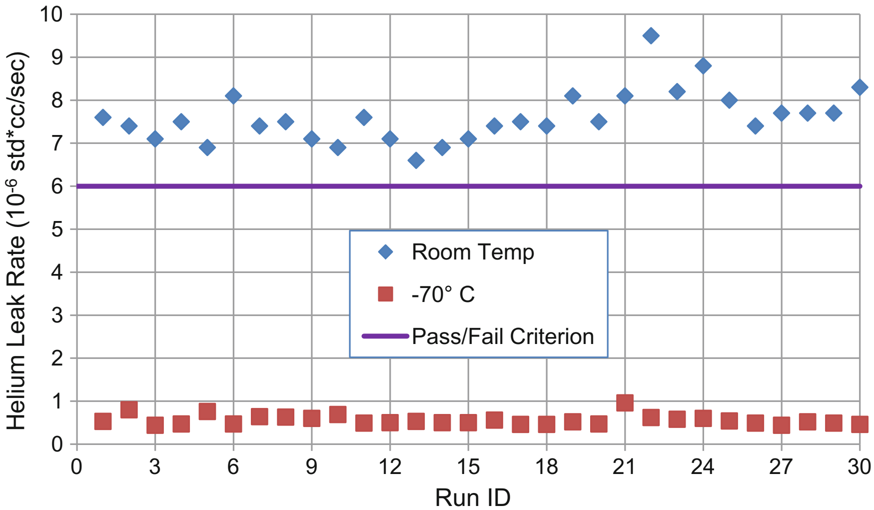


Fig. 12.14 Comparison of helium leak rates at room temperature and −70 °C for the same 30 plastic vials

12.4.6 Headspace Gas Analyzer

Headspace gas analyzer utilizes the technology of low energy laser absorption spectroscopy to measure the headspace gas inside a container for leak detection. The laser light is tuned in frequency to match the internal absorption frequency of the gas molecule to be detected. The laser light is passed through the headspace of a container. The absorption of laser light after it passes through the headspace is measured by a spectrometer. The frequency modulated laser is converted in the spectrometer to an amplitude modulation that is proportional to gas concentration. By comparing the concentration of the headspace gas in the tested container to that of a non-leak container, the CCI of the package can be determined. Figure 12.15 shows a simplified schematic diagram of a headspace analyzer using laser absorption spectroscopy.

Three molecules, oxygen, carbon dioxide, and water moisture, can be analyzed by the headspace analyzers currently available on the market. Analyzer that measures oxygen concentration can be used in a container that is packaged with nitrogen blanket headspace. Analyzer that measures carbon dioxide (or oxygen) concentration is applicable to a container that is stored under dry ice. And analyzer that measures the moisture concentration (as pressure reading) can be used in a container that is packaged under vacuum pressure.

The technology for moisture concentration analysis has been demonstrated to be suitable for CCI test of lyophilized drug which is usually packaged under vacuum to minimize moisture content inside the container for the prevention of caking. Table 12.3 shows results from an application of headspace analyzer in detecting leak on vials with different manipulations. The oxygen content in the lyophilized drug vials without leak is typically in the range of 2–4%. The results clearly confirmed the capability of the method to measure small leak on Sample F and large leak on Samples A, E, and G.

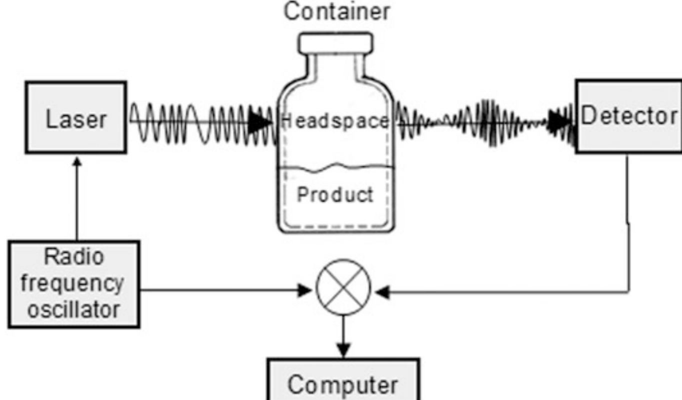


Fig. 12.15 A simplified schematic diagram of a headspace analyzer using frequency modulation spectroscopy technique

Table 12.3 Measured oxygen content from headspace analyzer in lyophilized drug vials

Sample	Oxygen (%)	Vial conditions
A	22.4	Small crack in glass vial
B	2.8	Normal sample
C	3.6	Normal sample, filled with 3 torr air
D	3.3	Normal sample, filled with 3 torr nitrogen
E	22.0	Stopper punctured by a large needle, left overnight
F	6.7	Stopper punctured by a small needle, left overnight
G	22.0	Open stopper for about 1 s, close again

Figure 12.16 shows results of another application of using headspace analyzer to evaluate the CCI of vials under 24 h storage of dry ice. Results from the stoppered and aluminum cap crimped vials showed the vials were intact with nominal oxygen concentration prior to and after dry ice storage. That from the vials with a 27G needle inserted through the stopper showed substantial leak with low oxygen concentration. And results from the stopper only vials showed variable leaks with wide range of oxygen concentration. Rubber stopper lost its sealing property under the cryogenic condition with dry ice. However, the crimping of aluminum cap compensated for the loss of rubber stopper's sealing property and was able to maintain the CCI of the vials.

Headspace analyzer is a non-destructive method as it utilizes the technique of laser light passing through the headspace without altering the package or impacting its contents. It is often used for 100% online leak detection due to its fast speed and non-destructive natures. However, the test is limited to package that is transparent and contains a targeted gas with its concentration changing by leak. The method would not be able to detect leak in a container packaged and stored under

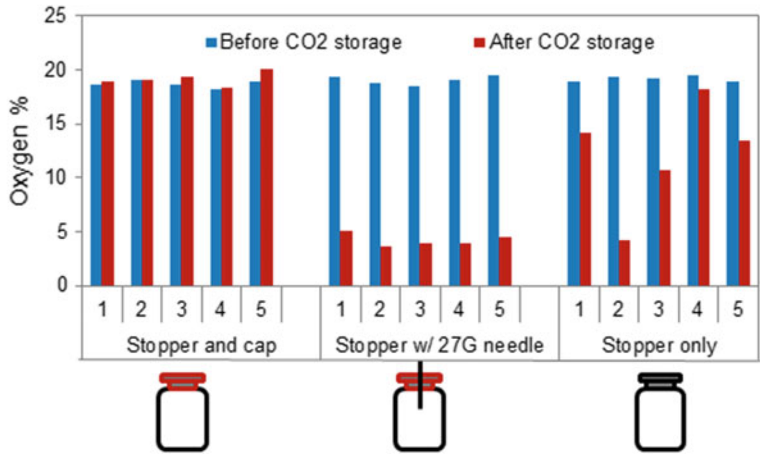


Fig. 12.16 Oxygen concentrations inside vials under 24 h storage of dry ice

atmospheric pressure. A leak in this container under atmospheric pressure would not result in a measurable change in the concentration of oxygen, moisture, or pressure. Another factor that could limit the application of this method is the availability of headspace. Prefilled syringe typically has a nominal headspace less than a few millimeters. With such a small clearance in headspace compounded with the allowable tolerance in fill volume, the syringe could be very close to liquid full. Therefore, there is a great risk that in a test the laser light will pass through the liquid phase and produce false result. A robust design of the detection setup has to be in place to prevent a false alarm when using headspace analyzer for syringe with small headspace.

12.5 Method Development, Qualification, and Validation

Validation and qualification can mean different activities for manufacturing process, analytical method, instrument, and equipment. It could also have different meanings for different companies. The process to get a CCI test method validated consists of the following activities: instrument and equipment validation, test method development and qualification, and finally test method validation. These steps and related elements are discussed in this section.

12.5.1 Instrument and Equipment Validation

It is recommended to validate the instrument and equipment used in test method prior to the method development work. Instrument and equipment validation includes three phases of qualification; installation qualification (IQ), operation qualification (OQ), and performance qualification (PQ). Instrument vendors typically have the IQ, OQ, and PQ documents to facilitate the validation process.

A laboratory usually has preventive maintenance to keep instrument calibration up to date. However, calibration alone lacks the benefits from the extra activities performed in IQ, OQ, and PQ thus may not be sufficient for the instrument to be used in CCI testing. For example, the transducer in pressure decay equipment is calibrated, but the performance of the equipment may not be appropriate due to a faulty Venturi tube or leak at internal tubing connections. In addition, instrument validation can be helpful when unexpected issue occurs during method transferring from site to site. For instance, successful instrument validation at both the originating and targeting sites can rule out instrument, utilities, or facilities as the cause of conflicting results.

The goal of IQ is to verify the delivered instrument is built in compliance with the designs and specifications, installed according to manufacturer's instructions, and located in a proper environment. In the IQ phase, the instrument is identified to have right model number, the valid certificates of calibration are included, an

adequate space is allocated at the installation site, the proper temperature and humidity controls for the environment are in place, and the utilities are within the required specifications and correctly connected to the instrument.

The instrument is demonstrated to function properly according to operational specifications during the OQ phase. Test cases with written procedures are performed to verify that data collection system, security and audit trails, control loops, alarm system, and functional operations all run as intended applications by instrument manufacturer. For example, when a package with a gross leak is assessed using manufacturer-supplied parameters in one test case, the leak should be identified on instrument screen, the alarm is activated if the reading exceeds the alarm limit, and the correct output of failure is recorded on instrument's storage device. The reliability of the instrument under normal operating parameters can also be tested and confirmed in OQ phase.

All elements for the operation of the instrument, including the connected peripheral devices such as computer and test chamber, are included during PQ testing. Tests are set up to verify the performance of an integrated instrument system according to known results, standards, or intended applications. Typically, a series of tests are executed and repeated at different time in PQ phase to check the consistency of the performance. Tests in PQ may resemble those in OQ, but the goals and acceptance criteria are different. The PQ is verified by successfully and repeatedly differentiating known leak and no-leak containers in different days with parameters from vendor's validation document or from a satisfactorily completed Factory Acceptance Test.

12.5.2 Test Method Development and Qualification

12.5.2.1 System Suitability for Test Method

System suitability is an important feature in a valid CCI method; however, it is often neglected in the development process. Thus, it is discussed in particular here to emphasize its importance.

The CCI test method is an integrated system and may consist of equipment, instrument, test chamber, tracer material, operation procedures, test parameters, positive and negative controls, samples to be tested, environmental chamber, and computer, etc. Even after a successful completion of method validation, occasionally the method may not work properly due to unexpected excursion such as leak at the connecting tubing or contamination at the analyzer. System suitability check is to ensure the integrated system is working properly ***at the time of test***. The check is normally confirmed by using qualified positive and negative controls. With the acceptance criteria of CCI test as defined and verified in the method development, the positive and negative controls shall consistently fail and pass, respectively.

System suitability is also essential for confirming method sensitivity and providing evidence that the test samples are actually challenged at the time of test. One example to demonstrate the importance of system suitability is related to the dye penetration test. Many documents (such as ISO, USP, and EP in Table 12.2) provide guidance on dye test conditions, yet no reference or suggestion on the necessity of positive control is mentioned. Therefore, many laboratories perform dye ingress tests following these documents without using any positive control. With the lack of positive control when a tested sample did not show dye penetration, it is possible that the leak was not actually challenged as no evidence on the ability of dye penetration was provided.

The use of controls is crucial but selection of controls can be challenging and difficult, especially if they need to be simple, easy to maintain, reliable, and robust. Recently, laser drill technology with its capability to produce definite leak size that resembles a real tortuous leak path has been widely employed as a way of creating leak samples for rigid primary containers. However, the laser-drilled parts are expensive, prone to clog, and with limited accuracy at small leak size (i.e., a huge tolerance as hole gets smaller). In addition, they are not reusable in most of the tests. Even if reusable, the holes need to be assessed and confirmed as they may be altered by the preceding leak test. For example, the hole may become bigger from the shock of high voltage in HVLD test or occluded by dust or particles during vacuum decay test. Extensive researches and studies sometimes are required to identify the adequate controls for the system suitability check of each specific method.

12.5.2.2 Development and Qualification Activities

Before a method can be implemented for in-process control, product release, or stability study, it has to demonstrate its capability to measure the intended properties. A series of activities take place during method development and qualification to identify, define, and analyze parameters of the method. The activities typically include assessment and establishment of method characteristics, evaluation and optimization of operational conditions, research and development of negative and positive controls, and determination and justification of acceptance criteria. These activities may be performed independently, jointly, in sequence, in multiple stages or in groups. Statistical studies like Design of Experiments can be used to screen and optimize the input test conditions and output test results. Based on knowledge gained through development activities, a CCI test can be established and qualified to demonstrate its capability for the intended use. It is essential that the development activities and results be documented, which can be the basis for making decision on qualification data.

The characteristics of a method to be assessed depend on method type and its intended application. For analytical methods, the classifications of method type and characteristics to be qualified are described in ICH Q2(R1) and USP <1225>. Some of these characteristics (such as the specificity) may not be totally relevant to the

physicochemical methods for CCI testing. Nevertheless, they can serve as guidance for the development and qualification of a CCI test method. Typical characteristics evaluated in the qualification of a CCI test include sensitivity, system suitability, precision (repeatability and reproducibility), robustness, detection limit, or quantitation limit for a quantitative test.

Method qualification should be performed at the completion of development based on a defined experimental design. A successful qualification depends on the understanding of method from the development process. Method capability is confirmed by demonstrating the success of expected outcomes with the predetermined acceptance criteria. The criteria used to determining the acceptability of the method should be specified, justified, and documented based on sound scientific and engineering principles for the intended purpose. Appropriate sample size of intact and defect samples, including mixed population of various types of defects and leak sizes if possible, should be used to achieve an appropriate method qualification. Data collection and analysis should include sufficient statistical confidence. When the targeted expectations are not met during qualification, the method should be reevaluated and redeveloped or different acceptance criteria should be established and justified. Method qualification for CCI test needs to be repeated when the design of a container is changed.

12.5.3 Test Method Validation

The purpose of method validation is to establish documented evidence that a specific and qualified CCI test method will consistently produce the predetermined acceptable performance in detecting leak to meet required container quality. Method validation is required prior to the implementation in GLP or GMP facilities for CCI testing.

Method validation is performed after method qualification is satisfactorily completed with the test parameters and acceptance criteria selected and optimized. After method is developed and qualified, it needs to demonstrate that the same performance attained in method qualification can be achieved reproducibly and repeatedly at different days using different instruments, analysts, and sites under GLP or GMP conditions. The characteristics identified and verified in method qualification should be included in the validation.

Written protocol and proper approvals with the established acceptance criteria from qualification should be used. Sufficient samples on intact containers from different batches as well as representative leaks close to realistic defects if possible should be used in validation. Again, the collected data should be statistically significant to demonstrate the suitability, repeatability, reliability and validity of the method.

12.6 Summary

Each CCI test method has its advantages and disadvantages. The selection of an appropriate CCI method depends on the purposes and requirements of the test. Table 12.4 provides a summary of the comparisons for the methods presented.

The lifecycle concept has been adopted by regulatory agencies for the development, qualification and validation of manufacturing processes. The CCI test method used for commercial or clinical production shall follow the similar approach for the lifecycle of a product. The method shall not only be monitored and maintained regularly, but also be modified, improved, and updated whenever innovations and advances in new leak detection technologies become available. Due to limited resource or low priority, many companies continue using the method that was developed and validated many years ago even after newer instrument with higher sensitivity and capability becomes available. Since the consequence on loss of container closure integrity in parenteral product is critical, it is important to employ the best method possible for providing patients with sterile products at the highest safety and quality standards.

Table 12.4 Comparisons of container closure integrity test methods

Method	Pro	Con
Microbial immersion	• Extremely small microorganism	• Media-filled sample (non-product filled) • Probabilistic • Long test time
Vacuum decay	• Sensitive • Repeatable, non-destructive • FDA consensus standard	• Defect clogged by large molecules in product
Dye ingress	• Convenient but long test duration • Repeatable • Detects clogs	• Hazardous waste/water consumption • Limited sensitivity • Destructive
HLVD system	• Clogs are detected • Potential for 100% online inspection	• Suitable positive control research • Potentially destructive • Wet leak path required
Helium leak test	• Extremely sensitive • Cryogenic temperature testing • Reliable	• Helium-filled sample • Complex setup • Test interfered by degassing
Headspace analyzer	• Potential for 100% online inspection • Non-destructive	• O_2, H_2O, and CO_2 detection only • Sufficient headspace clearance

References

1. ASTM F2338-09. Standard test method for nondestructive detection of leaks in packages by vacuum decay method. ASTM International, West Conshohocken, PA.
2. ASTM F2391-05. Standard test method for measuring package and seal integrity using helium as the tracer gas. ASTM International, West Conshohocken, PA.
3. Burrell B, Carver M, DeMuth G, Lambert W. Development of a dye ingress method to assess container-closure integrity: correlation to microbial ingress. PDA J Pharm Sci Technol. 2000;54(6):449–55.
4. Container closure systems for packaging human drugs and biologics. U.S. Department of Health and Human Services Food and Drug Administration, Guidance for Industry, May 1999.
5. Container and closure system integrity testing *in Lieu* of sterility testing as a component of the stability protocol for sterile products. U.S. Department of Health and Human Services Food and Drug Administration, Guidance for Industry, February 2008.
6. EP 3.2.9. Rubber closures for containers. European Pharmacopeia 7.0, European Directorate for the Quality of Medicines, Strasbourg, France.
7. EudraLex. The rules governing medicinal products in the European Union, Vol 4, EU guidelines to good manufacturing practice, medicinal products for human and veterinary use, Annex 1—Manufacture of Sterile Medicinal Products, European Commission.
8. Guazzo DM. Parenteral product container closure integrity testing, Chapter 14 of pharmaceutical dosage forms: parenteral medications. In: Neema S, Ludwig JD, editors. Formulation and packaging, 3rd ed, Vol 1. NY: Informa Healthcare; 2010.
9. ICH Q2(R1). Validation of analytical procedures: text and methodology. In: International conference on harmonization of technical requirements for registration of pharmaceuticals for human use.
10. ISO 8871-5:2005. Elastomeric parts for parenterals and for devices for pharmaceutical use—Part 5: functional requirements and testing.
11. ISO 8362-2:2008. Injection containers and accessories—Part 2: closures for injection vials.
12. ISO 8362-5:2008. Injection containers and accessories—Part 5: freeze drying closures for injection vials.
13. Kirsch L, Nguyen L, Moeckly C, Gerth R. Pharmaceutical container closure integrity II: the relationship between microbial ingress and helium leak rates in rubber stoppered glass vials. PDA J Pharm Sci Technol. 1997;51:195–202.
14. Kirsch L. pharmaceutical container/closure integrity VI: a report on the utility of liquid tracer methods for evaluating the microbial barrier properties of pharmaceutical packaging. PDA J Pharm Sci Technol. 2000;54(4):305–13.
15. Lin T, Hsu C, Kabakoff B, Patapoff T. Application of frequency-modulated spectroscopy in vacuum seal integrity testing of lyophilized biological products. PDA J Pharm Sci Technol. 2004;58(2):106–15.
16. Measurement Systems Analysis. Automotive Industry Action Group, 4th ed. June 2010.
17. McGinley C, Bigwarfe P, Thiel A, Olson K, Loe Ø, Loffredo D, Joseph J. Degradation of drug product caused by a leak detection instrument: mechanistic studies of degradation. Hospira, Inc., One 2 One Global Pharmaceutical Services, Lake Forest, IL 60045.
18. Orosz S Jr, Guazzo D. Glass vial finish defects, leak detection and product risk assessment. In: PDA annual meeting, packaging science Interest Group, March 16, 2010 Orlando, FL.
19. Pharmaceutical Package Integrity. Technical Report No. 27. PDA J Pharm Sci Technol 1988;52:S2, 35–7.
20. Sterile Drug Products Produced by Aseptic processing—Current Good Manufacturing Practice. U.S. Department of Health and Human Services Food and Drug Administration, Guidance for Industry, September 2004.
21. The Code of Federal Regulations. Title 21 food and drugs, Revised April 1, 2006.

22. USP <381>. Elastomeric closures for injection, United States Pharmacopeial Convention, Inc., Rockville, MD, December 2011.
23. USP <1207>. Sterile product packaging—integrity evaluation. United States Pharmacopeial Convention, Inc., Rockville, MD, December 2011.
24. USP <1225>. Validation of compendial procedures. United States Pharmacopeial Convention, Inc., Rockville, MD, December 2011.
25. Wolf H, Stauffer T, Chen S, Lee Y, Forster R, Ludzinski M, Kamat M, Godorov P, Guazzo D. Vacuum decay container/closure integrity testing technology. Part 1. ASTM F2338-09 Precision and Bias Studies. PDA J Pharm Sci Technol. 2009;63:472–88.
26. Wolf H, Stauffer T, Chen S, Lee Y, Forster R, Ludzinski M, Kamat M, Mulhall B, Guazzo D. Vacuum decay container/closure integrity testing technology. Part 2. Comparison to dye ingress tests. PDA J Pharm Sci Technol. 2009;63:489–98.

Chapter 13
Chemical Durability of Glass—Delamination

Holger Roehl, Philippe Lam and Dominique Ditter

Abstract Primary packaging containers used for the storage of parenteral drugs are designed to protect the medicinal product from the environment to ensure patient safety. Mainly borosilicate glasses are leveraged as the vial material of choice due to their excellent chemical durability and other additional benefits. Nevertheless, the formulation and its excipients can interact with the glass leading to an alteration of the surface. This interaction can result in ion leaching or glass corrosion. One prominent example is the occurrence of delamination which is the formation of glass flakes/lamellae. Such visible particles were the reason for several recalls within the last years. For that reason, an overview of general interaction mechanisms of the formulation with the glass surface and the root cause for delamination is presented within this chapter. Factors influencing the risk for delamination are discussed in detail as well as analytical techniques suited to investigate the impact on the properties of the primary packaging containers. The combination of the knowledge about the underlying root cause and the respective analytical tools to characterize the vial internal surfaces will help both the manufacturers of the vials and the pharmaceutical companies to establish a thorough control strategy to avoid the issue of delamination.

Keywords Glass · Primary packaging containers · Vials · Chemical durability
Leachables · Delamination · Analytical methods

H. Roehl (✉)
Late Stage Pharmaceutical and Processing Development, Pharmaceutical Development & Supplies, Pharma Technical Development Biologics EU, F. Hoffmann-La Roche Ltd., Grenzacherstrasse 124, 4070 Basel, Switzerland
e-mail: holger.roehl@roche.com

P. Lam
Pharmaceutical Processing and Tech. Dev., Genentech Inc., 1 DNA Way, South San Francisco, CA 94080, USA

D. Ditter
Site Manufacturing Science and Technology, Validation Engineering & Filling, Roche Diagnostics GmbH, Sandhofer Straße 116, 68305 Mannheim, Germany

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_13

13.1 Chemical Durability of Glass

Fused silica or fused quartz is a glass made out of pure SiO_2. It has outstanding properties like a low thermal expansion coefficient, high temperature, and high chemical resistance. It is inert against acids except hydrofluoric and hot phosphoric acid. But it has a main disadvantage which limits its usage as a primary packaging component, e.g., as a vial: Transformation, softening, and processing temperatures are all significantly higher compared to other glass types used for pharmaceutical applications. Therefore, additional raw materials have to be added to lower the processing temperature, mainly alkali oxide (Na_2O or K_2O, so-called network modifiers) [1]. The modifiers are mobile ions within the glass backbone formed by SiO_2 and other network formers and lead to a disruption of the network. This results in a lower processing temperature. The addition of network formers like B_2O_3 or Al_2O_3 helps to improve the chemical durability (borosilicate glasses). Depending on the desired chemical and physical properties, several more substances (metal oxides) can be added, e.g., coloring agents like iron or titanium in case of brown glass. To keep the elemental composition as tight as possible, the raw materials need to have a constant and (of course) high quality.

Glass corrosion and ion leaching

Glasses for pharmaceutical applications are typically made of borosilicate glasses with very high hydrolytic resistance (chemical durability). Depending upon the chemical properties of the drug product solution (formulation), different interactions of the liquid with the glass surface can be observed [1–4]:

- Is the pH lower than 7 (acidic), an H_3O^+ exchange will occur with the most mobile cations, i.e., typically with Na^+ or other network-modifying ions. As an inter-diffusion process, it is governed by a square-root time-law:

$$x = \text{const.} \cdot t^{1/2}$$

  Therefore, the growth of thickness x of the exchanged zone is rapid in the beginning and slows down continuously, until it stops (from a macroscopic point of view). The affected depth is only in the range of several ten nanometers, but a consequence of the described reaction is that the pH value of non-buffered systems will rise. Water For Injection (WFI) exhibits a pH of approx. 5–6 and therefore will show this effect. In addition, alkaline ions will leach into the drug solution and could interact with the excipients or with the API itself.
- OH^- ions in alkaline solutions ($pH > 7$) are able to split the structural units of silicate glasses directly (i.e., the siloxane bridges). During a basic attack, the backbone of a silicate glass is destroyed. This process proceeds proportional to time, that means that the process will continue and will not slow down:

$$x = \text{const.} \cdot t$$

The dissolution of the backbone has another important impact: Not only alkaline ions, but also all components of the glass can migrate into solution and can interact with the excipients and the drug molecule. One example is the formation of barium sulfate particles formed out of leached barium from the glass into a sulfate-containing formulation or the interaction of phosphate buffer systems with glass leachables [5].

– At neutral pH values (around pH = 7), a combined mechanism will be observed due to the autodissociation of water. At first, ion-exchange reactions occur similar like in acids. By that, the concentration of OH^- in the water raises leading to a start of a base attack.

Acids, bases, or other excipients of the formulation can react differently with the varying glass elements leading to a preferential dissolution of these components [6]. A backbone of chemically more stable materials is left behind. The result is a thin porous layer which can detach from the glass surface (delamination) [7]. Such phenomena have a high level of attention from both the authorities and the pharmaceutical companies worldwide [8, 9] and are the cause for several recalls within the last months/few years [10]. The basic knowledge to understand the root cause of delamination is therefore described in the following paragraphs.

13.2 Background/Definition of Delamination

Delamination is the peeling-off or detachment of thin glass layers from the inner surface of vials usually in the form of small lamellae ("flakes," see Fig. 13.1), which then can appear as particles in the product solution (see Fig. 13.2). These particles can be in the visible or sub-visible range. The phenomenon is not new and was first described in 1940 by Bacon et al. who reported the formation of flakes or spicules in solution after accelerated tests on soda lime glass bottles [11]. In 1953,

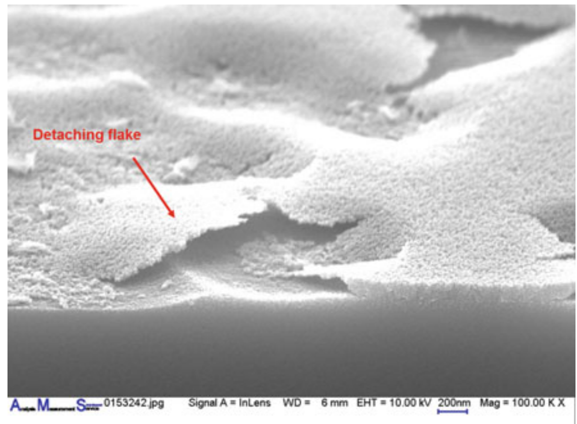


Fig. 13.1 Thin glass layers detached from the inner vial wall surface (*Source* Schott AG)

Fig. 13.2 Delaminated glass particle (flakes) in a placebo solution (drug formulation without API) (*Source* Schott AG)

Dimbleby recognized the seriousness of glass flakes in pharmaceutical products [12]. Nevertheless, delamination still caused a high number of recalls in the past few years [10]. Due to patient safety and the high costs involved for recalling a product from the market, it is of high interest to pharmaceutical companies to know the root cause for the occurrence of delamination. It is essential to avoid the issue by taking the right preventive actions.

13.3 Root Cause of Delamination

When evaluating published product recalls from 2010 to 2011, no common features/properties of the drug solutions could be identified as the root cause for delamination. The pH of the affected products ranged from 3.0 to 9.2, most of them being near the neutral point (pH = 7). Therefore, the effect is not restricted to either acidic or basic solutions alone. The product itself or the formulation of the drug contains sometimes only water (WFI) or small amounts of salt. More often polyvalent ions like citrates or phosphates are used as buffer systems.

Recent studies [13–23] revealed that the forming process of the vials has a major impact on delamination. For pharmaceutical applications, mainly Type I borosilicate glasses are used for vials due to their high chemical resistance. During the forming process (from canes to the final vials), hot gas flames are used for heating up the glass sufficiently to soften the glass and to enable forming. At temperatures

of approx. 1000–1200 °C, some elements/species of the glass evaporate, mainly borates. These components condense at cooler parts of the cane. In areas near the forming zone, the glass surface is still so hot that the condensates (element specific) diffuse into the glass. This changes the overall elemental composition of the glass near the surface in these areas. Additionally, a phase separation is taking place due to the change in chemical surface composition of the glass. Both processes result in boron-enriched areas exhibiting a different (lower) resistance of the glass against chemical attacks compared to the composition of the original borosilicate glass. Specific elements (e.g., boron or sodium) are selectively dissolved/eliminated from the network by a chemical attack of the formulation components leading to a porous and weak structure ("reaction layer," see Fig. 13.3). Additional stresses then can lead to the detachment of the thin porous layers resulting in delamination.

The overall result is the formation of two areas of the vial revealing an increased risk for delamination (see Fig. 13.4).

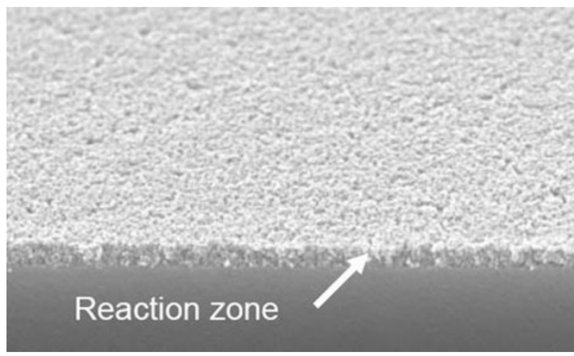


Fig. 13.3 SEM image of a reaction zone caused by selective dissolution of glass components [4] (*Source* Schott AG)

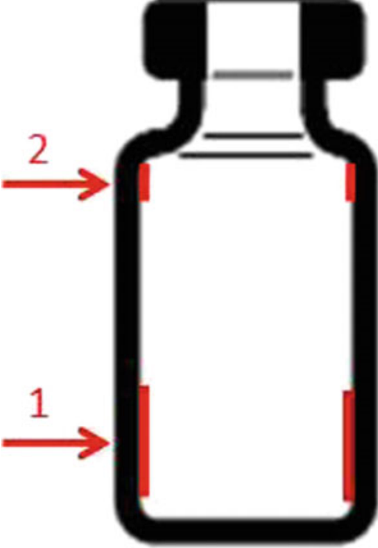


Fig. 13.4 Areas of the vial with increased risk for delamination [24]

The first zone is located at the wall closest to the bottom where the temperature gradients due to hot forming are the most extreme. The highest risk for delamination is located there. A second area is located close to the shoulder of the vial. But the effect is less pronounced due to the lower glass temperature and the shorter time used for the forming process. Usually, vials are not filled in a way that the liquid level is reaching that area. It could get important though if the vials are stored inverted for stability testing of the drug product.

The bottom itself also exhibits a different chemical composition compared to the original glass. Because of the evaporation of borates, it exhibits a higher SiO_2 content making it chemically more stable compared to unaltered surface areas and therefore less prone to delamination.

For syringes, the delamination risk is generally considered low due to the lower temperatures needed during the forming process.

13.4 Additional Factors Influencing Risk for Delamination

The forming process is the main root cause for delamination [24]. It is possible by a close control of the forming parameters to produce vials for which the risk for delamination is reduced to a minimum. Schott's Delamination Controlled DC vials® is one commercially available product.

Molded glass can be considered as a cheaper alternative to tubular glass in the context of delamination [25]. As delamination issues are associated with the hot forming process, the risk of delamination associated with molded glass may be considered lower due to the different production process. Delamination effects are still possible in molded glass, typically affecting the whole surface and not just certain risk areas, which needs to be considered in any glass change assessments due to delamination. However, molded glass is for a variety of other reasons often less favorably used compared to tubing glass.

In addition, several more factors than the production process have an impact on the delamination issue, which will be discussed in more detail in the following chapters.

13.4.1 Glass Composition

The glass composition has a direct influence on the hydrolytic resistance [26–30]. Mostly the boron and (to a lesser amount) the sodium content in the glass are critical for the occurrence of delamination. For example, because of the higher silicon content of 3.0 expansion glass compared to 5.0 expansion glass (the value is indication the thermal expansion coefficient α) 3.0 glass requires higher temperatures for forming which is associated with a higher risk for delamination. However, Duran glass has a lower borate content, which is generally associated with a lower

risk for delamination. Therefore, due to the lack of empirical evidence, which would allow for a more accurate risk assessment, a decision for the selection of the glass type must be achieved on a case-by-case basis. And it must always be kept in mind that delamination is the result of the specific interaction of the formulation liquid with the glass surface.

Another approach is to eliminate the likely main responsible element boron completely from the glass composition. This can be done by replacing boron by aluminum offering an aluminosilicate glass vial or by using pure quartz glass vial made solely from SiO_2. Both solutions would potentially remove the main root cause for delamination.

13.4.2 Coatings

A strategy to reduce the risk of delamination is the application of a coating on the inner vial surface. This is taking up the idea of changing the glass composition and thus eliminating the root cause for delamination. Different options are already available on the market and will be discussed briefly below.

13.4.2.1 SiO_2

Type I plus® vials from SCHOTT [31] and vials from SiO_2 Medicals are coated with a thin SiO_2 layer, which acts as a protective or a “victim” layer enhancing the chemical resistance and the lifetime of the used primary packaging container. Delamination is prevented because of the lack of boron. However, high pH solutions will dissolve this layer. The higher the pH the faster this process is. The advantage in this case is the time gained until the layer is dissolved. For that period of time, no attack on the original glass can occur. Additionally, the interaction of the formulation components with a large variety of glass elements is reduced to silicon (Si) and silicon oxide species.

13.4.2.2 Hydrophobic

TopLyo® vials from Schott or siliconized vials (with or without the combination of a heat treatment to create a baked-on silicone layer) exhibit a hydrophobic coating on the vial interior surface resulting in a contact angle to water of up to approx. 100°. The interaction with the formulation is therefore minimized.

13.4.3 Container Size

In general, vials with a higher nominal fill volume exhibit a bigger body diameter and an increasing wall thickness. Both factors lead to higher temperatures or longer

times at elevated temperatures associated for the forming of bigger vial sizes leading to a higher risk for delamination.

In contrast, small vials like 2 mL/2R vials reveal the highest surface to volume ratios. A chemical attack on the glass surface is more severe compared to bigger formats resulting in a higher delamination risk. During the experimental assessment of delamination, both small and big vial formats should be considered when applying a bracketing approach.

13.4.4 Ammonium Sulfate Treatment

The ammonium sulfate treatment is applied to remove alkaline ions from the glass surface. This results in a lower amount of sodium that can be exchanged with H^+ ions from the formulation/water. Therefore, such a treatment is normally used for pH-sensitive (non-buffered) solutions. During the treatment, the glass surface is chemically attacked and weakened, which lowers its chemical resistance, thus enhancing the risk of delamination.

13.4.5 Process Steps at the End User's Side

Not only the vial forming and processing steps at the manufacturers' end have an impact on delamination propensity. A lot of influencing factors have to be considered by the pharmaceutical companies using the vials for filling their medicinal products [32–35].

13.4.5.1 Formulation Composition

Several buffer systems (e.g., phosphate or citrate buffers) are reported to facilitate delamination [35–39]. Mechanisms are still poorly understood, and current knowledge is limited to descriptions of some general principles of glass corrosion and to some degree delamination specifically [40, 41].

Delamination is also observed with WFI or solutions with low ionic strength, and (as already described before) a generalization or prediction is difficult to be made because the root cause is still unclear [41].

In any case, high pH formulations are considered to exhibit a higher risk for delamination. OH^- ions attack the backbone of the glass network leading to dissolution of the surface. H^+ ions at low pH values typically result in ion-exchange processes not harming the glass integrity [42–44].

13.4.5.2 Lyophilization

The risk of delamination in lyophilized products is generally considered lower than with liquid products. This is mainly due to the low contact time of the liquid to the glass surface. Contact times encompass process times including holding times before the lyophilization step and time after reconstitution before application. In total, contact time ranges from hours to days which is normally not sufficient for the generation of the described reaction zone [45].

13.4.5.3 Primary Packaging Preparation

The preparation of glass primary packaging material for filling (depending on individual product and process) typically involves washing, drying, depyrogenation, and sterilization. Terminal steam sterilization is the preferred method of sterilization for WFI and (most) small molecule products. Each of these parameters, even though with different degrees of severity, may have an influence on glass delamination.

The influence of production practices such as pressure of water and the use of detergents during the washing step on glass delamination is considered low. But the residual moisture remaining in the containers after the drying (blowout) and pre-warming steps in the depyrogenation process is considered critical. Residual water in the vial, resulting from incomplete vial drying will result in generating water vapor during a depyrogenization or sterilization process, significantly attacking the glass matrix potentially similar to the described alkalinity mechanism [46, 47]. Adequate vial processing is key to minimize delamination risk, and this parameter may be even more critical than formulation composition.

As glass corrosion processes are temperature dependent and require humidity (resp. an aqueous environment), the combination of humidity and high temperatures as encountered during terminal sterilization by autoclaving poses a very high risk to promote delamination. In combination with a previous ammonium sulfate treatment, this can be considered as worst-case conditions.

13.4.5.4 Storage

Parenteral products are typically stored at room temperature and sensitive products at refrigerated conditions. Although these are very moderate temperatures, the very long exposure during shelf life allows glass corrosion to proceed slowly. Higher temperatures in general accelerate chemical reactions; consequently, the higher the temperature during storage of the filled container, the higher the potential risk for delamination [48, 49].

13.4.5.5 Transportation

Mechanical stresses during transport may have an impact on the stability of the reaction zone described above. Therefore, peeling-off of flakes may become more likely especially if mechanical stresses are applied to products that have already been stored for a longer amount of time.

13.5 Detection and Analysis

The success of detecting corroded containers during 100% visual inspection and AQL testing after Drug Product manufacturing is considered low due to the slow kinetics of delamination formation and the time point of visual inspection within weeks after container filling. The probability of detection during stability testing is higher due to the prolonged exposure time of the container to the solution. Thus, it is imperative to monitor "visible particles" on stability testing. Yet, the choice of the right primary packaging configuration has to be ensured by assessing of the suitability of the container before usage for a specific product. The propensity of delamination can be evaluated both in form of a risk assessment or via experimental testing, either with selected representative solutions or with the real formulation (with or without the API). Pharmacopoeial monographs give detailed recommendations for the setup of respective studies (see, e.g., EP 3.2.1 and USP <1660>). Assessing delamination via experimental testing requires the use of stress protocols and accelerated conditions to make this assessment manageable related to time. Yet, these conditions often poorly correlate to actual risk during long-term storage and cannot reliably predict on what will be observed during long-term storage.

Due to complexity of the system, relying on a single analytical tool is not considered sufficient. It is recommended to address different aspects with appropriate technologies focusing on the following topics:

- *Visual inspection of the drug solution*
  This would be based on Ph. Eur. 2.9.20 testing. Illumination from the bottom of the vial is helpful to visualize even small particles. A magnifying camera and documenting videos aid in the detection of small visible particles. The characteristic "blinking" of the flakes with this technique is a first hint for delamination. This is due to the nature of the flakes; they have a relatively large surface area but are very thin (normally <100 nm, see Fig. 13.2).
- If particles are present or if their presence is assumed, the solution can also be filtered and the particles are further analyzed, e.g., by using SEM/EDS to identify their chemical composition. This may help to distinguish between flakes, which originate from delamination, and other particles (dust, precipitations, etc.).

- An optical inspection of the emptied container identifies possible zones of a strong chemical attack of the glass surface. Areas, in which such attacks have occurred, can be visualized under special illumination as a milky ring-shaped features or colored (bluish) areas near the bottom of the vial. Especially the coloration is a strong indication for a high risk of delamination.
- Determination of glass leachables by ICP-MS/OES
  Delamination is the result of preferential dissolution of specific elements of the glass backbone/matrix, mainly boron. Therefore, analyzing the elemental content of the drug product solution is a method to detect whether delamination processes may have occurred. In particular, elements such as calcium, aluminum, silicon, boron, sodium, and potassium may be of particular interest as they are part of the composition of the glass network (if they are not an integral part of the formulation). However, attention has to be paid to the fact that elevated amounts of glass ions could just be the result of a strong corrosive attack and are not directly linked to delamination.
  Inductively coupled-plasma mass spectrometry (ICP-MS) is an ideal and very sensitive technique to quantify these elements in parallel in the parts-per-billion (ppb) or parts-per-million (ppm) range. If the ratios of the elements in solution differ from the calculation of the content of each element in the glass, then this is a hint for a preferential dissolution, which is a strong indicator for a potential delamination risk.
- The result of the preferential dissolution is the formation of the so-called reaction zone. The presence of such a zone can be visualized by cutting the vials and subsequent surface analysis by SEM.
- Optionally a Time-of-Flight-Secondary Ion Mass Spectrometry (ToF-SIMS) depth profile reveals the distribution of the glass elements inside the upper glass surface (first few ten nm). Preferential leaching of specific elements can be made visible, and the information obtained can be also be correlated with the ICP-MS/OES results.

Samples for the analyses can originate from real-time or accelerated studies. Studies using increased temperatures should carefully consider the selected temperature range. Levels exceeding 40 °C might trigger reactions which are (due to their activation energies) not favorable at room temperature (or below) resulting in misleading data.

13.6 Summary

Delamination is the peeling-off or detachment of thin glass layers from the inner surface of vials usually in the form of small lamellae (“flakes”), which then can appear as particles in the product solution. These particles can be in the visible or sub-visible range. Delamination is a phenomenon which can often be avoided by a few preventive actions. The quality of the glass vials including the production

conditions, glass composition and coatings are of high importance to ensure final drug product quality. Secondly, processing conditions of vials, including washing, depyrogenization, and sterilization need to be carefully assessed and managed to protect the vial from a corrosive attack. Thirdly, sound formulation and drug product development should consider formulation and primary packaging interactions, and long-term stability testing needs to assess visible and sub-visible particles, on stability. In case unexpected particulates are observed, further actions and analytical characterization of the solution and vial surface are required to assess whether delamination is the root cause.

Adequate product and process design are important to design quality into the product, complemented by a thorough control strategy.

References

1. Schott technical glasses. http://www.schott.com/d/epackaging/2fbc7180-e37c-4209-9eec-617ad9208e51/1.0/18.11.15_final_schott_technical_glasses_row.pdf.
2. Scholze H. Chemical durability of glasses. J Non-Cryst Solids. 1982;52(1–3):91–103.
3. Hench LL, Clark DE. Physical chemistry of glass surfaces. J Non-Cryst Solids. 1978;28 (1):83–105.
4. Conradt R. Chemical durability of oxide glasses in aqueous solutions: a review. J Am Ceram Soc. 91:728–35.
5. Ogawa T, Miyajima M, Wakiyama N, Teradab K. Effects of phosphate buffer in parenteral drugs on particle formation from glass vials. Chem Pharm Bull. 2013;61(5):539–45.
6. White WB, Zoitos BK. Theory of corrosion of glass and ceramics. EDS Noyes Publications; 1992. p. 2–28.
7. Li GG, Cao S, Jiao N, Wen ZQ. Classification of glass particles in parenteral product vials by visual, microscopic, and spectroscopic methods. PDA J Pharm Sci Technol. 2014;68(4): 362–72.
8. Ratnaswamy G, Piedmonte DM, et al. A case study of nondelamination glass dissolution resulting in visible particles: implications for neutral pH formulations. J Pharm Sci. 2014;103 (4):1104–14.
9. Watkins MA, Iacocca RG, Shelbourn TL, Dong X, Stobba-Wiley C. Impact of glass corrosion on drug substance stability. J Pharm Sci. 2014;103(8):2456–63.
10. FDA Recalls. FDA Recalls, ed. https://www.fda.gov/Drugs/drugsafety/DrugRecalls/default.htm.
11. Bacon, et al. J Am Ceram Soc. 1940;23(1):1–9.
12. Dimbleby V. Glass for pharmaceutical purposes. J Pharm Pharmacol. 1953;5(12):969–89.
13. Guadagnino E, Zuccato D. Delamination propensity of pharmaceutical glass containers by accelerated testing with different extraction media. PDA J Pharm Sci Technol. 2012;66 (2):116–25.
14. Rothhaar U, et al. Comparative delamination study to demonstrate the impact of container quality and nature of buffer system. PDA J Pharm Sci Tech. 2016;70:560–7.
15. Iacocca RG, et al. Factors affecting the chemical durability of glass used in the pharmaceutical industry. AAPS PharmSciTech. 2010;11(3):1340–9.
16. Sloey C, Gleason C, Phillips J. Determining the delamination propensity of pharmaceutical glass vials using a direct stress method. PDA J Pharm Sci Technol. 2013;67(1):35–42.
17. Guardagnino E, Zuccato D. delamination propensity of pharmaceutical glass containers by accelerated testing with different extraction media. PDA J Pharm Sci Technol. 2012;66 (1):116–25.

18. Haines D, Scheumann V, Rothhaar U. Glass flakes, pre-testing stops a big problem before it even starts. In: Contract Pharma, 2–7 June 2013.
19. Ennis RD, et al. Glass vials for small volume parenterals: influence of drug and manufacturing process on glass delamination. Pharm Dev Technol. 2001;6(3):393–405.
20. Rupertus V, Hladik B, Rothhaar U, Scheumann V. A quick test to monitor the delamination propensity of glass containers. PDA J Pharm Sci Technol. 2014;68(4):373–80.
21. Ditter D, et al. Evaluation of glass delamination risk in pharmaceutical 10 mL/10R Vials. J Pharm Sci. 6 Oct 2017. pii: S0022-3549(17)30673-1. https://doi.org/10.1016/j.xphs.2017.09.016. [Epub ahead of print].
22. Ditter D, et al. Evaluation of glass delamination risk in pharmaceutical 10 mL/10R vials. J Pharm Sci. 2018;107(2):624–37.
23. Ditter D, et al. Characterization of surface properties of glass vials used as primary packaging material for parenterals. Eur J Pharm Biopharm, 10 Jan 2018. pii: S0939-6411(17)31008-1.
24. Haines D, et al. Glass flakes. Contract Pharma, 5 June 2013. http://www.contractpharma.com/issues/2013-06/view_features/glass-flakes/.
25. Ennis RD, et al. Glass vials for small volume parenterals: influence of drug and manufacturing processes on glass delamination. Pharm Dev Tech. 2001;6(3):393–405.
26. Boltres B, Tratzky S, Kass C, Eichholz R, Nass P. There is still room for improvement: presentation of a neutral borosilicate glass with improved chemical stability for parenteral packaging. PDA J Pharm Sci Technol. 2016;70(4):346–52.
27. Cailleteau C, Angeli F, Devreux F, Gin S, Jestin J, Jollivet P, Spalla O. Insight into silicate-glass corrosion mechanisms. Nat Mater. 2008;7(12):978–83.
28. Iacocca RG, Allgeier M. Corrosive attack of glass by a pharmaceutical compound. J Mater Sci. 2007;42(3):801–11.
29. Iacocca RG, Toltl N, Allgeier M, Bustard B, Dong X, Foubert M, Hofer J, Peoples S, Shelbourn T. Factors affecting the chemical durability of glass used in the pharmaceutical industry. AAPS PharmSciTech. 2010;11(3):1340–9.
30. Schaut RA, Peanasky JS, DeMartino SE, Schiefelbein SL. A new glass option for parenteral packaging. PDA J Pharm Sci Technol. 2014;68(5):527–34.
31. Walther Marten. Schott type I Plus® containers for pharmaceutical packaging in thin films on glass. 2nd ed. Heidelberg: Springer; 2003.
32. Ennis RD, Pritchard R, Nakamura C, Coulon M, Yang T, Visor GC, Lee WA. Glass vials for small volume parenterals: influence of drug and manufacturing processes on glass delamination. Pharm Dev Technol. 2001;6(3):393–405.
33. Jiang G, Goss M, Li G, Jing W, Shen H, Fujimori K, Le L, Wong L, Wen ZQ, Nashed-Samuel Y, Riker K, Germansderfer A, Tsang P, Ricci M. Novel mechanism of glass delamination in type 1A borosilicate vials containing frozen protein formulations. PDA J Pharm Sci Technol. 2013;67(4):323–35.
34. Yamanaka S, Akagi J, Hattori M. Reaction of pyrex type borosilicate glass with water in autoclave. J Non-Cryst Solids. 1985;70(2):279–90.
35. Sloey C, Gleason C, Phillips J. Determining the delamination propensity of pharmaceutical glass vials using a direct stress method. PDA J Pharm Sci Technol. 2013;67(1):35–42.
36. Bacon FR, Raggon FC. Promotion of attack on glass and silica by citrate and other anions in neutral solution. J Am Ceram Soc. 1959;42(4):199–205.
37. Guadagnino E, Zuccato D. Delamination propensity of pharmaceutical glass containers by accelerated testing with different extraction media. PDA J Pharm Sci Technol. 2012;66(2):116–25.
38. Bacon FR, Raggon FC. Promotion of attack on glass and silica by citrate and other anions in neutral solution. J Am Ceram Soc. 1959;42(4):199–205.
39. Ogawa T, Miyajima M, Wakiyama N, Terada K. Effects of phosphate buffer in parenteral drugs on particle formation from glass vials. Chem Pharm Bull (Tokyo). 2013;61(5):539–45.
40. Bacon FR, Raggon RC. Promotion of attack on glass and silica by citrate and other anions in neutral solution. J Am Ceram Soc. 1959;42(4):199–205.

41. Wu S, et al. (2008) The effect of solution properties on the delamination of vials used in parenteral products, AAPS 79, Poster.
42. Bunker BC. Molecular mechanisms for corrosion of silica and silicate glasses. J Non-Cryst Solids. 1994;179:300–8.
43. Cailleteau C, et al. Insight into silicate-glass corrosion mechanisms. Nat Mat. 2008;7:978–83.
44. Geisler T, et al. Aqueous corrosion of borosilicate glass under acidic conditions: a new corrosion mechanism. J Non-Cryst Solids. 2010;356:1458–65.
45. Abdul-Fattah AM, Oeschger R, Roehl H, Bauer Dauphin I, Worgull M, Kallmeyer G, Mahler HC. Investigating factors leading to fogging of glass vials in lyophilized drug products. Eur J Pharm Biopharm. 19 Jun 2013.
46. Iacocca RG, et al. factors affecting the chemical durability of glass used in the pharmaceutical industry. AAPS Pharm Sci Tech. 2010;11(3):1340–9.
47. Iacocca RG (2011) The causes and implications of glass delamination. Pharmceut Technol, 1st Nov 2011. http://www.pharmtech.com/pharmtech/Analytics/The-Causes-and-Implications-of-Glass-Delamination/ArticleStandard/Article/detail/747427.
48. Bacon FR, Burch OG. Effect of time and temperature on accelerated chemical durability tests made on commercial glass bottles*. J Am Ceram Soc. 1940;23(1):1–9.
49. Bacon FR, Burch OG. Effect of time and temperature on accelerated chemical durability tests made on commercial glass bottles, II*. J Am Ceram Soc. 1941;24(1):29–36.

Chapter 14
Fogging

Holger Roehl, Philippe Lam and Dominique Ditter

Abstract The surface properties of primary packaging containers are determining their interaction with the parenteral drug product solution. This is not only restricted to ion leaching or corrosion effects but also impacting adsorption phenomena. The formulation can creep up the walls forming a thin liquid layer on the inside of the containers. This layer is getting visible as product residues on the vial walls after a lyophilization process. This mechanism is known under the term of "fogging". This chapter describes the underlying root causes and provides measures to avoid the issue.

Keywords Glass · Primary packaging containers · Vials · Lyophilization
Fogging · Surface properties · Marangoni effect

Fogging is a phenomenon observed after lyophilization of a pharmaceutical product [1, 2]. It is a result of deposits formed by thin liquid films from creeping flows (see Fig. 14.1). Though the overall amount of material is low, the freeze-dried material on the vial inner surface can be considered as cosmetic defect which could interfere with optical inspection or Container Closure Integrity (CCI, if the liquid is reaching the vial opening). This often results in higher, and often quite variable and unpredictable reject rates and thus, economical loss for the pharmaceutical companies.

H. Roehl (✉)
Late Stage Pharmaceutical and Processing Development, Pharmaceutical Development & Supplies, Pharma Technical Development Biologics EU, F. Hoffmann-La Roche Ltd., Grenzacherstrasse 124, 4070 Basel, Switzerland
e-mail: holger.roehl@roche.com

P. Lam
Pharmaceutical Processing and Tech. Dev., Genentech Inc., 1 DNA Way, South San Francisco, CA 94080, USA

D. Ditter
Site Manufacturing Science and Technology, Validation Engineering & Filling, Roche Diagnostics GmbH, Sandhofer Straße 116, 68305 Mannheim, Germany

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*, AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_14

Fig. 14.1 Glass with lyophilized product exhibiting fogging

This complex phenomenon is well known and has been reported in the scientific literature as the Marangoni effect [3, 4]. The most often cited example of Marangoni effect is the "tears or legs of wine" where thin streaks of wine or liquor are observed to climb the sides of the glass (legs), accumulating in pools which eventually become sufficiently large to fall back down under influence of gravity as droplets (tears) [5].

In general, the Marangoni effect refers to a class of fluid flow driven by surface tension gradients. The classical explanation for the tears of wine effect is that liquid flow stems from the surface tension gradient between the surface of the liquid in the glass and the upper edges of the wetted film present on the vertical wall. Presumably, due to faster local evaporation of the volatile component (alcohol) at the leading film edge, the concentration of alcohol is lower there, resulting in a higher local surface tension. As a result, liquid from the lower portion is "pulled" up the glass wall (flow). This flow can be sustained for some time, but if evaporation is eliminated, by covering the glass, then the tears of wine stops.

Surface tension gradients can also arise due to temperature gradients of the film covering the substrate [3] or due to the presence of surfactants. In the case of "fogging" of lyophilized pharmaceutical product vials, surfactants (such as polysorbate) in the liquid drug substance are responsible for the formation of surface tension gradients along the inner surface of the vial in the axial direction. Other components of the formulation may also contribute, but the surfactant is the main agent which substantially lowers the solution surface tension as compared to that of pure water.

Figure 14.2 depicts the meniscus region inside a liquid-filled glass vial. The solution surface is populated with surfactant molecules and exhibits a lower surface tension than pure water. A clean glass surface is normally hydrophilic and is typically coated with a thin film of water (hydration film). Therefore, the liquid meniscus region will exhibit a gradient of surface tension, ranging from low near the liquid surface where surfactant is present, to high on the hydration film of the glass. The surface tension gradient "pulls" the solution upwards, resulting in flow of the bulk liquid which will coat the vial surface as a thin film. This Marangoni flow occurs spontaneously, immediately after filling or shortly thereafter. Unlike the tears of wine, flow due to surfactant will cease once sufficient surfactant molecules diffuse into the hydration film to diminish the surface tension gradient below what is necessary to pull liquid against gravity.

Several flow patterns, illustrated in Fig. 14.3, are observed, often simultaneously: (1) a uniform front, (2) fingering patterns and, (3) branching patterns [6–11]. These are all manifestation of the same complex Marangoni flow phenomena. Fingering and branching are due to instabilities in the liquid spreading front.

The white deposits that characterize "fogging" are a result of the drying of the liquid film formed by Marangoni flows. The composition of the deposits is the same as that from liquid splashes during filling or sloshing during post-fill handling/ transport on the line. The extent of observed fogging (how much coverage) depends on a multitude of factors such as composition of the liquid, surface of the glass,

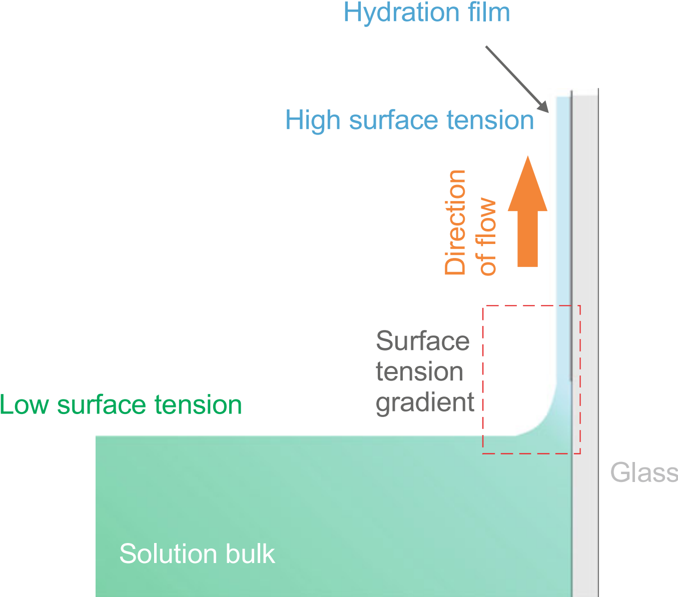
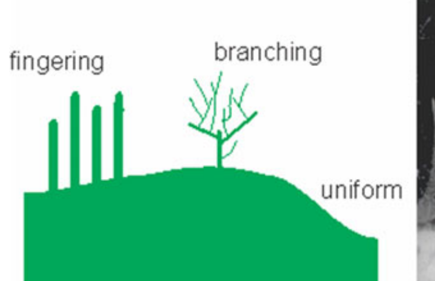


Fig. 14.2 Surfactant-driven Marangoni flow in a glass vial. This illustration is not drawn to relative scale. The initial hydration film thickness is much less than that of the resulting film due to Marangoni flow



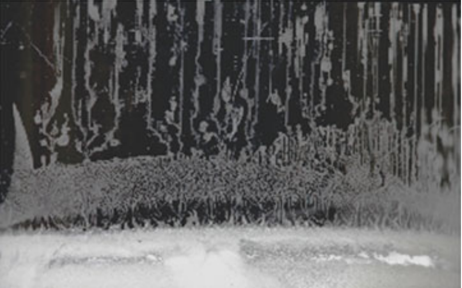


Fig. 14.3 Marangoni flow patterns and example of the resulting "fogging" on a lyophilized product vial

handling of the vial post-fill. In addition, the thickness of the deposits depends also on the amount of time the liquid film is allowed to drain before being frozen and dried.

Recent publications [1, 2] reported on the impact of formulation and process conditions on fogging including solution composition, temperature and glass treatments (washing/depyrogenation conditions). The two common factors essential for the occurrence of fogging are the presence of a surfactant in the formulation solution and the hydration film on the glass surface (low contact angles to water, increasing contact angles result in a lower degree of fogging). Diverse studies investigating the mechanism of solution creeping and film transfer through observing creeping behaviour of, e.g., a placebo solution containing a fluorescein dye, or charged nanoparticles in aqueous solutions along the interior glass surface of vials or containers, [14] are available in the literature. All other factors have no clear impact on fogging. This is why batch-to-batch, vial-to-vial and even variations within one single vial occur.

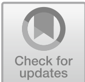
Furthermore, due to the complex interplay of the numerous factors, controlling fogging in a reliable manner is not possible with the standard components. The only robust solution to this problem is to use vials with a hydrophobic surface. This single change will eliminate fogging completely [12]. It is well known that siliconized glass (baked-on silicon) exhibits more hydrophobic surfaces and therefore shows poor wetting and larger contact angles with polar liquids as compared to untreated clean glass surfaces [2, 13]. Schott TopLyo™ vials are one standard preventing fogging completely. These vials typically show a contact angle >90° and consist of expansion 51 glass (referring to the thermal coefficient of thermal expansion α) combined with a completely transparent and nonporous hydrophobic layer that is applied by plasma impulse chemical vapour deposition (PICVD).

References

1. Bauer-Dauphin I, Mahler HC. A method for avoiding glass fogging of pharmaceutical containers during freeze drying. In: PCT International Application. Switz: F. Hoffmann-La Roche AG; 2010. 26 pp.
2. Abdul-Fattah AM, et al. Investigating factors leading to fogging of glass vials in lyophilized drug products. Eur J Pharm Biopharm. 2013.
3. Ludviksson E, Lightfoot EN. The dynamics of thin liquid films in the presence of surface-tension gradients. AIChE J. 1971;17(5):1166–73.
4. He S, Ketterson JB. Surfactant-driven spreading of a liquid on a vertical surface. Phys Fluid. 1995;7(11):2640–7.
5. Vuilleumier R, Ego V, Netlner L, Cazabat AM. Tears of wine: the stationary state. Langmuir. 1995;11:4117–21.
6. Cazabat AM, Heslot F, Carles P, Troian SM. Hydrodynamic fingering instability of driven wetting films. Adv Coll Interf Sc. 1992;39:61–75.
7. Cazabat AM, Heslot F, Troian SM, Carles P. Fingering instability of thin spreading films driven by temperature gradients. Nature. 1990;346:824–6.
8. Eres MH, Schwartz LW, Roy RV. Fingering phenomena for driven coating films. Phys Fluid. 2000;12(6):1278–95.
9. Troian SM, Wu SL, Safran SA. Fingering instability in thin wetting films. Phys Rev Let. 1989;62(13):1496–500.
10. Matar OK, Troian SM. The development of transient fingering patterns during the spreading of surfactant coated films. Phys Fluid. 1999;11(11):3232–46.
11. Troian SM, Herbolzheimer E, Safran SA. Model for the fingering instability of spreading surfactant drops. Phys Rev Let. 1990;65(3):333–6.
12. WO2010/115728A2: a method for avoiding glass fogging.
13. Roedel E, Blatter F, Buettiker JP, Weirich W, Mahler HC. Contact angle measurement on glass surfaces of injection solution containers. Pharm Ind. 2013;75(2):328–32.
14. Johnson DD, Kang B, Vigorita JL, Amram A, Spain EM. Marangoni flow of Ag nanoparticles from the fluid − fluid interface. J Phys Chem A. 2008;112(39):9318–23.

Part V
Processing Considerations

Chapter 15
Bulk Protein Solution: Freeze–Thaw Process, Storage and Shipping Considerations



Parag Kolhe and Sumit Goswami

Abstract Protein drug substance is typically frozen to enable manufacturing flexibility through prolonging the shelf life of drug substance and providing better biochemical stability. The process of freezing and thawing of bulk protein solutions poses several challenges. It is important to understand the process and define mitigation strategies to address these challenges. Just not the process but the choice of storage container can have an impact on stability and downstream operation of formulation during drug product process. Therefore, understanding the options currently available in terms of drug substance storage containers and how to balance the need based on specific scenarios is critical. Storage temperature is as important as the container the drug substance is stored at. Furthermore, shipping drug substance in frozen state requires thorough understanding of logistics and dependence of shipping temperature on stability. This chapter fundamentally looks at the freezing and thawing process, discusses phenomenon of cryoconcentration in various containers and mitigation strategies, consequence of not choosing appropriate storage temperature, and provides considerations for storage and shipping of drug substance.

Keywords Drug substance · Cryoconcentration · Freeze–thaw
Glass transition temperature · Stability · Shipping

15.1 Introduction

Protein bulk solutions are manufactured in drug substance manufacturing facility, filled in suitable storage container, typically frozen at the DS manufacturing facility, stored at recommended storage temperature, and shipped to DP manufacturing facility. Bulk protein solution freezing offers distinct advantages which include

---

P. Kolhe (✉) · S. Goswami
BioTherapeutics Pharmaceutical Sciences, Pharmaceutical R&D,
Pfizer Inc., 700 Chesterfield Parkway West, St. Louis, MO 63017, USA
e-mail: parag.kolhe@pfizer.com

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_15

extending DS shelf life and offer better biochemical stability during storage as well as during transportation (eliminates air–water interface and hence protects protein from denaturation).

Freezing bulk protein product offers substantial benefits but is not devoid of challenges. Freeze–thaw unit operation parameters, storage of bulk drug substance, shipping in frozen state, and logistics are some of the critical elements that need to be considered. It is essential that the phenomenon of freezing and thawing is understood well while designing the processes. Understanding various considerations and constraints during product development is important for successful scale-up, process development, developing design space around various process parameters, and instituting adequate control strategy for clinical and commercial manufacturing.

This chapter focusses on various factors associated with the storage of DS such as choice of DS storage temperature, commonly used DS storage containers, and considerations associated with their choices, robustness around freeze–thaw process, technical challenges, and mitigation strategies for cryoconcentration effects in various containers and shipping considerations in frozen state.

15.2 DS Storage Considerations

Important aspect of DS storage is choice of temperature. Temperature ranges that are typically used for storage of biologics DS are 2–8 °C (typically for short-term storage), −20, −30, −40, −55, and −70 °C. Although storage at 2–8 °C can be an option for certain types of DS, biologics DS are typically stored at frozen condition as bulk. Bulk storage of DS at frozen condition provides a number of important benefits. Some of these are listed below:

1. Longer shelf life than storage at 2–8 °C. Frozen storage allows reduction of the rate of physical and chemical degradation by minimizing molecular mobility and interaction with the container closure surface and thus slows down the reaction kinetics.
2. Reduces the risk of microbial contamination as frozen solution condition is not conducive to microbial growth [1]. Other than the extremophiles which are not commonly encountered in manufacturing facility, frozen storage conditions are detrimental to the growth of regular microbes. If the bioburden load of the released DS is within the acceptable limit (NMT 1 cfu/10 mL of DS), risk of contamination of frozen DS with newer microbes is really low.
3. Minimizes the potential risk of agitation/shaking-induced degradation during transportation. Agitation/shaking-induced destabilization of biologics is primarily mediated by the interfacial stress resulting from air–water interface. Partitioning of the molecules in the air–water interface may lead to exposure of hydrophobic patches on the molecules which may lead to aggregation. Storing of DS in frozen condition takes away the air–water (liquid) interface and thus minimizes the agitation/shaking-induced destabilization.

4. Bulk storage of frozen DS with longer shelf provides flexibility toward meeting the real-time need of manufacturing of DP (both clinical and commercial) and also reduces the overall cost of drug development. DP batch size and the need for manufacturing a batch are primarily driven by the clinical demand. Need for reloading (remanufacturing) a DP batch may show up as a sudden requirement during an ongoing clinical trial for various reasons, such as extension of clinical trial. In such situation, availability of bulk DS may reduce the requirement of multiple DS manufacture which provides flexibility as well as reduces the overall cost [2].

The next relevant question is how to decide what storage condition to choose while storing DS. Freezing is known to increase the storage stability of DS but does storing at each of the abovementioned temperature condition provide equal extent of protection against degradation? Does formulation of the DS have a role to play in the choice of frozen storage condition? Answers to these questions are intricately connected to the understanding of the phase behavior of water and how that behavior is influenced by the presence of solute. Understanding the phase behavior of water is essential as biologics are primarily formulated in aqueous system. Phase behavior of pure water describes the equilibrium process of freezing of liquid water into ice [3]. The temperature (0 °C) at which such transition (liquid to ice) starts to happen has been described as the freezing point. The presence of other excipients (or solute) in water causes changes in the phase behavior as well as depression of freezing point which has been described by Raoult's law. Changes in the phase behavior can be very complex depending on the type (crystallizable vs. non-crystallizable), number, and relative concentration of the solutes present in the system. Equilibrium phase diagrams are available for a number of binary systems, e.g., water/NaCl, water/trehalose [4–9]. A few phase diagrams are also available for some of the ternary system of water, e.g., water/glycerol/NaCl, water/DMSO/NaCl [4, 10–15]. Complexity of the phase behavior due to inclusion of excipients is apparent from the phase diagram of NaCl. Freezing of a normal saline system (0.9 wt% NaCl in water) causes water to come out of the bulk solution phase as ice crystal, and as a result, the concentration of NaCl increases considerably in the bulk phase. As the temperature of the system is brought down to −21.2 °C, the bulk phase experiences a 26-fold increases in salt concentration (23.3 wt%). At this temperature, referred as eutectic point the system is a complex equilibrium mixture of ice, hydrohalite ($NaCl{\cdot}2H_2O$), and saturated solution of saline [4]. Eutectic point also refers to the depressed freezing point of this water/NaCl system. This can be clearly understood from the phase behavior of water/NaCl binary system that storing at −20 °C may not be enough to achieve a truly frozen state [4].

Direct application of the equilibrium phase diagram is though limited from practical standpoint, as in reality a formulated DS hardly follows equilibrium behavior while undergoing freezing process. Changes associated with the phase behavior during freezing of aqueous solution (water containing other excipients or solutes) have been described by the time–temperature relationship diagram by Akyurt et al [16]. This relationship describes the non-equilibrium nature of the

freezing process which starts with supercooling of the system followed by ice nucleation. Ice nucleation initiates the process of freezing which continues until there is no more 'freezable' water available [3]. The process of ice formation (phasing out of water from bulk) increases the concentration of the solutes in the bulk resulting at cryoconcentration or freeze concentration. Cryoconcentration of the unfrozen matrix continues to increase until freezing is complete. Combined effect of increase in concentration and lowering of temperature increases the viscosity of the unfrozen bulk with a concomitant decrease in diffusion coefficient. When formation of ice crystal is practically complete, the concentration of the unfrozen mixture reaches to a maximum and the temperature beyond which no further increase in ice crystallization is obtained while measured under short time scale is called glass transition temperature (Tg′) [17, 18]. As the system passes through the Tg′, along the glass transition curve, the viscosity of the unfrozen bulk becomes considerably high to the point where the state is described as 'glass' (viscosity > 10^{10} Pa/S) [3, 18, 19].

Tg′ plays a critical role in the choice of frozen storage condition for biologics DS. Mechanistic understanding of why frozen storage increases the storage stability of DS may help us understand the relevance of Tg′. Different mechanisms have been proposed to describe cryoprotection. Formation of 'glass' like phase (thus increase in viscosity with a corresponding decrease in diffusion coefficient of the unfrozen bulk) beyond Tg′ has been proposed to provide kinetic stabilization through reduction in the rate of degradation (unfolding, aggregation) resulting from decrease in molecular mobility [1, 3, 17]. Another proposed mechanism is thermodynamic stabilization which is essentially mediated by preferential exclusion. As the freezing process continues along with other solutes, protein molecules also get partitioned into the unfrozen bulk. In solution phase, protein molecules are hydrated (surrounded by water molecule). Hydration of protein molecules has been postulated to be important for the structural stability [20, 21]. As the water crystalizes out during freezing, the hydration shell around protein molecule is disrupted due to removal of water, described as desiccation. Desiccation may lead to denaturation of protein. The presence of stabilizer molecules works against the loss of hydration shell around protein molecules via preferential exclusion mechanism. Preferential exclusion of the stabilizers destabilizes the denatured state of the protein and thus achieves thermodynamic stabilization [22, 23]. If thermodynamic stabilization was the only mechanism involved, then inclusion of stabilizer would be enough for providing stability at any frozen conditions. In practical scenario though, kinetic stabilization plays a critical role. Piedmonte et al. reported that long-term storage of an Fc-fusion protein at −30 °C in sorbitol-containing formulation showed aggregation [24]. Tg′ value of pure sorbitol as reported in the literature is −44 °C [25, 26]. It was also found that storage at −20 and −70 °C did not cause increase in aggregation. Similar finding was reported by Singh et al. for a monoclonal antibody in trehalose (Tg′ is −29 °C)-containing formulation [1]. In this study, Singh et al. reported that storage of the formulation at −20 °C showed aggregation of the mAb whereas storage at −10 °C did not show any noticeable change in aggregation level. These phenomena have been explained by primarily two different mechanisms—

kinetic stabilization and crystallization of the stabilizer. Storage at above Tg′ causes significant decrease in viscosity resulting at increase in molecular mobility. Change in viscosity just above Tg′ has been described by WLF equation (Williams–Landell–Ferry) [27]. This empirical equation describes that the change in viscosity above Tg′ (but in the in vicinity of Tg′) does not follow Arrhenius kinetics and shows much higher sensitivity to change in temperature. In accordance with this altered kinetics, it has been found that for a 3% sucrose solution storage at −20 °C causes 100-fold decrease in viscosity ($\sim 10^6$ to 10^4 cP) compared to while stored at −30 °C. Storing at −10 °C causes an additional 100-fold reduction in viscosity [17]. Decrease in viscosity allows increased level of molecular mobility which has been postulated to be one of the reasons for increase in protein aggregation at −20 and −30 °C for trehalose-containing and sorbitol-containing formulations respectively [1, 24]. Increase in molecular mobility facilitates interaction between the partially denatured protein molecules causing aggregation. It has been proposed that during freezing process interaction of protein molecules with the growing ice surface causes partial denaturation [28–30]. Singh et al. postulated that at slightly higher temperature than Tg′ (−20 °C) molecular mobility is high enough for the partially denatured molecules to interact and form aggregate. Crystallization of trehalose has also been proposed to be another cause for destabilization of the mAb at −20 °C. Sundaramurthi et al. reported that trehalose undergoes crystallization while stored at −18 °C for 3 days [31]. This process reduces the concentration of cryoprotectant in the amorphous phase, which once again, when the molecular mobility is high enough, may lead to increased intermolecular interaction of the denatured molecules causing aggregation. Lack of stability issue at −10 °C for trehalose-containing formulation has been proposed to be due to much higher molecular mobility allowing refolding of the denatured molecules. Although crystallization of trehalose happens at −10 °C, Singh et al. proposed that much higher molecular mobility at this elevated temperature favors the process of refolding over aggregation [1]. On the other hand, the decreased stability of the Fc-fusion protein in sorbitol-containing formulation stored above Tg′ has been described due to the crystallization process of sorbitol [24]. All these abovementioned observations clearly indicate that the storage of DS at a temperature above the Tg′ of the system may run a much higher risk of destabilization of the protein molecules during long-term storage under frozen condition. Importance of Tg′ also indicates that the choice of temperature for frozen storage is closely associated with the choice of formulation components (stabilizer, excipients, etc.) as the latter determines the Tg′ of the system. Target storage condition can be achieved if formulation components are carefully chosen considering long-term stability of both DS and DP (drug product).

For a given system, Tg′ is highly dependent on the constituent solutes/excipients. The main constituents of a standard formulated DS are buffer, excipients (stabilizer/cryoprotectant), and protein. Tg′ for a system can be calculated using the Fox equation which takes into account the Tg′ of the individual component along with their weight fraction as described in Eq. 15.1 [19].

$$1/\mathrm{Tg}' = w_1/\mathrm{Tg}'_1 + w_2/\mathrm{Tg}'_2 \quad (15.1)$$

Above equation describes the calculation of Tg′ for a two-component system, where w_1 and w_2 are the relative weight fractions of each component and Tg'_1 and Tg'_2 are the glass transition temperatures of each component, respectively. List of Tg′ for different solutes could be found in the following references [25, 32, 33]. Proteins do exhibit glass transition behavior as well. Tg′ for different proteins has been reported to be around −10 °C [34]. Colandene et al. reported that introduction of monoclonal antibody (mAb) into a buffer system causes increase in Tg′ and the increase was found to be positively correlated with the increase in mAb concentration [35].

15.3 Impact of Freezing Stress on Protein Stability

Need for obtaining longer storage life for biologics DS is of paramount importance from the standpoint of reducing the overall cost of drug development. Thus, DS freezing has become a critical step in the development of biotherapeutics. While benefits are well-understood and importance is well-established, formulation scientist needs to be aware of the impact of freezing stress on the stability of the biologics DS.

As the water molecules phase out as ice crystals, concentration of solute at the vicinity of ice front goes up which in turn increases the possibility of protein molecules interacting with the ice surface. Such surface interaction followed by adsorption on to the ice surface has been reported to cause structural perturbation (loss of native secondary/tertiary structure) leading to denaturation [28, 36]. Strambini et al. proposed that size of liquid water pool in the unfrozen matrix in equilibrium with ice has significant impact on ice surface-mediated perturbation of protein structure. For the given system they tested (formulation, protein, and temperature), it was observed that at water pool below 1.5%, the free energy for unfolding of protein molecules decreases progressively [28]. Decreases in water pool cause increase in ice surface area (ice growth) leading to higher adsorption of protein molecules. Once adsorbed on the surface denaturation is favored as it increases solvent accessible surface area and thus stronger interaction with the ice surface leading to further decrease in free energy. It has been found that the freezing processes that generate larger ice surface area are more prone to cause surface interaction-/adsorption-induced denaturation of protein [28, 37, 38]. Slower cooling rate may result at less perturbation, most likely due to the fact that molecules get more time to diffuse out of the growing ice front and formation of larger size ice crystals that have relatively lesser surface area. Interaction of protein molecules with ice can be also minimized by inclusion of surfactants in the formulation. Chen et al. reported the direct benefit of surfactant for protecting the protein molecules from ice surface interaction-induced denaturation [36]. Cause of adsorption of

protein molecules on ice surface has also been described entropically. It has been proposed that formation of ice causes ordering of water molecules along the ice surface which is entropically unfavorable. Adsorption of protein molecules on the ice surface frees up the water molecule into the bulk, a process which is entropically favorable [39, 40].

Cold denaturation and desiccation are natural consequence of freezing. Cold denaturation simply refers to the stress effect from lowering of temperature. Impact of high temperature on protein stability is well known. Exposing protein molecules to freezing temperature can also be stressful enough that may result at protein denaturation. Griko et al. reported the relationship between the Gibbs free energy of unfolding with temperature that ranged from low negative to high positive value for staphylococcal nuclease enzyme [41]. The relationship between free energy and temperature was found to be parabolic in nature which indicates free energy of unfolding became low at lower temperature. When the temperature was as low as −25 °C, the free energy of unfolding was negative at formulation pH of 7. Cold denaturation has been proposed to be a thermodynamic consequence of change in solvent property (dielectric constant of water at −25 °C is 109, whereas at 25 °C it is 79) [3], weakening of the strength of electrostatic and hydrophobic interaction, etc [3, 42–44]. Typically, cold denaturation happens somewhere between 0 °C and the temperature at which other freezing effects start to show up and the specific temperature at which it happens is related to formulation pH, the presence of other solutes, e.g., sugar, and also concentration of protein [41, 45–49]. Study with staphylococcal nuclease enzyme indicates that lowering of formulation pH further reduces the Gibbs free energy of unfolding at a given temperature and thus at lower pH cold denaturation happens at relatively higher temperature [41]. In the presence of denaturant (urea, guanidine HCl), the cold denaturation happens at higher temperature as well. Tang et al. found that in the presence of sugar and polyols, cold denaturation happens at a lower temperature [50]. They also found that at higher concentration protein self-stabilizes by reducing the cold denaturation temperature [50]. Cold denaturation has been thought to be reversible in nature and in the absence of other freezing stresses has been considered non-detrimental to the storage stability of the DS [3, 19]. Adrover et al. reported that increased hydration of the protein molecules is the cause of cold denaturation. By performing SAXS study on Yfh1 (mitochondrial human protein) that undergoes cold denaturation at neutral pH and at temperature high enough for experimentation, Adrover et al. found that cold-denatured molecules exhibited higher degree of hydration compared to their heat denatured counterparts [51]. It was hypothesized that increased hydration at lower temperature and thus tighter binding of water molecules to the amide bond hinders the conversion of the partially unfolded state back to their folded secondary structure.

Desiccation on the other hand results from perturbation of the hydration shell around the protein molecules. Water molecules form hydration shell around the protein surface through direct interaction, and such shell is essential for maintaining the three-dimensional fold of protein molecules as well as lubricating the protein surface allowing rapid exchange of hydrogen bonds with the backbone and side

chain of the protein molecules [21, 52]. As the freezing process continues, water molecules are removed out of the protein surface leading to desiccation of protein molecules. This may perturb the hydration shell of the protein molecules. Remmele et al. reported structural change of lysozyme (amide I bond frequency shift and bandwidth narrowing) in the absence of cryoprotectant, and this was attributed to loss of water (desiccation). The presence of sucrose was found to mitigate that effect [53] and was considered to be caused by the maintenance of hydration shell via preferential exclusion of sucrose.

Cryoconcentration during freezing may have significant impact on the stability of the protein molecule. The basic concept of cryoconcentration and how freezing rate (cooling rate) influences the cryoconcentration behavior have been described in the previous section. Cryoconcentration results from the partitioning of the unfrozen formulation components (buffer, excipient, protein, etc.) into the amorphous bulk that builds up within the growing ice crystals. As the freezing process continues, concentration of the formulation components in the unfrozen matrix may undergo several fold increases. This may lead to several consequences that are unwanted and detrimental to the stability of the protein molecules. Increase in concentration can become high enough for one or more of the formulation components to be supersaturated at a given location. This may lead to crystallization or precipitation of the component. Phosphate buffers are known to show such effect during freezing. Dibasic salt of sodium phosphate is much less soluble than the monobasic salt. Cryoconcentration during freezing may cause precipitation of the dibasic salt leading to decrease in pH. Sodium phosphate buffer has been shown to undergo a decrease in as much as 3 pH units (7 to 4) upon freezing (room temperature to −10 °C) [54–57]. For potassium phosphate buffer system, the effect is opposite and thus shows increase in pH due to precipitation of the monobasic salt. The shift in pH is more prominent with the increase in concentration of the buffer. Shift in pH may be detrimental to protein molecules that are susceptible to pH change. Degradation of β-galactosidase in sodium phosphate buffer has been reported by Pikal-Cleland et al., and this has been attributed to freezing-induced decrease in pH [58]. They also found a correlation between higher concentration of the buffer component and increased level of aggregation. Increase in protein concentration on the other hand was found to cause decrease in aggregation [58]. Pikal-Cleland et al. also reported that application of faster freeze–thaw process may possibly minimize the exposure to the shift in pH leading to reduction in the level of aggregation [58]. Susceptibility to pH shift may vary between proteins though. Kolhe et al. performed a systematic study to understand the pH change behavior of commonly used buffers and the impact of pH change on IgG2 aggregation [59]. While some of the buffers (phosphate, Tris) showed considerable change in pH upon freezing either due to crystallization or temperature-dependent change in pKa, no impact on the aggregation of the IgG2 could be identified that could be solely attributed to change in pH during freezing [59]. The presence of other crystallizable component such as NaCl has been found to further aggravate the formation of aggregates. Adverse effect of NaCl on protein (e.g., hGH) stability during freezing process has been reported in multiple studies [58–61]. Such effect was attributed to

either crystallization of NaCl (leading to salting out of protein) or specific anion interaction with protein surface [60]. In the presence of cryoprotectant such as sucrose, crystallization of buffer component and excipients has been found to be suppressed or minimized [17, 62].

Cryoconcentration can lead to liquid–liquid phase separation within the amorphous phase as the temperature decreases during freezing. This leads to formation of more than one amorphous phases and possible partitioning of the protein molecules within those amorphous phases. Resulting amorphous phases may not be equal in their cryoprotective property which may lead to inefficient cryoprotection of the protein molecules [17]. Heller et al. reported phase separation of a PEG–dextran mix during freezing, and when hemoglobin was formulated in that mix, it was found to be partitioned into the separated phases [63]. The presence of mannitol was found to minimize phase separation [64]. Most of the phase separation issue has been observed with solutions containing polymeric materials such as dextran, PEG, PVP. In-depth characterization of system containing protein and the most commonly used stabilizers in pharmaceutical industry is still pending.

15.4 Practical Aspects of DS Storage Container and Freeze–Thaw Systems

Selection of appropriate container as well as technology for freeze–thaw operation is dependent on the availability of specific technology in the manufacturing environment as well as volume of production, frequency of production along with related logistics. This section discusses available containers and freeze–thaw systems and factors that can influence the choice of the same.

15.4.1 Available Freeze–Thaw Systems and Containers

Freeze–thaw systems and accompanying containers intended for storage of DS are classified into two categories: controlled freeze–thaw operation (also termed as active system) versus uncontrolled freeze–thaw operation (also termed as passive system where freeze–thaw rates are dependent on environmental temperature and loading).

15.4.1.1 Controlled Freeze–Thaw Systems

Controlled freeze–thaw systems offer consistent freezing and thawing rates that can be applied to protein drug substance in intended container. The following section describes the currently available commercial systems capable of controlling the freezing and thawing rates for bulk protein solutions:

Celsius Pak® System

Celsius Pak® system comprises of bags that are constructed of S71 film which has product contact layer of EVAM (ethylene vinyl acetate copolymer, monomaterial) and sizes specifically designed for controlled freeze–thaw (REF—Celsius-Pak® validation). The offerings in the controlled freeze–thaw include 1, 2, 8.3, and 16.6 L Celsius®-Pak which can be frozen and thawed by using Celsius® freeze–thaw module. These modules are equipped with freeze–thaw module for Celsius®-Pak holding, temperature control unit and control panel (REF: Celsius FT100 Modular freeze–thaw system from Sartorius Stedim)

Cryovessel System

Cryovessel system uses identical system in terms of freeze–thaw operation where a temperature control module is used although it differs in the mechanism and design. Cryovessels can handle volumes in the range of 50–300 L and are made up of stainless steel. Cryovessels are jacketed and cylindrical in shape. CryoFin™ technology is used for the design of cryovessel where the sections of cryovessel are divided into eight longitudinal sections to facilitate dendritic ice growth and providing smaller identical sections in terms of heat transfer.

Zeta FreezeSystem®

Zeta freeze–thaw systems utilize heating/cooling unit called as FreezeController® which is attached to FreezeContainer® made out of stainless steel. FreezeContainer® is available in various volumes ranging from 10 through 300 L (pilot scale to manufacturing scale). The mechanism of heat transfer is different than cryovessel. An internal circulation system which is mounted on lids provides the uniform heat transfer to facilitate freeze–thaw operation.

15.4.1.2 Uncontrolled Freeze–Thaw Systems

In the space of uncontrolled freeze–thaw systems, there are few options that are available which include traditional carboys/plastic bottles to more sophisticated systems such as Celsius® FFT system. Typical traditional plastic bottles include bottles made up of HDPE (high-density polyethylene) and PP (polypropylene). Celsius® FFT uses ready to use container which includes S71 film bag and a protective shell. This system is available in 2, 4, 6, and 12 L offerings.

Both of these systems are designed for uncontrolled freeze–thaw in traditional freezers/cold room/ambient condition. Freezing and thawing rates can vary depending on type of freezer used, placement of container, capacity of freezer, etc. One of the advantages with the uncontrolled freeze–thaw system is the flexibility afforded with container size, especially for early stages of projects.

15.4.2 Considerations for Choice of DS Container

There are multiple factors that can influence the choice of DS container. Some of those factors are briefly discussed below.

1. *Size of the DS/DP batch*:
   For early phase clinical supply, in a given manufacturing campaign the total number of DP units required may be in the range of thousands. This may be achievable with a bulk DS container size in the range of around few liters. On the other hand, for later stage as well as commercial supply requirement may be large enough such that the bulk DS storage container size may be in the range of tens to hundreds of liters. Thus, DS manufacturing campaign is closely related to DP requirement, and thus, the choice of the DS container can be influenced by the size of the DS campaign and intended DS storage volume.
2. *Temperature of storage*:
   DS storage for biologics typically happens at sub-zero temperature. Frozen storage is a typical practice at DS level because it provides longer use period and also bulk storage at frozen condition is cheaper. Most common storage temperature for a well-behaved biologics DS is −20 to −30 °C. Other storage temperatures include −40/−70 ± 10 °C. Storage temperature chosen for a specific DS can be strongly influenced by the stability of the material as well as the formulation. Certain excipients prefer colder temperature than others. For example, −40 °C is preferred than −20 °C from stability standpoint when trehalose is present in the formulation as an excipient. Although storage of DS at 5 ± 3 °C is not so common, there might be certain cases where frozen storage is not preferred due to F-/T-related instability. For example, certain biologics may prefer to be stored at 5 ± 3 °C due to the absence of any sugar or polyol that usually provides protection against F/T stress. Choice of the DS storage container can thus be influenced by storage temperature because all types of material that are typically used for making DS container (e.g., bag) are not compatible with all ranges of temperature. A detailed discussion around what DS storage container to be chosen for a given storage condition is provided in the next section.
3. *Requirement of controlled F/T*:
   Requirement of controlled F/T may have a significant influence on the choice of the DS container closure system. Or, in other words, it can be said that choice of DS container may potentially preclude the possibility of using a controlled F/T system. All available DS containers are not compatible with a controlled F/T system. For example, bottles cannot be frozen or thawed in a controlled F/T system. Similarly, Flexboy® containers (from Sertorius Stedim) that contain no outer rigid container are not suitable for controlled F/T system. Controlled F/T systems are typically designed for stainless steel cryovessel or Zeta FreezeContainer system, Celsius®-Pak FC (Flexboy flexible container in a frame), and Celsius® FFT flexible containers (integrated rigid outer container),

and their design does not allow controlled F/T for a bottle or Flexboy® container.

4. *Sensitivity of the DS to pH shift*
   Consideration for leachables from plastic container and its impact on possible pH shift should be considered carefully. If the DS formulation is not properly buffered, there is a possibility of pH shift of the DS formulation which in turn may have adverse effect on the quality of the DS if it is sensitive to pH shift. If for any reason the formulation is not properly buffered and the DS is sensitive to pH shift, there might be a need for considering alternative storage options. Under this situation, one may consider using 316 stainless steel vessels (cryovessel, FreezeContainer, etc), while controlled F/T is intended. If controlled F/T is not a requirement, one may choose flexible containers that have relatively cleaner leachable profile.
5. *Compatibility of the material of construction with the DS*
   Compatibility of DS with the material of construction of the container is an important consideration which is typically assessed through stability testing and extractable/leachable assessment. Container wall in contact with the DS formulation may leach out specific chemical entities (leachables) during real-time storage. Nature of these leachables is dependent on the container type as well as the formulation matrix of the DS. Compatibility of the DS with the leachables is thus an important aspect to be considered while finalizing the choice of container. While assessment of stability during real-time storage provides useful information regarding compatibility, direct assessment of the effect of leachables on the critical quality attributes of the DS is carried out during later phase of development.

15.5 Design Space Considerations for Freezing and Thawing Process Parameters

15.5.1 Freezing–Thawing Rates and Robustness

Freezing rate is an important process parameter for bulk protein freezing. The achievable freezing rate is solely dependent upon the bulk solution volume as well as the equipment capability in manufacturing facility. Rapid ice crystal growth is generally favored since this will reduce interfacial area for ice denaturation as well as decrease the risk of adverse conditions known to occur in cryoconcentrated area. This may not be feasible for large-scale bulk solution freezing but may be achieved by selecting lower freezing temperature, thereby increasing the freezing rate.

Based on the available stability data, the thawing temperature can be chosen. Typically, thawing can be achieved at room temperature or at 2–8 °C for plastic bottles. For controlled rate freeze–thaw units, the thawing rate can be controlled and uniform thawing can be achieved through mixing during thawing. Caution should

be exercised for mixing during the thaw operation since excessive mixing can result into foaming, thereby denaturing protein on air–water interface. Plastic bottles can be thawed with or without mixing. Thawing without mixing may take more time compared to thawing with mixing. In addition, concentration gradients can be formed with thaw process with no mixing. All these factors should be taken into consideration for the development of thawing cycle.

Understanding robustness of protein to freeze–thaw stress is an important parameter that should be considered during product development. Aspects to consider are slow freezing–thawing rate versus fast freezing–thawing rate. Freezing and thawing protein solution rapidly can afford greater stability for proteins since protein does not remain in transition state (ice–liquid) for longer period of time. This may be feasible for smaller volumes of protein although for bulk protein solutions where the volumes can be as high as 300 L, in practical terms even if fast freezing rate is applied, the time required for freezing such large volumes can be high. Protein can be exposed to transition state for longer times, and the extent could be different depending upon the position in the container. Therefore, understanding freeze–thaw robustness of protein can be performed by employing 'worst case' scenario evaluation as a part of scale-down studies. Combination of slow freezing and slow thawing offers 'worst case' scenario in terms of stress experienced by protein since protein remains in transition state for longer period of time and gets exposed to ice–water interface. A study can be designed to understand the robustness of protein by subjecting the protein to repeated freeze–thaw cycles using slow freeze–slow thaw cycle.

Manufacturing process typically involves at least one freeze–thaw cycle (freezing at DS manufacturing and thaw at DP manufacturing site prior to manufacturing). For process flexibility, additional freeze–thaw cycles can be included. Justification for repeated freeze–thaw cycling and demonstration of no changes in product quality is important during product development to afford such flexibility. Repeated freeze–thaw cycling robustness studies by employing slow freeze–slow thaw cycle can provide assurance of product quality. Herein, a case study is presented to demonstrate the understanding of freeze–thaw robustness for a monoclonal antibody (mAb).

Figure 15.1 provides the information on freeze–thaw profiles that were used for this study. The details are described below:

- Slow freeze–slow thaw cycle (freezing: 10 h; thawing: 10 h)
- Regular freeze–regular thaw cycle (freezing: 4.8 h; thawing: 2.7 h)
- Fast freeze–fast thaw cycle (freezing: 3 h; thawing: 1 h)

Monoclonal antibody DS was subjected to maximum of 15 freeze–thaw cycles by using these three different freeze–thaw profiles. Soluble aggregates data is shown in Fig. 15.2 demonstrating that there was no impact due to 'worst case' freeze–thaw stress (slow cycle) when compared with regular and fast cycle. This data provides robustness of freeze–thaw rate parameter.

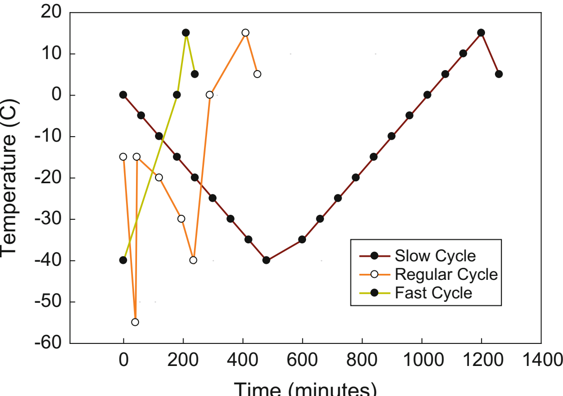


Fig. 15.1 Product temperature for various freeze–thaw profiles that were used to investigate the robustness of monoclonal antibody product quality upon repeated freeze–thaw cycles

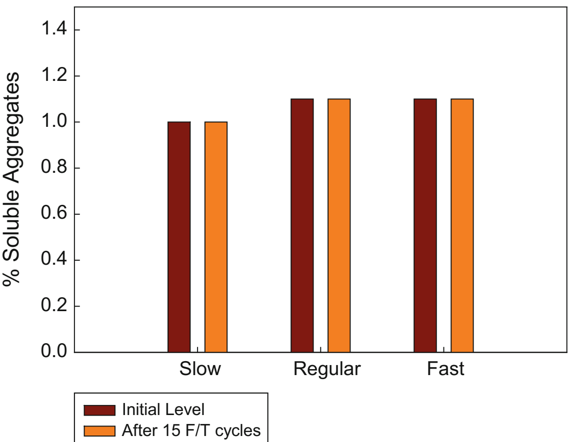


Fig. 15.2 Soluble aggregate levels when mAb was subjected to fifteen freeze–thaw cycle using three different profiles

15.6 Cryoconcentration Effects in Large-Scale Systems

As described in previous section, cryoconcentration is a consequence of freezing process. As water molecules phase out of solution in the form of ice crystal, concentration of the solutes increases in the unfrozen bulk which ultimately results at formation of ‘glass’ matrix. As the ice crystal grows, it causes increase in the solute concentration in the immediate vicinity of the ice surface, in other words at the ice–solution interface. If the progression of ice front happens at a rate slower than the rate of solute diffusion and convection away from the ice–solution interface, solutes are excluded from the growing crystals [65–67]. Exclusion from the ice surface increases the concentration of the solutes at the interface at a level that is much higher than the bulk giving rise to a concentration gradient. Depending on the dimension of the container (for frozen storage), such gradient may lead to a non-uniform distribution of the solute due to convective transport. Rodrigues et al.

reported that while formulated BSA was frozen in a 50 mL stainless steel container slower cooling rate caused protein molecules as well as the stabilizer (trehalose) to polarize near the bottom and radial center of the container [68]. Singh et al. also reported that freezing in large container leads to polarization of solute toward the bottom of the container [19]. For a small container (few milliliter storage volume), such polarization of solute concentration may not be that prominent. On the other hand while the cooling rate is faster, the rate of formation of ice crystal exceeds the rate of diffusive/convective transport of solutes. This may result at solutes getting entrapped within the growing crystals [68, 69]. While freezing happens at faster cooling rate, ice crystal grows in dendritic fashion [70], and at the completion of freezing, the average size of the ice crystal becomes much smaller than that results from slower cooling rate. Formation of dendrite disrupts the convective transport of the solute away from the ice front leading solute entrapment within the growing crystal [65, 66, 71–74]. Although trapped inside the growing ice crystal, cryoconcentration effect is still observed for entrapped solute. But such cryoconcentration effect is localized and rather minimizes the macrocryoconcentration across the container [75]. Rodrigues et al. reported that freezing of formulated BSA at a faster cooling rate causes much more uniform distribution (less polarization) of the solutes [68]. Miller et al. reported that storing of formulated IgG2 at a temperature higher than Tg′ showed less aggregation during long-term storage while freezing was done at faster rate. Although storage at temperature > Tg′ is expected to increase molecular mobility and thus aggregation. Miller et al. proposed that uniform distribution of the solute in the interdendritic space minimizes crystallization of the stabilizer and thus minimizes aggregation [70].

Nevertheless, cryoconcentrations are unavoidable for large volume protein bulk solution [75–82]. Several studies have evaluated extent of cryoconcentration in various passive and active systems. Three different systems are presented here to demonstrate the extent of cryoconcentration. Mitigation strategies are presented in subsequent section for various systems to ensure that cryoconcentration effects do not impact protein quality.

Extent of the cryoconcentration is dependent on the freezing rate. This was demonstrated by assessing osmolality in frozen bottles by taking various cores from cross section of the frozen bottles. Three different freezing rates were evaluated (5 to −70 °C, 5 to −40 °C, and 5 to −20 °C). Clear difference in the extent of osmolality observed when the solution was frozen from 5 to −70 °C as opposed to 5 to −40 °C and 5 to −20 °C depicting impact of freezing rate (Fig. 15.3). Observed 'bell-shaped' nature of concentration gradient is attributed to combination of heat transfer and freezing due to passive freezing. It is postulated that the heat transfer started from the sides of the bottle and as the freezing continues, ice excluded the solids resulting into solute concentration at the bottom due to density gradient.

Similar observations were made for Celsius bag freezing where these bags were subjected to control rate freezing [76]. Celsius bags with varying fill volumes either 2 L or 8 L were subjected to slow or fast freezing cycle by using identical formulations. Cross sections of the bags were taken which involved three vertical and

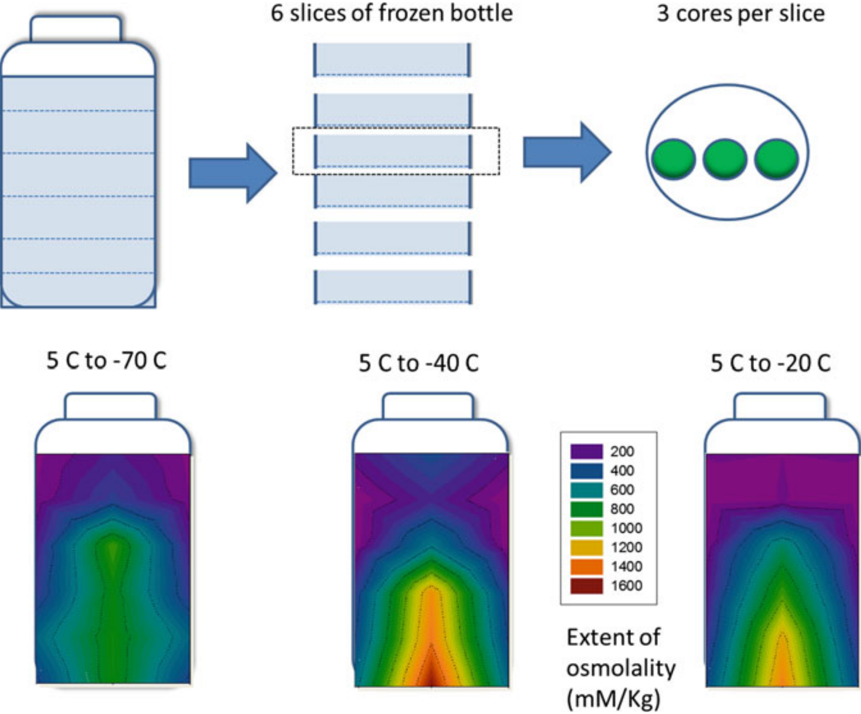


Fig. 15.3 Osmolality map of the cores taken after freezing bottle at various temperatures depicting effect of freezing rate

seven horizontal section assessments (Fig. 15.4a). Osmolality values suggest that solute concentration was observed in the bottom part of the bag for the middle vertical cross section. This suggests that the protein solution freezes from the active freezing plate (i.e., front and back section) and last part to middle cross section. Freezing process and density gradients due to solute concentration demonstrated cryoconcentration in the middle vertical cross section and at the bottom section of horizontal cross section. No significant difference in cryoconcentration was observed for 8 L bags despite differences in freezing rates (Fig. 15.4b).

Conversely, when the cryoconcentration effects were evaluated in Celsius bag filled with 2 L fill, significantly less cryoconcentration was observed suggesting that fill volume plays an important role. In addition, when two freezing rates were compared (slow vs. fast), impact of freezing rate was apparent for the low fill volume (Fig. 15.4b). Slow freezing provided slower ice formation resulting into a greater degree of polarization.

This pronounced effect of fill depth on cryoconcentration was also observed for cryowedge system (small-scale model for cryovessels). When 4 L protein solution was frozen in cryowedge and the samples were taken from top and bottom portion of the frozen block of cryowedge, it was apparent that the cryoconcentration was

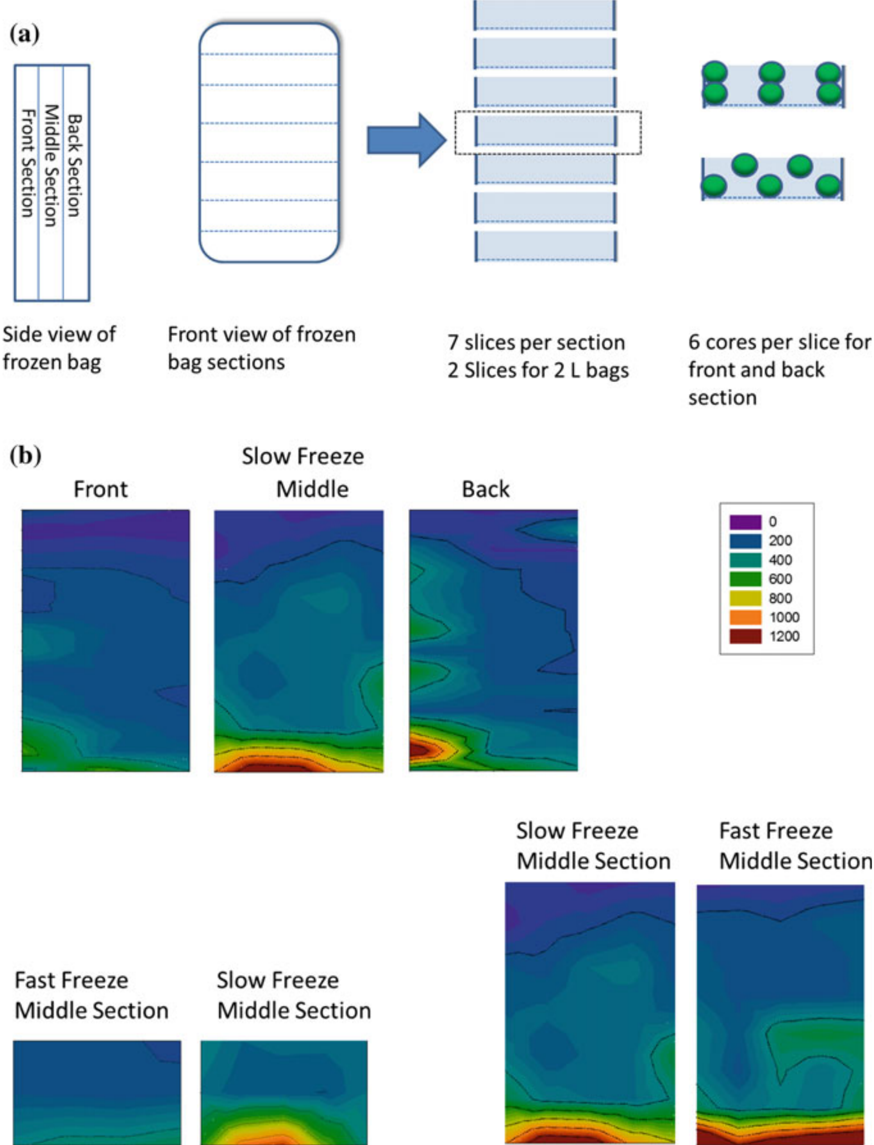


Fig. 15.4 **a** Methodology of obtaining cores from frozen Celsius bags. **b** Osmolality map of Celsius bags from the cores taken after subjecting to slow and fast freeze rates at various fill volumes

observed in the last point to freeze and at the bottom portion of the block confirming the observations of combination of freezing and density gradient due to solute conc. responsible for such behavior (Fig. 15.5). It is also important to note

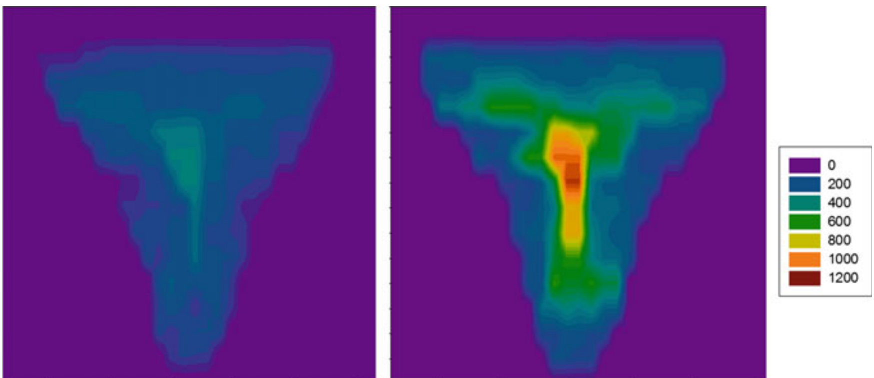


Fig. 15.5 Osmolality map of protein solution depicting effect of depth on cryoconcentration

that the fill depth can have pronounced effect on the extent of cryoconcentration observed as seen in the case of Celsius bags.

Studies which investigated cryoconcentration effects demonstrated that the cryoconcentration phenomenon in large-scale freezing process is unavoidable irrespective of it being an active or passive system. Active freezing system ensures that the freezing rate experienced by protein solution is constant each time as opposed to passive system although it cannot avoid cryoconcentration effects. It is important to note that these cryoconcentration effects get developed during freezing and stay the same in frozen condition until the container is thawed. Cryoconcentration can be detrimental to protein quality if not mitigated effectively especially after thaw. Subsequent section provides a closer look at potential effects of cryoconcentration upon thawing and mitigation strategies to tackle this challenge in various systems.

As the cryoconcentration effects happen as the solution freezes, it is important to have appropriate process controls during and after thawing to ensure that the cryoconcentration effects do not impact product quality. An illustration of impact of cryoconcentration effect is shown in Fig. 15.6 in plastic bottles. In this study, samples were taken after thawing the plastic bottle. It was observed that when the samples were taken without mixing the solution after thaw, significant increase in soluble aggregates was observed in the region where cryoconcentration was observed (bottom layer of the solution). Conversely, when the thawed solution was mixed well, no increase in soluble aggregates was observed indicating that effective mixing helped in obtaining acceptable product quality and mixing after thaw is an important step if the mixing during the thaw process is not feasible.

In another study where the thaw was performed by using controlled thaw process and the samples were taken from various locations after thaw [77]. Significant cryoconcentration was observed especially in the bottom–bottom part of the bag where the download of DS is intended to happen during DP formulation process.

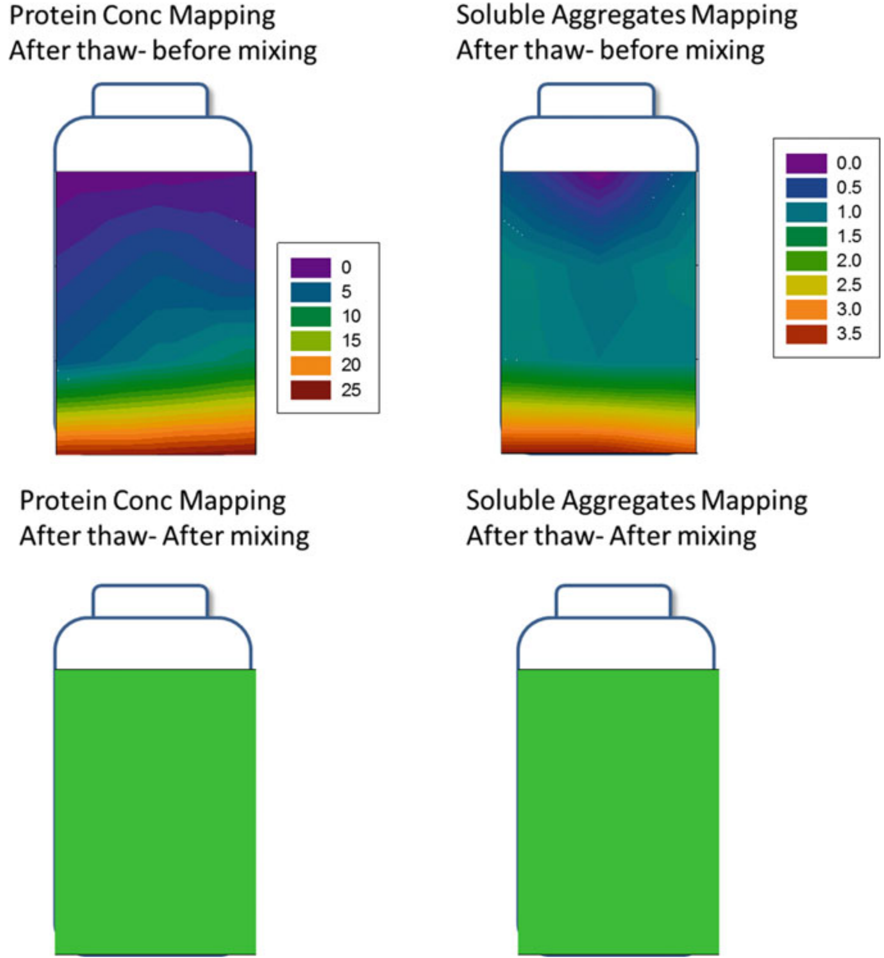


Fig. 15.6 Impact of cryoconcentration on soluble aggregate formation and importance of mixing after thaw

This observation is consistent with Kolhe et. al. with cryowedge system where significant cryococnentration was observed at the centre bottom part [78]. It is important to note that the Celsius bag was continuously rocking in one plane during the thaw process. This rocking process was not enough to eliminate the concentration gradient. Two different processes were employed to ensure efficient mixing of the contents. Data from two different mixing scheme (rocking in one plane horizontal stroke on cart) and combination of rocking and flipping is shown in Fig. 15.7. Rocking and flipping ensures that the protein concentration gradient is mixed well in all the planes hence making the solution homogeneous.

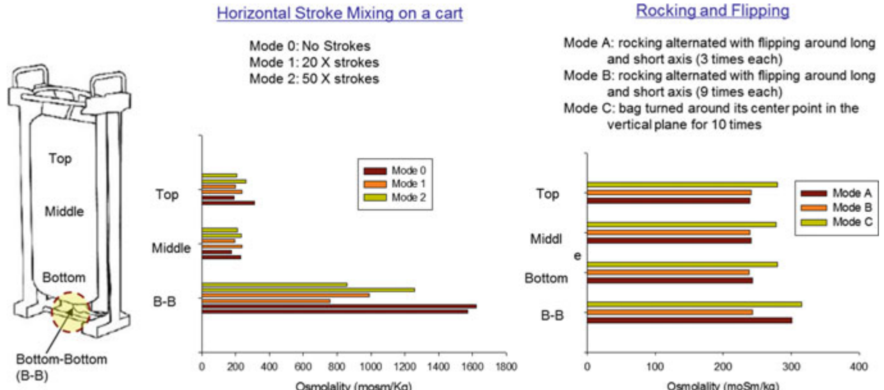


Fig. 15.7 Effect of mixing after thaw on protein concentration and effectiveness of mixing method

15.7 DS Shipping Considerations

Shipping of DS in frozen condition is an important aspect of process and needs to be carefully designed. DS shipping process, shipping container, and temperature ranges are important considerations and are highly dependent on the type of DS container used. The following aspects should be considered while defining the shipping process for given DS.

- DS Shipping temperature
  DS shipping temperature is an absolute important parameter that should be defined during the shipment. It is important to understand the stability of DS beyond its recommended storage temperature. For example, if the intended storage temperature for DS is −30 °C, it is critical that a range around −30 °C where DS is stable is identified. Efforts should be made to define the range in such a way that it is broad and allows for any temperature variations within that range during transportation.
- DS Container
  DS container plays an important role in defining the temperature range for transportation. Certain containers can afford transportation under dry ice (−70 °C or below) although some container may present unique challenge with transportation under dry ice. Plastic (e.g., HDPE bottles) as well as stainless steel container are good examples where shipment over dry ice is possible although systems utilizing EVA bags are not good candidates for dry ice shipment due to its proximity to cold crack temperature of the film. In addition, tunings can get brittle at these temperatures resulting into loss of integrity of the container/closure system.

15.8 Conclusion

Freezing and thawing of bulk drug substance are commonly used process in biotechnology industry. Freeze–thaw-induced instability due to exposure of proteins to ice–liquid interface, cryoconcentration effects, pH shifts, rate of freezing and thawing, excipient crystallization pose several challenges for the process development and tech transfer aspects. Several advance technologies are available to mitigate these challenges although these technologies are not perfect either. Fundamental knowledge of freezing and thawing processes and understanding of how various factors are interconnected can aid in designing these processes effectively.

References

1. Singh SK, et al. Frozen state storage instability of a monoclonal antibody: aggregation as a consequence of trehalose crystallization and protein unfolding. Pharm Res. 2011;28(4):873–85.
2. Kolhe P, Badkar A. Freeze-thaw of protein bulk drug substance. AAPS Sterile Prod Focus Group Ann Newsl. 2013:2–5.
3. Singh SK, Nema S. Freezing and thawing of protein solutions. In: Formulation and process development strategies for manufacturing biopharmaceuticals. Hoboken: Wiley; 2010.
4. Cocks FH, Brower WE. Phase diagram relationships in cryobiology. Cryobiology. 1974;11(4):340–58.
5. Angell CA. Liquid fragility and the glass transition in water and aqueous solutions. Chem Rev. 2002;102(8):2627–50.
6. Gayle FW, Cocks FH, Shepard ML. The H_2O-NaCl-sucrose phasediagram and applications in cryobiology. J Appl Chem Biotechnol. 1977;27:599–607.
7. Shalaev EY, Franks F. Equilibrium phase diagram of the water-sucrose-NaCl system. Thermochim Acta. 1995;255.
8. Green JL, Angell CA. Phase relationships and vitrification in saccharidewater solutions and the trehalose anomaly. J Phys Chem. 1989;93:2880–2.
9. Siniti M, Jabrane S, Letoffe JM. Study of the respective binary phase diagrams of sorbitol with mannitol, maltitol and water. Thermochim Acta. 1999;325:171–80.
10. Fahy GM. Analysis of “solution effects” injury. Equations for calculating phase diagram information for the ternary systems NaCl-dimethylsulfoxide-water and NaCl-glycerol-water. Biophys J. 1980;32(2):837–50.
11. Shepard ML, Goldston CS, Cocks FH. The H_2O-NaCl-glycerol phase diagram and its application in cryobiology. Cryobiology. 1976;13(1):9–23.
12. Jochem M, Korber C. Extended phase diagrams for the ternary solutions H_2O-NaCl-glycerol and H_2O-NaCl-hydroxyethylstarch (HES) determined by DSC. Cryobiology. 1987;24:513–36.
13. Shalaev EY, Kanev AN. Study of the solid-liquid state diagram of the water-glycine-sucrose system. Cryobiology, 1994;31:374–82.
14. Suzuki T, Franks F. Solid-liquid phase transitions and amorphous states in ternary sucrose-glycine-water systems. J Chem Soc Faraday Trans. 1993;89(17).
15. Chatterjee K, Shalaev EY, Suryanarayanan R. Partially crystalline systems in lyophilization: I. Use of ternary state diagrams to determine extent of crystallization of bulking agent. J Pharm Sci. 2005;94(4):798–808.

16. Akyurt M, Zaki G, Habeebullah B. Freezing phenomena in ice-water systems. Energy Conserv Manage. 2002;43:1773–89.
17. Bhatnagar BS, Bogner RH, Pikal MJ. Protein stability during freezing: separation of stresses and mechanisms of protein stabilization. Pharm Dev Technol. 2007;12(5):505–23.
18. Schenz TW. Glass transitions and product stability—an overview. Food Hydrocolloids. 1995;9:307–15.
19. Singh SK, et al. Large-scale freezing of biologics: a practitioner's review, Part 1—freezing mechanisms. BioProcess Int. 2009;7(9):32–44.
20. Franks F. Protein stability: the value of 'old literature'. Biophys Chem. 2002;96(2–3):117–27.
21. Chaplin M. Do we underestimate the importance of water in cell biology? Nat Rev Mol Cell Biol. 2006;7(11):861–6.
22. Carpenter JF, Crowe JH. The mechanism of cryoprotection of proteins by solutes. Cryobiology. 1988;25(3):244–55.
23. Franks F. Biophysics and biochemistry at low temperatures. 2012.
24. Piedmonte DM, et al. Sorbitol crystallization can lead to protein aggregation in frozen protein formulations. Pharm Res. 2007;24(1):136–46.
25. Levine H, Slade L. Thermomechanical properties of small carbohydrate-water glasses and 'rubbers': kinetically metastable systems at sub-zero temperatures. J Chem Soc Faraday Trans. 1988;84(8):2619–33.
26. Costantino H. Excipients for use in lyophilized pharmaceutical peptide, protein and other bioproducts. Lyophilization Biopharm, AAPS. 2004.
27. Williams ML, Landel RF, Ferry JD. The temperature dependence of relaxation mechanisms in amorphous polymers and other glass-forming liquids. JACS. 1955;77:3701–7.
28. Strambini GB, Gabellieri E. Proteins in frozen solutions: evidence of ice-induced partial unfolding. Biophys J. 1996;70(2):971–6.
29. Strambini GB, Gonnelli M. Protein stability in ice. Biophys J. 2007;92(6):2131–8.
30. Dong J, et al. Freezing-induced phase separation and spatial microheterogeneity in protein solutions. J Phys Chem B. 2009;113(30):10081–7.
31. Sundaramurthi P, Suryanarayanan R. Trehalose crystallization during freeze-drying: implications on lyoprotection. J Phys Chem Lett. 2010;1:510–4.
32. Levine H, Slade L. Principles of "cryostabilization" technology from structure/property relationships of carbohydrate/water systems: a review. Cryoletters. 1988;9:21–63.
33. Ablett S, Izzard MJ, Lillford PJ. Differential scanning calorimetric study of frozen sucrose and glycerol solutions. J Chem Soc, Faraday Trans. 1992;88(6):789–94.
34. Chang BS, Randall CS. Use of subambient thermal analysis to optimize protein lyophilization. Cryobiology. 1992;29:632–56.
35. Colandene JD, et al. Lyophilization cycle development for a high-concentration monoclonal antibody formulation lacking a crystalline bulking agent. J Pharm Sci. 2007;96(6):1598–608.
36. Chang BS, Kendrick BS, Carpenter JF. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. J Pharm Sci. 1996;85(12):1325–30.
37. Hsu CC, et al. Surface denaturation at solid-void interface—a possible pathway by which opalescent particulates form during the storage of lyophilized tissue-type plasminogen activator at high temperatures. Pharm Res. 1995;12(1):69–77.
38. Nema S, Avis KE. Freeze-thaw studies of a model protein, lactate dehydrogenase, in the presence of cryoprotectants. J Parenter Sci Technol. 1993;47(2):76–83.
39. Norde W. Adsorption of proteins from solution at the solid-liquid interface. Adv Colloid Interface Sci. 1986;25(4):267–340.
40. Sonnichsen FD, et al. Refined solution structure of type III antifreeze protein: hydrophobic groups may be involved in the energetics of the protein-ice interaction. Structure. 1996;4 (11):1325–37.
41. Griko YV, et al. Cold denaturation of staphylococcal nuclease. Proc Natl Acad Sci U S A. 1988;85(10):3343–7.
42. Franks F. Protein destabilization at low temperatures. Adv Protein Chem. 1995;46:105–39.

43. Jaenicke R. Protein structure and function at low temperatures. Philos Trans R Soc Lond B Biol Sci. 1990;326(1237):535–51; discussion 551–3.
44. Privalov PL. Cold denaturation of proteins. Crit Rev Biochem Mol Biol. 1990;25(4):281–305.
45. Brandts JF, Fu J, Nordin JH. Low temperature denaturation of chymotrypsinogen in aqueous solution and in frozen aqueous solution. In: Wolstenholme GEW, O'Connor M, editors. The frozen cell, A Ciba foundation symposium. London: J & A Churchill; 1970. p. 189–212.
46. Franks F, Hatley RHM. Low temperature unfolding of chymotrypsinogen. Cryoltters. 1985;6(3).
47. Privalov PL, et al. Cold denaturation of myoglobin. J Mol Biol. 1986;190(3):487–98.
48. Griko YV, Privalov PL. Calorimetric study of the heat and cold denaturation of beta-lactoglobulin. Biochemistry. 1992;31(37):8810–5.
49. Zhang J, et al. NMR study of the cold, heat, and pressure unfolding of ribonuclease A. Biochemistry. 1995;34(27):8631–41.
50. Tang XC, Pikal MJ. Measurement of the kinetics of protein unfolding in viscous systems and implications for protein stability in freeze-drying. Pharm Res. 2005;22(7):1176–85.
51. Adrover M, et al. The role of hydration in protein stability: comparison of the cold and heat unfolded states of Yfh1. J Mol Biol. 2012;417(5):413–24.
52. Zhang L, et al. Mapping hydration dynamics around a protein surface. Proc Natl Acad Sci U S A. 2007;104(47):18461–6.
53. Remmele RL Jr, Stushnoff C, Carpenter JF. Real-time in situ monitoring of lysozyme during lyophilization using infrared spectroscopy: dehydration stress in the presence of sucrose. Pharm Res. 1997;14(11):1548–55.
54. Gomez G, Pikal MJ, Rodriguez-Hornedo N. Effect of initial buffer composition on pH changes during far-from-equilibrium freezing of sodium phosphate buffer solutions. Pharm Res. 2001;18(1):90–7.
55. Murase N, Franks F. Salt precipitation during the freeze-concentration of phosphate buffer solutions. Biophys Chem. 1989;34(3):293–300.
56. Van Den Berg L. The effect of addition of sodium and potassium chloride to the reciprocal system: KH_2PO_4-Na_2HPO_4-H_2O on pH and composition during freezing. Arch Biochem Biophys. 1959;84:305–15.
57. Van Den Berg L, Rose D. Effect of freezing on the pH and composition of sodium and potassium phosphate solutions; the reciprocal system KH_2PO_4-Na_2-HPO_4-H_2O. Arch Biochem Biophys. 1959;81(2):319–29.
58. Pikal-Cleland KA, et al. Protein denaturation during freezing and thawing in phosphate buffer systems: monomeric and tetrameric beta-galactosidase. Arch Biochem Biophys. 2000;384 (2):398–406.
59. Kolhe P, Amend E, Singh SK. Impact of freezing on pH of buffered solutions and consequences for monoclonal antibody aggregation. Biotechnol Prog. 2010;26(3):727–33.
60. Pikal MJ, et al. The effects of formulation variables on the stability of freeze-dried human growth hormone. Pharm Res. 1991;8(4):427–36.
61. Sarciaux JM, et al. Effects of buffer composition and processing conditions on aggregation of bovine IgG during freeze-drying. J Pharm Sci. 1999;88(12):1354–61.
62. Shalaev EY, et al. Thermophysical properties of pharmaceutically compatible buffers at sub-zero temperatures: implications for freeze-drying. Pharm Res. 2002;19(2):195–201.
63. Heller MC, Carpenter JF, Randolph TW. Application of a thermodynamic model to the prediction of phase separations in freeze-concentrated formulations for protein lyophilization. Arch Biochem Biophys. 1999;363(2):191–201.
64. Heller MC, Carpenter JF, Randolph TW. Protein formulation and lyophilization cycle design: prevention of damage due to freeze-concentration induced phase separation. Biotechnol Bioeng. 1999;63(2):166–74.
65. Butler MF. Freeze concentration of solutes at the ice/solution interface studied by optical interferometry. Crystal Growth Des. 2002;2(6):541–8.
66. Korber C. Phenomena at the advancing ice-liquid interface: solutes, particles and biological cells. Q Rev Biophys. 1988;21(2):229–98.

67. Chen YH, Cao E, Cui ZF. An experimental study of freeze concentration in biological media. Food Bioprod Process. 2001;79(1):35–40.
68. Rodrigues MA, et al. Effect of freezing rate and dendritic ice formation on concentration profiles of proteins frozen in cylindrical vessels. J Pharm Sci. 2011;100(4):1316–29.
69. Ayel V, et al. Crystallisation of undercooled aqueous solutions: Experimental study of free dendritic growth in cylindrical geometry. Int J Heat Mass Transf. 2006;49:1876–84.
70. Miller MA, et al. Frozen-state storage stability of a monoclonal antibody: aggregation is impacted by freezing rate and solute distribution. J Pharm Sci. 2013;102(4):1194–208.
71. Wilkins J, Sesin SD, Wisniewski R. Large-scale cryopreservation of biotherapeutic products. Innov Pharm Technol. 2001;8:174–80.
72. Miyawaki O, Liu L, Nakamura K. Effective partition constant of solute between ice and liquid phases in progressive freeze-concentration. J Food Sci. 1998;63(5):756–8.
73. Wisniewski R, Wu V. Large-scale freezing and thawing of pharmaceutical products. In Biotechnology and biopharmaceutical manufacturing, processing preservation. Boca Raton: CRC Press;1996. p. 7–59.
74. Butler MF. Instability formation and directional dendritic growth of ice studied by optical interferometry. Cryst Growth Des. 2000;1(3):213–23.
75. Wisniewski R. Developing large-scale cryopreservation systems for biopharmaceutical products. BioPharm. 1998;11:50–6.
76. Kolhe P, Holding E, Lary A, Chico S, Singh SK. Large scale freezing of biologics: understanding protein and solute concentration changes in a cryovessel—Part II. Biopharm Int. 2010;23(7):40–9.
77. Kolhe P, Badkar A. Protein and solute distribution in drug substance containers during frozen storage as well as post-thawing: a tool to understand and define freezing-thawing parameters in biotechnology process development. Biotechnol Bioeng. 2009;27(2):494–504.
78. Kolhe P, Holding E, Lary A, Chico S, Singh SK. Large scale freezing of biologics: understanding protein and solute concentration changes in a cryovessel—Part I. Biopharm Int. 2010;23(6):53–60.
79. Maity H, Karkaria C, Davagnino J. Mapping of solution components, pH changes, protein stability and the elimination of protein precipitation during freeze-thawing of fibroblast growth factor 20. Int J Pharm. 2009;378(1–2):122–35.
80. Padala C, et al. Impact of uncontrolled vs controlled rate freeze-thaw technologies on process performance and product quality. PDA J Pharm Sci Technol. 2010;64(4):290–8.
81. Tschoepe M, Schmidt R. Impact of freeze/thaw processing on monoclonal antibody stability. In: Bioprocess International conference. 2008.
82. Webb SDW, Webb JN, Hughes TG, Sesin DF, Kincaid AC. Freezing biopharmaceutical using common techniques and the magnitude of bulk scale freeze-concentration. Bioprocess Int. 2002;15(5):22–34.
83. Wisniewski R, Wu VL. Large-scale freezing and thawing of biopharmaceutical products. In: Wu VL, Avis KE, editors. Biotechnology and biopharmaceutical manufacturing, processing, and preservation. Boca Raton: CRC Press; 1996. p. 7–60.

Chapter 16
Leachables and Extractables: From Regulatory Expectations to Laboratory Assessment

Michael Jahn

Abstract During manufacturing, storage and administration, a pharmaceutical drug product (DP) formulation and its constituents are in contact with a multitude of polymeric materials. Every such contact leads to leaching of chemicals from the material into the formulation. Health authorities demand from the pharmaceutical manufacturer to control leaching within an extractables/leachables (E/L) assessment as a measure to prevent the patient from undue risk. Such an E/L assessment is structured into different parts. Initially, the manufacturing, storage and administration process is mapped; i.e. all contact materials are listed together with relevant process parameters that might influence leaching. A risk evaluation scores the materials according to their risk. High-risk materials are subjected to laboratory E/L testing. Test conditions, i.e. incubation of materials, should reflect the conditions encountered in the process—too exaggerated incubation conditions leading to excessive extractables profiles that do not correlate with leachables profiles encountered during real contact should be avoided. Analysis of the incubation solutions within screening studies should cover comprehensively the physical–chemical properties of E/L compounds; typical analytical methods are headspace-GC–MS, GC–MS and LC–UV–MS. Quantitative evaluation of analytical data should be performed against analytical evaluation thresholds derived from toxicological safety thresholds. Compounds encountered above the threshold need follow-up activities, e.g. structural identification and toxicological assessment. Special considerations have to be applied and are discussed within this chapter with regard to E/L assessment of drug substance manufacturing materials, the interaction of leachables with process constituents, glass delamination, leaching of silicon oil and leaching from secondary packaging materials, adhesives and printing. In summary, a proper E/L assessment should enable the pharmaceutical manufacturer to choose manufacturing, storage and administration materials that do not pose an undue risk for patients with regard to leaching.

M. Jahn (✉)
Lonza Drug Product Services—Forensic Chemistry, Basel, Switzerland
e-mail: michael.jahn@lonza.com

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*, AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_16

Keywords Extractables · Leachables · Risk evaluation · Analytical evaluation threshold · Glass delamination · Silicon oil

16.1 Introduction

A pharmaceutical product officially—as described, e.g. in the patient information leaflet—is composed of the active pharmaceutical ingredient and the accompanying excipients. Unfortunately, this is a too simplistic view on the composition, as it neglects that chemical compounds (termed "leachables") will inevitably have contaminated the product during its manufacturing, storage and administration process through contact with and migration from polymeric materials.

Within this chapter, it is discussed how leaching occurs, and how it should be controlled to exclude a safety risk for patients when being treated with a pharmaceutical product.

16.1.1 Background

Throughout the manufacturing, storage and administration process of a pharmaceutical drug product (DP), a manifold of polymeric materials is in contact with the DP formulation and its constituents (in the following text simply summarized as "formulation"). During the manufacturing process, the formulation is filtered through microbial and sterile filters, and the formulation is often transferred through polymeric tubing. The DP is filled into and stored throughout the shelf life in a container (glass or plastic) with a polymeric closure (often a rubber stopper). The DP is often administered with the aid of disposable plastic materials, e.g. IV infusion sets or syringes.

The construction process of those plastic materials is complex. To guarantee the proper functionality of plastic materials they contain a polymeric matrix, e.g. polyethylene, polypropylene, polyvinyl chloride, together with plastic additives, e.g. antioxidants, plasticizers, slip agents, fillers or colourants. As these plastic additives have to perform a function, e.g. as antioxidants, they contain polar, e.g. phenolic, functional groups, which limit their compatibility with the unpolar polymeric matrix. Also, if degraded during compounding at high temperature (above the melting point of the polymer), they can form polar structural elements, e.g. carboxylates, which again have limited compatibility with the polymeric matrix [1]. The (often liquid) formulation in contact with the plastic material might on the other hand act as a good solvent for those chemicals, especially when containing surfactants or organic solvents. This leads to leaching, i.e. migration of the chemicals from the polymeric matrix onto its surface, and from there into the formulation. The final DP that is administered to the patient will therefore contain leachables.

16.2 Health Authority Expectations

Health authorities demand from pharmaceutical manufacturers to guarantee that leachables are not a safety concern for the patient. The US Code of Federal Regulations Title 21 [2, 3] demands that manufacturing equipment or containers and closures "… shall not be reactive, additive, or absorptive so as to alter the safety, identity, strength, quality, or purity of the drug … beyond the official or … established requirements.", where the attributes reactive and additive concern the phenomenon of leaching potentially leading to alteration of safety, quality and purity.

The FDA "Guidance for Industry: CCSs for Packaging Human Drugs and Biologics" [4] concludes that the control of leaching should correlate with the risk of leaching, which can be assessed by evaluating two contributing factors, i.e. the interaction of the formulation with the polymeric matrix (solid formulation exhibiting lower risk compared to liquid formulation) and the route of administration (oral administration being of a lower risk compared to inhalation or injection).

The EMA "Guideline on Plastic Immediate Packaging Materials" [5] gives guidance on the submission of data on leachables' control, which is depending on the risk factors "nature of an active substance", i.e. solid being less risky than liquid, and "route of administration", i.e. oral administration being less risky than inhalation or parenteral injection. Obviously, potential leaching from materials and formulations with higher risk demands for stricter control, e.g. by performing "extraction" and "migration" studies, as they are termed in this guideline.

In the USP chapters <1663> [6] and <1664> [7], the terms "extractables" and "leachables", respectively, are defined, and their control strategies are explained. Extractables are defined as "… organic and inorganic chemical entities that can be released from a pharmaceutical packaging/delivery system, packaging component, or packaging material of construction under laboratory conditions. Depending on the specific purpose of the extraction study, these laboratory conditions (e.g., solvent, temperature, stoichiometry, etc.) may accelerate or exaggerate the normal conditions of storage and use for a packaged dosage form. Extractables themselves, or substances derived from extractables, have the potential to leach into a drug product under normal conditions of storage and use". USP <1663> scientifically describes the two critical steps of extractables evaluation of a packaging material, i.e. generation of the extract and analytically testing the extract. The principles for extractables assessment can explicitly be applied also to manufacturing materials. Leachables are defined as "…organic and inorganic chemical entities that migrate from a packaging/delivery system, packaging component, or packaging material of construction into an associated drug product under normal conditions of storage and use or during accelerated drug product stability studies. Leachables are typically a subset of extractables or are derived from extractables". USP <1664> scientifically describes a leachables study outline, starting with the establishment of an analytical evaluation threshold based on a toxicological threshold, through analytical method development and validation considerations, as well as the correlation of leachables to extractables data.

Another guideline that is very useful to establish a control strategy for leachables is ICH M7 [8]. ICH M7 manifests a threshold of toxicological concern (TTC) of 1.5 μg for a lifelong daily intake of a genotoxic impurity and consults on how to appropriately increase the threshold for the case of less than lifetime exposure. It is explicitly stated in the guideline that its concepts are applicable to extractables/leachables (E/L) assessment.

16.3 Extractables/Leachables (E/L) Assessment

After a consultation of the appropriate guidelines, it should be clear if materials that will be used for a certain pharmaceutical application are of a potential risk with regard to leachables; if so, these materials have to be further assessed. A typical E/L assessment starts with a mapping of the DP manufacturing, storage and administration process and listing of all applied materials and is followed by a risk evaluation for each of those materials, which concludes if a material should be subjected to a laboratory E/L study.

16.3.1 Mapping of the Process

Typical DP process steps and examples of the applied materials are listed in Fig. 16.1.

Often the drug substance (DS) storage container is part of an E/L assessment of the DP manufacturing system. Other materials from the manufacturing process that are typically used and listed in the process map are microbial and sterile filters, gaskets, valves, connectors and seals. Storage of DP is performed after filling into CCSs (vials, pre-filled syringes, cartridges). The DP is finally administered to the patient, especially in the clinical phases, with the aid of administration devices (IV infusion sets consisting of infusion bags with catheters and in-line filters, disposable syringes, etc.).

It is important in this phase of E/L assessment to collect information from the different relevant stakeholders of the manufacturing, storage and administration process. Personnel from DS downstream processing and DS manufacturing should give input on materials used for and on storage conditions applied during DS storage. Colleagues from DP manufacturing process development should provide information on materials used during the fill/finish process as well as on their pre-treatment (sterilization, pre-flushing, etc.). Process conditions (badge size, maximum hold times, process formulations, etc.) should be established, because the applied parameters will influence the subsequent risk evaluation and potential laboratory testing conditions of the materials in question. The pharmaceutical development group should inform on the final DP formulation (concentration of the active pharmaceutical ingredient, as well as excipients, pH, etc.), on materials of

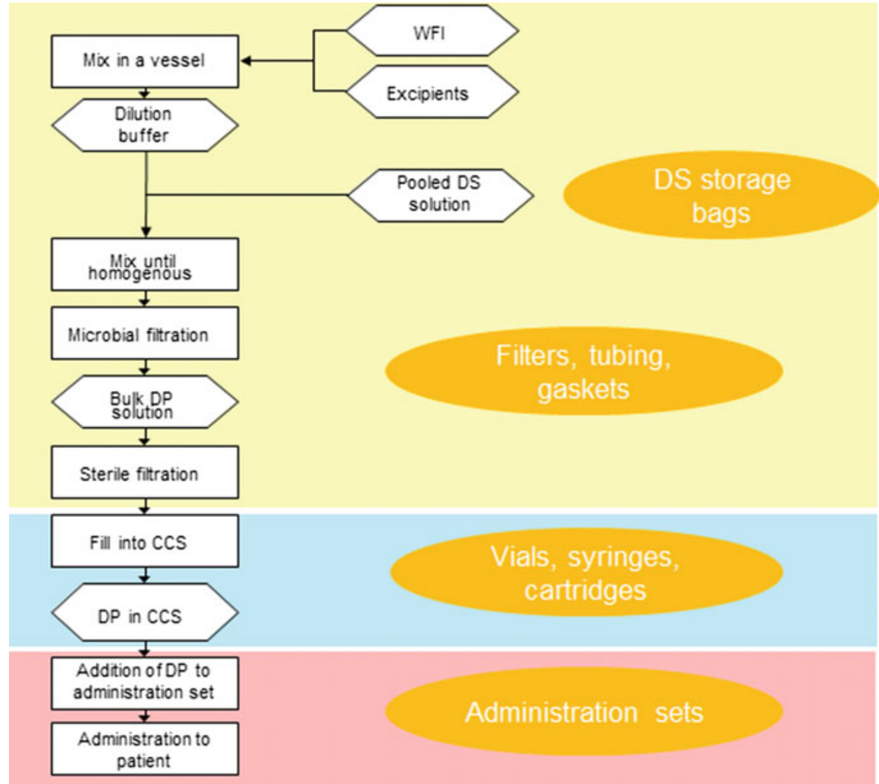


Fig. 16.1 Typical process steps/materials used during DP manufacturing, storage and administration

construction of the CCS (glass type, elastomeric closure, fluoropolymer liner, etc.), and on DP storage conditions (temperature, shelf life, etc.). From clinical development input on the posology (dosing strength, frequency, duration, etc.) is necessary; worst-case assumptions might be used, if the posology is not defined yet. Clinicians should inform on materials intended for DP administration (IV administration bags, disposable syringes, etc.), as well as on instructions for compounding (final concentration of DP in the vehicle, intermediate storage conditions in the pharmacy, etc.).

It has to be appreciated that the gathering of all these information is tedious and might take a considerable amount of time; however, a diligent collection of information in this initial phase of E/L assessment is important, as it influences the further risk evaluation and conclusions drawn from that.

16.3.2 Material Risk Evaluation

When determining the risk for the patient with regard to leaching from materials used during the manufacturing, storage and administration of pharmaceuticals, two fields of expertise have to contribute: chemistry and toxicology.

Expertise in chemical sciences is important to understand and evaluate the interaction of the formulation with the polymeric materials (i.e. what is the likelihood that leaching occurs, and that the leachables are present in the final DP that is administered to the patient). Factors that have to be taken into consideration are:

- The leachables' propensity of the formulation: the presence of organic solvents and/or surfactants and extreme pH excursions increase the leachables' propensity compared to purely aqueous formulations at neutral pH [9]. In general, liquid formulations can be regarded to have a higher leachables' propensity versus solid formulations (e.g. lyophilisates).
- The chemistry of the material: polymeric matrices like PVC contain high amounts of plastic additives that will leach [9], whereas fluoropolymers can be regarded as "clean" materials not releasing significant amounts of leachables. Fluoropolymeric liners (fluorotec coating) significantly reduce leaching from elastomeric stoppers in CCSs [10].
- The surface area of a contact material: for a contact with large surfaces (e.g. filter membranes), the total amount of leached substances is expected to be higher than from materials with low surface area (e.g. a polymeric seal ring).
- The change of the chemistry of materials during pre-treatment: sterilization, e.g. autoclaving or gamma irradiation, might modify the polymeric matrix and the plastic additives, contributing to additional leaching, whereas pre-flushing might remove leachables [11].
- The position of a material in the process indicates the possibility of clearance of leached compounds. This is of a higher significance for the risk evaluation of materials used during DS manufacturing, as clearance during DP manufacturing (e.g. adsorption to filter materials) is difficult to predict.
- Contact duration: transient contact during manufacturing exhibits a lower likelihood of contamination with leachables, as only the surface of the material is washed off, whereas long-term storage might allow migration of compounds from within the polymeric matrix onto the surface and from there into the formulation.
- Contact temperature: the temperature has an influence on the diffusion coefficients; i.e. leaching occurs predominantly at higher temperature, whereas in the frozen state no significant leaching will be observed.

Expertise in toxicological sciences and safety regulations is important to understand and evaluate the interaction of potential leachables in the formulation with the patient (i.e. what is the safety risk for a patient when treated with a medication that contains leachable compounds). Factors that have to be taken into consideration are:

- Information on contact materials: if laboratory studies (e.g. extractables studies performed by the materials' manufacturer) indicate that leaching of specific chemicals can be expected it should be evaluated by a toxicologist that toxicity thresholds for this chemical (e.g. permitted daily exposure, PDE) are not exceeded.
- Route of administration: safety requirements differ for different administration routes; orally inhaled and nasal drug products (OINDPs) have a higher degree of risk, as administered directly onto the sensitive and often affected organ (lung), compared to DPs that are administered parenterally. This is reflected in a lower safety concern threshold (SCT) for OINDPs (0.15 µg/day, as suggested by PQRI [12]) compared to parenterals (1.5 µg/day, as regulated by ICH M7 [8]).
- Frequency and duration of administration: OINDPs are administered with a high frequency (e.g. several times per day), whereas a cell therapy treatment might only occur once per lifetime. Potentially, genotoxic impurities have to be controlled in OINDPs therefore with higher scrutiny (i.e. at an SCT of 0.15 µg/day), whereas ICH M7 allows for an increased toxicity threshold for less than lifetime treatment. Less than lifetime treatment also happens during early phases of clinical development, where patients are additionally closely monitored in the hospital environment; a risk evaluation might therefore conclude that at early clinical phases reduced leachables assessment of materials (e.g. platform approach) is required.
- Patient population: if a medication is intended to be used in children, leaching from materials has to be evaluated from a safety perspective differently than for an adult patient population. Chemicals like benzyl alcohol are supposed to have a different effect when administered to children (i.e. potentially causing acidosis when metabolized to benzoic acid) compared to adults (where benzoic acid is not of a dramatic safety concern) [13].

Appropriate risk evaluation procedures (e.g. component categorization, material or component FMEA) can be applied to define the risk for a material. The conclusion should be that high-risk materials have to be submitted to laboratory E/L studies to support the use of specific contact materials in a specific manufacturing process, product packaging or delivery system. The risk evaluation should be documented and it should provide appropriate justification, if laboratory studies are not considered necessary.

The two following examples illustrate the conclusions drawn from such a risk evaluation:

- Microbial and sterile filters used during manufacturing of a biopharmaceutical DP: the formulation is liquid and contains a surfactant (i.e. strong interaction of the formulation with the filter materials), and the filters have large contact surfaces, and the formulation is injected intravenously into the patient. The risk evaluation likely comes to the conclusion that the filters have to be tested in a laboratory study (if appropriate information from the filter manufacturer is not already available).

- Food grade polypropylene container closure system (CCS) for tablets: information and food grade certificates on CCS materials/plastic additives are available, the dosage form is solid, and the tablets are administered orally. The risk evaluation might come to the conclusion that the CCS material poses a very low risk to patients with regard to leachables, and therefore a laboratory study might not be necessary.

16.3.3 Laboratory E/L Testing

When the material risk evaluation comes to the conclusion that a material, that shall be used for a specific application during DP manufacturing, storage and administration, is of an increased risk with regard to leachables, it is likely that the material is subjected to laboratory E/L testing (an alternative is to rely on extractables data delivered by the material's manufacturer).

16.3.3.1 Analytical Evaluation Threshold (AET)

A typical first step is to establish an analytical evaluation threshold (AET) that correlates to a pre-defined safety threshold, e.g. the threshold of toxicological concern (TTC) specified in ICH M7 [8].

The TTC (or another appropriate safety concern threshold) can be correlated to the AET, which translates the acceptable intake (amount) into an analytical concentration.

$$\mathrm{AET} = \mathrm{TTC} * (1/\mathrm{V}) * (1/\mathrm{n}) * \mathrm{F}_{\mathrm{Process}} * \mathrm{C}_{\mathrm{work\ up}}$$

AET Analytical evaluation threshold
TTC Threshold of toxicological concern
V Volume administered into a 50 kg patient. The dose administered to the patient might vary with patient weight; i.e. patients with higher patient weight are administered higher doses/volumes compared to patients with lower patient weights. A 50 kg mass is considered standard for safety evaluation purposes. Therefore, the AET is typically not adjusted for patient mass-dependant dosing, but rather the AET is calculated by taking into account the maximum dose/volume that will be administered to a 50 kg patient.
n Maximum number of administrations per day for a 50 kg patient (see explanation above on patient weight)
$F_{Process}$ Factor considering parameters relevant to the manufacturing/administration/testing process
$C_{work\ up}$ Concentration factor due to sample work-up

The factor $F_{Process}$ is different from 1 if the manufacturing/administration process cannot be directly simulated during testing (examples are given below).

The factor $C_{work\ up}$ has to be chosen such that the AET is resulting in a concentration level compatible with the reporting limit of the analytical method; i.e. the reporting limit should be lower than the AET.

The following two examples illustrate the calculation of the AET:

- A vial, filled with 4 mL of a formulation that is administered twice a day (administration volume: 3.8 mL), is subjected to E/L testing. A TTC of 1.5 µg is established (here, collaboration with a toxicologist might be advantageous to indeed apply an appropriate threshold). During leachables sample work-up, the analytes are up-concentrated by a factor of 10, e.g. by extracting 10 mL of formulation with an organic solvent, which is evaporated to 1 mL and then subjected to analysis (e.g. by GC–MS). The AET is calculated to

  $$\mathrm{AET} = 1.5\,\mu\mathrm{g} * (1/3.8\,\mathrm{mL}) * (1/2) * 1 * 10 = 2.0\,\mathrm{ppm}$$

  This threshold should not pose a problem to the analytical method GC–MS, where a reporting limit at a concentration level of an order of magnitude lower should be easily achievable (if not dealing with extreme matrices).
- During the manufacturing of this formulation (same dosing as above), a sterile filter is used and subjected to E/L testing, where the same filter (size, mode of sterilization and flushing) is incubated for a specified time (e.g. as described in the manufacturing instructions) with 1 L of formulation. In the process, the eluent of the filter is diluted in a surge tank of 0.5 L (i.e. it has to be assumed under worst-case considerations that the total amount of filter leachables is present in the initial 0.5 L). Sample work-up is as described above. The AET is calculated to

  $$\mathrm{AET} = 1.5\,\mu\mathrm{g} * (1/3.8\,\mathrm{mL}) * (1/2) * (0.5\,\mathrm{L}/1\,\mathrm{L}) * 10 = 1.0\,\mathrm{ppm}$$

  Again, the test conditions are compatible with the GC–MS method, which should have a much lower reporting limit than the AET.

16.3.3.2 Preparation of E/L Samples

The interaction of E/L samples of the formulation with materials varies with the contact time, which is transient (i.e. minutes to days) during the manufacturing process and during administration to the patient, and which is long term (several years) upon storage in a CCS. Sample preparation for laboratory E/L testing should simulate the actual contact time. Examples:

- If a manufacturing process is limited in the manufacturing instructions to a maximum hold time of 48 h, the manufacturing materials should be incubated during E/L testing with a formulation for this specified time.

- If the shelf life of a DP formulation in a CCS is specified for 24 months leachables samples in this CCS should be stored up to 24 months with intermediate pull points (typically at 3 months, 6 months and 12 months). Additionally, the CCS should be assessed in an extractables study, where exaggerated incubation conditions (storage at elevated temperature; formulation with higher solvent strength) are applied. It is important to avoid too exaggerated conditions, which would lead to an excess of extractables above the AET, with no correlation of the extractables profile with the leachables profile.

Biopharmaceutical formulations are often not analytically accessible for E/L testing, because the active protein is present at much higher concentration (up to ~150 mg/mL, i.e. 150,000 ppm) than the AET at which E/L compounds have to be detected (usually in the low ppm region). Therefore, alternative E/L test solutions (surrogate solutions), e.g. the placebo formulation not containing the biopharmaceutical active, or a surfactant solution with relatively high surfactant concentration (e.g. 0.1% aqueous polysorbate 20), might be used.

It is important to fill blank samples and treat them in the same way as the E/L samples.

16.3.3.3 Choice of Analytical Methods

Once the E/L samples and blanks have been generated, they are worked up and analysed by trace analytical means. E/L compounds are organic molecules (e.g. plastic additives and their degradation products) that migrate during contact from polymeric surfaces to the formulation, as well as inorganic impurities. Suitable work-up and trace analytical methods to detect organic and inorganic E/L compounds are:

- GC–MS after organic extraction for the detection of low molecular weight (~40–600 g/mol), volatile to semi-volatile analytes with low to medium polarity. Quantification using MS detection enables structural elucidation in case compounds are quantified above the AET. Typical reporting limits, established with an external reference compound, e.g. butylated hydroxytoluene (BHT), are in the range of ~0.1 ppm (after work-up).
- Headspace coupled to GC–MS (HS–GC–MS) for the detection of volatile analytes. Sample work-up and up-concentration is not feasible, because the target analytes—volatile compounds—would be evaporated and thus would escape detection. Therefore, aqueous samples are directly transferred into headspace vials and analysed. Organic samples are not compatible with HS–GC–MS. Quantification using MS detection enables structural elucidation in case compounds are quantified above the AET. Typical reporting limits, established with an external reference compound, e.g. toluene, are in the range of ~0.1 ppm.

- LC–UV–MS analysis for the detection of E/L compounds with high variability in molecular weight ($\sim$100–2000 g/mol) and polarity. Sample analysis can be performed by direct sample injection or after suitable sample pre-treatment. Often, in screening analyses only the UV response is used for quantification, because certain excipients in a surrogate or placebo formulation might strongly interfere with the MS method (e.g. polysorbates). Typical reporting limits, established with an external reference compound, e.g. butylated hydroxytoluene (BHT), are in the range of $\sim$0.1 ppm. MS is applied for structural elucidation in case compounds are quantified that exceed the AET.
- Elemental analysis (EA) for the detection of inorganic E/L compounds. ICH Q3D [14] demands that the elements Cd, Pb, As, Hg, Co, V, Ni, Li, Sb and Cu are assessed in the final (parenteral) DP. As there are many potential sources of contamination with these elements during the complex DS and DP manufacturing, storage and administration process, those elements are only assessed as E/Ls from specific materials if their presence is suspected. To assess inorganic leachables in a holistic approach, covering all of the different steps that lead to the final DP that is administered to the patient, the DP itself should be analysed. ICP-MS and ICP-OES are the methods of choice, and their reporting limits are variable with the respective element and with the formulation matrix.

16.3.3.4 E/L Compounds Above AET

If organic E/L compounds are quantified above the AET, follow-up work is necessary. The analytes have to be structurally identified; this might be accomplished by simple mass spectral database comparison, or by more sophisticated analytical and chemical methods, that need well-experienced and well-equipped personnel. This identification process should be finalized with a chromatographic and spectroscopic comparison with an authentic reference standard.

The accurate quantity of the E/L compound, measured ideally with the aid of the authentic reference standard, together with data on posology should be submitted to a toxicologist, who will judge if the E/L compound is of a safety risk to the patient. In the rare cases where this occurs, the material in question will likely be exchanged. As some of the here described studies might use exaggerated sample preparation conditions (e.g. during extractables studies or during simulation studies) it might also be considered to assess, if the respective E/L compound is present at a significant concentration, e.g. derived from a permitted daily exposure, in the final DP. Specific methods (e.g. single ion monitoring (SIM), MS/MS, UV at maximum absorption, specific elemental analysis) can then be applied for quantification. As per toxicological assessment the compound was deemed potentially harmful, a validated sample work-up and analysis method should be applied for its quantification.

16.3.4 Special Considerations

The workflow for an E/L assessment, as outlined above, is rarely a routine process. In the following sections, some special challenges are depicted.

16.3.4.1 Leachables During Drug Substance (DS) Manufacturing

During conventional small molecule drug substance (DS) manufacturing by chemical synthesis of the active pharmaceutical ingredient (API) assessment of leachables from manufacturing equipment is considered obsolete, because purification steps, e.g. re-crystallization, will effectively reduce leachables, and storage of the API (= DS) is often in the solid state, with minimal potential for interaction with plastic surfaces leading to leaching. During biotechnology DS manufacturing of—usually liquid—protein/antibody formulations a manifold of polymeric contact surface areas will release leachables. Although there are process steps applied for the purification of the protein—e.g. affinity liquid chromatography, ion exchange chromatography, hydrophobic interaction chromatography, size exclusion chromatography, diafiltration [15]—which enable the removal of process-related impurities and cell culture components, it should be evaluated if E/L compounds of interest are also cleared with high rates. Usually, the materials' manufacturer is testing their materials for extractables, by, e.g. following the BPOG protocol [16]; such a study, which should be accessible to the DS manufacturer, might reveal if leachables with safety concern could enter the process stream and if those need monitoring and control.

Material risk evaluation for biotechnology DS manufacturing materials is tricky. Numerical risk evaluation with thresholds used for further assessment decisions (e.g. follow-up E/L laboratory study versus relying on manufacturer's extractables data) are useful, but it has to be acknowledged that the scoring is somehow arbitrary. The results of this risk evaluation should be reviewed by an E/L expert.

Also, leachables testing of DS manufacturing materials is not trivial: Establishing an AET is complicated due to uncertainties regarding, e.g. the fate of leachables during further downstream processing (are they cleared or up-concentrated with the protein?). Analytical testing with authentic DS pools might either not be possible due to their unavailability or difficult due to their complex matrix.

If the analytical methods are established and validated, it might be useful to study leaching into the DS process fluids in a holistic manner, by performing leachables screening in the final DS. As outlined, above hurdles regarding the often high protein concentration in the DS with the implication of uncertain analyte recovery during work-up and analysis have to be overcome.

16.3.4.2 Interaction of Leachables with Process Constituents

Leachable compounds are mainly assessed with regard to their safety risk for patients. However, they might also be detrimental to process performance or to the quality of the final DP.

It was previously observed that cytotoxic leachables from single-use bioprocess equipment caused poor cell growth during fermentation [17]. The interaction of the API with leachables from following process steps must also be taken into consideration. We have observed that gamma-sterilized materials can release reactive oxygen species (e.g. peroxides) into the process stream, causing oxidation of the protein (data not published). Leaching inorganic elements might cause protein aggregation, as seen, e.g. with residual tungsten in pre-filled syringes originating from tungsten pins used during syringe fabrication [18]. Organic leachables from the stoppers of administration bags caused incompatibility with the protein dulanermin during potential freeze/thaw cycles [19].

It is very difficult to predict and proactively study any of those interactions of leachables with process constituents, and it is likely that only root cause investigations following OOS/OOE results would identify the culprit.

16.3.4.3 Glass Delamination

Another detrimental phenomenon that is caused by leaching is delamination from glass CCSs. Two possible mechanisms of delamination are described, i.e. leaching of ions (e.g. sodium) from the glass surface into the DP formulation, or interaction of formulation constituents with the glass surface. Both mechanisms cause a porous inner surface of the glass vials or ampules with subsequent release of glass flakes, which might not be observed during visual inspection of the final filled units, because the delamination process is rather slow, and therefore might only occur during long-term storage. The different parameters (glass composition, container forming process, container pre-treatment, sterilization conditions, DP formulation) that might contribute to glass delamination should be wisely chosen to prevent surprises in a late project phase [20].

16.3.4.4 Silicone Oil

Silicone oil is applied as a lubricant on the inner surface of pre-filled syringes to minimize the injection force that is needed, e.g. for administration with an autoinjector. During elongated storage of the DP in the pre-filled syringe as well as when applying shear stress, e.g. during transportation, the silicone oil will transfer (leach) into the DP formulation and form silicone droplets [21]. A follow-up interaction of the droplet surfaces with the protein might lead to aggregation and formation of sub-visible particles potentially inducing immunogenicity. During

leachables assessment, the “leaching” of silicone might be neglected, because the chemical itself is not of toxicological concern. However, if the protein is known to be sensitive to the presence of silicone droplets (this should be assessed during formulation development) the siliconization process should be carefully established, by finding a compromise between the functionality of the device and the quality and stability of the contained DP.

16.3.4.5 Secondary Packaging Materials, Adhesives and Printing

When selecting materials for E/L assessment, it is important to consider also secondary packaging, adhesives and printing inks as a potential source of contamination. Especially, flexible bags that are used during administration might be packed in overwraps with a direct contact of the bag material with the overwrap foil, thereby enabling leaching from the secondary packaging into the contained solution that is administered to the patient. Often even multi-layered foils—if not constructed especially for this purpose—are not migration barriers. This also causes leaching of chemicals used in adhesives to stick labels on the outer side of plastic bags; adhesive chemistry is complex! Also, printing inks applied to the outside of flexible bags and other polymeric containers will contribute to a contamination with leachables. Especially, the volatile components of the printing ink formulation with high diffusion coefficients will migrate through the polymeric material (if no dedicated migration barrier is added). Another source for printing ink leachables might be the manufacturing and printing process of the foils, when during stacking the inner surface is in direct contact with the outer printed surface. It is therefore important to perform leachables assessment on the final packed, labelled and printed materials.

16.4 Summary and Conclusion

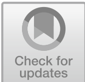
During manufacturing, storage and administration, leachables will contaminate medications and will ultimately be administered to the patient. It is of importance to control leaching for compliance and patients’ safety reasons. This might be done by exclusive risk evaluation (which should be based on scientific rational) or by laboratory E/L testing of materials. To get meaningful E/L results, it is important to obtain input from the different stakeholders of the CMC and clinical experts. It is then important to properly select testing conditions and correlate the analytical results of a study to a safety risk with regard to leachables to the patient.

Acknowledgements The author would like to acknowledge the countless contributions of Andreas Zerr, Lonza AG, to increasing the knowledge on extractables and leachables.

References

1. Zweifel H. (ed) Plastics additives handbook. Munich: Hanser Publisher. 2000.
2. Food and Drug Administration (FDA). 21 CFR Sec. 211.65, Equipment construction. 2016.
3. Food and Drug Administration (FDA). 21 CFR Sec. 211.94, Drug product containers and closures. 2016.
4. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER) and Center for Biologics Evaluation and Research (CBER). Guidance for industry—container closure systems for packaging human drugs and biologics. 1999.
5. European Medicines Agency, Committee for Medicinal Products for Human and Use (CHMP) and Committee for Medicinal Products for Veterinary Use (CVMP). Guideline on plastic immediate packaging materials. 2005.
6. United States Pharmacopeia. <1663> Assessment of extractables associated with pharmaceutical packaging/delivery systems.
7. United States Pharmacopeia. <1664> Assessment of drug product leachables associated with pharmaceutical packaging delivery systems.
8. International Conference on Harmonization (ICH) M7. Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk. 2014.
9. Teasdale A, Jahn M, Bailey S, Feilden A, Taylor G, Corcoran ML, Malick R, Jenke D, Stults CLM, Nagao LM. Controlled extraction studies applied to polyvinyl chloride and polyethylene materials: conclusions from the ELSIE controlled extraction pilot study. AAPS PharmSciTech. 2014;16:664–74.
10. Sharma B, Bader F, Templeman T, Lisi P, Ryan M, Heavner GA. Technical investigation into the cause of the increased incidence of antibody-mediated pure red cell aplasia associated with Eprex®. Eur J Hosp Pharm. 2004;5:86–91.
11. Curry W, Conway L, Goodfield C, Miller K, Mueller RL, Polini E. Reducing the risk of contamination of sterile parenteral products via ready-to-use closure components. AAPS PharmSciTech. 2010;11:1572–9.
12. Product Quality Research Institute Leachables and Extractables Working Group. Safety thresholds and best practices for extractables and leachables in orally inhaled and nasal drug products. 2006.
13. American Academy of Pediatrics. Benzyl alcohol: toxic agent in neonatal units. Pediatrics. 1983;72:356–8.
14. International Conference on Harmonization (ICH) Q3D. Guideline for elemental impurities. 2014.
15. Shukla AA, Hubbard B, Tressel T, Guhan S, Low D. Downstream processing of monoclonal antibodies—application of platform approaches. J Chromatogr B. 2007:28–39.
16. Ding W, Madsen G, Mahajan E, O'Connor S, Wong K. Standardized extractable testing protocol for single-use systems in biomanufacturing. Pharm Eng. 2014;34:1–11.
17. Hammond M, Nunn N, Rogers G, Lee H, Marghitoiu AL, Perez L, Samuel YN, Anderson C, Vandiver M, Kline S. Identification of a leachable compound detrimental to cell growth in single-use bioprocess containers. PDA J Pharm Sci Tech. 2013;67:123–34.
18. Bee JS, Nelson SA, Freund E, Carpenter JF, Randolph TW. Precipitation of a monoclonal antibody by soluble tungsten. J Pharm Sci. 2009;98:3290–301.
19. Chang JY, Xiao NJ, Zhu M, Zhang J, Hoff E, Russell SJ, Katta V, Shire SJ. Leachables from saline-containing IV bags can alter therapeutic protein properties. Pharm Res. 2010;27:2402–13.
20. Ennis RD, Pritchard R, Nakamura C, Coulon M, Yang T, Visor GC, Lee WA. Glass vials for small volume parenterals: influence of drug and manufacturing processes on glass delamination. Pharm Dev Technol. 2001;6:393–405.
21. Basu P, Krishnan S, Thirumangalathu R, Randolph TW, Carpenter JF. IgG1 aggregation and particle formation induced by silicone-water interfaces on siliconized borosilicate glass beads: a model for siliconized primary containers. J Pharm Sci. 2013;102:852–65.

Chapter 17
Biotherapeutic Drug Product Manufacturing and Process Development



Daniel Dixon and Anthony Gudinas

Abstract This chapter describes a typical biologics drug product manufacturing process and typical work to support that process. Unit operations addressed include dilution strategies and mixing, sterile filtration, filling, capping, and inspection, with a separate discussion on hold time monitoring. As each unit operation is discussed, manufacturing considerations and typical laboratory study designs are also introduced. A knowledge-based process design feeds into a robust risk management program, which in turn supports the Quality by Design paradigm. As a program progresses to later stages, the focus of the Quality by Design paradigm becomes demonstrating control of the process (through the control strategy) and proving consistency (through Process Performance Qualification).

Keywords Drug substance dilution · Sterile filtration · Filling
Hold times · Quality by Design

17.1 Overview

At-scale drug product manufacturing of protein-based drugs typically follows a standard flow simplified in Fig. 17.1. Drug substance is typically shipped from either the substance manufacturing site or a long-term storage location to the fill site under frozen conditions. The drug product manufacturing process begins with thawing the drug substance. The drug substance is then pooled into a suitable container and diluted with buffer, if required. The formulated bulk drug product is sterile filtered prior to filling into an appropriate container (vial or prefilled syringe). Lyophilized products are loaded into the lyophilizer, and the cycle is executed at

D. Dixon (✉) · A. Gudinas
BioTherapeutics Pharmaceutical Sciences, Pharmaceutical R&D,
Andover, MA 01810, USA
e-mail: Dan.Dixon@pfizer.com

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_17

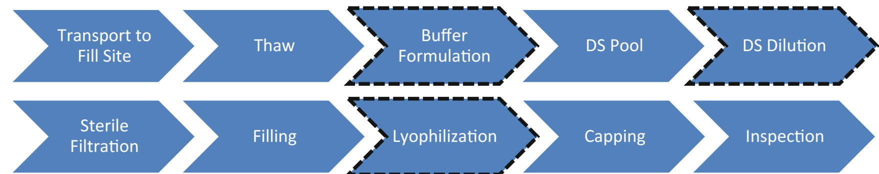


Fig. 17.1 Typical protein drug product manufacturing process. Dashed boxes indicate optional steps

this stage. Following filling (for liquid products) or lyophilization (for lyophilized products), vials are capped and inspected. At this point, the lot is ready for release pending analytical release testing and quality review of batch documentation.

Often, "process development" is approached from a purely scientific perspective. While a strong understanding of the relative sensitivities of the drug product material is critical to designing a manufacturing process, it is not the only factor. Additionally, equally critical considerations to manufacturing include: microbial control, compliance, and process reproducibility as well as economic drivers such as batch size and facility throughput. A process must be designed with the manufacturing environment in mind to minimize potential microbial risks by minimizing manipulations that may introduce contamination and with suitable equipment and consumable materials to prevent non-viable contamination. During execution, the process is typically checked at predefined points to ensure it is progressing as intended or measure the current state to define future actions through in-process controls. These controls often include pH, osmolality, active concentration, and bioburden. Process reproducibility can be impacted in several ways, including equipment capabilities and process simplicity. Process simplicity leads to reduced opportunity for human error during execution.

Expectations around procedures, facilities, and personnel are described in detail in ICH Q7 (International Conference on Harmonization [8]. The details of specific at-scale manufacturing processes are supported by development work, either at laboratory scale or at full scale, and a robust risk identification and mitigation strategy. ICH Q8(R2) [9], ICH Q9 [10], and ICH Q10 [7] work together to outline Quality by Design principles through pharmaceutical development, risk management, and quality systems. This chapter will walk through the manufacturing unit operations and highlight areas of focus and particular importance. This chapter will assume a liquid product, as lyophilization specific concerns are covered elsewhere (please see Chap. 17, "Lyophilization: Process Design, Robustness and Risk Management").

17.2 Transport and Thaw

There are two primary concerns during transportation of drug substance. The protection of the active materials during transport is critical as is the maintenance of the product temperature within predefined acceptable ranges. Both of these aspects are a function of the design of the shipping container and can be qualified independently from the actual product being shipped. Protection usually includes aspects such as protection from impact, light, and contamination. In general, early-stage programs should rely on shipping configurations that have been demonstrated to be fit for purpose in their ability to maintain the product temperature within the target range for the required duration of time while providing adequate physical protection for the contents against foreseen handling, which should consider potential delays in transit.

As drug substance is typically stored frozen, adequate storage capacity is required at the drug product manufacturing site to warehouse the drug substance within the storage temperature specifications prior to manufacturing. Typically, the first step of the drug product manufacturing process is the thaw of the drug substance. Thawing mechanism is dependent on (1) drug substance container type (e.g., bottles or bags), (2) volume within each container, (3) sensitivity of the active material to stresses encountered during thaw, and (4) availability of any specialized equipment at the drug product manufacturing site. For a more detailed discussion of considerations for drug substance storage, refer to Chap. 13 ("Bulk Protein Solution: Freeze Thaw Process, Storage and Shipping Considerations").

17.3 Pooling and DS Dilution

Post-thawing, the drug substance is pooled into a vessel or disposable container of appropriate size. To minimize manufacturing process time for drug substance, any required dilution buffer should be prepared in advance and ready for use. Buffer is added to the drug substance to reach the final target concentration based on the dilution strategy and the result of an in-process concentration determination. Any buffer added to the manufacturing process should be confirmed to meet relevant in-process controls (such as pH and osmolality) and passed through a 0.22-micron filter to minimize the risk of microbial contamination.

17.3.1 Dilution Strategy

In many cases, the drug substance and bulk drug product contain different concentrations of protein. This may be due to products that provide multiple dosage form presentations by varying active concentration, or reducing storage and

shipping volumes by increasing active concentration. If this is the case, the drug substance must be diluted during the drug product manufacturing process.

Buffers used during drug product manufacturing can be broadly split into two categories: (1) excipient modifying buffers and (2) dilution buffers. If the formulations of the drug substance and drug product are not the same, a modifying buffer is required. Use of a modifying buffer is preferred to adding dry excipients directly to a drug substance pool, as mixing speeds and durations can be selected to ensure dissolution of any dry materials without risk to potential shear degradation of the drug substance.

The quality of excipients used in protein formulations must be considered to minimize the impact of potential trace impurities (e.g., trace levels of aldehydes, peroxides, and/or metal ions which may lead to oxidation) [15]. Additionally, multiple vendors may be desirable for key excipients, in which case comparable quality is required [3].

Polysorbate surfactants have received significant attention in the literature due to potential for surfactant degradation and the potential impact of the degraded materials on the active product [13, 22]. From a manufacturing perspective, several potential strategies can be employed to mitigate potential risks. These include: (1) purchasing small volume containers to enable single use or (2) qualifying/validating application of a nitrogen overlay after each use. Application of small volume aliquots is more straightforward in a manufacturing environment. To comply with expectations around identification sampling from each container, it would be recommended to utilize a single sacrificial container for provisional acceptance of the bulk lot, followed by samples from the individual containers collected at the point of use if required. Opening of containers to be used in manufacturing should be avoided until the point of use to minimize the risk of polysorbate degradation.

In general, the preferred order of dilution in this case would be to (1) pool the drug substance, (2) add the modifying buffer at a predetermined ratio to yield the correct excipient concentration (potentially resulting in a range of protein concentrations from lot to lot), and (3) dilute the composition-modified pool to the final protein concentration. The composition of the modifying buffer should balance the solubilities of the augmented excipients with the degree of dilution of the drug substance. The two buffer approach, while requiring more facility time and effort to manufacture, provides flexibility with respect to lot-to-lot variability in drug substance concentration, while maximizing consistency of excipient composition from drug product lot to drug product lot.

The dilution strategy from the potentially variable drug substance concentration to formulated drug product must consider several aspects:

1. Magnitude of the dilution (large difference vs. small),
2. Volume of solution relative to working volume of vessel/container,
3. Accuracy of concentration determination method and time required for testing,
4. Single lot of drug substance or multiple, and
5. Mechanism for ensuring product homogeneity.

In general, minimizing the number of times a process is interrupted for microbiological and analytical testing is desirable. In a manufacturing environment, a typical UV/Vis absorption assay performed in a QC laboratory can cause a 2 h (or more) interruption in the process. Performing this testing multiple times may challenge the overall process hold time and facility schedule without significantly improving process robustness.

For a large dilution (e.g., the drug substance makes up less than 70% of the final volume), an initial dilution can be made based on the theoretical concentration (either from the Certificate of Analysis of the drug substance, or a weighted average of the concentrations of multiple lots). Using this theoretical concentration and pooled drug substance weight, an initial dilution can be performed to an intermediate point that is closer to the final concentration (e.g., 80% of the final theoretical weight). The proximity of the initial dilution to the final target should take into account the variability of the concentration determination measurement. After mixing, samples are collected to confirm homogeneity and determine the concentration at that point to use for calculation of the final dilution. If data has been generated to qualify that a solution is homogeneous when subject to a set of mixing conditions, then homogeneity does not need to be confirmed provided the mixing time, speed, and volume are within the previous qualification. Qualification of homogeneity is typically demonstrated near the process validation stage as commercial batch sizes and process parameter ranges are finalized, while earlier phase clinical manufacturing confirms homogeneity for each batch.

If the drug substance makes up greater than approximately 70% of the final volume, a single dilution from the drug substance pool to the final volume is recommended upon determination of the concentration of the pooled drug substance through an in-process control.

A potential exception to the above strategy comes when there is poor confidence in the composite calculated concentration. This lack of confidence can either be due to high variability of the analytical method or pooling of many different lots of drug substance over a range of concentrations. Under these circumstances, one should define a more conservative approach to the dilution process to ensure the material is not over or under diluted during the manufacturing process, thus potentially failing specification.

The pooled drug substance must be mixed to homogeneity prior to sampling. In general, the higher the mixing speed, the more quickly the solution becomes homogeneous. The choices of the mixing conditions are generally restricted on the aggressive side by seeking to minimize shear degradation, splashing, foaming, and air entrainment. Mixing conditions may also be influenced by the volume of solution. While it is preferable to design a vessel that remains within its working volume for the entire range of batch sizes, from the addition of drug substance through the 100% formulated solution under all circumstances, this is not always possible when required to work with existing equipment or for products with a significant batch size range. A scenario may exist (particularly if there is a large dilution between drug substance and drug product) where the drug substance pool is not above the minimum working volume of the vessel. Vessel choice should also ensure that the maximum batch size does not exceed the maximum working volume of the vessel.

In these cases, there are two methods to exceed the minimum working volume prior to mixing: (1) charge the vessel with a fixed amount of buffer prior to substance addition or (2) add the drug substance followed by buffer. The advantage of the first option is that mixing can initiate immediately after drug substance addition. The advantages of the second option are that it is more straightforward to define the weight of the drug substance pool and fewer manipulations are required. In the spirit of maximizing simplicity, the preferred option would be to identify (or design and purchase) a vessel that can mix across all required volumes. The second choice would be to add the drug substance first prior to an initial dilution to exceed the minimum working volume of the selected vessel.

Homogeneity can be defined in several ways. Often pH, osmolality, and protein concentration are measured. One can question the value of a pH measurement if the solutions that are mixed are drug substance lot(s) and buffer, each of nominally the same pH. Osmolality primarily measures the highest concentration of formulation excipients. In most protein formulations, these are readily soluble compounds (such as salts and polysaccharides/polyols). Additionally, if the drug substance and drug product are the same composition, then the only differences in osmolality would be due to additional ions carried through the drug substance purification process via the Donnan effect [20]. In either case, the osmolytes are small materials and would be expected to mix fairly quickly, relative to significantly larger protein molecules. As a result, the worst-case material to test for in determining homogeneity is generally the concentration of active ingredient.

Two strategies can be employed for ensuring homogeneity—(1) confirming homogeneity through in-process controls and (2) qualifying/validating mixing conditions that have been demonstrated to yield a homogeneous solution over the full range of batch sizes. For early-stage clinical programs which have not defined a commercial manufacturing process (such as batch size and equipment design), implementation of in-process controls will confirm that the process results in a homogeneous solution prior to progressing through manufacturing. Separately, a mixing study can be executed on the largest volume of solution at the slowest mixing speed (worst case) to define conditions that will result in homogeneity, which would typically be executed in preparation for process validation. Process parameters within the conditions defined in such a study would not be required to be challenged for homogeneity on a routine basis.

One popular method for confirming homogeneity is to collect samples from different locations within the pooled solution at a point in time (e.g., top, middle, and bottom of the vessel). In employing this method, care must be taken to ensure that the samples collected are representative of the solution at that particular location, particularly if long lengths of tubing are required to be submerged. Another drawback of this method is the opportunity for contamination by having an operator directly above the solution in a Grade C, down-flow area. The airflow in these areas would naturally wash over the operator and toward the solution. While downstream filters will mitigate contamination risk, having operators over open solutions is undesirable.

Another method is to collect two samples from the same location at different mixing times. If the concentration is unchanged between the two time points, then the solution can be considered homogeneous. This can minimize intrusions into the manufacturing vessel, particularly if aseptic sampling manifolds are employed.

When diluting, acceptance criteria should be predefined for the allowable variability between different in-process measurements. This should also take into account the variability of the concentration assay, as well as the variability allowed in the finished product specification (typically $\pm 10\%$). Additionally, an acceptance criterion should be defined for proximity to the target concentration, as a precaution against error in executing the concentration measurement. One method is to apply the following two equations:

$$\frac{\text{Smallest Value}}{\text{Largest Value}} > 0.95$$

$$0.95 < \frac{\text{Average of Measurements}}{\text{Target Value}} < 1.05$$

These equations ensure that all values are within 5% of each other and the average is within 5% of the target. Not meeting these criteria may indicate that:

1. The solution is not homogeneous.
2. The concentration measurement is not robust (or was not performed correctly).
3. There was an error in:
   a. The starting concentration value.
   b. The amount of buffer added.
   c. The weight of the bulk container (e.g., not everything that was on the balance during the tare was still on the balance).

At the end of the formulation process, the protein concentration should be confirmed and the solution is often passed through a bioburden reduction filter to reduce the challenge to the downstream sterile filters and to enable a hold prior to sterile filtration.

17.3.2 Considerations

The mechanism of mixing at manufacturing scale may lead to particulate formation or protein degradation in some instances. Commonly used bottom-mounted magnetic mixers with silicon carbide bearings were shown to impact the integrity of an IgG1 protein resulting in an increase in sample turbidity, in one example [2]. This particle generation was not observed for top-mounted mixers [11].

Material compatibility studies should include all product contact materials and should consider the surface area to volume ratio at the large scale.

These experiments are best performed at laboratory scale with surface area to volume ratios in excess of the worst-case scenario (preferably a multiple of the ratio) as a function of time exceeding the maximum allowable manufacturing time. Common materials include stainless steel, platinum-cured silicone, gasket materials (such as EPDM, PTFE, and platinum-cured silicone), and any plastics used in storage or sampling bags. When possible, samples of actual in-process manufacturing materials should be tested. Analytical tests should include stability-indicating assays that are expected to show change during storage (e.g., measurement of high molecular weight and low molecular weight species) as well as any other tests that may be expected to be sensitive to potential degradation. For example, the degree of oxidation should be measured after contact with stainless steel for proteins known to be sensitive to metal-catalyzed oxidation.

During compatibility studies with plastics, the concentration of any surfactants in the formulation should be monitored as a function of time to ensure that the solution exposed to those materials has the intended surfactant levels after worst-case exposure. Additionally, plastics should be evaluated for any potential extractables and leachables, and steps may be required to mitigate leachable impact on the finished product.

17.4 Laboratory-Scale Mixing Studies

There are several unit operations in a commercial manufacturing process that require a mixing step. For example, a mixing step after DS is pooled, a mixing step during buffer preparation, and a mixing step during dilution (if necessary) of DS to 100% formulated DP. All of the mixing steps are designed to ensure homogeneity of the protein and other excipients in the formulation. Typically, the commercial manufacturing site has established mixing speeds and times for the different mixing steps. Scaled-down laboratory process qualification (LPQ) studies are executed to compare a common parameter in the mixing process, such as shear rate at the tip of the mixing impeller, to ensure the commercial mixing parameters are within an established design space. The shear rate imparted on the solution by the mixing mechanism is a function of the diameter of the mixer, the rotational speed, and the power number of the mixer (which takes into account mixer geometry). Mixing speed at scale should be restricted by (1) allowable shear (determined from laboratory-scale experiments) and (2) prevention of splashing, vortexing, foaming, and air entrainment (confirmed at scale, with allowable ranges obtained using either model solutions or during development studies). Conditions in the laboratory process qualification studies are set to be more aggressive than the commercial process to ensure the LPQ studies effectively define the range of parameters within which protein quality is maintained. The shear rate at the impeller tip can be calculated for the range of mixing speeds and impeller designs of the commercial process, with LPQ mixing speeds chosen to surpass the shear rate developed in the manufacturing process by a safety factor. If protein quality is maintained in the LPQ

study, then the mixing conditions of the commercial process are within the design space that will ensure protein quality. The shear rate (γ (1/s)) at the tip of an impeller for a given vessel diameter and impeller diameter is [16]:

$$\gamma = \frac{\pi \cdot \text{impeller diameter} \cdot \text{impeller speed (rpm)}}{\left(\frac{\text{vessel diameter-impeller diameter}}{2}\right)}$$

For example, if a commercial process uses an 80 cm diameter vessel for a 400 L batch size with a 14 cm impeller and 120 rpm impeller speed, the shear rate would be 160/s. An LPQ study using a scaled-down 2 L vessel with a diameter of 11 cm and impeller diameter of 6 cm could be run at 100, 200, 300, and 400 rpm resulting in a shear rate range of 754–3015/s. If the protein quality from the laboratory study is within specifications after exceeding the mixing time and mixing rate of the commercial process, then it can be concluded the LPQ study indicates the commercial mixing parameters will result in satisfactory protein quality from a shear perspective. This study does not, however, ensure the commercial mixing parameters do not induce splashing or foaming, or produce a homogeneous formulation. Such assurances, if required, are the result of actual at-scale mixing studies during engineering runs that confirm the mixing parameters produce a homogeneous formulation without splashing or foaming. Surrogate solutions may be considered with appropriate characterization that homogeneity of the surrogate is representative of the active materials under consideration.

17.5 Sterile Filtration

Parenteral drug product formulations are required to be sterile. This can be accomplished either through terminal sterilization or in-process sterilization during manufacturing. As biologics are typically not compatible with terminal sterilization, the manufacturing process relies on filtration of the liquid drug product through a sterilizing-grade filter with a nominal pore size of ≤ 0.2 μm. Two types of filters are used in manufacturing processes, a bioburden reduction filter and a sterilizing-grade filter. The purpose of a bioburden reduction filter is to minimize the level of viable microorganisms in a manufacturing process stream. A bioburden reduction filter is used in process steps that do not require sterile output; rather, they are used to ensure the bioburden load of the drug product is below established control specifications prior to sterile filtration. Sterilizing filters ensure that the downstream process stream is sterile. This is ascertained by confirming that the solution presented to the sterilizing filters is low bioburden, less than 10 CFU/100 mL (colony forming units) or equivalent, that the filter has been demonstrated to provide at least an 8 log removal of the challenge organism *Brevundimonas diminuta* during validation studies, and that the filters have been demonstrated to be integral both prior to use and after use. Open collection of bioburden samples

carries a risk of contamination during sampling that could potentially lead to batch failure. One mitigation strategy would be to employ closed sampling methods to reduce the exposure of the bioburden sample to the environment (or operators) prior to analysis. Redundant series filters are used during sterile filtration as a risk mitigation strategy to ensure sterility in the event one filter is compromised. While finished product is tested for sterility after manufacturing, the sampling for this testing is not from a statistically significant portion of the lot and would only be expected to detect gross contamination.

17.5.1 Bioburden Reduction Filters

Bioburden reduction filters are used to reduce bioburden load at steps in the manufacturing process where sterility is not a requirement. For example, if formulated drug product is held overnight prior to filling, it can be filtered into a sterile vessel using a bioburden reduction filter. Commonly, bioburden reduction filters are the same membrane type and pore size as sterilizing filters and only differ from sterilizing filters in the controls placed around the filtration step (such as filter integrity testing and confirmation of challenge bioburden). A bioburden reduction filtration frequently precedes the sterile filtration step in a manufacturing process to ensure a low bioburden load on the sterile filters. Bioburden is measured by sampling and evaluation using a validated test method. Levels are monitored and confirmed during process qualification batches.

17.5.2 Filter Requirements for Early-Stage Processes

In preparation for early-phase clinical manufacturing, a filter must be selected for a manufacturing process. There are several factors involved in selecting a filter: the compatibility of the protein and formulation with the filter, batch size, membrane chemistry, and operating parameters of the process. Studies that demonstrate a filter is compatible with the product, and the filtered product meets the expected acceptance criteria, should be executed to verify a filter is suitable. These studies can include: protein adsorption, surfactant adsorption, effects on protein quality from multiple filtrations, and flux determination [to define the appropriate filter size (s) for proposed batch size(s)]. In addition, data from filter manufacturers is used to determine filter suitability for a specific process. Filter manufacturers are required to perform tests according to compendial methods to qualify the filter for use with pharmaceutical products. These tests include evaluation of leachables and extractables, ability to retain bacteria, and properties to confirm filter integrity typically using water as a challenge stream.

When a filter is selected for a process, it needs to be properly sized for the intended batch size. Filter sizing can be determined by executing a laboratory V_{max}

test, defined as the maximum filterable volume through a filter of specific area, to determine the flux of the drug product through the filter and the filter area required to filter the batch volume in a reasonable time period. The challenge volume/filter area used in the V_{max} test should be representative of the batch volume/filter area proposed for the commercial process to obtain the most accurate test data.

An alternative during early product development is to leverage a platform process approach where a single filter type is chosen that is compatible with multiple compounds for the purpose of delaying product-specific filter validation until the proposed commercial process is defined. A platform approach can be used after a sufficient amount of data is collected on multiple compounds which demonstrate a degree of robustness where no impact on protein quality is determined from protein adsorption, multiple filtrations, and membrane compatibility studies. Furthermore, if a new compound can be identified to be comparable in solution properties, concentration, excipient types and levels, then a risk-based approach can be applied to validation areas such as microbial retention and extractable and leachable testing during early clinical stages.

17.5.3 Filter Integrity Testing (FIT)

A post-use integrity test must be performed on the sterilizing filter in a manufacturing process to verify the filter is integral to comply with FDA regulations. To comply with European Commission Annex 1, the sterilized filter should also be integrity tested prior to use [4] and is strongly recommended by FDA [6]. Filter integrity testing determines if there are any defects in the filter membrane that might compromise the filter's ability to retain microorganisms. Filter manufacturers set water-wet integrity test specifications for filters and include this information with the filter's certificate of quality. If a product-specific filter integrity test specification needs to be established, the user or the manufacturer can execute the testing using validated methods and appropriate documentation, determining the difference between integrity test results of the challenge stream relative to water [1]. The two most common types of integrity tests are gas diffusion through a wetted membrane, or forward flow, and bubble point test of a wetted membrane. Gas diffusion measures the diffusion of gas molecules across a wetted filter under constant pressure and is proportional to filter surface area. Diffusion is generally preferred for large filters or filters with high bubble points. Bubble point measures the pressure required to push the wetted solution out of the largest pores in the membrane, is independent of filter surface area, and is correlated to vendor performed microbial retention testing. A water-wet integrity test is usually sufficient for Phase 1 manufacturing, provided the filters can be suitably wetted with water for a post-sterilization/preuse integrity test and that water can be flushed out of the filter without diluting the active material used to fill the initial portion of the batch. The integrity test verifies that the test filter has characteristics that have been shown by the manufacturer to retain bacteria.

The level of filter qualification increases from Phase 1 to Phase 3 clinical manufacturing and process validation runs. A risk assessment should be executed prior to full-scale engineering runs to identify any gaps in the filtration process that require additional testing at the development scale or full-scale engineering and qualification runs. A risk assessment will analyze factors and determine the probability, severity of occurrence, and then decide if additional experimentation is needed to provide more supporting data and reduce risk. ICH Q9 provides guidance on applying quality risk management principles to development and manufacturing.

17.5.4 Sterile Filtration Validation

Sterile filtration validation studies are typically required to be completed prior to process validation studies. Filter validation starts with defining the full-scale manufacturing process: process scale, time of filter exposure to the process stream, operating temperature and pressures, filter surface area, and type of process flow. This information will be used to design the appropriate tests by the user or the filter manufacturer for filter validation. The testing required to perform filter validation typically includes: product-specific integrity test parameters (if required and not already measured), bacterial retention, membrane compatibility, leachables and extractables.

17.5.5 Bacterial Retention

Bacterial retention demonstrates that the sterilizing membrane is capable of retaining *B. diminuta* bacteria (*B. diminuta*) (American Type Culture Collection (ATCC) 19146) in the presence of the drug product stream. Testing is conducted on a laboratory scale using 47 mm membrane disks. The use of 47 mm disks for bacterial retention testing allows for scaled-down simulation of the established filtration process. Three different lots of 0.2–0.22 μm membrane (test filters) are tested. A single 0.45 μm membrane is also tested as a control. Prior to retention studies, *B. diminuta* is added to an aliquot of DP solution to demonstrate viability of the bacteria, which determines the next steps during the retention study.

Acceptable performance for the 0.2–0.22 μm membrane is demonstrated by the membrane retaining all *B. diminuta* organisms when challenged with $\geq 1 \times 10^7$ CFU (colony forming units) per cm^2. The test organism is prepared and assessed for purity and identity. It is then inoculated into the DP. A sample is taken and the concentration of the inoculum and challenge fluid determined. The inoculated DP is filtered, and the filtrate is monitored for *B. diminuta.* It is expected that *B. diminuta* is present downstream of the 0.45 μm membrane, but not downstream of the 0.22 μm membranes.

17.5.6 Membrane Compatibility

Membrane compatibility studies assess whether exposure of drug product to the filter membrane impacts the characteristics and performance of the filter (the target is "no impact"). These characteristics include: water permeability (flow rate), product bubble point, weight, and appearance. The testing is done to determine if the pharmaceutical product is chemically compatible with the filter membrane and if exposure to the product alters the pore size, therefore altering the retention capabilities. Membranes from three lots are wetted with water and tested for bubble point. The membranes are then wetted with product and retested. The membranes are then statically exposed to the product for a period that brackets the maximum manufacturing time allowed by the process/manufacturing location. The membranes are then retested, and the results are analyzed to determine if the product has altered the filter membrane.

17.5.7 Extractables Testing

Extractables testing is performed on filters to determine whether filter-related components can be removed under aggressive exposure conditions, such as the use of a solvent or exposure to high temperature or high/low pH. Extractables represent the worst case in terms of number of compounds identified and levels of those compounds because of the aggressive exposure conditions. Leachables are a subset of extractables and represent components that can migrate from the filter during exposure of the process stream under normal process conditions. Extractable data can be generated by the filter manufacturer or the filter user. Ideally, the filter manufacturer can provide a list of possible extractable components, execute testing, and provide an analysis of the extractables. These extractables should be identified and quantified. Methods to identify and quantify extractables include reversed-phase high-performance liquid chromatography (RP-HPLC), liquid chromatography with mass spectrometry (LC–MS), and gas chromatography with mass spectrometry (GC–MS). Extractables testing of filter material using actual drug product, however, is not practical. The extractable compounds one could expect from filters consist of organic polymers or monomeric materials, surfactants, plasticizers, or any raw material used in the filter manufacturing process. Since the concentration of drug product and/or excipients is much greater than the potential filter extractables, it is difficult to develop a practical analytical method due to assay interference. Therefore, instead of testing the filter with actual drug product/placebo, the filter is tested using a model solvent approach.

Testing is performed using representative worst-case conditions and model solvent streams based on the product's formulation and process for the purpose of extractable substances analysis. Examples of model solvent stream(s) could include water, denatured ethanol, and low and high pH-adjusted solutions. The extraction

solutions are analyzed using a variety of methods (listed above) to determine the absence or presence of compound(s) and to quantify the total amount of extractables. In some cases, a specific identification is not possible, and these results are reported as a class of compound (e.g., “polyacrylate-related compounds”). The potential human toxicological impact must be evaluated for any identified extractable.

Because extractable testing is based on a model solvent approach, one could potentially apply the same extractables study across multiple programs, provided:

1. The model solvent bracket accurately describes all challenge solutions.
2. The extractables study was performed on a “worst-case” preparation of filters (maximum sterilization time, maximum hold time, maximum filter area to solution challenge).

If testing is based on this approach, extractables data for filters prepared within the bracketed parameters would be justified for future programs without need for additional study.

17.5.8 Leachables Testing and Flush Volume Determination

Leachables testing is performed to determine the minimum flush volume required to remove filter leachables from the drug product. Leachables testing is conducted using product or placebo under normal use conditions and can be challenging due to assay interference, as product ingredients exist at much higher concentrations than leachables. A feasibility study is first performed with the product to see if the analytical techniques intended to be used for leachables analysis demonstrate no matrix interference. These results determine if the product or placebo can be utilized for the study.

The study design typically involves filtering the product through the intended filter and collecting aliquots of filtrate which are analyzed for presence of leachables (nonvolatile, semi-volatile, or volatile components and metals). The minimum flush volume required for leachable reduction must then be compared to the results of the filter adsorption study. The chosen flush volume must not only remove leachables, but also demonstrate that the surfactant and/or product have adequately saturated the filter where no decrease in concentration is observed. The product requirement could be 2 L–7 L depending on process and filter. The flush volume must be implemented in manufacturing in a fashion that minimizes risks for potential microbial contamination of the filters and any sterile downstream portions of the filter assembly.

17.5.9 Filter Sterilization

Filters or filter assemblies must be sterilized prior to use, and the filter sterilization process is validated to verify that it has no impact on filter performance. The filter manufacturer's literature contains information regarding temperature/pressure limitations and the recommended sterilization process. The most common methods of sterilization are steam-in-place (SIP), autoclaving, irradiation, and gas sterilization. SIP is used for cartridge-type filters in housings rated for 30 psig or 2 bar. The filter is assembled and steam is passed through the filter for a validated time and temperature that ensures sterility. Autoclaving involves steam sterilization at 121–135 °C for 30–60 min depending on the filter's materials of construction and the qualification of the autoclave used. Filters should be placed inside two breathable microbial barriers to avoid contamination post-sterilization. Gas sterilization uses ethylene oxide as the sterilizing gas. Filters are placed in a breathable microbial barrier that allows penetration of the gas for sterilization and removal of residual gas when the process is complete. Sterilization by irradiation is accomplished by gamma radiation or electron beam radiation, generally by a third-party vendor. The advantages of irradiation are filters are dry, and there is no residual gas or water. A filter integrity test is performed, post-sterilization, with the filter in place to confirm the sterilization process did not compromise the integrity of the filter. Circumstances could occur when a filter might need resterilization. In this case, it should be confirmed with the filter manufacturer that the filter is compatible with multiple sterilization cycles and the process is validated.

17.6 Filling, Stoppering, and Capping

In the manufacturing process flow, once filter sterilized, material is ready for aseptic filling. Aseptic filling has three primary outputs and concerns: (1) The required amount of product is within the primary container, (2) has been dispensed there aseptically, and (3) the container has been appropriately sealed to ensure sterility of the contents over the shelf life of the product. This filling assumes that the depyrogenation of the vials has already been qualified, ensuring that the vials are sterile.

Filling is nearly always performed on a weight (rather than volume) basis. Weight is easy to measure accurately and quickly. In contrast, volume targets must be converted to fill weight by applying the density of the solution due to the lack of robust methods for accurately measuring volume directly within an aseptic manufacturing area.

17.6.1 Fill Weight Tolerance

The choice of fill tolerances should account for machine capabilities, as well as the required contents to meet the label claim, as described by USP<1>. From a machine capability perspective, most filling equipment establishes three sets of criteria around fill weight (all apply both above and below target):

1. Ready for filling—indicates that the machine is targeting the center of the fill range.
2. Machine adjustment—correct for minor drift, but still within the overall acceptance criteria.
3. Segregation/reject limit—measurement is outside of tolerance and action is required.

These limits interact in the following fashion:

1. During the setup operation, the filling lines are purged and the machine attempts to reach the target fill weight. This is defined as successful when a predetermined number of consecutive measurements fall within the upper and lower "transition" limits.
2. During routine manufacturing, any weight checks within the "machine adjustment" ranges require no action.
3. During routine manufacturing, weight checks between the "machine adjustment" and "segregation/reject limit" are subject to local procedure for machine adjustment. For example, some facilities require consecutive measurements within this range to trigger a machine adjustment (which can be done manually or through the machine automation). Once the adjustment is verified to bring the fill weights within the desired range, filling resumes.
4. During routine manufacturing, weight checks outside of the reject limits activate local procedures for how to determine the scope of the deviation. This is usually restricted to units filled by the needle in question, unless needle identity of the vials has been lost (e.g., they are on an accumulation table). For fill lines that perform 100% fill weight confirmation, the impacted unit is only the individual unit that failed the weight check.

Fill weights are monitored in one of two ways—in-line and offline. For in-line weight checks, a weighing mechanism is incorporated into the filling machine and monitors weight at a predefined frequency. The equipment software evaluates the weight check data against the preprogrammed acceptance criteria and automatically adjusts the machine, if needed. Offline fill weight checks are performed by operators, again at a specified frequency, and the results are manually evaluated with manual adjustments to the machine, if required. The table below shows how filling tolerances can be shown for two hypothetical target fill weights applying arbitrary (but often representative) criteria.

	Value	Weight 1 (g)	Weight 2 (g)
Reject (high)	+3%	0.515	10.30
Adjust (high)	+2%	0.510	10.20
Release (high)	+1%	0.505	10.10
Target		0.500	10.00
Release (low)	−1%	0.495	9.90
Adjust (low)	−2%	0.490	9.80
Reject (low)	−3%	0.485	9.70

In the "Weight 1" example, the difference between the target and adjust limit is only 0.010 g and to the reject limit is only 0.015 g (or approximately 15 μL, assuming a density of 1 g/mL). Limits at this level may be influenced by the reproducibility of the filling. Applied to this example, the question becomes, "Can the filler dispense with less than 10 μL of variability?" In the case of the limits applied above to a 0.5 mL fill, the answer may very well be "no" and wider tolerances must be established. Fill equipment manufacturers provide recommended diameters for tubing and fill needles to minimize variability across a lot while still allowing the equipment to run at the target fill rate without excessive splashing. Additionally, the action of the filling mechanism typically includes a "pull back," meaning that after completing the dispensing stroke, the fill pumps reverse slightly to withdraw the solution from the end of the fill needle. This prevents additional droplets from falling into some containers but not others, as well as reducing the incidence of droplets coming into contact with the neck of a vial as the needle is being withdrawn from the vial.

In the example above, the proposed fill tolerances mean that the acceptable range (no machine adjustment required) around the target fill weight is 0.40 g for the "Weight 1" and 0.020 g for "Weight 2." In this case, one may investigate whether more narrow criteria are desirable for the high fill weight, as this range is more easily attainable at manufacturing scale.

The choice of fill weight (or volume) target depends on the interplay of three questions:

1. How are in-process weight checks performed?
2. What is the hold-up volume of the container?
3. Is there a minimum required delivered dose?

In general, there are two types of in-process fill weight measurement—"to contain" and "to deliver." These two paradigms differ in how the weight check is performed. A "to contain" fill weight defines the fill weight as the difference between an empty and full container. Almost all online fill weight measurements are "to contain." Offline fill weight measurements where the container is rinsed and dried to yield an empty container can also be considered "to contain." An example of "to deliver" would be a destructive weight check of a prefilled syringe where the intended delivery mechanism was used to dispense contents into a tared weigh boat. "To deliver" is more representative of how an end user would approach the

materials, whereas "to contain" is more representative of how the filling machine was actually set up.

The difference between "to contain" and "to deliver" is described in the second question above—the hold-up volume of the container. "To deliver" represents what someone can readily remove from the container, while "to contain" describes how much material was actually filled into the container in the first place. Because of their design, prefilled syringes can have hold-up volumes as low as 10 μL. Conversely, large vials with larger diameter stoppers can have hold-up volumes that reach 150–200 μL. For vials, the hold-up volume is impacted by the geometry of the inside of the stopper, the surface area of glass, the geometry of the shoulder of the vial, and the surface tension of the solution. This is complicated when one considers that within an individual lot of containers, there is slight variation in these geometries (presumably within the tolerances stated on the material specifications). Across multiple lots, this variability certainly exists. Similar, low levels of variability exist in stopper geometries within a lot and across multiple lots. Because of this variability, experiments to measure hold-up volume should include a statistically meaningful number of units (preferably fully processed, including sterilization and any drying of stoppers that would occur during routine production). Understanding the average and worst-case hold-up volumes for a product is critical to defining the appropriate fill weight target as the hold-up volume represents the material that will be put into the vial at the manufacturing site that the patient cannot use.

To comply with USP<1> (Volume in Container), the volume that can be withdrawn from a container *must be greater than the nominal volume*. This means that the goal is for every unit to contain *at least* the labeled dose (nominal volume). Conversely, ICH Q8(R2) states that the quantity of any manufacturing overage must be justified. Excessive overages are undesirable per USP<1151>. Considering these guidances, the easiest way to define the fill weight target is to simply offset it from the nominal volume by the sum of the hold-up weight and the reject limit. The lower reject limit can be expressed by the simple equation below.

$$\textit{Lower Reject Limit} = \textit{Nominal Weight} + \textit{Hold Up Weight}$$

The table below updates the prior example by assuming that what was previously taken as the target fill weight is actually the nominal volume. In this example, the hold-up weights are representative of a prefilled syringe for the "Weight 2" column and a 10–15 mL tubing vial for the "Weight 1" column. The number of significant figures and rounding practices must also be taken into account, which explains why, for the high fill weight, the lower reject limit exceeds the nominal plus holdup by 0.01 g. When comparing the data below, from the table above, the fill target is increased by 0.031 and 0.47 g for the two configurations, respectively, when accounting for the container hold-up and meeting the nominal label claim.

	Value (%)	Weight 1 (g)	Weight 2 (g)
Reject (high)	+3	0.547	10.78
Adjust (high)	+2	0.542	10.68
Release (high)	+1	0.536	10.57
Target		0.531	10.47
Release (low)	−1	0.526	10.37
Adjust (low)	−2	0.520	10.26
Reject (low)	−3	0.515	10.16
Holdup		0.015	0.15
Nominal		0.500	10.00

17.6.2 Filling Mechanism

Many different technologies for filling have been commercialized. These include:

1. Time–pressure,
2. Peristaltic pump,
3. Rotary piston pump,
4. Non-rotating piston pump, and
5. Rolling diaphragm.

The mechanism for time–pressure filling is an orifice of fixed diameter, a surge vessel at a fixed pressure, and a pinch clamp or valve that opens for a variable duration of time. A peristaltic pump defines fill volume as the volume displaced from a fixed diameter of tubing through the variable rotation of the pump head. The final three mechanisms (rotary piston, non-rotating piston, and rolling diaphragm) involve a piston pump pulling in solution by fixed displacement, and then expelling it by movement of the piston. The differences between the three lie in how the solution flow is managed and the design of the piston.

Rotary piston pumps manage solution flow by rotating the piston between the inlet and outlet corresponding to the down (intake) or up (expel) stroke. Non-rotating piston pumps follow a similar principle, but without the rotation aspect of the piston. Solution flow from inlet to outlet is managed external to the piston by either a block or pinch valve. In both cases, the tolerances between the piston and wall are generally very tight and the piston is typically sealed and lubricated by wetting with product. High viscosity or high solid content solutions may dry in the sealing region, leading the pump to seize.

A rolling diaphragm pump utilizes a flexible gasket to isolate all but the head of the piston from the product stream. As the piston does not rotate, solution flow is managed externally, as in non-rotating piston pumps. Because of the gasket, the piston does not require additional lubrication for its motion which eliminates the risk of pump seizure due to solution drying.

In addition to shear and material compatibility, particulate generation can be a significant consideration impacted by pumping [21]. In the case of the filling pumps, this concern is more severe as the fill pumps are beyond any points of mitigation during manufacturing (e.g., filters). Nayak showed that during recirculation, 1–10 μm particulate levels in an IgG1 solution increased the least in peristaltic and time–pressure pumps. Rotary piston pumps, and particularly ceramic rotary piston pumps, showed the greatest increase in particulate levels. Based on microflow imaging (MFI), these particles were determined to be proteinaceous, and the root cause was hypothesized to be the relatively higher shear in rotary piston pumps compared to other pumping mechanisms [14]. Laboratory-scale studies can be executed that demonstrate the proposed commercial filling parameters will not have an effect on protein quality by replicating the commercial filling parameters of needle inner diameter, fill volume, and fill frequency. A design space can be determined by using a range of filling parameters in the laboratory study that bracket the commercial process parameters and perhaps identify filling parameters that determine edge of failure. It must be verified at full scale that the commercial filling parameters are within the design space and have no impact on protein quality.

17.7 Stoppering and Capping

Once filled, stoppers are placed in the vials. Key aspects of this step are the machinability of the stoppers (they are compatible with the filling machine and are available at the point of stoppering) and the alignment of the machine to robustly insert them into the vials. Stopper placement is a manufacturing step that only has one of two outcomes: (1) The stoppers are fully inserted into the vials, or (2) they are not. As the stopper forms the primary sterile barrier between the contents of the vial and the outside world, the stopper must be selected to be compatible with the vial and be demonstrated to make a robust seal once a cap is placed.

Capping is similar to stopper placement in terms of outcomes, but has a few additional considerations. The aluminum cap must be appropriately crimped around the head of the vial to ensure the seal between the vial and stopper is maintained throughout the shelf life of the product. The act of crimping the seal must not damage the neck of the vial.

Support for the integrity of the vial–stopper–cap system is provided by Container Closure Integrity Testing, either using a microbial bath or dye ingress method. This testing supports not only the component combination, but also the manufacturing equipment assembly of the components and must be repeated for every combination of components and manufacturing fill line.

17.8 Inspection

After filling and capping, the finished product undergoes visual inspection to segregate unacceptable units. Defects can range from product related, unintended extrinsic materials, or components of the container/closure. Inspection can be performed in a manual, semi-automated, or fully automated fashion. If visual inspection does not occur immediately after capping and the materials are stored under refrigerated conditions, the product should be re-equilibrated to room temperature prior to inspection to minimize the impact of condensation on the exterior of the product on inspection.

Manual visual inspection is performed by qualified operators manually holding finished product alternately against black and white backgrounds under appropriate lighting to maximize the ability of operators to detect defects because of the high contrast of the environment (see USP<790>). Semi-automated inspection still relies on operators to visually detect defects, but the product is presented to the operators in an automated or semi-automated fashion at a fixed rate and rotation against black and white backgrounds. In this paradigm, the operator would stop the inspection line to remove identified defects.

Automated visual inspection fully removes the human aspect of the inspection. Human eyes are replaced by a series of cameras at a number of stations that inspect for specific defects. This is accomplished through a combination of lighting, spinning of the product, and abrupt stopping of the rotation. Additionally, the inspection machine may automatically recycle the product through stations in the event of equipment faults or invalid results. This can lead to additional exposure to inspection conditions. Automated visual inspection must be demonstrated to be *at least as* sensitive as manual visual inspection through the validation of the equipment recipe and periodic requalification with standard product-specific challenge sets. As proteins tend to be at least somewhat photosensitive, stability during inspection as a function of several passes should be evaluated, particularly focusing on light-related degradation products.

Manual and semi-automated inspection personnel are qualified based on their ability to identify known rejects within a training set of materials. These same challenge sets are used to qualify automated systems. The goal of an automated inspection is to be at least as sensitive as a human inspector. In practice, this leads to tuning of the automated systems to balance increased sensitivity with an increased level of false rejects.

17.9 Hold Times

Hold times during manufacturing must be spread over each of the individual activities. The overall hold time for the process then becomes the sum of these individual steps. One way to define storage conditions is to classify any holds under

active refrigeration as "refrigerated" and any time outside of a refrigerator as "room temperature." Broadly, hold time typically is split between "room temperature" and "refrigerated," each with limits.

The biggest flaw in this paradigm is that it does not take into account product temperature, but rather uses the temperature of the surroundings. For large volumes or jacketed steel vessels, the amount of time required to equilibrate to a temperature condition under passive cooling/warming can be significant (on the order of days in some cases). The biggest advantage for this mechanism is the simplification of the documentation process—something is either being cooled or it is not. This also simplifies the execution perspective—manufacturing batch records can be built with defined limits for when to apply which hold time and how long certain operations may take.

Another approach would be to define product temperature ranges and divide the hold conditions into "2–8 °C" and ">8 °C." In this paradigm, the product temperature would have to be actively measured to identify which hold time should be used at each point. The advantage of this paradigm is that it more directly aligns with stability data and reflects the actual temperature of the product. The disadvantage comes in the difficulty of implementation at manufacturing scale. One could design a hybrid approach where kinetics of solution equilibration at varying environments could be measured for worst-case situations (e.g., largest batch sizes for cooling and smallest batch sizes for warming) and consider those times when assigning process limits.

Unless the product is particularly temperature sensitive, the approach of defining hold times based on environmental conditions is preferable as it is less prone to human error in a manufacturing environment and is less intrusive from a product perspective. Particularly, sensitive products may require active cooling during manufacturing. This should be avoided unless absolutely necessary as it adds significant complexity to managing the manufacturing process. Chilled vessels collect condensation from the environment that will drip onto the floor and require mitigation. Lowering temperature increases product viscosity (significantly, in the case of high concentration antibodies), which will impact performance of any filtration steps and may impact performance during filling. Additionally, time must be defined for moving the vessels from a formulation area to a non-adjacent fill area, which will require draining of any cooling jacket, moving the vessel, and reattaching to a new cooling source.

In addition to purely temperature-related considerations, exposure to light should be evaluated, as manufacturing processes may occur over several days [18]. Light exposure has commonly been shown to lead to degradation of proteins [12, 17], but often at levels far exceeding light encountered during a routine manufacturing process. Light during routine manufacturing may have an impact on protein stability for some molecules. This photosensitivity is linked to protein, light source/bulb type, and exposure duration [19].

Extractables and leachables from process materials (beyond the sterilizing filters, discussed earlier) are another area that deserves consideration [18]. While filtration extractables are typically tested, process components, such as stainless steel vessels

or silicone tubing, can often be evaluated based on a risk management paradigm that assesses the duration and magnitude of exposure against the extractables inherent to the contact material.

17.10 Risk Management and Quality by Design

Risk management is based on understanding the potential interaction between the execution of a manufacturing process and the resulting product. Once one adequately understands the risks in a manufacturing operation, one can identify potential mitigation strategies to manage those risks. Prior to evaluating a process, one must identify the criteria for success, namely which quality attributes are most important. Per ICH Q8(R2), a critical quality attribute (CQA) is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality [9]. For biologic drug products, critical quality attributes typically are categorized as safety (e.g., sterility), purity (e.g., high molecular mass species), identity (e.g., peptide map), and potency (e.g., bioactivity).

These concepts have been formalized in the Quality by Design paradigm, which is predicated on risk understanding and risk management. Any risk-based approach must be grounded in detailed product and process understanding. Then one can accurately assess potential interactions between process parameters and quality attributes, and the potential process variability and failure modes. The value of any risk assessment output is directly related to the quality of knowledge that is used as the foundation for the assessment. In this way, risk assessments function as supporting element of an overall knowledge management strategy, rather than as the foundation.

Progression from earlier to later phases of clinical development is typically accompanied by an increase in manufacturing scale and batch size. For early manufacturing, a broad understanding of potential risks and a conservative process design frequently yield a manufacturing process that is “fit for purpose.” Upon identifying the intended commercial manufacturing process, more detailed and formal risk management approaches should be employed building on the knowledge gathered at earlier stages of the program.

There are multiple paths to product and process understanding. Data can be generated at-scale or using laboratory scale-down models. When possible, scientific relationships between parameters should be defined. In cases where scientific relationships are overly complex or poorly understood, or when the number of variables is significant, statistically designed experiments can be utilized to generate empirical mathematical models following a Design of Experiments (DOE) paradigm. One should exercise caution when building a DOE experimental matrix that the parameters and ranges being examined are potentially linked in a way that is not otherwise describable and that the allowed variability in individual parameters reasonably accommodates the proposed allowed variation in the process.

With modern statistical software, one can very quickly generate an experimental design. However, without careful planning the design may not provide robust conclusions due to shortcomings.

ICH Q8(R2) identifies a flowchart of a potential Quality by Design approach. In that example, one starts with a target product profile and identifies potential critical quality attributes. This can be thought of as the first steps of the roadmap—"what do I want, and how do I know if I succeeded?" After these phases, one performs a risk analysis and may choose to develop a formal design space—"what if something varies, and how much can it vary before I am no longer certain if I will succeed?" Common tools for performing risk assessments and their typical application are shown in the table below. The goal of these exercises is to determine critical process parameters (CPP), defined as a parameter that must be controlled within a "limit, range, or distribution to ensure the desired product quality." In practice, a risk assessment must define the degree of variability that is reasonable to consider as an assumption. These are frequently taken to be just outside of proposed batch record control limits (which would be considered the normal operating range, or NOR), but can be widened with supporting data. The range of parameters known to yield acceptable materials provides the proven acceptable range, or PAR, which encompasses the NOR.

System	Acronym	Description	Example
Cause and effect matrices	C&E	Evaluate strength of relationships between process parameters and quality attributes. In C&E matrices, typical process ranges are only used as guidance. The output is typically a list of process parameters that have a moderate to strong correlation with one or more CQAs	For many monoclonal antibodies, time at room temperature results in an increase in high molecular mass species. Depending on the sensitivity of the antibody, this can either be a moderate or strong relationship
Failure mode effect (and criticality) assessment	FME(C)A	An FMEA identifies the impact of potential variability of process parameters on quality attributes. For each process parameter that exceeds the threshold from the C&E matrix, feasible modes of failure are identified that would cause the parameter to exceed the NOR (or PAR). These are evaluated for severity (degree of impact on each potentially impacted	Cumulative hold time at room temperature can be exceeded due to: 1. Operator error (inadequate tracking within batch records) 2. Equipment failure One may assume that the magnitude of the delay is 50% more than the NOR (e.g., 10-day cumulative hold with a 7-day limit). For the hypothetical monoclonal antibody:

(continued)

(continued)

System	Acronym	Description	Example
		CQA), occurrence (how often the failure mode can occur), and, optionally, detectability (can the failure mode be corrected prior to impacting the process up to no way of knowing if failure mode occurred) An FMECA build on an FMEA by adding the "criticality" element which would assess whether the combination of severity, occurrence, and detectability require additional scrutiny during manufacturing, perhaps even implementing controls to ensure the parameter remains at the target range or level. One output of a criticality assessment is the definition of the critical process parameters	– The severity may be moderate (expected to have an impact, but will not fail specification). – The occurrence may be "infrequent" for operator error (e.g., reasonable scheduling and batch record controls), and "rarely" for equipment failure (strong maintenance schedule, long history of rare failure of utilized equipment) – The detectability is likely moderate in both cases, as exceeding the hold time may not be discovered until after impacting the product

Process understanding and risk assessment are expected to be an iterative process. During early clinical manufacturing, the formulation and process scientists likely do not have a full understanding of the interaction of all process parameters with critical quality attributes over a wide range. The degree of variability within the process may also not be fully understood, as the number of batches is low. As a result, early risk assessments may be heavily influenced by suspected interactions and the process will be run in a conservative fashion to guarantee success. Due to this uncertainty, certain parameters (common examples include room temperature hold time, mixing speeds and times, and filter flush volumes) may initially appear to be a relatively high risk, but will evaluated in more detail as the program progresses.

Early-stage risk assessment should seek to identify gaps in knowledge and develop strategies for assessing and minimizing those potential failure modes. When possible, scale-down experiments should be designed to either confirm the relationship or demonstrate a range in which the process parameters do not adversely impact the process and CQAs. For some parameters (such as confirmation that mixing conditions yield a homogeneous solution), at-scale work is more appropriate.

As a program proceeds toward commercialization, the proposed manufacturing process becomes more defined. Additionally, data from prior manufacturing history and laboratory and at-scale experiments provides input into re-evaluating the proposed commercial manufacturing process. These data may support a wider range of

process parameters than those used during earlier phases, allowing for a more efficient and robust manufacturing process. A final risk assessment after Process Performance Qualification (PPQ) batches assesses the manufacturing process that will be described in regulatory fillings and commercialized, including a final determination of any critical process parameters.

17.11 Control Strategy

While the initial phases of risk management focus on the interaction of process parameters with quality attributes, the later phases flip the understanding. The question changes from "How does Process Parameter A interact with CQAs X, Y, and Z?" to "For CQA X, which Process Parameters A, B, and C must be controlled to ensure that the CQA remains with the target range or limit?" Certain important but non-critical quality attributes may also be evaluated in this fashion.

A detailed control strategy begins by determining which process steps and parameters have the potential to impact each critical quality attribute and how control of that attribute is assured. These may include aspects such as process design, in-process testing, release testing, and critical material attributes. In total, these process controls "address variability to assure quality of the product" [5].

To take sterility as an example, one can clearly identify a number of different mechanisms by which sterility is assured during a GMP manufacturing operation. A partial list of such controls is shown in the table below:

Step	Control	Rationale
Raw materials	Purchase of excipients and components that are low in bioburden	Ensuring microbial control throughout a process is desirable and simplified by minimizing the introduction of microbes
Hold times	Restrict hold times of unfiltered material, particularly at room temperature	Minimize risk of growth of any contamination by sterilizing materials in a timely fashion
Sterile filtration	Filter integrity (IPC or critical material attribute)	Filter integrity is monitored post-sterilization, prior to use, as well as post-use. This ensures that the sterilizing filter is integral over the course of the manufacturing process
Sterile filtration	Redundant filters (process design)	Redundant sterilizing filters mitigate the risk of a true filter failure by providing a second, equivalent filter
Sterile filtration	Prefiltration bioburden (IPC)	The bioburden of the challenge solution is demonstrated to be within the levels for which that filter has been validated to retain microbes
Release testing	Sterility assay	Confirms that gross contamination is not present in final product

As with other aspects of the Quality by Design paradigm, a control strategy evolves with growing product and process understanding. Early-phase clinical programs may rely on a platform-level control strategy that is "fit for purpose" for the phase of development and may not be explicitly defined. As a product moves toward commercialization and routine production, the control strategy becomes more formalized, detailed, and specific to the product in question.

17.12 Additional Validation and Process Qualification Considerations

The Process Performance Qualification (PPQ), formerly referred to as process validation (PV), strategy must support the intended *commercial* manufacturing process. Per FDA guidance, "effective process validation contributes significantly to assuring drug quality" and this validation is a "legally enforceable requirement" [5]. The goal of this validation is to "establish scientific evidence that the process is reproducible and will consistently deliver quality products" [5].

Validation is a documented program that provides a high degree of assurance that a specific process or system will consistently produce a result meeting predetermined acceptance criteria. Validation is attained through understanding the product and process, showing reproducibility at scale, and documenting the reproducibility. The development of the process should be governed by the principles of Quality by Design (QbD): a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management.

While the bulk of the information will be generated from at-scale manufacturing runs, information from previous qualification studies, at-scale studies, and laboratory-scale studies with a demonstrated tie to the commercial scale can be leveraged as supporting data. In general, Process Performance Qualification requires a minimum of three consecutive successful batches, performed under protocol with predefined acceptance criteria. For products with multiple presentations or multiple filling suites within a manufacturing site, a rationally designed bracketing strategy can reduce the overall number of runs by examining worst-case scenarios (such as extremes in batch size or fill volume). The rationale for any bracketing strategy should be documented and include justification for the identification of any worst-case parameters (batch size, dosage strength, fill volume, etc.).

The purpose of Process Performance Qualification is to demonstrate that a process is reproducible, robust, and ready for commercial manufacture. In this context, early-phase clinical processes are typically not validated. Early clinical programs are generally material constrained and run at a smaller scale than the

intended commercial batch size. Those processes are typically designed to be fit for purpose and phase appropriate.

The design of any PPQ strategy should consider the desired commercial process. One certainty about commercial manufacturing is that variability is assured. When considering PPQ strategy, one must understand the anticipated variability (which can be strengthened through the risk assessment process), determine potential extreme acceptable combinations of parameters and process, and ensure that data has been generated to demonstrate that product manufactured in such a fashion meets all required quality acceptance criteria. In this arena, a robust risk assessment process will identify whether there are gaps in the overall process understanding that must be closed prior to initiation of PPQ.

The consequences of failing a PPQ are very high. It is *highly* unadvisable to perform challenges during PPQ that have not been previously supported or known to be very low risk. Thus, it is generally desirable to perform a demonstration or challenge batch prior to PPQ to confirm that no issues are expected during PPQ. This batch should be designed to fill any gaps in knowledge. If such a batch is performed, it must be scheduled such that any samples collected can be analyzed and all data evaluated prior to progressing to PPQ.

Parameters that may be challenged during a Process Performance Qualification campaign include:

Unit operation	Challenge	Comments
Thawing of DS	Time between thaw and use	Temperature and duration based on product stability
Thawing of DS	Refreeze/thaw	Potential optional path for storage of drug substance not required for an individual batch
Manufacturing of dilution buffer	Hold times of buffer	Hold times are nearly always challenged during PPQ
Overall process	Maximum hold time	Compatibility with maximum holds for all process steps should be evaluated (both under refrigerated and ambient conditions)
Mixing conditions	Shear and homogeneity	Small batch size, high mixing speeds, and long times show shear compatibility. Large batch sizes with low mixing speeds and short times challenge solution homogeneity. Alternately, these characteristics can be qualified offline in stand-alone studies
Bulk filtration	Refiltration	Allowable reprocessing in the event of failing filters or potential sterility breaches post-filtration

Aspects *not* typically challenged during Process Performance Qualification include (but are not limited to):

Unit operation	Not challenged	Comments
Facility set points	Airflow patterns, sterilization parameters, standard operating procedures, calibration	A Process Performance Qualification is designed to confirm that the process can be operated within an existing facility. The validation of facility, equipment, sterilization, and personnel is not challenged
Overall process	Media fill restrictions	The manufacturing process must comply with any restrictions in place from media fills, including overall filling time and acceptable interventions. If the existing media fill program is inadequate for a given process, the media fill program should be augmented to bracket the proposed process prior to PPQ
Buffer formulation	Excipient concentration	Formulation robustness studies are leveraged to demonstrate that the product is robust to the anticipated variability of excipient concentrations
Filling	Fill volume	The fill volume is not intentionally challenged. All PPQ batches should intend to manufacture material at the target fill volume

During a PPQ campaign, sampling is significantly greater than would be required during either clinical or commercial production. These samples should be tested for impact of any challenges using assays that would be most stability indicating based on the stress. For monoclonal antibodies, this will frequently include measurement of high and molecular weight species, protein concentration, and assessment of subvisible particles (particularly after shear challenges).

Hold times are also typically challenged during PPQ. In these batches, each step is held for the longest allowable time (or just beyond). Process holds that may include multiple allowable temperatures (typically room temperature and refrigerated) challenge both in series. These maximum hold batches are sampled at the beginning and end of each hold to demonstrate that (1) product quality was not impacted by the hold, as measured by key stability-indicating assays, and (2) microbial control was maintained. Testing of the finished product demonstrates that, even with the maximum cumulative hold, all the product met all specifications.

A successful PPQ campaign results in:

1. All samples and assays meeting predefined acceptance criteria.
2. Finished product meeting all proposed commercial specifications.
3. A statistical measurement demonstrating adequate process capability (when warranted)
4. A data set that can support anticipated variability during commercial manufacturing.
5. A report that collects and presents all data in a traceable form and can be used as a source document in regulatory applications.
6. Information that can be used as inputs into the continuous process verification plan.

As PPQ is generally restricted to a small number (typically 3) of batches with an expected level of variability and the data shows the process at a fixed point in time. At the conclusion of PPQ, critical aspects of a process are identified that should be monitored and trended as a function of time to ensure that a process remains within its validated state and does not drift. This stage is referred to as continuous process verification and is considered by regulatory agencies to be a part of the overall validation process [5].

17.13 Conclusion

Most biologics drug product manufacturing processes follow a similar manufacturing process. This chapter has stepped through the unit operations included in a typical manufacturing process and discussed key considerations for each step. Additionally, concepts around quality risk management and process validation were discussed in the context of drug product manufacturing.

References

1. Antonsen H, Awafo V, Bender J, Carter J, Conway R, Egli S, et al. Sterilizing filtration of liquids. Technical Report 26. PDA J Sci Technol. 2008;62(S-5):2–60.
2. Brukl L, Hahn R, Sergi M, Scheler S. A systematic evaluation of mechanisms, material effects, and protein-dependent differences on friction-related protein particle formation in formulation and filling steps. Int J Pharm. 2016:931–45.
3. Chang D, Chang R-K. Review of current issues in pharmaceutical excipients. Pharm Technol. 2007 (May):56-66.
4. European Commission. Volume 4, Annex 1—manufacture of sterile medicinal products. Brussels; 2008.
5. FDA. Guidance for industry—process validation: general principles and practices. Rockville, MD; 2011.
6. FDA. Guidance for industry: sterile drug products producted by aseptic manufacturing current good manufacturing practice. Rockville, MD; 2004.

7. International Conference on Harmonization. Q10: pharmaceutical quality system. 2008.
8. International Conference on Harmonization. Q7: good manufacturing practice guide for active pharmaceutical ingredients. 2000.
9. International Conference on Harmonization. Q8(R2): pharmaceutical development. 2009.
10. International Conference on Harmonization. Q9: quality risk management. 2005.
11. Ishikawa T, Kobayashi N, Osawa C, Sawa E, Wakamatsu K. Prevention of stirring-induced microparticle formation in monoclonal antibody solutions. Biol Pharm Bull. 2010;33(6): 1043–6.
12. Kerwin B, Remmele R Jr. Protect from light: photodegradation and protein biologics. J Pharm Sci. 2007;96(6):1468–79.
13. Kishore R, Kiese S, Fischer S, Pappenberger A, Grauschopf U, et al. The degradation of polysorbates 20 and 80 and its potential impact on the stability of biotherapeutics. Pharm Res. 2011:1194–210.
14. Nayak A, Colandene J, Bradford V, Perkins M. Characterization of subvisible particle formation during the filling pump operation of a monoclonal antibody solution. J Pharm Sci. 2011;100(10):4198–204.
15. Patro S, Freund E, Chang B. Protein formulation and fill-finish operations. Biotechnol Ann Rev. 2002:55–84.
16. Paul E, Atiemo-Obeng V, Kesta S, editors. Handbook of industrial mixing: science and practices. Wiley; 2004.
17. Qi P, Volkin D, Zhao H, Nedved M, Hughes R, Bass R, et al. Characterization of the photodegradation of a human IgG1 monocloncal antibody formulated as a high-concentration liquid dosage form. J Pharm Sci. 2009;98(9):3117–30.
18. Rathore N, Rajan R. Current perspectives on stability of protein drug products during formulation, fill and finish operations. Biotechnol Prog. 2008;24(3):504–14.
19. Sreedhara A, Yin J, Joyce M, Lau K, Wecksler A, Deperalta G, et al. Effect of ambient light on IgG1 monoclonal antibodies during drug product processing and development. Eur J Pharm Biopharm. 2016;100:38–46.
20. Steele A, Arias J. Accounting for the Donnan effect in diafiltration optimization for high-concentration UFDF applications. BioProcess Int. 2014:50–4.
21. Tyagi A, Randolph T, Dong A, Maloney K, Hitscherich C, Carpenter J. IgG particle formation during filling pump operation: a case study of heterogeneous nucleation on stainless steel nanoparticles. J Pharm Sci. 2009;98(1):94–104.
22. Zhang L, Yadav S, Demeule B, Wang YJ, Mozziconacci O, Schoeneich C. Degradation mechanisms of polysorbate 20 differentiated by 18O-labeling and mass spectrometry. Pharm Res. 2017:84–100.

Chapter 18 Line Sterilization Considerations and VHP

Ulla Grauschopf, Katherine Thomas, Joerg Luemkemann, Sebastian Schneider, Ada Hui, Y. John Wang and Kirk Eppler

Abstract Biological drug products are usually filled into the primary packaging container under aseptic conditions. Modern fill–finish plants often employ containment installations like isolators to ensure the highest level of sterility assurance for the product. The industry standard for containment decontamination is the treatment with vapor phase hydrogen peroxide. Although being very efficient in sterilizing the containment, its oxidizing potential makes it a threat to the drug product filled after the decontamination cycle. The following chapter elaborates on the determination of scientifically meaningful product protection measures. It connects the concept of molecule-specific sensitivity assessment to the determination of VPHP residuals in the containment and to the uptake of VPHP from the atmosphere surrounding the drug product during the fill–finish process.

Keywords Vapor phase hydrogen peroxide (VPHP) · Product impact Sensitivity assessment · VPHP quantification · VPHP uptake study

18.1 Introduction

Biological drug products are usually filled into the primary packaging container under aseptic conditions because they cannot be terminally sterilized like many small molecules.

U. Grauschopf
Device Development, F. Hoffmann La Roche, Basel, Switzerland

K. Thomas · J. Luemkemann (✉) · S. Schneider
Pharmaceutical Development & Supplies, F. Hoffmann La Roche, Basel, Switzerland
e-mail: joerg.luemkemann@roche.com

A. Hui · Y. John Wang
Late Stage Formulation & Process Development, Genentech, South San Francisco, USA

K. Eppler
Process Development Engineering, Genentech, South San Francisco, USA

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*, AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_18

Modern fill–finish plants often employ containment installations like Restricted Access Barrier Systems (RABSs) or isolators (in the following referred to the general term containment) to ensure the highest level of sterility assurance for the product.

Containments require regular decontamination to ensure the absence of microorganism on all inner surfaces. The industry standard for containment decontamination is the treatment with vapor phase hydrogen peroxide (VPHP). Although being very efficient in killing viable microorganisms on the inner surfaces of the containment, its oxidizing potential makes it a potential threat to the drug product filled after the decontamination cycle.

Residuals of the VPHP, if present in the containment atmosphere or desorbing from parts of the product path during filling operations, will potentially enter the drug product during the fill and finish process

Depending on the product-specific sensitivity for hydrogen peroxide, the damage to the drug product from the residuals of the VPHP can be seen immediately or developed over the shelf life of the product.

This chapter gives an overview about measures that can be taken to characterize product-specific hydrogen peroxide sensitivity and to avoid inappropriate exposure of sensitive drug products to VPHP in a typical containment fill–finish environment. Strategies to define aeration levels for removal of hydrogen peroxide from the atmosphere as well as subsequent filling production conditions are presented.

18.2 Decontamination with Vapor Phase Hydrogen Peroxide

Vapor phase hydrogen peroxide (VPHP) is a commonly used agent for the decontamination of modern (especially contained) aseptic fill–finish installations. VPHP has several advantages compared to other disinfectants or sterilizing gases (such as alcohols, chlorine dioxide, peracetic acid, formalin, ethylene oxide) [1]. It is more reliable than the agents mentioned above in terms of reproducible decontamination result, easier to use, less corrosive, less toxic to humans, easier to remove from the containment, and less pollutant to the environment than alternative agents [2]. One of the main characteristics of VPHP is its strong oxidizing property—it reacts quickly with substances in the environment and typically breaks down to water and oxygen, leaving only non-toxic residues on all surfaces in the containment installation. These residues do not interfere with the environment or the pharmaceutical process.

Hydrogen peroxide is brought into the gaseous phase by vaporization or nebulized by a spray nozzle and subsequently introduced into the containment to kill viable microorganisms. It is critical that all relevant surfaces are exposed to the peroxide to ensure a high sterility assurance level of the containment. Only if the VPHP can act on the microorganism under defined conditions (e.g., temperature, relative humidity, hydrogen peroxide concentration in the vapor, exposure time),

a reliable kill rate (log reduction of living microorganisms) can be achieved [3]. It is very critical to define robust conditions to make sure that during routine operation, a reliable decontamination can be achieved. After the decontamination, residual VPHP must be removed from the containment. Usually, the full process, consisting of conditioning (introduction of the vapor to the containment to the specified concentration), decontamination (working time of the vapor), and the following aeration (exhaust/removal of the vapor) of the containment is referred to as a decontamination cycle. At the end of a decontamination cycle, the containment is in a sterilized state and routine production (e.g., aseptic fill–finish operations) can proceed. The efficiency of the decontamination cycle has to be proven during cycle development and validation. It is an industry standard for decontamination cycles to achieve six log reductions of viable spores of *Geobacillus stearothermophilus* on all relevant surfaces and materials of the containment [4]. To demonstrate that the processing conditions reproducibly lead to sterile surfaces, spore strips with 10^6 living spores specifically designed for hydrogen peroxide testing are placed throughout the containment (especially in known or proposed worst-case positions). The efficacy of the decontamination process is shown by the absence of microbiological growth derived from the spore strips when they are removed and tested at the end of the cycle—to increase the sterility assurance, extended exposure periods can be used during the decontamination phase. The main focus of decontamination cycle validation over the last decade has been the assurance of sterility of the containment by consistent preconditioning and the treatment with a reproducible amount of VPHP over a defined time to ensure sufficient contact time of the vapor with the surfaces.

The aeration phase after the decontamination has until recently been solely a question of operator protection. The maximum allowable concentration for hydrogen peroxide in the air in rooms or/open containments that people work in is regulated in most countries to be 0.5 ppm [5]. It has been a common practice to confirm that the VPHP concentration has fallen below this threshold by chemical and/or electrochemical indicators (e.g., Draeger tubes etc.) and subsequently released the containment for operation.

Unfortunately, biopharmaceutical formulations are often less tolerant toward the oxidizing potential of hydrogen peroxide. Concentrations that can safely be tolerated by operators can substantially damage drugs that are filled in the containment directly after the threshold of 0.5 ppm is reached in the primary aeration phase. For these sensitive formulations, an additional aim of cycle development is to reliably achieve conditions at the end of the aeration phase that allow for filling of the drug with no impact to the product characteristics.

Over the recent years, it has been learned that residual amounts of VPHP can be desorbed over extended periods into the atmosphere of the containment from the material such as HEPA filter and other—especially polymer-based—materials of construction, inside the containment installation. Advanced measurement techniques (refer to Sect. 18.4) revealed that the decay curve for VPHP below the 0.5 ppm concentration value is typically exponential. This leads to very long aeration time if the goal is set to achieve VPHP concentrations in containment equivalent to that in the regular environment outside the containment.

Techniques have been shown to be able to reduce aeration time. Heating up the air in the containment is relatively easy and leads to faster desorption of hydrogen peroxide from the surfaces of the containment. Even more effective is the application of catalysts (e.g., platinum; manganese dioxide) that speed up the decay of VPHP at the end of the decontamination phase dramatically.

18.3 Product-Specific H_2O_2 Sensitivity Assessment (Spiking Studies)

Currently, biopharmaceutical manufacturers are moving protein drug product filling operations out of traditional clean rooms into containments for the reason of improved sterility assurance and operator comfort. A key step in the development of contained fill and finish processes is to determine the sensitivity of the drug product formulation when exposed to hydrogen peroxide. This sensitivity is essential to determine the amount of acceptable residual hydrogen peroxide within the VPHP isolator system for the protein drug products to be manufactured. A simple strategy for such a sensitivity assessment is to perform a hydrogen peroxide spiking study. During such studies, specified amounts of hydrogen peroxide solution can be spiked into protein formulations, which are then assessed for immediate effects on critical quality parameters and in addition are placed on stability for long-term assessment. Throughout the assessment, protein oxidation and other qualities in the formulations can be monitored to determine a maximum tolerated level of peroxide that does not trigger unacceptable changes in drug product quality including oxidation. The results from hydrogen peroxide spiking studies can be used as guidance for the development of a VPHP decontamination cycle for the isolator.

Additionally, uptake studies (where vials filled with protein or surrogate solution are exposed to VPHP inside the isolator) are valuable in determining the amount of hydrogen peroxide that can enter and dissolve into the drug product. Many factors such as vial size, vial opening diameter, fill volume, time between liquid fill and stopper closing, interruptions, residual VPHP level, airflow, location of the vial can affect the hydrogen peroxide uptake rate and the dissolved amount. A thorough assessment combining the drug product formulation sensitivity data (spiking studies) with the dissolved hydrogen peroxide amounts absorbed into a specific primary packaging configuration (uptake studies) can assure a fill and finish process that does not lead to unacceptable oxidation triggered by residual hydrogen peroxide.

Among all amino acids, methionine is the most susceptible to hydrogen peroxide oxidation [6]. Though histidine and tryptophan are also subject to oxidation, they require that the oxidant be free radicals that are generated through light or transition metals such as iron or copper [7]. For this reason, the reaction between methionine and hydrogen peroxide is our main focus. Because monoclonal antibodies are the main stakeholder of the biotechnology products nowadays and more data are available from the same Fc methionine residues in different antibodies [8] and

formulations, the kinetics of methionine oxidation are illustrated by monoclonal antibody drug products [9]. Besides the methionine residues within the protein-based drug product, formulation excipients can also react with hydrogen peroxide as described later. Therefore, in a comprehensive spiking study, it is desirable to monitor the amount of protein oxidized, the overall integrity of the protein, the concentration of remaining hydrogen peroxide, and any other change in excipients as a function of time and temperature. Of these, determination of the concentration of hydrogen peroxide would be most readily achievable by using the commercially available fluorometric Amplex UltraRed Assay reagent. This assay allows determination of hydrogen peroxide down to 10 ng/mL. Oxidation of protein can be determined by peptide mapping or reverse phase high performance liquid chromatography (RP-HPLC). For mAb specifically, separation of Fc from Fab after cleavage at the hinge region allows sensitive detection of methionine oxidation in Fc. The protein A column [10] was also found to be useful in the following Fc methionine oxidation.

In a spiking study, ideally, one would conduct the stability study until the measured content of hydrogen peroxide reaches an undetectable level, <5 ng/mL, and then to assess the degree of protein oxidation. Hydrogen peroxide spiked into the drug solution may persist for long period of time if the number and amount of oxidation sensitive reactants within the drug product formulation are low. Typically, one would aim for a fast data readout because the drug development schedule would not allow waiting for complete exhaustion of hydrogen peroxide. Therefore, it is essential to understand the kinetics of the reaction between methionine and hydrogen peroxide in order to predict with assurance the degree of protein oxidation under complete hydrogen peroxide removal.

18.3.1 Kinetics of Hydrogen Peroxide Reacting with Methionine [9]

This reaction can be written as:

$$\text{Met} + \text{H}_2\text{O}_2 \rightarrow \text{Met-O(Oxidized Met)}$$

With the reaction rate equation:

$$-\text{d[Met]}/\text{dt} = -\text{d}[\text{H}_2\text{O}_2]/\text{dt} = \text{d[Met-O]}/\text{dt} = \text{k[Met]}[\text{H}_2\text{O}_2]$$

where [Met] is the concentration of Met residues in the protein formulation and $[H_2O_2]$ is the concentration that is spiked into the samples. When the protein concentration is in excess (i.e., $[\text{Met}] \ggg [H_2O_2]$), the Met concentration remains unchanged and the first-order rate equation can be reduced to:

$$-d[H_2O_2]/dt = k'[H_2O_2] \tag{18.1}$$

where

$$k' = k[Met] \tag{18.2}$$

Or it can be written as:

$$\ln[H_2O_2]_t = -k'_t + \ln[H_2O_2]_{t0} \tag{18.3}$$

where $[H_2O_2]_{t0}$ and $[H_2O_2]_t$ are the concentrations of H_2O_2 at the initial time point and at another specified time point, respectively. The assumption that the protein concentration is always in excess compared with the hydrogen peroxide concentration is reasonable (in these spiking studies) because the amount of protein is always much greater than the hydrogen peroxide that is spiked in. By using mAb as an example, the concentration of 10 mg/mL mAb, which has a MW of 150 kDa, is equal to 0.066 mM with two chains each having Met 252 and Met 428 residues, which results in a total concentration of 0.26 mM (0.066 $\times$ 4) of Met residues that can be potentially oxidized by hydrogen peroxide. Whereas hydrogen peroxide has a MW of 34 Da, it can range from 0.00029 mM at 10 ng/mL to 0.029 mM at 1000 ng/mL. Based on this comparison of concentration, it is obvious that mAb, or the [Met], is far greater than that of hydrogen peroxide, and remains fairly constant throughout the kinetic study. Therefore, the decline of hydrogen peroxide concentration follows the pseudo-first-order kinetics (Eq. 18.1), where the straight lines were observed in the semilog plots as shown in Fig. 18.1. Based on mathematic modeling, such straight line could be observed even if [Met] is only 4$\times$ the $[H_2O_2]$. In addition, the reaction rate constant is proportional to the Met concentration, which is proportional to the protein concentrations (Eq. 18.2). The half-lives for the reaction under 10, 30, and 100 mg/mL mAb could be estimated to be about 2.2, 0.74, and 0.22 month, respectively. By knowing the reaction rate, one can design the study with appropriate duration; e.g., for a drug product with 100 mg mAb/mL, the study will be over in 1 month as hydrogen peroxide may decline to undetectable level in four half-lives, e.g., one month, whereas, with 10 mg mAb/mL, the study will take ten times longer, 10 months, for hydrogen peroxide to reach undetectable level. For prediction of reaction rate based on Arrhenius plot, the average activation energy of the reaction for hydrogen peroxide with L-methionine was approximately 38 kJ/mol, and that with a monoclonal antibody, 59 kJ/mol [9].

18.3.2 Extent of Fc Methionine Oxidation in Monoclonal Antibodies

In mAbs, there are two methionine residues on the Fc portion that are most reactive, and it was reported that Met 252 is about twice as reactive as Met 428 [9]. When the

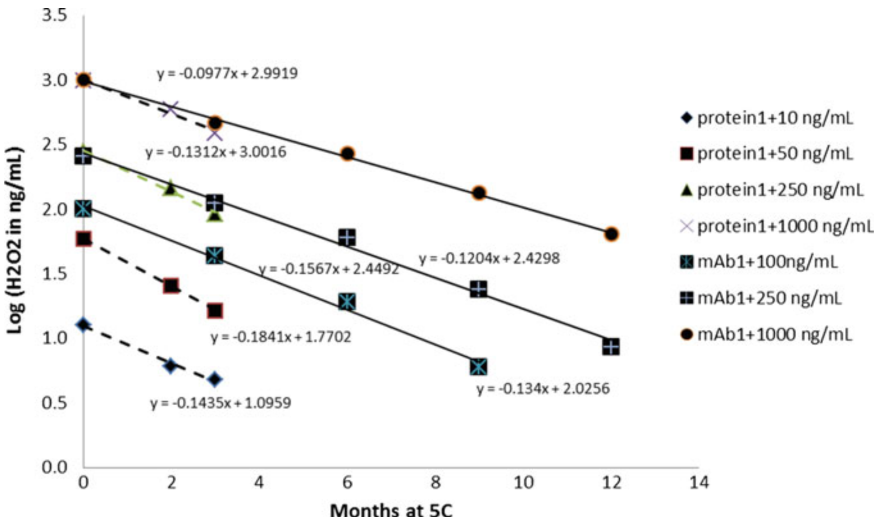


Fig. 18.1 Pseudo-first-order kinetic reactions of H_2O_2 consumption by Met oxidation observed for various concentrations of H_2O_2 spiked-in mAb1 formulation (10 mg/mL protein concentration) represented by solid lines; and in small protein formulation (5 mg/mL) by dashed lines

spiked-in hydrogen peroxide is consumed exclusively by the methionine, we can calculate the total amount of methionine oxidized. From Fig. 18.2, one can estimate the total amount of Met 252 oxidation based on the mAb concentration and the spiked-in concentration of hydrogen peroxide. For example, in drug product of 40 mg/mL mAb, Met 252 oxidation will reach about the 1% level by 200 ng/mL of hydrogen peroxide [9].

18.3.3 Lyophilized Drug Product

The aforementioned kinetics and predicted extent of oxidation were based on protein in liquid product. Limited experiences with lyophilized product exist. It was observed that typically the majority (50–80%) of the spiked-in hydrogen peroxide was removed during the lyophilization processes (unpublished results). The remaining hydrogen peroxide can react with protein in the lyophilized state. Its kinetics in one case showed a first-order loss of hydrogen peroxide, but not in others (unpublished results). One needs to be cognizant about the duration of hydrogen peroxide exposure, while protein is still in liquid state prior to lyophilization as the reaction can be fast. Sometimes, this period of time can be many hours wherein oxidation or other change may take place.

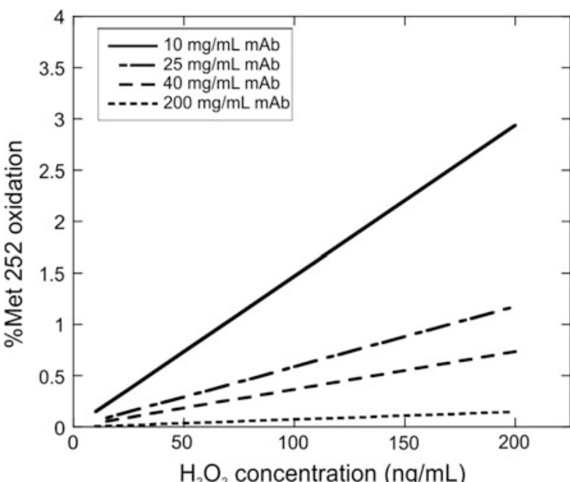


Fig. 18.2 Theoretical percent Met 252 oxidation based on protein concentration and a ratio of 2:1 for Met 252 to Met 428 oxidation [9]

18.3.4 Effect of Formulation Excipients on Hydrogen Peroxide Degradation

As reported [9], the rate of hydrogen peroxide decline was in one occasion faster than others. It was determined that formulations with trehalose or sucrose exhibited faster rate of hydrogen peroxide decline than those without. The faster rate could be mitigated by the addition of a chelating agent, EDTA, suggesting the catalytic effect of impurities (e.g., metal ions) present in these excipients. Excipients such as free methionine undoubtedly will neutralize the effect of hydrogen peroxide. Free methionine, 1 mM, was used in formulation as an antioxidant; the reaction rate of this methionine was about 10× faster than the rate of reaction with mAb 25 mg/mL concentration [9]. Therefore, it can be assumed that free methionine as an antioxidant will react with nearly all hydrogen peroxide and spare the methionine residue in the protein.

Because recently new products are mainly antibodies, small proteins received less attention. Nevertheless, the experiences gained from mAb would be applicable to small proteins. In a case of a small protein (ca 22kD with three reactive methionine residues) formulation, at 5 mg/mL, rates of hydrogen peroxide decline were faster than the rates from 10 mg/mL mAb (Fig. 1). The disparity could be explained based on the higher molar concentration of reactive methionine residues in small protein, 0.46 mM, than in mAb, 0.26 mM. The molar quantity of hydrogen peroxide loss resulted in a corresponding molar quantity of the oxidized protein (Fig. 3). Although this formulation contains an antimicrobial preservative, phenol, and polysorbate 20, neither accelerated the degradation of hydrogen peroxide nor increased the amount of protein oxidation. In a subsequent study, oxidation of an antimicrobial preservative was closely examined. Benzyl alcohol (92500 µM, 1%) was present in the drug product formulation of another small protein (ca 42kD with

multiple methionines) at low concentration (2.25 μM, 0.09 mg/mL). About 23 and 0.3 μM benzaldehyde (oxidized form of benzyl alcohol) was generated from spiked-in hydrogen peroxide concentration at 1000 and 200 ng/mL (representing 29 and 6 μM, respectively) in 3 months, at 25 °C. At 5 °C, 0.4 and 0.3 μM benzaldehyde was generated under the same conditions. Concomitantly, protein was oxidized 40 and 5% when exposed to 1000 and 200 ng/mL hydrogen peroxide at 25 °C (unpublished results). Because of very low protein concentration, significant portion of protein was oxidized under the study conditions with these high concentrations of hydrogen peroxide. After one month at 5 °C, only about 10% of the spiked-in peroxide could be detected. Compared with the other products described previously (Fig. 18.1), this rate was too fast for peroxide consumed by low concentration of protein. Thus, the fast rate could only be attributed to the oxidation of benzyl alcohol, and indeed corresponding amount of benzaldehyde was found. This example illustrates the fact that benzyl alcohol, like free methionine, effectively reacted with hydrogen peroxide and consequently reduced the amount of protein oxidation. Even though methionine residue in protein could be much more reactive than benzyl alcohol, methionine residue was overpowered by the high concentration of benzyl alcohol (about 41,100×). Because the portion of benzyl alcohol affected was very small, benzyl alcohol assay did not detect any loss in all test samples with different concentrations of hydrogen peroxide.

Polysorbate 80 degradation was observed in a study; however, loss of polysorbate 20 was not detected (unpublished results), and thus, the sensitivity of polysorbate 80 could be attributed to its unsaturated hydrocarbon chain of oleate components, not to the polyoxyethylene (POE) components.

In conclusion, when measuring hydrogen peroxide in the protein sensitivity study, the utmost concern is the presence of antioxidant such as methionine and antimicrobial preservative, such as benzyl alcohol. Besides protein that can be affected, polysorbate 80 is also susceptible (Fig. 18.3).

18.4 Determination of VPHP in the Containment After Aeration

Around 2005, the principal device for measuring residual levels of hydrogen peroxide inside containment after a decontamination cycle was the Polytron low concentration (LC) sensor, manufactured by Draeger Industries. This sensor, with a range of 0.1–20 ppm, was effective for the majority of small molecule (and vaccine) pharmaceutical uses. Research was begun on what were safe levels of hydrogen peroxide for biotech products, with the intent of using containments for filling biologic drug products. The product sensitivity assessments (refer to Sect. 18.3) were used as a target, and various production scale containments were tested to determine what VPHP levels could be safe for a production environment. Early tests indicated that some products required hydrogen peroxide levels to be less than 10 ng/ml so that protein oxidation levels could be acceptable. With this in mind,

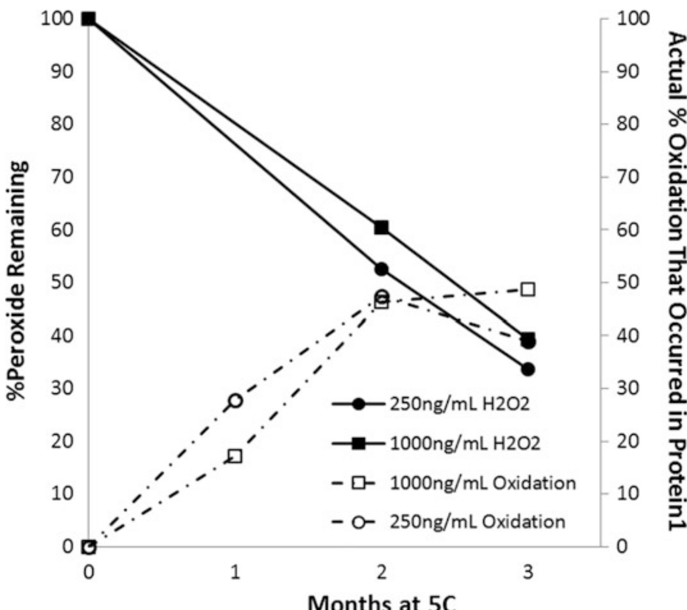


Fig. 18.3 Correlation between protein oxidation and loss of H_2O_2 in protein 1 formulation (5 mg/mL protein concentration) spiked with 250 and 1000 ng/mL H_2O_2 and stored at 5 °C. Theoretical calculation is based on the molar consumption of H_2O_2 equals to the molar quantity of protein oxidized. At 1000 ng/mL, % protein oxidation is expected

it was determined that residual VPHP levels would need to be below 10 ppb (0.010 ppm) for safe line stoppages during filling operations. Based on this need, an assessment for improved methods for measuring residual VPHP in the containment atmosphere was conducted.

Initial research showed that this was not going to be an easy task. Due to the unstable nature of hydrogen peroxide, it is not possible to buy a cylinder of calibration gas or other hydrogen peroxide standard. The Draeger detectors do not use hydrogen peroxide to calibrate (15% error), as their sensors have a cross-sensitivity with SO_2, so a calibration cylinder of SO_2 gas may be used as the standard (a surrogate). Since most users of hydrogen peroxide are only concerned with the human safety levels (Lit 17.2.5) of the gas, very low levels (<0.1 ppm) are not of general interest, and thus most manufacturers of measuring equipment have not been concerned about measuring in our range of interest.

The standard laboratory method for measuring hydrogen peroxide in liquid is the Horseradish Peroxidase Assay (HRP), using Amplex Red, manufactured by Invitrogen. A more sensitive reagent, Amplex Ultra Red (same principle as Amplex Red), was used to achieve more resolution at the lower end of the scale. This was coupled with a robust sampling method by absorbing hydrogen peroxide in large volume of air from the containment into small amount of water, and thus the hydrogen peroxide level in the air can be determined. Using this method, residual

VPHP level from about 125 ppb down to about 5 ppb (0.125–0.005 ppm) could be determined. This assay was good as an interim method, but it was quickly realized that it had many limitations. It did not provide real time data, it required many intermediate steps including multiple dilutions, a fluorescent plate reader needed to be kept calibrated, and the overall method was subject to operator error.

A search was initiated for a robust, pharmaceutical-grade hydrogen peroxide measuring instrument. From 2006 to 2009, five different instruments were evaluated, manufacturers and inventors were interviewed, and tests were run between a select few of the instruments. Finally, a custom hydrogen peroxide measuring device was purchased from Picarro, Inc., (Santa Clara; CA.) a company known for their environmental gas measuring systems, which was determined to give the best combination of accuracy, precision, ease of use, and robustness. The Picarro unit uses cavity ringdown spectroscopy (CRDS) in a unit about the size of an industrialized PC. The biggest concern with the Picarro is the lack of a calibration method. The second choice was an Aero-Laser AL2021 (Aero-Laser, Germany). While this unit was able to be calibrated and gave a better lower end reading, it was too complicated for day-to-day use on the production floor in a pharmaceutical facility.

The Picarro CRDS unit was tested on recently constructed production containment and was found to have a very good correlation with the HRP assay method (Fig. 18.4).

During start-up of new containments, many tests were run to measure the residual VPHP in the atmosphere. Tests on materials of construction (MOC) showed that hydrogen peroxide absorbed minimally into PTFE* tubing, but strongly interacted with silicone tubing due to high permeability. By connecting a long Teflon tube and a long power cord to the Picarro, it was possible to move it around to measure the air in the containment at many different locations. The containment was entered either through a double-ended tri-clamp bulkhead fitting or by cutting a hole in a finger in one of the gloves. Residual VPHP levels were measured and compared in various locations in several different containment systems.

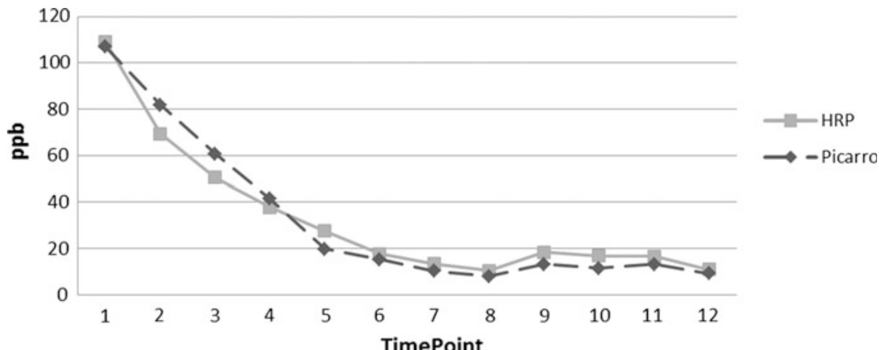


Fig. 18.4 Comparison of readings of the Picarro instrument with the VPHP determination by the Amplex Red method

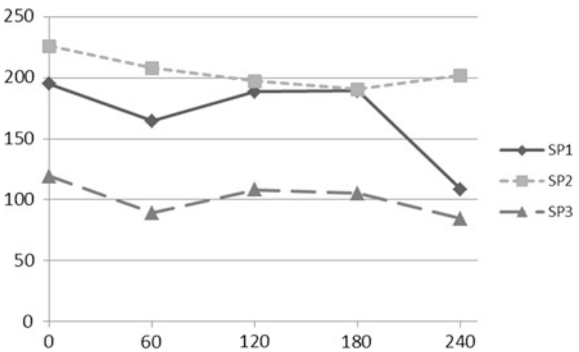


Fig. 18.5 VPHP concentrations at three sampling points overtime

Early tests showed variations in the VPHP residuals inside the containment of about 30 ppb, whether measured near the depyrogenation tunnel, near the fill nozzles, or near the lyophilizer. Because the product exposure (unstoppered vial) occurs mostly in the area between the filling nozzles and the stopper placement device, that was chosen as the primary location for residual atmospheric measurements (Fig. 18.5).

The preferred mounting location for a VPHP residual sensor is near the fill nozzles. A poor location for the VPHP sensor seems to be in the return air duct, especially if the air velocity is higher than recommended by the sensor manufacturer [11]. While this can give an aggregate reading for multiple areas of a sectioned containment, the velocity issue can make the sensor read inaccurately.

While measuring VPHP levels during cycle development, the worst-case conditions when product may be exposed should be considered. After the decontamination cycle, there are typically many steps that need to be executed prior to beginning the product filling. Because the drug product is at risk while in the containment, even when inside silicone tubing (refer to Sect. 18.5), all of the various process steps should be examined to reduce the potential impact to the product.

18.5 Testing for Vapor Phase Hydrogen Peroxide Absorption

Due to the unknown mechanisms of VPHP uptake into biotech products, a series of studies were initiated to determine the amount of hydrogen peroxide that could migrate into the final drug product container. Initial tests measured residual VPHP absorbing into an open vial of drug product and branched out into other potential product contacting surfaces. For fill–finish operations, reasonable allowances for production stoppages without discarding product are of importance. A 60 min interruption was the target, with the assumption that any vial that has been filled but not stoppered was at risk for hydrogen peroxide uptake. As illustrated by Fick's first

law of diffusion, the amount of diffusion into a liquid is based on the hydrogen peroxide differential between the environment and the liquid, the distance between the environment and the liquid, and the surface area of the liquid. Because hydrogen peroxide has a huge solubility in water (~280,000 ng/ml, per Henry's law of solubility), it can be assumed that almost all peroxide in the direct environment of the liquid surface will migrate into the liquid.

Early studies showed that Fick's law was not a definitive model for predicting the uptake of VPHP into the liquid in a vial, but a place to start. By exposing WFI-filled vials to the containment's environment for a known time, and assaying the vial's contents by the Horseradish Peroxidase Assay, uptake rates for various vial fill configurations could be determined. It was quickly verified that the vial's geometry and fill volume would play a role in the final hydrogen peroxide concentration in the vial (Figs. 18.6 and 18.7).

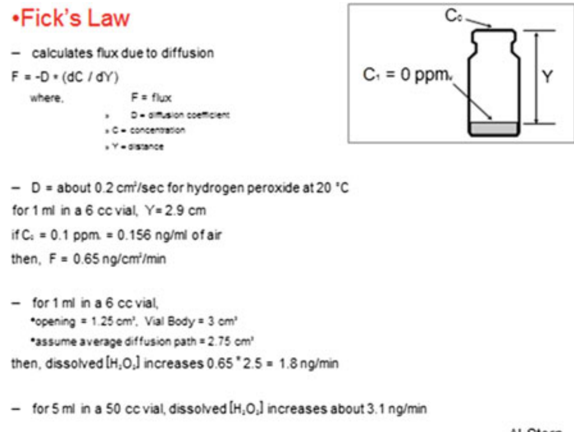


Fig. 18.6 Application of Fick's law on the fill volume of 1 ml in a container of the geometry of a 6 ml vial

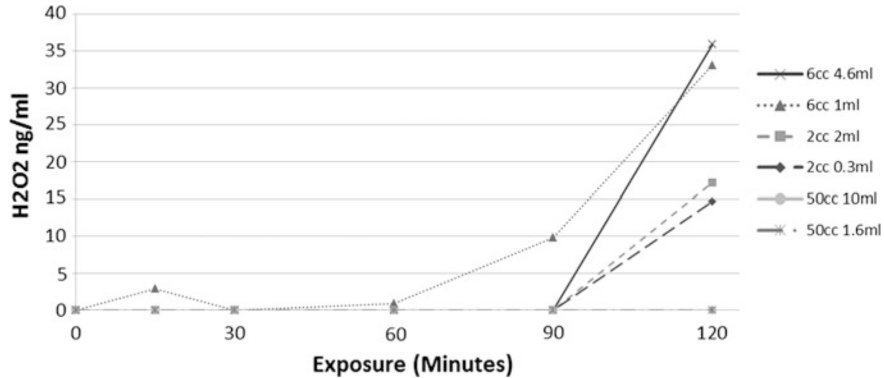


Fig. 18.7 VPHP uptake in different sized vials and fill volumes

An initial assumption was made that WFI was a good model for VPHP uptake, as biotech products are mostly water. However, once full-scale testing began, it was found that the drug product does not absorb VHP at the same rate as WFI, sometimes faster, sometimes slower (Fig. 18.8).

The first tests for product uptake involved products with a large fill volume. These large volumes benefit by diluting the hydrogen peroxide. Studies were executed to look into the amount of VPHP picked up by the empty vial and stopper before being filled, and determined the amounts to be trivial relative to the amount picked up by the liquid. However, when investigating smaller volumes (<1 ml), the effect of the vial and stopper had a major impact. Studies showed that an open empty vial circulating on a filler infeed buffer conveyor for a few hours could pick up more hydrogen peroxide than the exposed liquid would over a short period of time (5–10 min).

Early tests of materials of construction (MOC) showed several materials used within the containment were not the best in terms of hydrogen peroxide uptake, and probably contributed to the slow decrease of VPHP levels inside the containment (Fig. 18.9).

The MOC studies kept a focus on silicone tubing. Tests were run on the VPHP uptake of tubing before and after sterilization in place, as well as the transmission of hydrogen peroxide through the tubing into the drug product. Tubing was installed after the decontamination cycle was completed, and it was found that residual VPHP in the atmosphere would still transmit through the tubing into the product. These studies pointed out that drug product in the fill tubing is at risk after a

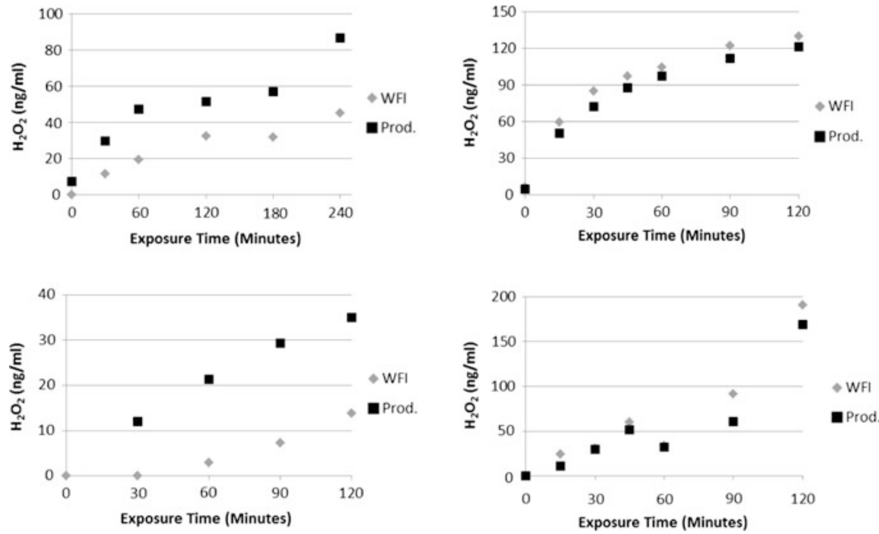


Fig. 18.8 Different mAb formulations (from top left to bottom right: mAb A, E, D, C); uptake rates versus water for injection (different vial configurations and fill volumes lead to different WFI uptake rates)

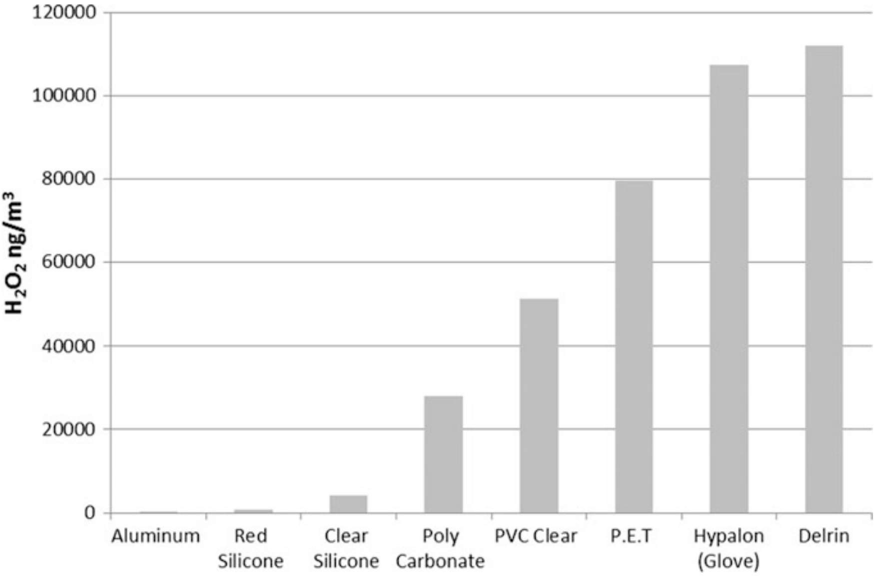


Fig. 18.9 VPHP uptake rate (ng/cm^3) in various materials of construction

stoppage as well, even if it was not installed before the VPHP decontamination cycle. The first sample and samples 8–12 were outside the isolator when this experiment was run (Fig. 18.10).

Due to the high affinity of hydrogen peroxide for water, a study was done on the potential VPHP uptake at the tip of a nozzle (Fig. 18.11). Various sizes and shapes of menisci were tested under similar conditions, and the lowest surface area of product exposed to the environment at the meniscus led to the least amount of hydrogen peroxide uptake (Fig. 18.12).

18.6 Vapor Phase Hydrogen Peroxide Absorption Testing in a Scale-Down Containment

Performing VPHP absorption tests in the production environment leads to major challenges in terms of production line utilization, related cost, and overall duration of the absorption experiments. It is very time consuming to prepare a filling line to start absorption studies, because the containment needs to go through all the preparations steps of a VPHP decontamination to achieve the specific VPHP levels and concentration decay curve that is typical for the containment. It is obviously attractive to simulate the large production containments with a smaller representative model. Ideally, the smaller model (scale-down model—DSM) is able to create a controlled environment in terms of VPHP concentration, air velocity, relative humidity, and temperature. It should be able to mimic the VPHP decrease

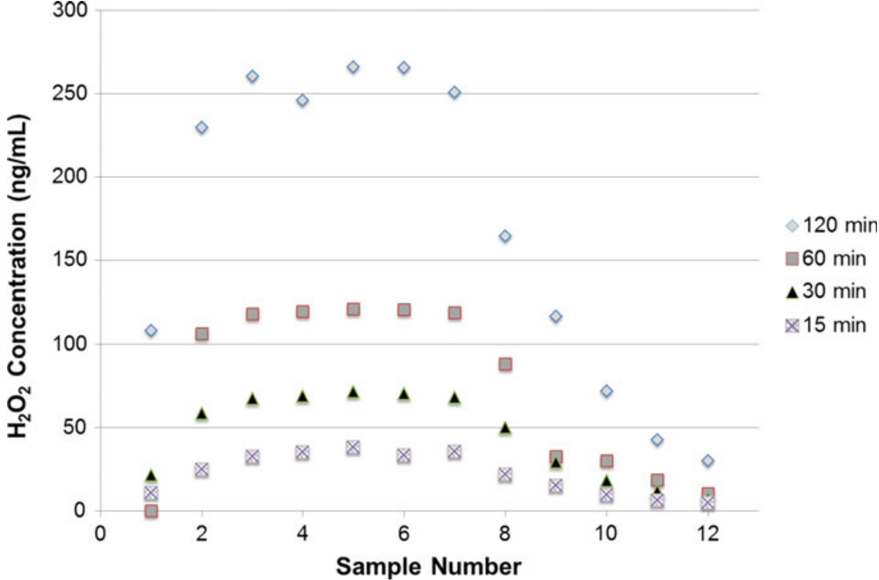


Fig. 18.10 VPHP uptake through a typical silicone fill tubing (6.0 mm ID × 1.2 mm wall) at 500 ppb VPHP in the environment

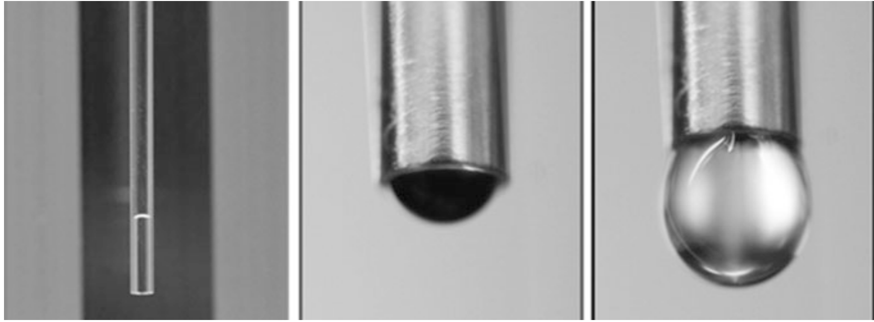


Fig. 18.11 Menisci of drug product solution at the tip of the filling nozzle

curve of larger aseptic fill–finish containments to allow for representative hydrogen peroxide uptake studies. To technically achieve a representative VPHP concentration profile over time in the DSM, very small amounts of liquid hydrogen peroxide are vaporized and introduced into the air handling system of the DSM. By adjusting the vaporization rate, distinct peroxide levels in the atmosphere of the DSM can be achieved.

The miniaturization of the DSM is limited by the fact that a unidirectional airflow is needed to simulate cleanroom class A (ISO 5) airflow characteristics, so typically a DSM is a small containment (comparable to a sterility test isolator)

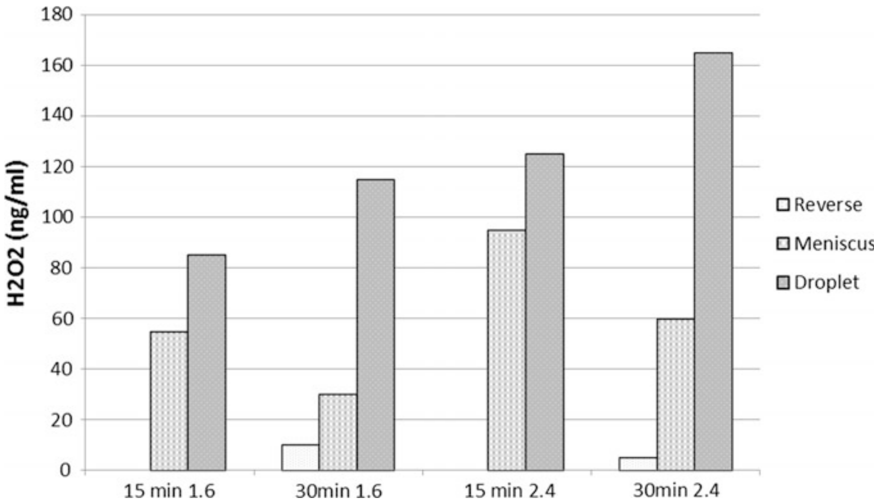


Fig. 18.12 Hydrogen peroxide uptake into the drug product solution at differently shaped menisci of drug product solution at the tip of the filling nozzle

which is equipped with control systems for the above-mentioned process parameters.

The first step in establishing a DSM for a distinct containment is to determine the line-specific aeration kinetic (decrease in peroxide concentration). For the concentration range below 100 ppm, a “Picarro” (cavity ringdown spectroscopy) system is a suitable method to determine the time-dependent concentration decrease (refer to Sect. 18.4).

Figures 18.13 and 18.14 indicate the VPHP decay kinetics in the air of the filling line and the DSM. The measurements start at the end of the aeration phase, when the containment goes back into its normal mode of operation.

It can be concluded that in the VPHP concentrations follow the same trend and the concentrations are in the same order of magnitude. The VPHP concentrations in the DSM are tuned to be slightly higher to simulate a worst case in the context of uptake experiments (Fig. 18.15).

To compare the VPHP uptake kinetic in the filling line and the DSM, 2.25 ml prefilled syringes filled with 1 mL WFI were placed (unstoppered) in both containments under aseptic filling conditions for up to 240 min. Peroxide absorption ended by stoppering the syringes at distinct time points, and the hydrogen peroxide concentration in the liquid was determined by the Amplex Red assay. Additionally, empty syringes were exposed, filled with 1 ml of WFI after the exposure, and analyzed in parallel to find out if hydrogen peroxide adsorption to the primary packaging and subsequent desorption into the WFI played an important role in this experiment.

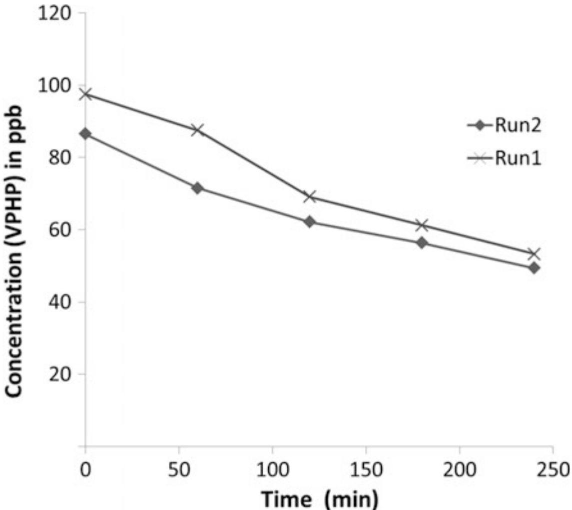


Fig. 18.13 The graph represents the VPHP concentration in the atmosphere of a production line containment during the development of the VPHP decontamination cycle

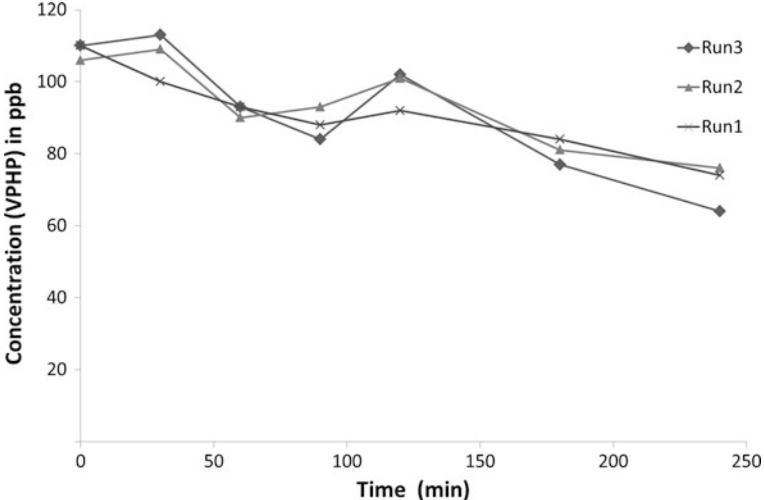


Fig. 18.14 The graph represents the VPHP concentration in the atmosphere of the scale-down containment tuned to mimic the hydrogen peroxide decrease curve of the production line containment

To gain additional insight, the trial in the DSM was extended to cover VPHP absorption into a placebo buffer and a monoclonal antibody formulation (Fig. 18.16).

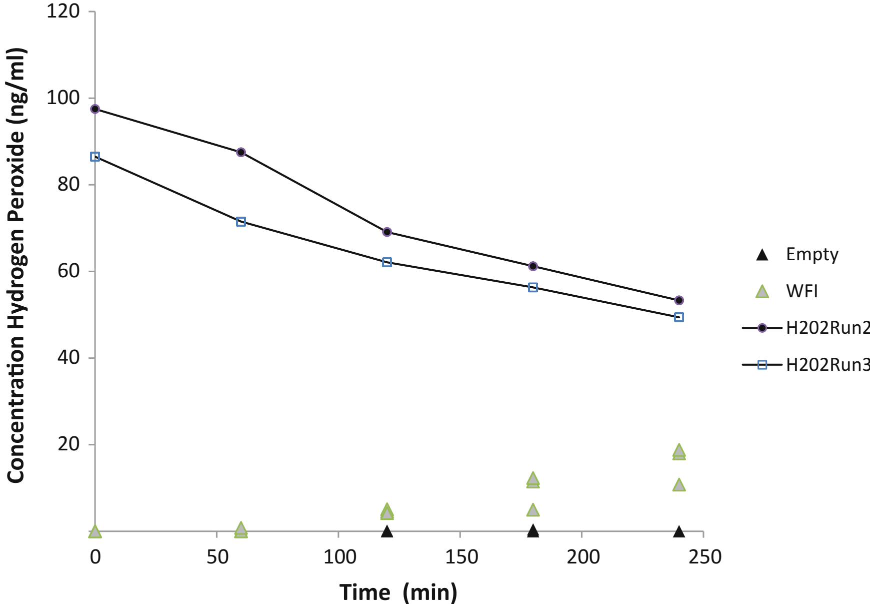


Fig. 18.15 The graph represents the VPHP concentration in the atmosphere of a production line in comparison with the hydrogen peroxide uptake into empty and filled syringes

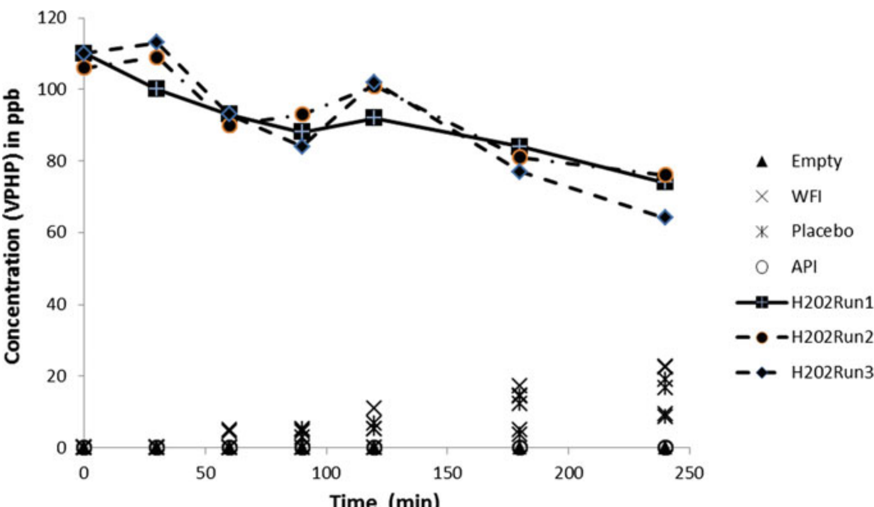


Fig. 18.16 The graph represents the VPHP concentration in the atmosphere of the scale-down model containment in comparison with the hydrogen peroxide uptake into empty and filled syringes

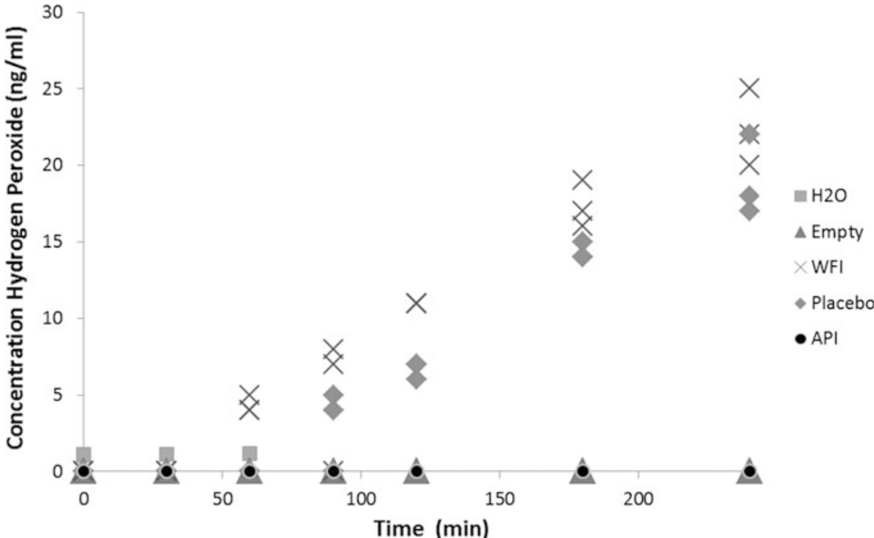


Fig. 18.17 The graph represents the hydrogen peroxide uptake into empty and filled syringes and the found amounts in the HRP assay post exposure

In the given VPHP concentration and exposure time, the adsorption to the primary packaging item did not play a role in the overall hydrogen peroxide uptake. Nevertheless, it could be shown that the amount of hydrogen peroxide present in the empty and water for injection (WFI)-filled syringes was very comparable in the experiment conducted in the filling line and in the DSM (Fig. 18.17).

To further elucidate VPHP absorption behavior of different solutions, we compared the uptake of WFI, placebo, and the full mAb formulation. In this case, the VPHP was not detectable in the mAb formulation, but showed increased concentrations in line with the exposure time in WFI and placebo solutions. We concluded that the formulation experiences comparable VPHP uptake, but the peroxide is not detectable after the experiment due to the reaction with the mAb molecule (refer to Sect. 18.4).

18.7 Summary

We could show that production scale aeration profiles can be mimicked in a DSM leading to equivalent exposure conditions and subsequently comparable amounts of VPHP absorption. Empty syringes picked up no VPHP over the tested time and exposed VPHP concentrations. WFI-filled syringes and the placebo-filled syringes started to pick up measurable amounts of VPHP after 60 min.

In terms of the absorption rate of different liquids, it can be stated that for the formulation buffer and WFI tested in this experiment, no relevant difference could be detected; nevertheless, a comparability of absorption behavior should be established. No peroxide could be detected in API-filled syringes, as the hydrogen peroxide was reacting with the highly concentrated mAb (refer to Sect. 18.4).

The information that can be gathered by DSM experiments mainly relates to recommendations with regard to exposure time for open drug product solutions and packaging materials. It is also an appropriate way to connect the formulation sensitivity (determined in the spiking studies) with packaging configuration and filling line-specific hydrogen peroxide absorption.

18.8 Conclusion

Vapor phase hydrogen peroxide (VPHP) is considered industry standard for the decontamination of containments. Unfortunately, certain biological drug products are very sensitive to traces of hydrogen peroxide. Care has to be taken to avoid product oxidation by absorption of residuals of VPHP from the decontamination/aeration phase. A thorough assessment combining the drug product formulation sensitivity data (spiking studies) with the dissolved hydrogen peroxide amounts into a specific primary packaging configuration (uptake studies) can assure a fill and finish process that does not lead to unacceptable oxidation triggered by residual hydrogen peroxide.

To ensure product safety, VPHP concentrations at the end of the aeration phase can be validated for an individual containment to ensure that product exposure by VPHP absorption does not exceed accepted levels. Very sensitive measurement techniques like cavity ringdown spectroscopy, near-infrared laser spectroscopy, and the application of the Amplex Red assay can support the validation of the aeration directly at the production line or studies in representative scale-down models. In any case, the knowledge about the stability behavior of the molecule introduced into a decontaminated containment should be available.

References

1. McDonnell G, Denver Russell A. Antiseptics and disinfectants: activity, action, and resistance. Clin Microbiol Rev. 1999 Jan;12(1):147–79. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC88911/.
2. Unger-Bimczok B, Kottke V, Hertel C, Rauschnabel J. The influence of humidity, hydrogen peroxide concentration, and condensation on the inactivation of *Geobacillus stearothermophilus* spores with hydrogen peroxide vapor. J Pharm Innov. 2008;3:123–33.
3. Hultman C, Hill A, McDonnell G. The physical chemistry of decontamination with gaseous hydrogen peroxide. Pharm Eng. 2007;27(1):22.

4. Sigwarth V, Stärk A. Effect of carrier materials on the resistance of spores of *Bacillus stearothermophilus* to gaseous hydrogen peroxide. PDA J Pharm Sci Technol. 2003;57(1) (January/February 2003).
5. Deutsche Forschungsgemeinschaft DFG. MAK- und BAT-Werte-Liste 2014, Senatskommission zur Prüfung gesundheitsschädlicher Arbeitsstoffe, Mitteilung 50; VCH.
6. Manning MC, Patel K, Borchardt RT. Stability of protein pharmaceuticals. Pharm Res. 1989;6(11):903–18.
7. Ji JA, Zhang B, Cheng W, Wang YJ. Methionine, tryptophan, and histidine oxidation in a model protein, PTH: mechanisms and stabilization. J Pharm Sci. 2009;98(12):4485–500.
8. Chumsae C, Gaza-Bulseco G, Sun J, Liu H. Comparison of methionine oxidation in thermal stability and chemically stressed samples of a fully human monoclonal antibody. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;850(1–2):285–94.
9. Hui A, Lam XM, Kuehl C, Grauschopf U, Wang YJ. Kinetic modeling of methionine oxidation in monoclonal antibodies from hydrogen peroxide spiking studies. PDA J Pharm Sci Technol July/Aug. 2015;69:511–525. http://doi.org/10.5731/pdajpst.2015.01059.
10. Loew C, Knoblich C, Fichtl J, Alt N, Diepold K, Bulau P, Goldbach P, Adler M, Mahler HC, Grauschopf U. Analytical protein a chromatography as a quantitative tool for the screening of methionine oxidation in monoclonal antibodies. J Pharm Sci. 2012;101(11):4248–57.
11. Draeger Safety hydrogen peroxide Compendium. https://etapii.com/upload_file/H2O2-Compendium-2016_02_23.pdf

Chapter 19
Lyophilization: Process Design, Robustness, and Risk Management

Daniel Dixon, Serguei Tchessalov and Bakul Bhatnagar

Abstract This chapter briefly reviews the formulation literature with a focus on defining formulations that can support aggressive lyophilization. Each step in the lyophilization process is reviewed with considerations for scale-up with an emphasis on mathematical modeling (enabled by equipment characterization) and application of process analytical technologies, when possible. A scientific approach to process robustness relying upon grouping of process parameters to provide worst case conditions is described, and the consequences of grouping these parameters is discussed. Process robustness, assessed in this fashion, can then support the definition of the process design space and inform risk management activities.

Keywords Freeze drying · Primary drying · Secondary drying
Mathematical modeling · Process analytical technologies (PAT)
Lyophilizer characterization · Process robustness · Quality by design

19.1 Introduction

Preservation of water-sensitive materials by freeze—drying received deserved attention in the past few decades. There are many review papers [1–5] that outline principles and advances in freeze-drying (lyophilization). The review papers focused mostly on research topics while the purpose of this chapter is to outline "good practices" that authors developed over the course of their work in pharmaceutical industry and applied to routine clinical and commercial manufacture of lyophilized materials.

D. Dixon (✉) · S. Tchessalov · B. Bhatnagar
BioTherapuetics Pharmaceutical Sciences, Pharmaceutical R&D, Pfizer, Inc,
1 Burtt Rd, Andover, MA 01810, USA
e-mail: ddixon1016@yahoo.com

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_19

19.2 Formulation

Detailed formulation strategies and expanded lists of formulation excipients used in freeze-drying have been published [6–8]. In general, formulation of lyophilized products should consider several aspects:

1. Long-term stability of the dried product in the formulation. Ideally this stability supports room temperature storage during distribution and storage by the end-user.
2. Short-term stability of the liquid formulation during manufacturing, as well as after reconstitution during final dose preparation.
3. Ability to reproducibly lyophilize the product, hopefully with a cycle of "short" duration. Ideally, the cycle would be robust to process deviations without compromising long-term stability.

To accomplish these goals, one should *not* start with a formulation designed for liquid stability and attempt to lyophilize it. This may lead the formulator to select excipients that may have undesirable properties from either stability or processing standpoints (or both). Rather, at its base, a lyophilized formulation typically includes:

- A buffering species at fairly low level (e.g., 10–20 mM). Succinate, histidine, and tris are three popular buffers depending on the desired product pH.
- A primary stabilizer. This is frequently sucrose. Trehalose has also been used, but can cause other issues, such as crystallization during frozen drug substance storage [9–11]. For sucrose, a mass ratio of greater than one part sucrose to one part protein has been shown to provide optimal stability [12, 13] or a molar ratio of 360:1 [14]. Many reports have been published in the literature comparing trehalose and sucrose [15–18]. In most of these studies, the stability of the examined molecule may be slightly improved in the presence of either sucrose or trehalose, but the differences are generally not significant from a long-term stability of a commercial product perspective. A brief comparison of sucrose and trehalose is shown below:
  - Sucrose—less expensive than trehalose.
  - Trehalose—less susceptible to acid hydrolysis under acidic conditions [18].
- In the case of low-active content cakes, a bulking agent. This is often glycine or mannitol, both of which can crystallize under appropriate formulation and process conditions [19–21]. In the case of higher concentration active ingredients (e.g., greater than 50 mg/mL protein), a bulking agent is not required as the cake has sufficient mass to prevent blow out, and crystallization of bulking agents would be inhibited by the additional dry excipients and active [22, 23].
- Additional materials at low levels to protect against specific degradation pathways. For example, polysorbate-80 has been seen to improve the clearing of gas bubbles after reconstitution [24]. Polysorbates may also prevent aggregation of proteins during lyophilization and reconstitution [18, 25].

Many companies have developed formulation "platforms" to minimize the development investment for early phase clinical programs [26]. These can be as restrictive as predefined formulation options that are tested for suitability, or slightly more flexible in only defining a fixed set of experiments and a few options to select from. For a rationally constructed platform, this provides many significant benefits. These include:

- Easier identification of product stability. In a rationally designed platform, if the only thing that changes is the identity of the active ingredient, and possible slight variations in active concentration, the stability of a new program under accelerated conditions can be evaluated against previously developed materials to verify that the degradation rate is within the range of experience [27].
- Reduced labor and development in defining the initial dosage form. Limiting options and defining specific studies provide more focus to the initial development activities. The output from these studies is more consistent from program to program, harmonizing expectations across the organization.
- More robust process development. If the product is in the same formulation as existing products and has been shown to have similar glass transition and collapse properties, then preexisting process development can be supportive for the new product, assuming comparable product configuration and total dried mass.
- Streamlined tech transfer. Reducing the number of potential excipients and components during an early stage can result in time savings for procurement and qualification of GMP supplies to support clinical manufacture. Additionally, platform programs which utilize a common fill site can often omit executing engineering activities prior to clinical manufacture.
- Simplified initial regulatory filings. From a CMC perspective, if the formulation and development studies are conserved, template text can be developed to describe the formulation and manufacturing processes.

Platform formulations (and formulation strategies) provide a starting point for development and must be demonstrated to be suitable for implementation for each new molecule [26]. The platform does not replace product development, it just provides a prescriptive sequence of studies and testing to demonstrate acceptability. In this way, a platform can be determined to be "fit for purpose."

A well-designed platform will also take into account aspects of the initial target product profile (TPP). Examples of this may include:

- While the formulation may be somewhat fixed by the platform approach, the final choice of fill volume and container/closure must consider dose preparation and the proposed clinical dose ranges.
- If a lyophilized dosage form does not fit the TPP, then a strategy must be defined to develop a liquid dosage form in parallel with clinical activities, including the appropriate cross-over and comparability studies to implement the liquid dosage form for later clinical development.

In the event that a platform formulation strategy is unsuccessful, the formulation scientist must be cognizant of the impact of formulation decisions on the eventual manufacturing process. From a manufacturing perspective, a shorter cycle is favored over a longer one. Shorter cycles can substantially reduce the cost of manufacturing. For busy manufacturing sites, shorter cycles are easier to fit into a full schedule, help increase the total output of the site, and may even allow production strategies that may otherwise not be feasible. While shorter cycles are strongly desired, successful cycles are required. A short cycle that does not reproducibly yield suitable product is not acceptable.

Keeping the desire for efficient equipment utilization in mind, there are a number of decisions a formulation scientist can make that have a direct impact on the final result. Some of these include:

- Inclusion of additional excipients that reduce the overall glass transition temperature of the matrix [28]. Sodium chloride has a very low glass transition temperature, and is very difficult to crystallize unless the ratio of sodium chloride to that of the other excipients is greater than 2 [29]. Formulations requiring additional tonicity should consider disaccharides or bulking agents rather than sodium chloride to enable more efficient lyophilization, as these excipients will suppress the composite glass transition temperature [30].
- Concentrations of low glass transition temperature excipients are above the minimum required to achieve the desired outcome. This is the same effect as described in the bullet above.
- Incomplete annealing of crystallizing excipients [19].

Some example lyophilized formulations for commercial materials are shown below in Table 19.1:

Table 19.1 Examples of several commercial lyophilized formulations

	Herceptin [31]	Enbrel [32]	BeneFIX [33]	Remicade [34]
Protein	420 mg/vial	25 mg/mL	50–600 IU/mL	100 mg/vial
Buffer	9.5 mg/vial L-histidine HCL 6.1 mg/vial L-histidine	10 mM tromethamine (Tris)	8 mM L-histidine	6.1 mg/vial dibasic sodium phosphate, dihydrate 2.2 mg/vial monobasic sodium phosphate, monohydrate
Stabilizer	381 mg/vial trehalose	10 mg/mL sucrose	0.8% sucrose	500 mg/vial sucrose
Bulking agent	n/a	40 mg/mL mannitol	208 mM glycine	n/a
Surfactant	1.7 mg/vial polysorbate-20	n/a	0.004% polysorbate-80	0.5 mg/vial polysorbate-80
Other	n/a	n/a	0.234% sodium chloride[a]	n/a

[a]Sodium chloride is added in the reconstitution solution

Once a formulation is defined, the frozen properties of that formulation must be characterized to enable the design of a suitable lyophilization cycle. The critical instrument in this arena is differential scanning calorimetry (DSC). The DSC should be used to generate a roadmap of the temperatures that may play a role in the performance of the formulation. For purely amorphous formulations, the main deliverable is the glass transition temperature of any amorphous phases. For partially crystalline formulations, additional information includes nucleation temperatures, temperature conditions for crystal growth (annealing), and eutectic (or secondary) melting temperatures. For example, sodium chloride must be cooled below approximately −40 °C to enable nucleation and facilitate crystallization on annealing at temperatures between −35 and −30 °C. During drying, these formulations must remain below the secondary melting temperature of approximately −21 °C.

Conclusions from DSC experiments should be confirmed by freeze drying microscopy (FDM). Microscopy experiments visualize the physical consequence of exceeding the temperatures defined during DSC. If one considers the glass transition temperature as the range of temperatures over which the viscosity of a material changes several orders of magnitude from an essentially rigid glass to a flowing viscous liquid, then the collapse temperature is the temperature at which the viscosity has reduced enough to allow for flow over the timescale of observation. Due to temperature dependence of viscosity, collapse occurs more rapidly the further away from the glass transition temperature. This concept has implications on method induced variability during FDM. For example, if one applies a temperature ramp for the duration of an FDM experiment, the ability to identify the point at which a structural change was initially observed may be compromised. The recommended best practice for FDM would be to proceed stepwise at fixed intervals between temperatures and hold under isothermal conditions to observe any potential structural changes in the sample. The spacing of the isothermal steps can vary based on experiment and DSC data, and sequential experiments can focus on temperature ranges of interest through smaller step changes for increased resolution of observed structural changes.

Depending on the viscosity of the product, determination of collapse using a temperature ramp can yield results that are up to 5 °C (or more) above the collapse temperature defined by "isothermal" FDM. This is particularly impactful if the formulation has a relatively low glass transition temperature (e.g., below −25 °C) as a difference in allowable product temperature of 5 °C will significantly impact the duration of primary drying. Table 19.2 shows a comparison of collapse temperatures for low solid content, sucrose-based formulations measured by a ramping method (0.5 °C/min) versus an isothermal method in two different laboratories (data previously unpublished).

While traditional lyophilization practice has been to lyophilize the product below the collapse temperature, one can challenge that, if the product is well understood, then lyophilization above collapse may be suitable [35–38] and even preferable in some instances [39–41]. For many partially crystalline formulations, this has been the typical practice. In these cases, the crystalline material provides a scaffold for the amorphous phase to coat. Because of the crystalline scaffold, there are no visual

Table 19.2 Accuracy of collapse measurement by freeze-drying microscopy: comparison of ramping and isothermal methods

			"Isothermal" FDM	"Ramp" FDM	
Material #	Dry content (%)	Tg′	Tcollapse	Tcollapse	ΔTcollapse
1	2.35	−36.0	−35.0	−33.9	1.1
2	3.96	−35.0	−34.6	−23.2	11.4
3	6.51	−34.8	−34.5	−30.0	4.5
4	10.34	−33.3	−31.0	−24.1	6.9
5	11.63	−33.2	−32.0	−24.1	7.9
6	13.52	−34.7	−33.0	−25.5	7.5
7	14.24	−33.8	−33.5	−26.6	6.9
8	14.46	−33.1	−33.0	−31.3	1.7

signs of macroscopic collapse. At a minimum, one must demonstrate that no critical quality attributes are impacted by lyophilization above collapse, if this strategy is to be employed. Relevant product quality assays may include (but are not necessarily limited to): reconstitution time, residual moisture, quality attributes impacted by interfacial stress (such as size-exclusion chromatography), and measurements of subvisible and visible particles.

19.3 Process Development and Transfer

The product temperature profile over the course of a lyophilization cycle defines the thermal history of that material. Maintaining a consistent thermal history from vial to vial, lot to lot, and lyophilizer to lyophilizer is a robust, scientifically sound mechanism for maximizing the probability of success in process transfers. Recipe set points are a critical output in their impact on defining the product temperature profile. For different lyophilizers, this often means that different set points may be required to yield a comparable product temperature profile. In these circumstances, it is more appropriate to adjust the recipe considering the product temperature profile rather than maintain an existing recipe that will generate a different profile [42].

To properly implement this strategy, laboratory scale process development should take into consideration the ultimate clinical or commercial manufacturing equipment. This ensures that the conclusions drawn from development studies can be applied to production scale. The additional mass within a production scale lyophilizer means that temperature equilibration generally takes longer relative to laboratory scale, so additional time must be added to steps where the product is expected to equilibrate to a temperature. Additionally, the heat transfer efficiency will likely be different between laboratory and manufacturing scale, particularly for edge vials [43].

The target lyophilization cycle at scale must consider both the formulation as well as equipment capabilities [44]. Typical batch record limits for allowable shelf temperature variability are ±3 °C, up to ±5 °C for some lyophilizers. Pressure tolerances vary more significantly across manufacturing scale lyophilizers, with some lyophilizers holding within ±10 mT, while others having ±50 mT allowed variability. The level of allowed process variation must be considered when designing the lyophilization process, particularly during the primary drying stage.

19.3.1 Ramp Rates

In general, refrigeration systems on laboratory equipment are oversized, relative to their production scale counterparts. At laboratory scale, a lyophilizer may be able to maintain adequate homogeneity at ramp rates up to 1 °C/min. At production scale, because of the mass of the shelves and products and the distance from the point of shelf inlet fluid temperature measurement to the actual inlet of the shelves, product homogeneity across a given lot is often compromised above ramp rates of approximately 0.5 °C/min. Higher ramp rates may lead to differences of 2–5 °C between shelf inlet and outlet in production scale lyophilizers, which would manifest as differences in shelf surface temperature [44]. As a result, laboratory studies should utilize ramp rates of no more than 0.5 °C/min to minimize variability introduced during process transfer. Using ramp rates in this range during development studies mean that the ramp rate likely will not have to be changed upon scale-up.

Some formulations and products may require faster ramp rates to minimize the impacts of interfacial sensitivity and phase separation [45], forcing a compromise to homogeneity for product stability. In these cases, critical temperatures and rates should be identified to ensure suitable product, and the consequences of this data must be evaluated to determine impact on product robustness, preferably at laboratory scale.

19.3.2 Freezing Temperature

Many large lyophilizers cannot reach shelf temperatures much below −50 °C. One should ensure that there are no transitions that are required to be met below this temperature during laboratory scale development (this can be as simple as examining a DSC thermogram). In general, the final freezing step should be the same for laboratory and production lyophilizers. During process scale-up, hold time at freezing temperature for the commercial dryers is normally programmed longer by 1–2 h to account for differences in size and thermal inertia.

19.3.3 Annealing

Annealing steps can serve two different purposes, depending on the design of the formulation:

- Growth of crystals for phase-separated crystallizing excipients (such as mannitol, glycine, and sodium chloride).
- Increase the size of ice crystals through Ostwald ripening, which in turn will result in a larger pore during drying, and thereby also increase the sublimation rate.

Annealing occurs substantially more rapidly at higher temperatures [46]. One caution is to select annealing conditions that are safely below any relevant melting temperatures (as defined in DSC experiments). This means ensuring all products stay below about −5 to −10 °C to avoid the onset of water (or eutectic phase) melting in the case of amorphous formulations or formulations containing mannitol or glycine, and below about −30 °C (well below the eutectic melting temperature) for formulations containing sodium chloride. While annealing is common in formulations containing crystalline excipients, it is less common in purely amorphous formulations.

In the case of high protein concentration products (e.g., >50% of the dry cake weight is protein), the composite glass transition temperature may be above −15 °C, meaning that annealing would not be expected to impact the cake structure significantly without risk of partial melting of some vials, and thus annealing is not expected to provide significant benefit.

Annealing steps should be performed at the same temperature for laboratory and production scale processes. The duration of the annealing hold at production scale would be expected to be longer than laboratory scale to: (1) account for the additional time required for product equilibration at the annealing temperature and (2) to compensate for the smaller initial pore size due to the increased degree of supercooling for materials manufactured in clean-room production environments rather than uncontrolled laboratory environments. From a practical point of view, the duration of annealing at commercial scale should be extended by 1–2 h from laboratory scale if the solids content is no more than 10% and by 2–3 h if the solids content is above 10%. Additionally, annealing temperatures closer to the glass transition temperature require a longer time to achieve the same desired annealing effect (pore structure or degree of bulking agent crystallinity) than annealing conditions further above the glass transition temperature [46].

Consistency in crystalline content, both within a single batch, as well as across multiple batches and scales, can be measured by powder X-ray diffraction. Success can be defined as having a comparable polymorph distribution (preferably without any hydrates, as in the case of mannitol, which may form a hemihydrate) with comparable peak areas for dominant polymorphs. This can be confirmed more quantitatively by powder DSC after the conclusion of the process, where the glass transition temperature and the temperature and enthalpy of melting of crystalline

components can be compared. Comparable glass transition temperatures and enthalpic recoveries show a comparable composition of the amorphous phase(s) with respect to moisture content and crystallizing excipients (amorphous mannitol, glycine, or sodium chloride will reduce the glass transition temperature). Measuring specific surface area of the lyophilized product can confirm comparable pore structure.

At the conclusion of annealing, the product should be cooled below any relevant transition temperatures for condenser cooling and vacuum initiation. Prior to increasing the shelf temperature to the primary drying conditions, the vacuum level should be stabilized to the set point.

19.3.4 Primary Drying

The primary drying stage of a lyophilization process is typically the longest and most sensitive step. At this stage, heat is applied to the product to support the direct conversion of phase-separated ice to water vapor by sublimation. This process is coupled heat and mass transfer and, with appropriate product and equipment characteristics, can be modeled reasonably accurately [42, 47]. At its most simplified level, primary drying in a vial can be described by Eq. 19.1:

$$\frac{S_{in}\left(P_{Sub} - P_{Chamber}\right)}{R} = \frac{k_{heat} S_{Out}\left(T_{Shelf} - T_{\Pr oduct}\right)}{\Delta H_S} \tag{19.1}$$

S_{in} and S_{out}	surface area on inside and outside of vial
P_{sub} and $P_{Chamber}$	vapor pressure of water at the sublimation interface and chamber pressure
T_{Shelf} and $T_{Product}$	shelf inlet and product temperatures
ΔH_S	heat of sublimation of water
R	cake resistance to mass transfer
k_{heat}	overall effective heat transfer coefficient

In this equation, the three modes of heat transfer (conduction, convection and radiation) are included in a single heat transfer coefficient. As a result, the heat transfer coefficient of edge vials is higher than the heat transfer coefficient of center vials due to radiation received from the relatively warmer chamber walls [43]. This heat transfer coefficient is a function of the lyophilizer, vial size, vial geometry (particularly bottom gap height), vial glass type (tubing vs. molded), position on shelf (radiation) and chamber pressure (gas conduction, convection). The worst-case locations are typically the corners of the shelf (high heat due to maximum radiation) and the center of the shelf or location nearest an internal condenser (lowest or negative radiation from the condenser coils). The heat transfer coefficient of a corner vial may be 30–40% greater than that of a center vial under some circumstances, particularly at low shelf temperatures [43].

The cake resistance to mass transfer is a function of both the formulation and product thermal history, including solids content, degree of supercooling, degree of crystallinity (if relevant), dried layer thickness, phase separation (surface skin) and any collapse that may have occurred. Recall that the degree of supercooling is greater in a low particulate environment relative to laboratory scale. For un-annealed, purely amorphous materials at solids content of 10% and above, this can lead to a 75% increase in mass transfer resistance moving from laboratory to production scale (data unpublished), particularly at relatively high protein concentrations. If annealing is applied for high ($\geq 10\%$) solids formulations, the increase in resistance (resistance scale-up factor) could be as high 50%. For low solids formulation, if annealing step is implemented, we assume resistance will only increase by about 25% when transferring process from laboratory to GMP controlled conditions. This increase in cake resistance will reduce the sublimation rate under a given heat input as it is more difficult for water molecules to navigate from the sublimation interface to the bulk phase, resulting in an increase in product temperature (reduced cooling from the reduced sublimation rate).

Supplementing Eq. 19.1 with equations to describe the vapor pressure of ice as a function of temperature and the heat transfer from the bottom of the vial to the sublimation interface, one can design a relatively simple mathematical model to calculate the relationship between process inputs (shelf temperature, chamber pressure, and time) for a given combination of product, vial, and lyophilizer (heat transfer coefficient, mass transfer resistance, cake height) to calculate the theoretical product temperature as a function of time during primary drying [48–50]. Simplification of mathematical models requires making assumptions and accepting the error or uncertainty that comes with those assumptions. In these cases, simplifications required to enable utilization of a simple spreadsheet-based model ("LyoModeling Calculator" for one example [51]) may introduce temperature inaccuracies of up to approximately 1 °C and underestimate the duration of primary drying by about 15–20%. As long as these shortcomings are known and understood, modeling provides an ability to examine a series of potential process conditions without any material cost to hone in on ones that are desirable for further examination. Such modeling can be used to generate a theoretical process design space in silico, which can be supported by selected experimental data.

In general, sublimation proceeds from the top of a frozen product to the bottom in an approximately planar fashion. Due to heterogeneity of dry layer mass transfer resistance, however, primary drying time could vary between different vials, even adjacent vials that would be otherwise expected to be identical. Determining the endpoint of primary drying and ensuring the entire batch has completed is a critical point of implementation of process analytical technologies (PAT). Several methods are available for end point determination of primary drying: dew point, product temperature, gas plasma spectroscopy, TDLAS, comparative pressure measurement (Pirani vs. capacitance manometer), condenser temperature and pressure, and pressure rise test [52] with last three methods available on the commercial scale. More recently, mass spectrometry has been applied to lyophilizers, both to monitor progress of primary and secondary drying, as well as detection of silicone oil leaks within the chamber [53–55].

If shelf temperature and pressure are maintained constant during primary drying, product temperature will continue increasing due to increase in dry cake resistance. If product temperature reaches critical temperature, one should decrease the shelf temperature or pressure or both to maintain a product temperature below the critical value [56–58].

During transfer of a process across lyophilizers, the product temperature profile during primary drying is a critical factor to maintain consistency between laboratory and commercial scales. There is always desire to measure actual product temperatures at commercial scale to ensure that product remains within acceptable design space. This, however, becomes more difficult if the receiving lyophilizer utilizes automated loading and unloading, as those systems tend to be incompatible with wire thermocouples. Production facilities in general are reducing the use of wire thermocouples as the thermocouples increase the risk of microbial contamination. While temperature monitoring is not always possible during production batches, understanding the product temperature profile during technical batches is critical in evaluating success during tech transfer and may require novel PAT technologies to accomplish.

19.3.5 Secondary Drying

While primary drying is the bulk sublimation of phase-separated water, secondary drying is the removal of adsorbed water by diffusion. This desorption is a function of both time and product temperature. While secondary drying occurs to some degree during sublimation stage (primary drying), the desorption rate at low shelf temperatures (typical for primary drying) is low. That is why the shelf temperature is typically raised during secondary drying (unless it is the same as during primary drying). While shelf temperature is the major factor during secondary drying, the chamber pressure is not generally considered a significant factor when pressure control is maintained by injection of dry nitrogen [59–61]. Secondary drying typically occurs in shelf temperature ranges of 25–45 °C. The duration of secondary drying is optimized to yield moisture within the target range across the entire batch. For most protein-based formulations, the duration of secondary drying is 6–9 h targeting residual moisture level below 1%. While the specific surface area of products during aseptic manufacture is typically higher compared to the laboratory scale, theoretically resulting in faster desorption rate due to a reduced desorption distance, secondary drying usually occurs more slowly at large scale due to lags in product temperature equilibration with shelf temperature. As a result, during scale-up, the duration of secondary drying may be extended by an hour or two to account for the longer time required for the product to equilibrate with the shelf. The ramp rate between primary and secondary drying may impact the final appearance of amorphous products. Ramp rates greater than or equal to 0.5 °C/min may lead to microscopic collapse, which would manifest itself as a slightly shrunken cake due to product temperature potentially exceeding the glass transition temperature during

fast ramp. Reducing the ramp rate to 0.1–0.15 °C/min [62] (ensuring the rate of glass transition temperature increase due to desorption is faster than the rate of product temperature increase) would mitigate this cosmetic difference. Researchers have begun exploring the mechanisms of cake shrinkage and cracking [63]. In the authors' experience, ramp rates of 0.2 °C/min between primary and secondary drying for most protein-sugar formulations has yielded acceptable product with minimal shrinkage. For partially crystalline materials, this ramp rate is not as critical, as the crystalline scaffold will prevent macroscopic consequences for exceeding the glass transition temperature.

If the primary drying condition is in the appropriate temperature range for secondary drying, secondary drying can be performed as an extension to the primary drying conditions (also referred to as "single step drying"). This is often feasible with high protein concentration or partially crystalline formulations.

Optimization of the secondary drying process typically focuses on reaching a target moisture range. This can be accomplished in two ways:

1. Multiple sequential cycles with variable secondary drying conditions. This is a material- and time-intensive approach.
2. Utilization of either a "sample thief" or sequential stoppering within the lyophilizer. Both of these scenarios require specialized equipment and are typically only available at laboratory scale. The difference between the two is that the sample thief method removes vials from the lyophilizer, while the sequential stoppering uses the thief arm to press down the stoppers to prevent further desorption.
3. Mathematical modeling [64, 65]. Historically, modeling of secondary drying has been more challenging than primary drying due to the input coefficients required, and the more complicated relationship between the process inputs and moisture desorption rates [65].

Recent advances in the mathematical understanding of secondary drying are guiding a simplified model that can be implemented into readily available spreadsheet format [66]. These advances will enable utilizing modeling to identify critical experiments to perform for the understanding of secondary drying optimization, in a manner similar to the proposed strategies for utilization of modeling to guide primary drying development and design space construction.

19.3.6 Stoppering and Unloading

Once secondary drying has completed, the chamber is typically backfilled with sterile, dry nitrogen prior to seating the stoppers. The backfill pressure is usually in the range of 0.7–0.9 atm to prevent stopper pop up and assist in maintaining container/closure integrity. As the use of automated loading and unloading systems has expanded, vials that stick to the shelf above has become a more significant risk. To mitigate this, one company has developed a Teflon coating for the bottom of the

shelves to prevent stopper sticking [67]. Once stoppered, the product can be unloaded. Unloading temperature is product specific; however, the room temperature (20–25 °C) is most commonly used to avoid condensation on the outside of cold vials. Additionally, some configurations (such as dual chamber cartridges or vails) may require aseptic stopper placement outside of the lyophilizer. In these cases, the relative humidity of the manufacturing area must be adequately controlled to minimize moisture uptake of lyophilized materials prior to stoppering, and the moisture uptake across the batch should be understood.

19.3.7 Process Analytical Technologies (PAT)

Many techniques have been developed to monitor the lyophilization process, each with their own advantages and disadvantages. These can either be in situ or online/offline after the process is complete.

19.3.7.1 Product Temperature Monitoring

If the product temperature profile is correlated to the suitability (including stability) of the product, then measurement of that product temperature profile provides extremely valuable information. The traditional technique for product temperature measurement has been the use of wire thermocouples or resistance temperature detector (RTD). These thermocouples and RTDs are robust, inexpensive and can be easily calibrated over the temperature range of interest. They are not without disadvantages though. Wire thermocouples and RTDs can transmit heat into the monitored vial and act as a point of nucleation during freezing. In commercial production, they require a significant amount of manual intervention by operators and are very difficult to use in conjunction with auto-loading equipment. Due to sterility concerns of having a wire over open containers during production, wire thermocouples are only placed in edge vials during routine production, which represent the highest heat condition within the lyophilizer (highest product temperature and shortest drying time). As a result of these drawbacks, they are generally not feasible for routine manufacturing use in many cases.

One interesting, emerging alternative to wire thermocouples is Tempris probes. These passive probes communicate with a transducer in the 2.4 GHz frequency range. As they are submerged within a product vial, they still provide a potential nucleation site. Because they do not have a wire, they are less intrusive during commercial manufacturing. Data show that readings from Tempris probes are comparable with thermocouple readings [68, 69].

Both wire thermocouples and Tempris probes monitor individual vials, thus applying those results to the bulk of a batch involves an extrapolation. The LyoFlux (tunable diode laser adsorption spectroscopy, TDLAS) monitors the transport of water vapor from the product chamber to the condenser through the duct piece

between the two chambers. With careful calculation and measurement of heat transfer coefficients, the results from the LyoFlux can calculate a bulk average product temperature profile [70]. Alternately, a pressure decrease test (PDT, [71]) can also be used for the estimation of batch average product temperature.

19.3.7.2 Endpoint of Primary Drying

Primary drying is typically the longest step of the lyophilization process. If the process is advanced from the primary drying hold to the secondary drying condition prior to the completion of sublimation, the product is at risk for meltback or collapse. As a result, confirmation of the endpoint of primary drying is critical (when considered with other routine process data) to ensure that the process executed properly.

Comparative pressure measurement is an extremely robust and inexpensive method for determination of endpoint of primary drying [52, 72]. Comparative pressure measurement relies on two pressure transducers in the product chamber—a capacitance manometer and a Pirani gauge. The capacitance manometer measures pressure by the current generated from a pressure-dependent deflection of a thin membrane and is independent of the composition of the gas it is measuring. A Pirani gauge measures pressure by the thermal conductivity of the gas that it is in contact with. The thermal conductivity of water vapor is approximately 60% higher than that of air or nitrogen. Pirani gauges are calibrated using dry air or nitrogen. During primary drying, the chamber is saturated with water vapor, resulting in a Pirani gauge reading 60% higher [52] than the capacitance manometer reading. As sublimation concludes, the concentration of water vapor in the chamber decreases as it is transported to the condenser. The lyophilizer bleeds in sterile nitrogen to maintain the pressure set point. This results in the Pirani reading moving toward the reading of the capacitance manometer, indicating the end of primary drying.

19.3.7.3 Product Moisture Monitoring

Product moisture monitoring can generally be classified as either online or offline. Currently, there are no accurate mechanisms to monitor product moisture for an individual vial in situ during a lyophilization process. Upon conclusion of a process, many alternatives have been developed to assess residual moisture.

The most common method is Karl Fischer titration, which involves a moisture-dependent chemical reaction of iodine. Karl Fisher titration is a destructive test that is typically performed in a QC environment on a very small number of samples (relative to the lot size). With appropriate controls, the method is easy to validate, very reproducible, and accurate. As water is a reactant in the method, no external calibration is required. Disadvantages include that sample preparation is time consuming and requires organic solvents.

Loss-on-drying methods also exist for residual moisture measurement. While these methods avoid the use of solvents and do not require a calibration standard, they are not without shortcomings. Lyophilized material is typically very hygroscopic; thus, extreme caution is required during sample preparation to minimize the adsorption of additional water. In low-humidity glove-boxes, these dried powders often show a high degree of electrostatic charge, adding complexity to sample preparation. A standard thermogravimetric analyzer is comprises of a balance and a furnace. This equipment may not be able to differentiate between water vapor and other volatile components. Other manufacturers have developed equipment with water-specific sensors to measure water vapor in a heated sweep gas. In either case, the temperature profile required for suitable moisture measurement will need to be developed and verified.

In a typical manufacturing process, where fewer than five samples are tested, this provides assurance that the process did not go significantly deviate from the intended target, but is not statistically powered to demonstrate the moisture homogeneity of the lot.

A popular alternative to Karl Fisher titration is the use of near infrared spectroscopy (NIR), focusing on the water band at approximately 5100 cm^{-1}. NIR methods are much faster, non-destructive and require no sample preparation. The speed and non-destructive nature of NIR makes feasible for higher sample load applications. Because the output of NIR is an absorbance, a calibration curve must be generated correlating that absorbance to water concentration. The generation of this curve is not trivial, as the production of samples at known moisture values can be a stumbling point. Those calibration samples are verified by Karl Fisher titration.

The implementation of NIR in a manufacturing process can take several forms, including:

- Offline measurement in a QC environment.
- At line measurement of selected samples using either handheld or benchtop units.
- Incorporation of a 100% inspection system into the process flow.

Any of the above forms can be performed either by directing the NIR beam at a location within the cake, or through the headspace of the vial (based on the design of the calibration curve). Alternate containers may also be compatible with NIR moisture measurement with appropriate method development.

Lyophilized cake porosity and dimensions had no effect on NIR measurement when cake height and diameter exceeded the NIR penetration depth [73]. Since last point of primary drying is at the bottom of cake, one could assume that residual moisture at the end of primary drying is highest toward the bottom of vial. This is consistent with the theoretical calculations provided in [74]. For conservative NIR measurement, therefore, pointing beam toward the bottom of vial will be a preferable choice.

Implementation of 100% NIR inspection as part of the manufacturing process should weigh the value of a 100% inspection against the cost of an increase in materials falsely identified as rejects. This relies on understanding the impact of

elevated moisture on other critical quality attributes. Products that are very sensitive to residual moisture or are at risk of failing moisture specifications during routine manufacture should implement an aggressive strategy of monitoring moisture results. For products that are robust to moisture and always fall within a narrow range, such monitoring adds significant cost of false rejects with minimal additional value.

NIR can provide significant value in several situations:

- Demonstration of homogeneity across and between Process Performance Qualification batches.
- Identification of outliers that can be assayed by Karl Fischer titration for actual moisture value.
- Situations when it is desirable to measure moisture and product quality from the same vial.

19.3.8 Equipment Characterization and Modeling

As the field of lyophilization has evolved, more scientists are developing process models based on the heat and mass transfer equations that describe the lyophilization process, particularly for the primary drying (sublimation) phase. In order to satisfy these equations, product- and equipment-based coefficients and constants must be carefully measured. A model is more robust and effective if both the sending and receiving lyophilizers have been characterized using the same general protocol. For laboratory equipment, this is usually a fairly straightforward exercise, from a resource and scheduling perspective. Production scale equipment is more difficult to manage, from scheduling, planning, and execution perspectives. Defining the minimum number of experiments required to collect this data becomes critical, particularly to minimize the time required and impact on production equipment. From an equipment perspective, the characterization protocol would include the following experiments (at a minimum) [42, 75]:

1. Minimum controllable pressure as a function of sublimation rate and maximum sublimation rate the lyophilizer can support.
2. Heat transfer coefficients of desired vials as a function of chamber pressure.
3. Homogeneity of the shelf surface temperatures during ramps, and the impact on product temperature heterogeneity as a function of ramp rate.

Minimum controllable pressure as a function of sublimation rate supports just how aggressive of a lyophilization cycle a machine can handle. At the high end, the sublimation rate is capped by (1) heating of the condenser to greater than −40 °C, or (2) choked flow [76]. At the low end, this experiment is an assessment of the rate of mass transfer from the chamber to the condenser as a function of chamber pressure.

Heat transfer coefficients (K_v) as a function of pressure and position (edge vs. center) should be measured for any vials of interest [43]. These experiments can also measure the impact of tray bottoms (if they are also used during production), which can restrict heat transfer by as much as 50% in the authors' experience.

Homogeneity during both heating and cooling ramps can help identify heterogeneity that may be experienced by some vials due to thermal gradients across shelves during more aggressive ramping. In general, homogeneity is preferred across the batch, preferably keeping product temperature within 1–2 °C across each other across a load, particularly during the freezing step if relying on uncontrolled nucleation.

One of the key outputs of equipment characterization is that it can enable transfer between two characterized lyophilizers primarily utilizing modeling to estimate target manufacturing process conditions. In addition to minimizing the at-scale work required to confirm process transfer, modeling can be used to correlate data generated across multiple lyophilizers, including process robustness assessments [77].

19.3.9 Process Robustness

As a program progresses through clinical development, the understanding of the process and product must also increase [78]. This concept fits within the quality by design paradigm of deepening the understanding of the process design space required to yield acceptable product.

During early clinical development, material is limited. Additionally, the number of batches and the size of these batches are small (relative to commercial production). As a result, most formulation and process scientists will adopt a conservative "make sure it works" approach. The process is biased to more conservative conditions to ensure that each batch is acceptable and to minimize potential clinical supply disruptions. Platform formulation approaches can provide significant de-risking during this phase [26, 27]. If a new program is in the same (or highly similar) formulation as previous programs at a comparable protein concentration, then development data for those processes can be highly supportive in providing confidence in the robustness of a new cycle. While that data are strongly supportive, it does not directly substitute for knowledge of the current molecule, formulation, and process.

As a project proceeds through clinical development, the program must validate the manufacturing process. For commercial manufacturing, the focus shifts slightly from "make sure it works" to "make sure it works, and is efficient." Demands on commercial equipment to meet desired production schedules mean that the desired cycle is the shortest one that can robustly be executed. To support this, a more detailed understanding of the process design space is required, including considerations for the range of process parameters that may be feasible at production scale (accounting for the characterization work that has been performed). This target commercial cycle must be defined prior to validation (clearly), but also prior to process robustness assessment.

Prior to execution of process robustness, several decisions must be made:

1. At what scale will the process robustness studies be performed?
2. What is the overall strategy?
   a. Design of experiments (DOE)
   b. One factor at a time (OFAT)
   c. Multiple Factors per experiment (hybrid). This strategy can include combination of extreme process parameters that define maximum product temperature design space (e.g., high/high or low/low shelf temperature and chamber pressure).
3. How much stability is required, and what assays will be monitored?

The simplest strategy (from a process support perspective) would be to perform process robustness at scale. This is generally not feasible, both from material requirement and scheduling perspectives. At-scale work should generally focus on aspects that cannot be adequately scaled down to laboratory scale and may have product impact.

Most formulation and process scientists rely on laboratory scale robustness studies to support production processes. The scope of these studies must be carefully crafted to support a wide range of potential process deviations during routine manufacture. The primary impact of these process deviations would be to alter the product temperature profile. Thus, a laboratory robustness study should seek to define the range of potential product temperature profiles that yield suitable product. This focus on product temperature profile reflects the direct correlation between product temperature profile and finished product critical quality attributes. The impacts of process parameters such as shelf temperature and chamber pressure are significant only in how they impact the product temperature profile. For example, different combinations of shelf temperature and chamber pressure can yield comparable product temperature profiles, and thus comparable stability. Numerous strategies exist for designing process robustness studies. One significant consideration into defining the strategy for robustness studies lies in how that data will be utilized to support the manufacturing process. Once the product is in routine manufacturing, the design space defined during robustness studies will be heavily leveraged to support deviations in manufacturing, so colleagues responsible for the ongoing commercial support must understand and be able to properly apply the conclusions of these studies to future process deviations.

One popular, and somewhat controversial, method is to employ statistical design to create a sequence of experiments that simultaneously varies multiple parameters to examine their impact upon process outputs, commonly referred to as design of experiments. These experiments can be very useful in scenarios where (1) relationships between parameters and outputs are unknown or poorly understood, (2) the interactions between parameters are unknown, or (3) there are too many process parameters to study individually. While application of DoE has become more common in supporting lyophilization processes [79], one can question the choice of parameters and ranges examined. For example, during primary drying, the

relationship between chamber pressure and shelf temperature as inputs on product temperature as an output can be calculated; thus, varying these independently from each other would lead to additional experiments that would only serve to confirm the previously described mathematical relationship without providing additional information. The duration of primary drying is only one of two things: long enough to complete sublimation for the entire batch or not. If sublimation is complete, then additional soak under those conditions only serves as a start to secondary drying, the impact of which depends on the relative temperatures of primary and secondary drying. If sublimation is not complete, the cycle generally will not be acceptable. Thus, primary drying time should be considered a dependent variable that is defined by the duration required to complete sublimation plus a suitable safety factor.

As discussed previously, a lyophilization cycle can be broken down into three distinct phases: (1) generation of the frozen matrix (freezing and annealing), (2) sublimation (primary drying), and (3) desorption (secondary drying). When one examines a lyophilization process, one can frequently define "worst-case" or "extreme" conditions for each step. Progressing through the lyophilization cycle, one can identify combinations of these conditions that result in "high" or "low" extremes. The degree of variability considered during these studies for shelf temperature and chamber pressure should exceed the anticipated variability encountered during routine manufacturing. How far in excess of the anticipated manufacturing variability should take into account the robustness of the baseline cycle and product. For example, ±5 °C and 20 mTorr may be a reasonable starting point.

When considering the ranges for consideration, one must consider and avoid known failure modes. For example, if the freezing temperature is not low enough, ice crystallization may be incomplete or sub-optimal. The consequences discussed earlier in this chapter for each of the processing steps should be considered as well.

For the following examples, consider two types of formulations: amorphous and partially crystalline, in evaluating process robustness design. Table 19.3 walks through each step in the lyophilization process and describes the primary impact of varying parameters.

Examining Table 19.3, one can group the conditions together as shown below based on their impact to the product (Table 19.4).

Since smaller pores during freezing would serve to increase the product temperature during primary drying by increasing the cake resistance [46], one can further group "small pores" with "high product temperature" to make "worst-case high temp" and "large pores" with "low product temperature" to make "worst-case low temp." Using this logic, for an amorphous formulation, one could simplify a robustness study to examine the high and low temperatures at high and low moisture, resulting in a total of four lyophilization cycles plus one control at the target set points to examine the streamlined design space. If the edge of process failure is near this design space, an additional cycle approaching the edge of failure, or is just beyond the edge of failure may provide insight into the product quality consequences of operation under these conditions. This is a significant simplification of the 14 parameters listed in Table 19.3.

Table 19.3 Impact of variability in process parameters on the quality of final product

	Amorphous Formulation		Partially crystalline Formulation	
Step	High temperature/ pressure, or fast rate, or long duration	Low temperature/ pressure, or slow rate, or short duration	High temperature/ pressure, or fast rate, or long duration	Low temperature/ pressure, or slow rate, or short duration
Freezing ramp rate	Fast rate, more supercooling, smaller ice crystals, smaller pores	Slow rate, less supercooling, larger ice crystals, larger pores	Fast rate, more supercooling, smaller pores	Slow rate, less supercooling, larger pores
Freezing temperature	High temperature, incomplete freezing (under extreme variability)-low Tg′	No impact	High temperature, incomplete nucleation of crystalline species	No impact
Annealing ramp rate	Fast rate, less Ostwald ripening [46]	Slow rate, more Ostwald ripening (larger pores)	Fast rate, less crystallization	Slow rate, more crystallization
Annealing temperature	High temperature, more Ostwald ripening	Low temperature, less Ostwald ripening	High temperature, more crystallization[a]	Low temperature, less to no crystallization[b]
Annealing time	More Ostwald ripening → larger ice crystals	Less Ostwald ripening → smaller ice crystals	More crystallization	Less crystallization
Refreeze rate	Less Ostwald ripening	More Ostwald ripening	Less crystallization	More crystallization
Refreeze temperature	Closer to Tg′ (or Tc)	Further from Tg′ (or Tc)	Closer to Tg′ (or Tc)	Further from Tg′ (or Tc)
Primary drying ramp	Fast rate, more heat	Slow rate, less heat	Fast rate, more heat	Slow rate, less heat
Primary drying temperature	High temperature, more heat	Low temperature, less heat	High temperature, more heat	Low temperature, less heat
Primary drying duration	Longer than required, no impact	Shorter than required—likely cycle failure	Longer than required, no impact	Shorter than required—likely cycle failure
Chamber pressure	High pressure, more heat	Low pressure, less heat	High pressure, more heat	Low pressure, less heat
Secondary drying ramp	Fast rate, closer to Tg (or collapse)	Slow rate, further from Tg	Fast rate, minimal impact	Slow rate, minimal impact

(continued)

Table 19.3 (continued)

	Amorphous Formulation		Partially crystalline Formulation	
Step	High temperature/ pressure, or fast rate, or long duration	Low temperature/ pressure, or slow rate, or short duration	High temperature/ pressure, or fast rate, or long duration	Low temperature/ pressure, or slow rate, or short duration
Secondary drying temperature	Lower moisture	Higher moisture	Lower moisture[c]	Higher moisture[c]
Secondary drying time	Lower moisture	Higher moisture	Lower moisture	Higher moisture
Secondary drying pressure	Minimal impact	Minimal impact	Minimal impact	Minimal impact

[a]This assumes that the higher temperature annealing remains below the eutectic melting temperature of the crystalline phase

[b]For mannitol formulations, annealing temperature may impact the polymorph distribution of crystalline mannitol, including mannitol hemihydrate. In general, it is preferable to select conditions that minimize the formation of mannitol hemihydrate [80], if possible

[c]For mannitol formulations, elevated temperatures during secondary drying have been shown to lead to dehydration of mannitol hemihydrate and recrystallization anhydrous polymorph [81]. For these formulations, the impact of secondary drying on mannitol hemihydrate levels must be understood

As the product formulation has a significant impact on lyophilization process performance, one should consider certain formulation robustness aspects when defining process robustness. Several potential examples are described below:

– In a partially crystalline/partially amorphous formulation, reducing the ratio of crystallizing excipient may reduce the overall level of crystallinity, thus potentially impacting the stability of the final product due to the increased presence of the crystalline excipient.
– Varying buffer concentration could increase the risk of preferential crystallization of one buffer species, potentially shifting the pH in the frozen state.
– Increasing the concentration of low glass transition temperature formulation components may substantially reduce the composite glass transition temperature. This may impact product performance during aggressive drying cycles.
– Fast ramp rates to secondary drying exceed the glass transition temperature, leading to elevated residual moisture.

The potential impacts of formulation robustness should consider anticipated process variability considering the goal of both studies is to identify ranges where the formulation and process will be successful. At one extreme, one may conclude that the magnitude of formulation variability is unlikely to impact the process, and can be discounted from a risk perspective. On the other extreme, the perceived risk may justify performing additional work to combine worst-case formulation and process conditions as part of the assessment.

Table 19.4 Summary of process parameters to be utilized in the generation of a broad design space

	Amorphous formulation			Partially crystalline formulation		
	Cooling/heating rate	Temperature/pressure	Time	Cooling/heating rate	Temperature/pressure	Time
	Low-fast conditions/cycle			Low-fast conditions/cycle		
Freezing						
Small pores (or lower SSA—for amorphous formulations) or low crystallinity (for partially crystalline formulations)	Fast	Low shelf temperature	Longer duration of annealing	Fast	Low shelf temperature	Longer duration of annealing
Primary drying						
High product temperature	Fast	High shelf temperature + high chamber pressure	NA[a]	Fast	High shelf temperature + high chamber pressure	NA[a]
Secondary drying						
High water content	Fast (risk of exceeding Tg′/Tc)	Low shelf temperature	Shorter duration	Fast	Low shelf temperature	Shorter duration
	High-slow conditions/cycle			High-slow conditions/cycle		
Freezing						
Large pores (or lower SSA—for amorphous formulations) or high crystallinity (for partially crystalline formulations)	Slow	High shelf temperature	Longer duration of annealing	Slow	High shelf temperature	Longer duration of annealing
Primary drying						
Low product temperature	Slow	Low shelf temperature + low chamber pressure	NA[a]	Slow	Low shelf temperature + low chamber pressure	NA[a]
Secondary drying						
Low water content	Slow	High shelf temperature	Longer duration	Slow	High shelf temperature	Longer duration

[a]The duration of primary drying should be sufficient to ensure sublimation has completed in all vials and should not be a variable independently examined during design space construction

Once robustness cycles are completed, they are enrolled in a stability program to demonstrate that the product is robust to the process deviations examined. One should consider which critical quality attributes (besides residual moisture) have been shown to be impacted by storage of dried materials when selecting the assays for stability monitoring. For most proteins, these assays will include measures of high and low molecular mass species. Specific activity assays may be useful if they show low variability and provide insight that structural assays do not. Additional assays may be selected based on knowledge of the particular product, its degradation routes, and key stability-indicating learnings during product development.

One of the goals of the "high moisture" cycles is to generate product at or just outside of the proposed commercial moisture specification [27]. Clinical manufacturing experience may have only yielded lots at below 1% residual moisture, while a proposed specification may be 2%. While moisture is typically a specification of lyophilized products, one must consider what this specification means. A critical quality attribute is defined as an attribute that has an impact on the "safety, efficacy, or purity" of a pharmaceutical product [78]. One could make the argument that residual moisture, in and of itself, does not impact safety, efficacy or purity. The primary impact of increased residual moisture is the reduction of the glass transition temperature of the dried matrix [3]. As a result of the reduced glass transition temperature, materials within the amorphous phase (including protein) have increased mobility, resulting in an acceleration of degradation reactions and interactions that do alter the efficacy and/or purity of the product. In this respect, residual moisture is a marker for potential future degradation or issues during the execution of the lyophilization cycle.

19.3.10 Process Performance Qualification/Process Validation

Process Performance Qualification (PPQ) is required of the commercial dosage form. Traditionally, this requires a minimum of three consecutive batches (depending on the number of configurations required) and is performed at-scale. Assuming process robustness has been suitably demonstrated at laboratory scale and correlated to full scale, varying cycle parameters is not performed during PPQ batches. Lyophilization cycles executed at the process set points confirm the reproducibility of the commercial process and the laboratory scale work demonstrates that acceptable variation of those parameters does not negatively impact process performance.

One aspect to consider during PPQ is lyophilizer load. For aggressive cycles, full load represents a worst-case challenge to the equipment ability to maintain chamber pressure under the maximum sublimation load. Conversely, for low sublimation processes, a lower load may challenge the ability of the gas bleed/pressure control system to maintain the pressure within the required variability. A lower load could result in higher product temperature and faster drying [82].

19.3.11 Quality by Design and Risk Management

Quality by design, as defined in ICH Q8R, Q9, and Q10, refers to the combination of process parameters that will yield suitable product, in contrast to "Quality by QC," which implies that the success of a process is defined by the end of production testing [78, 83, 84]. In a quality by design paradigm, the process robustness studies support the definition of the process design space [85]. These studies provide direct data on the proven acceptable range (PAR) and potentially identify edge(s) of failure. This design space should account for both product and equipment limitations [86]. The normal operating ranges (NORs) (typically batch record process acceptance criteria) should be within the PAR, preferably with enough space between the two concepts to support process deviations.

Risk management is fundamental to the quality by design philosophy. A typical risk management process includes the following steps [83]:

1. Correlation between process parameters and critical quality attributes (typically a cause and effect matrix), leveraging robustness data.
2. Determination of the degree of variability for the process parameters (failure mode and effect analysis), based on the commercial equipment and process.
3. Correlation of this variability to potential impact to critical quality attributes, leveraging robustness data.
4. Scoring the severity, detectability, and occurrence of variability that may impact a CQA and compiling those results to define a Risk Prioritization Number (RPN).
5. Based on the composite scores and predefined scoring criteria, assess whether any process steps require mitigation or accept any residual risk.

In examining the "occurrence" concept, typical failure modes considered should be within the realm of reasonable possibility that may be acceptable. For example, using a risk assessment to define the acceptability of major refrigeration breakdown probably does not add much value to the knowledge base. On the other hand, assessing the impact of pressure deviations outside the acceptable batch record limits represents a high probability of occurring in the future.

From ICH Q8R2, a CQA is "a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality." ICH Q8R2 further defines a critical process parameter (CPP) is "a process parameter whose variability has an impact on a critical quality attribute and therefore should be monitored or controlled to ensure the process produces the desired quality" [78]. Process parameters that do not impact a CQA cannot be critical process parameters. On top of that, process parameters which, over the range of variability considered, do not impact CQAs enough to compromise the desired product quality can also be considered non-CPP's. Based on this interpretation, in certain instances, it is feasible for a lyophilization process to not have any CPPs, as the knowledge space supports any anticipated process variability without impact to CQAs.

An experimental design that balances scientific knowledge to guide the selection of key experiments, potentially augmented with a rationally thought out design of experiments to characterize areas with unknown interactions, followed by a formal risk assessment process to tie all of the work together and identify potential gaps yields a thorough understanding of the lyophilization process. The decision to capture this information in a quality by design-based regulatory filing is, however, a business decision on the part of the sponsor to balance the potential benefits of such a filing against any potential risks. At a minimum, the gathered and categorized knowledge should lead to an enhanced support of the lyophilization process within a license application.

19.4 Conclusion

This chapter focuses on providing practical advice on the lyophilization process, particularly from a manufacturing and design space perspective. While lyophilized formulation development was not discussed in detail here, the formulation has a significant impact on the lyophilization process. Utilization of equipment characterization and mathematical modeling can significantly facilitate process scale-up and/or transfer. The correlation of processes across multiple lyophilizers can enable direct application of robustness studies performed at laboratory scale to support the manufacturing scale process. By understanding the interaction of multiple process parameters and steps on the resulting lyophilized product, the number of experiments required to support process robustness studies can be reduced to support worst-case scenarios. These data provide inputs into the Risk Management paradigm and can be used to support the manufacturing process design space.

References

1. Wang W. Lyophilization and development of solid protein pharmaceuticals. Int J Pharm. 2000;203:1–60.
2. Nail SL, Jiang S, Chongprasert S, Knopp SA. Fundamentals of freeze-drying. Pharm Biotechnol. 2002;281–360.
3. Pikal MJ. Freeze drying. Encyclopedia of pharamceutical technology. USA: Informa Healthcare; 2006. p. 1807–33.
4. Bhatnagar B, Tchessalov S, Lewis L, Johnson R. Freeze drying of biologics in encyclopedia of pharmaceutical science and technology. 4th ed. New York: Taylor and Francis; 2013. p. 1673–722.
5. Auffret GLAT, Shalaev EY, Speaker SM, Teagarden DL. Freeze-drying concepts: the basics. In: McNally EJ, Hastedt JE, editors. Protein formulation and delivery. 2nd ed. Florida: CRC Press; 2007. p. 177–95.
6. Carpenter JF, Pikal MJ, Chang BS, Randolph TW. Rational design of stable lyophlized proteins: theory and practice. Pharm Res. 1997;969–75.

7. Nema S, Washkuhn RJ, Brendel RJ. Excipients and their uses in parenteral products. PDA J Pharm Sci Technol. 1997;166–71.
8. Constantino HR. Excipients for use in lyophilized pharmaceutical peptide, protein, and other bioproducts, in lyophilization of biopharmaceuticals. Arlington, VA: AAPS Press; 2004. p. 139–228.
9. Sundaramurthi P, Suryanarayanan S. Trehalose crystallization during freeze-drying: implication on lyoprotection. J Phys Chem Let. 2010;510–4.
10. Singh SK, Kolhe P, Mehta AP, Chico SC, Lary AL, Huang M. Frozen state storage instability of a monoclonal antibody: aggregation as a consequence of trehalose crystallization and protein unfolding. Pharm Res. 2011;28(4):873–85.
11. Connolly BD, Le L, Patapoff TW, Cromwell MEM, Moore JMR, Lam P. Protein aggregation in frozen trehalose formulations: effects of composition, cooling rate, and storage temperature. J Pharm Sci. 2015;104(12):4170–84.
12. Wang BQ, Tchessalov S, Cicerone MT, Warne NW, Pikal MJ. Impact of sucrose level on storage stability of proteins in freeze-dried solids: II. Correlation of aggregation rate with protein structure and molecular mobility. J Pharm Sci. 2009;3145–66.
13. Wang BQ, Tchessalov S, Warne NW, Pikal MJ. Impact of sucrose level on storage stability of proteins in freeze-dried solids: I. Correlation of protein-sugar interaction with native structure preservation. J Pharm Sci. 2009;3131–44.
14. Cleland JL, Lam X, Kendrick B, Yang J, Yang TH, Overcashier D, Brooks C, Hsu C, Carpenter JF. A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody. J Pharm Sci. 2001;90(3):310–21.
15. Pikal MJ, Rigsbee D, Roy ML, Galreath DK, Karl J, Wang B, Carpenter JF, Cicerone MT. Solid state chemistry of proteins: II. The correlation of storage stability of freeze-dried human growth hormone (hGH) with structure and dynamics in the glassy solid. J Pharm Sci. 2008;97(12):5106–21.
16. Luthra S, Pikal MJ. Stabilization of lyophilized pharmaceuticals by control of molecular mobility: impact of thermal history. In: Jameel F, Hershenson S, editors. Formulation and Process Development Strategies for Manufacturing Biopharmaceuticals. Hoboken, NJ: Wiley; 2010. p. 521–48.
17. Duddu SP, Zhang G, Dal Monte PR. The relationship between protein aggregation and molecular mobility below the glass transition temperature of lyophilized formulations containing a monoclonal antibody. Pharm Res. 1997;14(5):596–600.
18. Carpenter JF, Pikal MJ, Chang BS, Randolph TW. Rational design of stable lyophilized protein formulations: some practical advice. Pharm Res. 1997;14(8):969–75.
19. Kasraian K, Spitznagel TM, Juneau J, Yim K. Characterization of the sucrose/glycine/water system by differential scanning calorimetry and freeze-drying microscopy. Pharm Dev Tech. 1998;233–9.
20. Lueckel B, Bodmer D, Helk B, Leuenberger H. Formulations of sugars with amino acis or mannitol: influence of concentration ratio on the properties of the freeze-concentrate and the lyophlizate. Pharm Dev Tech. 1998;325–36.
21. Schneid S, Riegger X, Gieseler H. Influence of common excipients on the crystalline modification of freeze-dried mannitol. Pharm Tech. 2008;178–84.
22. Dixon DA, Tchessalov S, Barry AB, Warne NW. The impact of protien concentration on mannitol and sodium chloride crystallinity and polymorphism upon lyophilization. J Pharm Sci. 2009;3419–29.
23. Liao X, Krishnamurthy R, Suryanarayanan R. Influence of the active pharmaceutical ingredient concentration on the physical state of mannitol—implications in freeze-drying. Pharm Res. 2005;11(11):1978–85.
24. Krishnan S, Pallitto M, Nagle S, Crampton SL, Speed Ricci M, Cao W, Lin H, Xie Y. USA Patent WO2007014073 A3, 2008.
25. Chang BS, Kendrick BS, Carpenter JF. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. J Pharm Sci. 1996;85(12):1325–30.

26. Warne NW. Development of high concentration protein biopharmaceuticals: the use of platform approaches in formulation development. Eur J Pharm Biopharm. 2011;78(2):208–12.
27. Tchessalov S, Dixon DA, Barry AB, Warne NW. Lyophilization cycle robustness strategy. USA Patent US20090324586 A1, 2009.
28. Chang BS, Randall CS. Use of subambient thermal analysis to optimize protein lyophilization. Cryobiology. 1992;29:632–56.
29. Osterberg T, A. Fatouros A, Mikaelsson M. Development of a freeze-dried albumin-free formulation of recombinant factor VIII SQ. Pharm Res. 1997;892–8.
30. Her LM, Deras M, Nail SL. Electrolyte-induced changes in gralss transition temperatures of freeze-concentrated solutes. Pharm Res. 1995;12(5):768–72.
31. Genentech. Herceptin prescribing information. [Online]. Available: https://www.gene.com/download/pdf/herceptin_prescribing.pdf. Accessed 29 June 2017.
32. Amgen, Inc. Product monograph—Enbrel (Etanercept) [Online]. Available: https://www.amgen.ca/products/~/media/5d0a40b2b8774fb5994190f97daf7fbd.ashx. Accessed 29 June 2019.
33. Pfizer, Inc. Product monograph—BeneFIX (coagulation factor IX). [Online]. Available: http://www.pfizer.ca/sites/g/files/g10017036/f/201410/BENEFIX_PM_E_153056_27Dec2012.pdf. Accessed 29 June 2017.
34. Janssen Biotech. Remicade prescribing information [Online]. Available: https://www.remicade.com/shared/product/remicade/prescribing-information.pdf. Accessed 29 June 2017.
35. Tchessalov S, Dixon DA, Warne NW. Lyophilization of pharmaceuticals above collapse temperature. USA Patent WO. 2010;2010017296:A1.
36. Chatterjee K, Shalaev ESR. Partially crystalline systems in lyophilization: II withstanding collapse at high primary drying temperature and impact on protein recovery. J Pharm Sci. 2005;809–20.
37. Johnson R, Kirchhoff C, Gaud J. Mannitol-sucrose mixtures—versatile formulations for protein lyophilization. J Pharm Sci. 2002;914–22.
38. Wang DQ, Hey JM, Nail SL. Effect of collapse on the stability of freeze-dried recombinant factor VII and a-amylase. J Pharm Sci. 2004;93(5):1253–63.
39. Schersch K, Betz O, Garidel P, Bassarab S, Winter G. Systematic investigation of the effect of lyophilizate collapse on pharmaceutically relevant proteins I: stability after freeze-drying. J Pharm Sci. 2010;99(5):2256–78.
40. Schersch K, Betz O, Garidel P, Muehlau S, Bassarab S, Winter G. Systematic investigation of the effect of lyophilizate collapse on pharmaceutically relevant proteins, part 2: stability during storage at elevated temperatures. J Pharm Sci. 2012;101(7):2288–306.
41. Schersch K, Betz O, Garidel P, Muehlau S, Bassarab S, Winter G. Systematic investigation of the effect of lyophilizate collapse on pharmaceutically relevant proteins III: collapse during storage at elevated temperatures. Eur J Pharm Biopharm. 2013;85(2):240–52.
42. Tchessalov S, Dixon DA, Warne NW. Principles of lyophilization scale-up. Am Pharm Rev. 2007;88–91.
43. Pikal MJ, Bogner R, Mudhivarthi V, Sharma P, Sane P. Freeze-drying process development and scale-up: scale-up of edge vial versus center vial heat transfer coefficients, Kv. J Pharm Sci. 2016;105:3333–43.
44. Sane S, Hsu C. Considerations fro successful lyophlization process scale-up, technology transfer and routine production. In: Jameel F, Hershenson S, editors. Formulation and process development strategies for manufacturing biopharmaceuticals. Hoboken: Wiley; 2010. p. 797–826.
45. Heller MC, Carpenter JF, Randolph TW. Manipulation of lyophilization-induced phase separation: implications for pharmaceutical proteins. Biotechnol Prog. 1997;13(5):590–6.
46. Searles JA, Carpenter JF, Randolph TW. Annealing to optimize the primary drying rate, reduce freezing-induced drying heterogeneity, and determine Tg′ in pharmaceutical lyophilization. J Pharm Sci. 2001;872–87.

47. Pikal MJ, Roy ML, Shah S. Mass and heat transfer in vial freeze-drying of pharmaceuticals: role of the vial. J Pharm Sci. 1984;1224–37.
48. Pikal MJ. Use of laboratory data in freeze drying process design: heat and mass transfer coefficients and the computer simulation of freeze drying. J Parenteral Sci and Tech. 1985;39 (3):115–37.
49. Sheehan A, Liapis AI. Modeling of the primary and secondary drying stages of the freeze drying of pharmaceutical products in vials: numerical results obtained from the solution of a dynamic and spatially multi-dimensional lyophilization model for different operational policies. Biotech and Bioeng. 1998;60(6):712–28.
50. Hottot A, Vessot S, Andrieu J. Determination of mass and heat transfer parameters during freeze-drying cycles of pharmaceutical products. PDA J Pharm Sci Tech. 2005;59(2):138–53.
51. SP Scientific. LyoModeling calculator. 2016. [Online]. Available: http://www.spscientific.com/LyoCalc/Lyocalculator.html. Accessed 3 July 2017.
52. Patel SM, Doen T, Pikal MJ. Determination of end point of primary drying in freeze-drying process control. AAPS PharmSciTech. 2010;73–84.
53. Meissner U, Satahl H, Steiinkellner D. Detection of silicone oil leakages in freeze dryers. PDA J Pharm Sci Technol. 2011;65(5):481–4.
54. Barresi AA, Pisano R, Fissore D, Rasetto V, Velardi SA, Vallan A, Parvis M, Galan M. Monitoring of the primary drying of a lyophilization process in vials. J Pharm Sci. 2009;48 (1):408–23.
55. Connelly JP, Welch JV. Monitor lyophilization with mass spectrometer gas analysis. PDA J Pharm Sci Technol. 1993;47(2):70–5.
56. Tang X, Nail SL, Pikal MJ. Freeze-drying process design by manometric temperature measurement: design of a smart freeze-dryer. Pharm Res. 2005;685–700.
57. Adams GDJ, Ramsay JR. Optimizing the lyophilization cycle and the consequences of collapse on the pharmaceutical acceptability or Erwinia L-Asparaginase. J Pharm Sci. 1996;1301–5.
58. Tchessalov S, Warne NW. Lyophlization methods and apparatuses. USA Patent WO 2008042408 A2. 2008.
59. Pikal MJ, Shah S, Roy ML, Putman R. The secondary drying stage of freeze drying: drying kinetics as a function of temperature and chamber pressure. Int J Pharm. 1990;203–17.
60. Searles JA, Aravapalli S, Hodge C Effects of chamber pressure and partial pressure of water vapor on secondary drying in lyophlization. AAPS PharmSciTech, 2017.
61. Nail SL. The effect of chamber pressure on heat transfer in the freeze drying of parenteral solutions. PDA J Pharm Sci Technol. 1980;34(5):358–68.
62. Tang XPMJ. Design of freeze-drying processes for pharmaceuticals: practical advice. Pharm Res. 2004;191–200.
63. Ullrich S, Seyferth S, Lee G. Measurement of shrinkage and cracking in lyophlized amorphous cakes. Part I: final product assessment. J Pharm Sci. 2015;104:155–64.
64. Liapis A, Bruttini R. A theory for the primary and secondary drying stages of the freeze drying of pharmaceutical crystalline and amorphous solutes: comparison between experimental data and theory. Sep Technol. 1994;144–55.
65. Pikal MJ, Cardon S, Bhugra C, Jameel F, Rambhatla S, Mascarenhas WJ, Akay HU. The nonsteady state modeling of freeze drying: in-process product temperature and moisture content mapping and pharmaceutical product quality applications. Pharm Dev Technol. 2005;17–32.
66. Sahni EK, Pikal MJ. Modeling the secondary drying stage of freeze drying: development and validation of an excel-based model. J Pharm Sci. 2017;106(3):779–91.
67. IMA-Pharma. Lyophilization process—fusion and fusion plus [Online]. Available: http://www.ima-pharma.com/Product/EN/Products-F575/Product_Insights-S872/Lyophilization_Process-T889/FUSION%E2%84%A2_%26_FUSION_PLUS_%E2%84%A2-Q894.html. Accessed 29 June 2017.

68. Schneid S, Gieseler H. Evaluation of a new wireless temperature remote interrogation system (TEMPRIS) to measure product temperature during freeze drying. AAPS PharmSciTech. 2008;729–39.
69. Wilbur B. Process development of a dual-chambered syringe. San Diego, CA. 2012.
70. Schneid SC, Gieseler H, Kessler WJ, Pikal MJ. Non-invasive product temperature determination during primary drying using tunable diode laser adsorption spectroscopy. J Pharm Sci. 2009;98:3406–18.
71. Pisano R, Fissore D, Barresi A. A new method based on the regression of step response data for monitoring a freeze-drying cycle. J Pharm Sci. 2014;1756–65.
72. Nail SL, Johnson W. Methodology for in-process determination of residual water in freeze-dried products. Dev Bioblogical Stand. 1992;74:137–51.
73. Lin TP, Hsu CC. Determination of residual moisture in lyophilized protein pharmaceuticals using a rapid an non-invasive method: near infrared spectroscopy. PDA J Pharm Sci Technol. July August 2002;196–205.
74. Sadikoglu H, Liapis AI, Crosser OK, Brurrini R. Estimation of the effect of product shrinkage on drying times, heat input and condenser load of the primary drying stages of the lyophilization process in vials. Drying Technol. 1999;17(10):2013–35.
75. Rambhatla S, Tchessalov S, Pikal MJ. Heat and mass transfer scale-up issues during freeze-drying, III: control and characterization of dryer differences via operational qualification tests. AAPS PharSciTech. 2006;7(2):E61–70.
76. Searles JA. Observation and implication of sonic water vapor flow during freeze-drying. Am Pharm Rev. 2004;8(2):58–69.
77. Tchessalov S, Warne NW. Lyophlization: cycle robustness and process tolerances, transfer and scale up. Euro Pharm Rev. 2008;3:76–83.
78. International Conference on Harmonization, “Q8(R2): Pharmaceutical Development,” 2009.
79. Sundaram J, Shay YHM, Hsu CC, Sane SU. Design space development for lyophilization using DOE and process modeling. BioPharm Int. 2010;23(9):26–36.
80. Mehta M, Bhardwaj SP, Suryanarayanan R. Controlling the physical form of mannitol in freeze-dried systems. Eur J Pharm Biopharm. 2013;85(2):207–13.
81. Yu L, Milton N, Groleau EG, Mishra DS, Vansickle RE. Existence of mannitol hydrate during freeze-drying and practical implications. J Pharm Sci. 1999;88(2):196–8.
82. Patel SM, Jameel F, Pikal MJ. The effect of dryer load on freeze drying process design. J Pharm Sci. 2010;99(10):4363–79.
83. International conference on harmonization, Q9: quality risk management, 2005.
84. International conference on harmonization, Q10: pharmaceutical quality system, 2008.
85. Glodek M, Liebowitz S, McCarthy R, McNally G, Oksanen C, Schultz T, Sudararajan M, Vorkapich R, Vukovinsky K, Watts C, Millili G. Process robustness—a PQRI white paper. Pharm Eng. 2006;26(6):1–11.
86. Nail SL, Searles JA. Elements of quality by design in development and scale-up of freeze-dried parenterals. BioPharm Int. 2008;44–52.
87. International conference on harmonization, Q7: good manufacturing practice guide for active pharmaceutical ingredients, 2000.
88. Patro S, Freund E, Chang B. Protein formulation and fill-finish operations. Biotechnology Annual Review. 2002;55–84.
89. Chang D, Chang R-K. Review of current issues in pharmaceutical excipients. Pharm Technol. May 2007;56–66.
90. Kishore R, Kiese S, Fischer S, Pappenberger A, Grauschopf U, Mahler H-C. The degradation of polysorbates 20 and 80 and its potential impact on the stability of biotherapeutics. Pharmaceutical Research. 2011;1194–210.
91. Zhang L, Yadav S, Demeule B, Wang YJ, Mozziconacci O, Schoeneich C. Degradation mechanisms of polysorbate 20 differentiated by 18O-labeling and mass spectrometry. Pharm Res. 2017;84–100.
92. Steele A, Arias J. Accounting for the Donnan effect in diafiltration optimization for high-concentration UFDF applications. BioProcess Int. 2014;50–4.

93. Brukl L, Hahn R, Sergi M, Scheler S. A systematic evaluation of mechanisms, material effects, and protein-dependent differences on friction-related protein particle formation in formulation and filling steps. Int J Pharm. 2016;931–45.
94. Ishikawa T, Kobayashi N, Osawa C, Sawa E, Wakamatsu K. Prevention of stirring-induced microparticle formation in monoclonal antibody solutions. Biol Pharm Bull. 2010;33 (6):1043–6.
95. Paul E, Atiemo-Obeng V, Kesta S, editors. Handbook of industrial mixing: science and practices. Hoboken: Wiley; 2004.
96. European commission. Volume 4, Annex 1—manufacture of sterile medicinal products. Brussels, 2008.
97. FDA. Guidance for industry: sterile drug products producted by aseptic manufacturing current good manufacturing practice. Rockville, MD, 2004.
98. Antonsen H, Awafo V, Bender J, Carter J, Conway R, Egli S, Feeser T, Jornitz M, Kearns M, Levy R, Madsen R, Martin J, McBurnie L, Meissner L, Meltzer T, Pawar V, Phelan M, Stinavage P, Vega SNH, Witchey-Lakshmanan L. Sterilizing filtration of liquids. Technical report 26. PDA J Sci Technol. 2008;62(S-5):2–60.
99. Tyagi A, Randolph T, Dong A, Maloney K, Hitscherich C, Carpenter J. IgG particle formation during filling pump operation: a case study of heterogeneous nucleation on stainless steel nanoparticles. J Pharm Sci. 2009;98(1):94–104.
100. Nayak A, Colandene J, Bradford V, Perkins M. Characterization of subvisible particle formation during the filling pump operation of a monoclonal antibody solution. J Pharm Sci. 2011;100(10):4198–204.
101. Rathore N, Rajan R. Current perspectives on stability of protein drug products during formulation, fill and finish operations. Biotechnol Prog. 2008;24(3):504–14.
102. Kerwin B, Remmele R Jr. Protect from light: photodegradation and protein biologics. J Pharm Sci. 2007;96(6):1468–79.
103. Qi P, Volkin D, Zhao H, Nedved M, Hughes R, Bass R, Yi S, Panek M, Wang D, Dalmonte P, Bond M. Characterization of the photodegradation of a human IgG1 monocloncal antibody formulated as a high-concentration liquid dosage form. J Pharm Sci. 2009;98(9):3117–30.
104. Sreedhara A, Yin J, Joyce M, Lau K, Wecksler A, Deperalta G, Yi L, Wang Y, Kabakoff B, Kishore R. Effect of ambient light on IgG1 monoclonal antibodies during drug product processing and development. Eur J Pharm Biopharm. 2016;100:38–46.
105. FDA. Guidance for industry—process validation: general principles and practices. Rockville, MD, 2011.
106. Shalaev EY, Wang W, Gatlin LA. Rational choice of excipients for use in lyophilized formulations in protein formulation and delivery. 2nd ed. Boca Raton: FL; 2008. p. 197–217.
107. Chang BS, Kendrick BS, Carpenter JF. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. J Pharm Sci. 1996;85:1325–30.
108. Jiang X-R, Song A, Bergelson S, Arroll T, Parekh B, May K, Chung S, Strouse R, Mire-Sluis A, Schenerman M. Advances in the assessment and control of the effector functions of therapeutic antibodies. Nat Rev Drug Disc. 2011;10:101–10.
109. Capon D. Designing CD4 immunoadhesins for AIDS therapy. Nature. 1989;525–31.
110. Aggarwal S. What's fueling the biotech engine—2012 to 2013. Nat Biotechnol. 2014;32 (1):32–9.
111. Mohler K, Torrance DS, Smith C, Goodwin R, Stremler K, Fung V, Madani H, Widmer M. Soluble tumor necrosis factor (TNF) receptors are effective therapeutic agents in lethal endotoxemial and function simultaneously as both TNF carriers and TNF agonists. J Immunol. 1993;151(3):1548–61.
112. Ducore JM, Miguelino MG, Powell JS. Alprolix (recombinant factor IX Fc fusion protein): extended half-life product for the prophylaxis and treatment of hemophilia B. Expert Rev Hematol. 2014;7(5):559–71.

113. Powell JS, Josephson NC, Quon D, Ragni MV, Cheng G, Li E, Jiang J, Li L, Dumont JA, Goyal J, Zhang X, Sommer J, McCue J, Barbetti M, Luk A, Pierce GF. Safety and prolonged activity of recombinant factor VIII Fc fusion protein in hemophilia A patients. Blood. 2012;119(13):3031–7.
114. Shapiro A. Development of long-acting recombinant FVIII and FIX Fc fusion proteins for the management of hemophilia. Expert Opin Biol Ther. 2013;13(9):1287–97.
115. Rath T, Baker K, Dumont J, Peters R, Jiang H, Qiao S-W, Lencer W, Pierce G, Blumberg R. Fc-fusion proteins and FcRn: structural insights for longer-lasting and more effective therapeutics. Crit Rev Biotechnol. 2013.
116. Wu B, Sun Y-N. Pharmacokinetics of peptide-Fc fusion proteins. J Pharm Sci. 2014;103:53–64.
117. Hermeling S, Crommelin D, Schellekens H, Jiskoot W. Structure-immunogenicity relationships of therapeutic proteins. Pharm Res. 2004;22(6):897–903.
118. Dintzis H, Dintzis R, Vogelstein B. Moleculare determinants of immunogenicity: the immunon model of immune response. Proc Natl Acad Sci USA. 1976;73(10):3671–5.
119. Shimamoto G, Gegg C, Boone T, Queva C. Peptibodies: a flexible alternative format to antibodies. mAbs. 2012;4(5):586–91.
120. Cines D, Yasothan U, Kirkpatrick P. Romiplostim. Nat Rev Drug Discov. 2008;7:887–8.
121. Molineux G, Newland A. Development of romiplostim for the treatment of patients with chronic immune thrombocytopenia: from bench to bedside. Br J Haematol. 2010;150(1):9–20.
122. Huang C. Receptor-Fc fusion therapeutics, traps, and MIMETIBODY technology. Curr Opin Biotechnol. 2009;20:692–9.
123. Lindzen M, Carvalho S, Starr A, Ben-Chetrit N, Pradeep C, Loestler W, Rabinkov A, Lavi S, Bacus S, Yarden Y. A recombinant decoy comprising EGFR and ErbB-4 inhibits tumor growth and metastasis. Oncogene. 2012;31:3505–15.
124. Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Burova E, Ioffe E, Huang T, Radziejewski C, Bailey K, Fandl JP, Daly T, Wiegand SJ, Yancopoulos GD, Rudge JS. VEGF-Trap: a VEGF blocker with potent antitumor effects. PNAS. 2002;99:11393–8.
125. Kim ES, Serum A, Huang J, Manley CA, McCrudden KW, Frischer JS, Soffer SZ, Ring L, New T, Zabski S, Rudge JS, Holash J, Yancopoulos GD, Kandel JJ, Yamashiro DJ. Potent VEGF blockade causes regression of coopted vessels in a model of neuroblastoma. PNAS. 2002;99:11399–404.
126. Kimchi-Sarfaty C, Schiller T, Hamasaki-Katagiri N, Khan M, Yanover C, Sauna Z. Building better drugs: developing and regulating engineered therapeutic proteins. Cell. 2013;34 (10):534–48.
127. Dumont J, Low S, Peters RT, Bitonti A. Monomeric Fc fusions: impact on pharmacokinetic and biological activity of protein therapeutics. Biodrugs. 2006;20(3):151–60.
128. Economides A, Carpenter L. Cytokine traps: multi-component, high-affinity blockers of cytokine action. Nat Med. 2003;9:47–52.
129. Nimmerjahn F, Ravetch J. Fcgamma receptors as regulators of immune responses. Nat Rev Immunol. 2008;8:34–47.
130. Jacobs C, Beckmann M, Mohler K, Maliszewski C, Fanslow W, Lynch D. Pharmacokinetic parameters and biodistribution of soluble cytokine receptors. Int Rev Exp Pathol. 1992;34B:123.
131. Yu H-K, Lee H-JAJ-H, Lim I-H, Moon J-H, Yoon Y, Yi L, Kim S, Kim J-S. Immunoglobulin Fc domain fusion to apolipoprotein(a) kringle V significantly prolongs plasma half-life without affecting its anti-angiogenic activity. Protein Eng Des Sel. 2013;26 (6):425–32.
132. Shiga Y, Oshima Y, Yoshinori K, Sugimoto A, Tamaki N, Murata D, Takeuchi T, Sato A. Recombinant human lactoferrin-Fc fusion with an improved plasma half-life. Eur J Pharm Sci. 2015;67:136–43.
133. Jazayeri JA, Carroll GJ. Fc-based cytokines. Biodrugs. 2008;22(1):11–26.

134. Valee S, Rakhe S, Reidy T, Walker S, Lu Q, Sakorafas P, Low S, Bitonti A. Pulmonary administration of interferon beta-1a-fc fusion protein in non-human primates using an immunoglobulin transport pathway. J Interferon Cytokine Res. 2012;32(4):178–84.
135. Dixon W, Hyrich K, Watson K, Lunt M, Galloway J, Ustianowski A, Symmons D. Drug-specific risk of tuberculosis in patients with rheumatoid arthritis treated witn anti-TNF therapy: results from the British Society for Rheumatology Biologics Register (BSRBR). Ann Rheum Dis. 2010;69(3):522–8.
136. Tubach F, Salmon D, Ravaud P, Allanore Y, Goupille P, Breban M, Pallot-Prades B, Pouplin S, Sacchi A, Chichemanian R, Bretagne S, Emilie D, Lemann M, Lorthololary O, Marriette X. Risk of tuberculosis is higher with anti-tumor necrosis factor monoclonal antibody therapy than with soluble tumor necrosis factor receptor therapy: the three-year prospective French Research Axed on Tolerance of Biotherapies registry. Arthritis Rheum. 2009;60(7):1884–94.
137. Hunt L, Emery P. Etanercept in the treatment of rheumatoid arthritis. Expert Opin Biol Ther. 2013;13(10):1441–50.
138. Peppel K, Crawford D, Beutler B. A tumor necrosis factor (TNF) receptor-IgG heavy chain chimeric protein as a bivalent antagonist of TNF activity. J Exp Med. 1991;174:1483–9.
139. Zhang J, Carter J, Siu S, O'Neill J, Gates A, Delaney J, Mehlin C. Fusion partners as a tool for the expression of difficult proteins in mammalian cells. Curr Pharm Biotechnol. 2010;11 (3):241–5.
140. Carter P. Introduction to current and future protein therapeutics: A protein engineering perspective. Exptl Cell Res. 2011;317:1261–9.
141. Kumagai Y, Fujita T, Ozaki M, Sahashi K, Ohkura M, Ohtsu T, Arai Y, Sonehara Y, Nichol JL. Pharmacodynamics and pharmacokinetics of AMG 531, a thrombopoiesis-stimulating peptibody, in healthy Japanese subjects; a randomized, placebo-controlled study. J Clin Pharmacol. 2007;47(12):1489–97.
142. Sathish J, Swaminathan S, Bielsky M-C, de Haan L, French N, Govindappa K, Green J, Griffiths C, Holgate S, Jones D, Kimber I, Moggs J, Naisbitt D, Pirmohamed M, Reichmann G, Sims J, Subramanyam M, Todd M, Van Der Laan J, Weaver RJ, Park BK. Challenges and approaches for the development of safer immunomodulatory biologics. Nat Rev Drug Disc. 2013;12:306–24.
143. Grinyo J. An integrated safety profile analysis of belatacept in kidney transplant recipients. Transplantation. 2010;90:1521–7.
144. Chen X, Zaro J, Shen W-C. Pharmacokinetics of recombinant bifunctional fusion proteins. Expert Opin Drug Metab Toxicol. 2012;8(5):581–95.
145. Strohl W. Optimization of Fc-mediated effector functions of monoclonal antibodies. Curr Opin Biotechnol. 2009;20:685–91.
146. Jefferis R. Glycosylation as a strategy to improve antibody-based therapeutics. Nat Rev Drug Discov. 2009;8:226–34.
147. Kaneko Y, Nimmerjahn F, Ravetch J. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. Science. 2006;313:670–3.
148. Stavenhagen J, Gorlatov S, Tuaillon N, Rankin C, Li H, Burke S, Huang L, Johnson S, Koenig S, Bonvini E. Enhancing the potency of therapeutic monoclonal antibodies via Fc optimization. Adv Enzyme Regul. 2008;48:152–64.
149. Shoji-Hosaka E, Kobayashi Y, Wakitani M, Uchida K, Niwa R, Nakamura K, Shitara K. Enhanced Fc-dependent cellular cytotoxicity of Fc fusion proteins derived from TNF receptor II and LFC-3 by fucose removal from Asn-linked oligosaccharides. J Biochem. 2006;140:777–83.
150. Matsuda K, Kubota T, Kaneko E, Iida S, Wakitani M, Kobayashi-Natsume Y, Kubota A, Shitara K, Nakamura K. Enhanced binding affinity for FcgammaRIIIa of fucose-negative antibody is sufficient to induce maximal antibody-dependent cellular cytotoxicity. Mol Immunol. 2007;44(12):3122–31.
151. Kellner C, Derer S, Valerius T, Peipp M. Boosting ADCC and CDC activity by Fc engineering and evaluation of antibody effector functions. Methods. 2014;65:105–13.

152. Houde D, Peng Y, Berkowitz S, Engen J. Post-translational modifications differentially affect IgG1 conformation and receptor binding. Mol Cell Proteomics. 2010;9:1716–28.
153. Nagashima H, Kaneko K, Yamanoi A, Motoi S, Konakahara S, Kohroki J, Masuho Y. TNF receptor II fusion protein with tandemly repeated Fc domains. J Biochem. 2011;149 (3):337–46.
154. Yeung Y, Leabman M, Marvin J, Qiu J, Adams C, Lien S, Starovasnik M, Lowman H. Engineering human IgG1 affinity to human neonatal Fc receptor: impact of affinity improvement on pharmacokinetics in primates. J Immunol. 2009;182:7663–71.
155. Zalevsky J, Chamberlain A, Horton H, Karki S, Leung I, Sproule T, Lazar G, Roopenian D, Desjarlais J. Enhanced antibody half-life improves in vivo activity. Nat Biotechnol. 2010;28 (2):157–9.
156. Czajkowsky D, Hu J, Shao Z, Pleass R. Fc-fusion proteins: new developments and future perspectives. EMBO Mol Med. 2012;4:1015–28.
157. Scallon B, Tam S, McCarthy S, Cai A, Raju T. Higher levels of sialylated Fc glycans in immunoglobulin G molecules can adversely impact functionality. Mol Immunol. 2007;44 (7):1524–34.
158. Davis P, Abraham R, Xu L, Nadler S, Suchard S. Abatacept binds to the Fc receptor CD64 but does not mediate complement-dependent cytotoxicity or antibody-dependent cellular cytotoxicity. J Rheum. 2007;34(11):2204–10.
159. Bruhns P, Iannascoli B, England P, Mancardi D, Fernandez N, Jorieux S, Daeron M. Specificity and affinity of human Fcgamma receptors and their polymorphic variants for human IgG subclasses. Blood. 2009;113:3716–25.
160. Wang Q, Chen K, Liu R, Zhao F, Gupta S, Zhang N, Prud'homme G. Novel GLP-1 fusion chimera as potent long acting GLP-1 receptor agonist. PLoS ONE. 2010;5:e12734.
161. Vafa O, Gilliland G, Brezski R, Strake B, Wilkinson T, Lacy E, Scallon B, Teplyakov A, Malia T, Strohl W. An engineered Fc variant of an IgG eliminates all immune effector functions via structural perturbations. Methods. 2014;65:114–26.
162. Lee J-H, Yeo J, Park HS, Sung G, Lee SH, Yang SH, Sung YC, Kang J-H, Park C-S. Biochemical characterization of a new recombinant TNF receptor-hyFc fusion protein expressed in CHO cells. Protein Expr Purif. 2013;87:17–26.
163. Ishino T, Wang M, Mosyak L, Tam A, Duan W, Svenson K, Joyce A, O'Hara D, Lin L, Somers W, Kriz R. Engineering a monomeric Fc domain modality by N-glycosylation for the half-life extension of biotherapeutics. J Biol Chem. 2013;288(23):16529–37.
164. Ying T, Chen W, Feng Y, Wang Y, Gong R, Dimitrov D. Engineered soluble monomeric IgG1 CH3 domain: generation, mechanisms of function, and implications for design of biological therapeutics. J Biol Chem. 2013;288(35):25154–64.
165. FDA.gov. Guidance for industry: immunogenicity assessment for therapeutic protein products. August 2014 [Online]. Available: http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm338856.pdf. Accessed 24 Mar 2015.

Chapter 20
Scientific Approaches for the Application of QbD Principles in Lyophilization Process Development

Vinay Radhakrishnan, Penny Davis and David Hiebert

Abstract Lyophilization (or freeze drying) is an important manufacturing capability for delivering high-quality parenteral drug products. Significant lyophilization process knowledge based on established scientific fundamentals and understanding of the good freeze drying practice principles exists within the pharmaceutical industry. This cumulative scientific knowledge should be leveraged across the portfolio of both small and large molecules and serves as the basis to gain process and product understanding. By using the scientific principles of lyophilization, good freeze drying practices and existing product knowledge, a risk-based approach to process development will facilitate focused efforts to increase product and process understanding to decrease those risks and identify which parameters are critical for product quality. Ultimately, this approach driven by scientific principles results in the development of a robust control strategy for a given product. The process whereby this occurs is based on principles of Quality by Design (QbD) as defined in ICH Q8 (R2) Pharmaceutical Development. This chapter brings together the technical knowledge of the lyophilization process, well-established scientific lyophilization principles and elements of QbD to provide guidance on approaches to develop a robust manufacturing process and support a regulatory filing strategy for drug products, while maximizing the freedom to operate. While it incorporates QbD elements that align with ICH Q8 (R2), Q9, Q10, and Q11, the goal is to define a knowledge space based on robust scientific evidence and principles. Having this knowledge and data will provide the basis to justify the control strategy and lyophilization process description in the dossier that presents the critical product quality attributes and a process control strategy that is defined by the critical process parameters to ensure consistent product quality.

V. Radhakrishnan (✉)
Pfizer Inc., 700 Chesterfield Parkway West, Chesterfield, MO 63017, USA
e-mail: vinay.radhakrishnan@pfizer.com

P. Davis
Pfizer Inc., 1 Burtt Road, Andover, MA 01810, USA

D. Hiebert
Pfizer Inc, 100 Route 206 North, Peapack, NJ 07977, USA

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_20

Keywords Lyophilization · Freeze-drying · Quality by Design (QbD) Control strategy · Risk assessment · Process development · Product temperature Quality control · Process parameter · Regulatory flexibility

20.1 Introduction

A traditional approach to regulatory strategy for the commercialization of products focuses on operating controls, end product testing, and process validation to demonstrate quality control of the manufacturing process. Within this approach, the control strategy and process description may inadvertently include information that is not critical for delivering quality of the drug product. In other words, if there is uncertainty regarding the importance of a quality attribute (QA) or process control, it will likely be interpreted as a required element of the control strategy subject to regulatory review and approval. This may result in overly constrictive regulatory commitments that are not based on scientific rationale or considered important/critical for delivering product quality.

Rather than testing quality into the product, an alternative approach emerged to ensure product quality using risk-based approaches and product and process understanding. This approach is referred to as Quality by Design (QbD) and was introduced by ICH Q8 (R2) "Pharmaceutical Development" [1]. QbD as defined by ICH Q8 (R2) is a systematic approach to development that begins with predefined objective and emphasizes product and process understanding and process control, based on sound science and quality risk management. Determining the interaction between input variables and process parameters in a multidimensional approach facilitates the creation of a robust process providing assurance of delivering expected product quality. In addition to a robust manufacturing process and quality assurance, additional benefits include improved lifecycle management of the product with regard to regulatory flexibility and quality risk management.

The objectives of this chapter are the following:

- Provide an overview of the science and risk-based approach to lyophilization (or freeze drying) process development and control strategy.
- Demonstrate application of principles of QbD and lyophilization scientific principles to process development and control strategy design.
- Discuss experimental design and studies which will provide process and product understanding to further evaluate the lyophilization process and control strategy.
- Discuss the benefits of applying QbD tools to lyophilization process development and control strategy.

20.2 Application of Quality by Design Principles to Lyophilization Development

Figure 20.1 illustrates the development of a drug product beginning with the quality requirements of the product to ensure efficacy and safety to the patient. This approach utilizes knowledge and scientifically based risk management approaches to design a robust lyophilization process and ending with a control strategy focusing on parameters and testing demonstrated to be critical for product quality. Defining a control strategy by those elements proven to be critical, it is anticipated that the regulatory flexibility will exist for non-critical parameters or within the range of success proven for the critical parameters.

20.3 Quality Target Product Profile

The Quality Target Product Profile (QTPP) is an outline of desired product characteristics and is used to design quality into the product and process [1]. There may be other non-quality related components of the QTPP that are needed for product development, such as cost of goods and number of strengths or presentations. While each product will have a unique profile, it is expected that lyophilized products will have the same or similar profiles. An example of elements to be included in a lyophilized product QTPP is provided below:

- Indication,
- Shelf life and storage condition,
- Duration/frequency of dosing,
- Route of administration,
- Dosage form,
- Dosage range,
- Strengths,
- Fill volume,
- Injection volume,
- Number of injection sites,
- Primary package (components—e.g., for a syringe include needle gauge, needle length),
- Cost of goods,
- Safety/local irritation.

Once the QTPP is established, initial product and process knowledge can be acquired using several QbD tools (see Fig. 20.1). Initially, there will be some degree of process and product understanding from which to build upon. Leveraging platform process operations and well-established scientific lyophilization principles may also contribute to that knowledge.

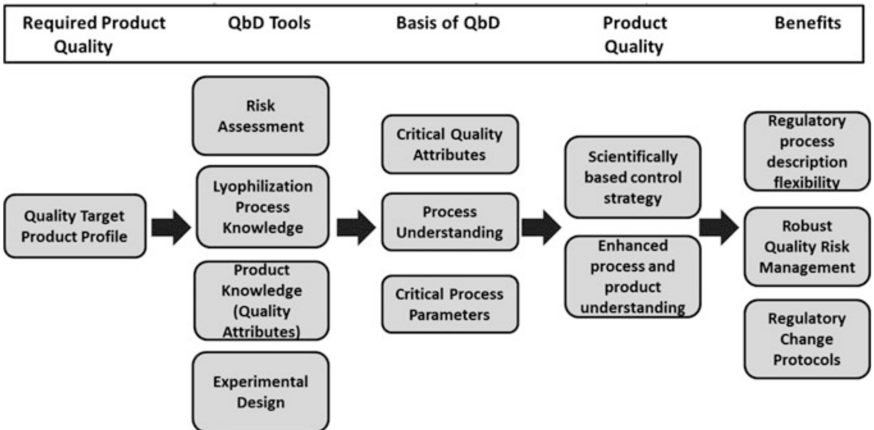


Fig. 20.1 Path to QbD during process development

20.4 Quality Attributes (Product Knowledge)

In addition to initial process knowledge, it is important to identify product QAs (quality attributes) based on molecule type, general lyophilized product attributes, compendial or monograph references, nonclinical and initial characterization studies [2–6]. Typical product quality attributes for a lyophilized product will include pH, appearance of cake and reconstituted solution, reconstitution time, residual moisture, particulate matter and sterility, among other QAs. Along with identification of the QAs, it is important to understand the relationship between QAs. For example, a certain amount of residual moisture in the lyophilized cake may lead to increased formation of product aggregates in biotherapeutics or degradation products in biotherapeutics and small molecule entities.

20.4.1 Critical Quality Attributes

Once QAs have been identified, their criticality must be determined in order to facilitate process development and identification of the process parameters which have impact on those QAs. A critical quality attribute (CQA) is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality. The following quality attributes are typically considered critical for a lyophilized drug product:

- Appearance of cake and reconstituted solution,
- pH,
- Reconstitution time,
- Residual moisture,
- Potency,
- Concentration or protein content for biotherapeutics,
- Particulate matter,
- Content uniformity,
- Product purity- and impurity-related quality attributes,
- Sterility,
- Endotoxin.

Determining the criticality of a QA may be performed using principles of risk assessments as outlined in ICH Q9, “Quality Risk Management.” Multiple risk assessment tools for determining CQAs can be used, such as risk ranking or preliminary hazards analysis. The use of a particular risk assessment tool may depend on the desire to assess certain factors as part of criticality such as uncertainty, severity, or probability of occurrence. When using the risk ranking tool, factors with assigned numerical values may include: uncertainty, severity of impact on efficacy, and functionality demonstrated in terms of efficacy, pharmacokinetics (PK), and safety. The following equation will provide a continuum of criticality for the list of product QAs [7]. Quality attributes having higher criticality scores will be considered critical based on the degree of impact on safety and/or efficacy and degree of uncertainty about the relationship to safety and/or efficacy.

$$\text{Criticality (Risk score)} = \text{Impact} \times \text{Uncertainty}$$

Variations of this equation may be developed to individually account for specific impact on safety, PK and efficacy. During early development, very little may be known about the relationship between QAs and PK, safety and efficacy, and thus, the uncertainty value for several QAs may be high. Throughout clinical development, information may be available based on platform or literature knowledge of the same molecule type (e.g., mAb). This information may be sufficient to perform an initial CQA assessment which will become an iterative process as further knowledge from clinical/nonclinical studies and additional analytical data becomes available, facilitating additional rounds of experiments. As knowledge is accumulated for QAs throughout development, it is expected that the criticality assessments should yield lower ranking for several QAs as a result of decreased uncertainty values and a higher degree of confidence about the impact of QAs to PK, safety, and/or efficacy.

20.5 Lyophilization Process Knowledge

Prior to reviewing lyophilization information, it is important to examine the formulation and container closure that can play a key role in process design. Platform formulations are typically used in the development of small and large molecules where, based on the molecule class and type, a standard set of excipients at defined concentrations are used to assess initial stability. Based on acceptable stability, these formulations can be advanced toward commercial product development. When the formulations are similar, there is a high probability of the physical and thermal properties of the formulations being similar, which provides an ability to leverage prior knowledge during lyophilization process development.

The container closure for lyophilized products can be vials with rubber closures, dual chamber vials (Acto-vials), or dual chamber syringes or cartridges. For this chapter, the presentation in vials with rubber closure is considered. The principles outlined below can be utilized for any other container closure as long as the same principles of heat and mass transfer are applied.

20.5.1 Process Overview

The process flow for lyophilization is provided in Fig. 20.2.

The following unit operations comprise the lyophilization process step: loading, freezing, annealing (if required), primary drying, secondary drying, and stoppering.

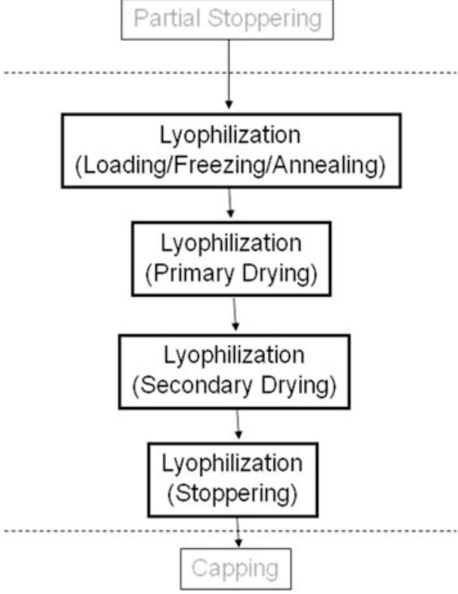


Fig. 20.2 Process flow for lyophilization process

The filled and partially stoppered vials are loaded onto the shelves in a freeze drier. The vials are frozen, and if needed, annealing step is applied. The frozen material is then subjected to primary drying in which the chamber is evacuated to a set pressure and the shelf temperature is increased to a set temperature and held for a period of time. The water is removed by direct conversion of ice to water vapor (process of sublimation). Once primary drying is completed, secondary drying is initiated where the shelf temperature is ramped to a higher temperature and held for a period of time, often under the same evacuated chamber pressure. Unfrozen water is typically removed during this step by desorption. Once secondary drying is completed, the shelf is cooled to ambient temperature and the chamber is aerated with sterile gas. At a certain chamber pressure (close to atmospheric pressure), the shelves are collapsed and the stoppers are fully seated in the vials. The chamber is further aerated to atmospheric pressure, and the vials are then unloaded from the chamber for capping.

A lyophilization cycle is developed where specific process parameters need to be established for the steps outlined below [8, 9]. This chapter will focus on the freezing, primary drying, and secondary drying lyophilization steps. Although annealing is not discussed, relevant concepts from this chapter can be applied to the annealing step of the lyophilization process. In order to define process parameters, it is important to understand the factors that ensure successful completion of each of these steps.

20.5.1.1 Freezing

The goal is to completely solidify the product, both the water and product fractions. Water solidifies as pure ice, and then, the product phase solidifies as a eutectic mixture of solids or more commonly as a glass. When the solute phase solidifies as a glass, there is a temperature-dependent transition from low to high viscosity as the temperature drops. The key time limit for successful freezing is the minimum duration of the freezing hold step which is intended to deliver complete solidification of both water and solute phase in all vials in the freeze dryer. There is no scientific rationale to specify or experimentally verify the maximum duration of freezing. Once the product is frozen, the product will remain in this solid state based on near-zero molecular mobility (on the time scale of the freeze drying process). The material science of the solidified product leads to the conclusion of negligible risk of long-term storage (several additional hours) of a frozen product at extremely cold temperatures.

20.5.1.2 Condenser Cooling Step

The goal of condenser cooling is to prepare the condenser surface to adsorb water vapor, to act as a trap for water, and to protect the vacuum pump(s) from water contamination. Essentially, the condenser serves as the water pump for the freeze

drying process. The general sequence entails cooling the condenser at the completion of the freezing hold step. Meanwhile, the shelf and product are maintained at the temperature achieved at the end of freezing, in order to keep the product in the frozen state. The duration of the condenser cooling step is not critical. The intent of this step is to reduce the temperature below a logical threshold, resulting in low vapor pressure of water in the condenser vessel. The vapor pressure of water at the ice-condenser interface is intended to be low relative to the vapor pressure of water at the ice interface in the product on the shelves, thereby establishing the pressure differential that ensures vapor flow into the condenser. One example might be to cool the condenser until the temperature is below −40 °C, where the equilibrium vapor pressure in the condenser vessel is 98 mTorr. As the condenser continues to cool, the vapor pressure will be reduced further. In this manner, the water in the environment of the condenser vessel is captured as solid ice onto the cold condenser surfaces. It is reasonable to assert that as long as the condenser is properly cooled, for example below −40 °C, then the condenser cooling is not a critical part of the description of the successful freeze drying process. Modern freeze drying equipment uses refrigeration system design to deliver this heat removal, and proper lyophilization cycle design selects heat input to the product to ensure a "cold" condenser throughout the process. This discourse assumes the condenser is properly cooled throughout the freeze drying process execution.

20.5.1.3 Evacuation Step

The next step in the logical sequence is to reduce the freeze dryer pressure. Once the condenser surface is prepared to act as the water trap (see condenser cooling step above), the control system initiates the evacuation step. The vacuum pump(s) are activated, generally given a brief period to "warm up," the valve isolating the chamber from the condenser is opened (for equipment designed with this feature), and the valve(s) separating the pumps from the condenser are opened. The gases in the chamber and condenser then flow toward the vacuum pump draw point, as the pumps perform the job of gas removal. As the gaseous contents of the freeze dryer vessel(s) naturally flow toward the vacuum draw point in the condenser, the majority of the water vapor in this gas mixture is extracted by condensing as ice on the cold condenser surfaces. The refrigeration system then removes the heat generated during this deposition and phase transition process. In this manner, the vacuum pumps are protected from water contamination and primarily are removing non-condensable gas components from the gaseous environment of the freeze dryer vessel(s). The logical endpoint of the evacuation step is the achievement of the pressure threshold required for the sublimation process. During the evacuation step, the shelf and product temperature are the same as the temperature achieved at the end of the freezing step, thereby maintaining the product in the fully frozen state. It is reasonable to assert that as long as the evacuation end point is achieved, the evacuation step is not a critical part of the description of the successful freeze drying process. This discourse assumes that the description of controlling the

chamber pressure at the proper recipe setpoint implies the success of the evacuation step.

The time required for the condenser cooling and evacuation steps is not critical, given that the product is maintained in a static state as a fully frozen solid material. Generally, the timescale of condenser cooling and evacuation is negligible relative to the time required for physical change of the fully solidified product.

20.5.1.4 Primary Drying

Once the chamber has been evacuated and the pressure threshold needed for sublimation is achieved, primary drying can begin. The goal is to completely sublime the frozen ice from the solid material. This is achieved by warming the frozen product under low pressure, well below the triple point for water. The timeframe for this step is based on the minimum duration of the primary drying hold time required to deliver complete sublimation of ice from all vials in the freeze dryer. The premise is execution of sublimation while maintaining the cake structure (product phase) delivered in the freezing step. There is no requirement to specify the maximum duration of primary drying. Once the ice in the product is sublimed, the product will remain in this partially dried state based on near-zero molecular mobility of the solid-state product phase at the primary drying hold temperature. The material science of the solidified and partially dried product leads to the conclusion of negligible risk of long-term exposure (several additional hours) of a partially dried product at intermediate heating conditions used in primary drying.

20.5.1.5 Secondary Drying

The goal during secondary drying is to remove the adsorbed/unfrozen water from the solid material. This is achieved by further warming the partially dried product under low pressure. The temperature for secondary drying is dependent on the maximum heat the product can withstand prior to degradation—the higher the temperature, the more efficient is the removal of the adsorbed moisture. The timeframe for successful secondary drying depends on the minimum duration of the secondary drying hold step, at a particular shelf temperature, to deliver sufficient desorption of bound water from the partially dried matrix for all vials in the freeze dryer such that they meet the moisture specification. The premise is the execution of desorption while maintaining the cake structure delivered in the freezing and primary drying steps. Generally, there is no requirement to specify the maximum duration of secondary drying from the water removal perspective. Once the bound water in the product is desorbed, the product will remain in this dried state based on near-zero molecular mobility and negligible chance of rehydration in normal process execution. The material science of the solidified and dried product leads to the conclusion of negligible risk of long-term storage (several additional hours) at aggressive heating conditions used in secondary drying as long as the product is

stable under these conditions. Prior knowledge obtained from product stability or pilot studies can be used to evaluate the impact of prolonged high heat input during secondary drying on potency or degradant formation.

20.6 Lyophilization Equipment Qualification

In the execution of the freeze drying process, the lyophilization equipment and associated control system regulate the timed sequence of two key process parameters: shelf temperature and chamber pressure. The lyophilization recipe is used by the control system to regulate the process. Generally, control systems deliver shelf temperature control against a measurement of shelf temperature considered representative of the actual shelf temperature. This representative shelf temperature is often measured by a resistance temperature detector (RTD): a temperature device inserted into a thermowell in the shelf manifold inlet piping. The measurement of the shelf inlet probe is then used as feedback into an algorithm that alternatively activates a heater or refrigeration to enable tight control against the setpoint. If the measured temperature is below the setpoint, then heat introduction is provided, if greater than the setpoint, then heat removal is provided. This results in a natural variation as the temperature oscillates around the setpoint. This variation is equipment-specific, is based on the heating and cooling algorithm, and defines the capability of shelf temperature control for the freeze dryer. This control then becomes the baseline expectation to be factored in the design space. Clearly, the ranges for shelf temperature control in the design space must respect this baseline control capability. The shelf temperature ranges established in the recipe will need to be wider than this control capability to encapsulate the equipment capability well within the proven success range of the process.

Similarly, the equipment controls chamber pressure based on the balance of sterile gas introduction against the constant removal of non-condensable gases by the vacuum pump, and the constant removal of water vapor by the condenser. The equipment and control system use an algorithm quite similar to shelf temperature control, generally using a capacitance manometer or nearly equivalent gauge mounted on the chamber vessel as the feedback measurement for the pressure control algorithm. The control is a bit more complex as there are two pumping systems—condensable gas pumping system and non-condensable gas pumping system. As part of the condensable gas, or water vapor pumping system, there are variations in both water vapor and heat removal by the cycling refrigeration system. As part of non-condensable gas pumping system, inert gas such as nitrogen can be introduced via a bleed valve to control total chamber pressure. The variation in chamber pressure control is often a bit wider than the shelf temperature control, and the oscillations may vary according to the trajectory of the freeze drying cycle, with tight control during some portions of drying, and larger variations at other times. This is a natural outcome of proportional–integral–derivative (PID) style control with its associated mathematical complexities. Nevertheless, the expected variations

established while drying actual product rather than empty chamber studies should be used as the baseline expectation for the demonstration of the proven acceptable range. The recommendation for encapsulating this natural variation well within the verified success zone applies for chamber pressure control similar to shelf temperature control.

20.6.1 Defining Success in Freeze-Drying

Successful freeze-drying is verified primarily by a detailed study of cake appearance and achievement of moisture targets. For a given experiment, freeze drying is considered successful if the intended cake appearance is achieved for the majority of the batch in the process execution and all CQAs are within specification. A prerequisite to the evaluation of successful freeze drying is agreement on the expected cake appearance prior to the execution of experiments. Often the agreed-upon cake appearance is documented in a "Defect Library" that provides a visual guidance for the range of possible cake appearance outcomes, from acceptable to failure, as an aid to the visual inspection process. The 100% visual inspection of lots is considered to be the most sensitive verification of the success of freeze drying. An alternative to the photographed defect library is a control group that contains the range of cake appearance in an organized presentation of vials, ranging from acceptable to samples that need to be rejected.

20.6.2 Lyophilization Cycle Development Based on Good Freeze-Drying Principles

The process is controlled using a lyophilization cycle that needs to be developed based on the product, container closure, and freeze dryer. The lyophilization cycle is initially defined following good freeze drying principles [10]. Tang and Pikal reviewed prior experience in freeze-drying of proteins and developed recommendations for each step of the lyophilization process: freezing, primary drying, and secondary drying [10]. Cooling rates during freezing, freezing temperature, and heating rates during secondary drying were chosen based on published recommendations. While these recommendations pertain to biotechnology-related products, these principles can be applied to any type of product including small molecules.

Based on extensive data, Tang and Pikal provided a general guidance on development of the lyophilization process based on scientific principles or good freeze drying practices (GFDP). Primary containers (e.g., vials) filled with drug product solutions are loaded onto the shelf that is cooled and maintained at 5 °C. After loading, the vials are allowed to come to 5 °C by holding for at least 15–30 min.

The shelf temperature was decreased at the rate of about 1 °C/min to below −40 °C (the temperature is selected to ensure that the solution is frozen). The vials are held below −40 °C for 1–2 h (longer time may be needed based on fill volume). If annealing is needed, the product was warmed to a product temperature of 10–20 °C above glass transition temperature in the frozen state (Tg') and held for 2–5 h [10]. This step is needed to crystallize excipients and/or for increased ice crystal size (due to 'Oswald ripening'). The shelf is then cooled to a final shelf temperature of approximately −50 °C at about 0.5 °C/min and held for 2–3 h (duration is based on fill volume) [10].

The condenser is then cooled (if the condenser refrigeration is separate from shelf refrigeration) to below −60 °C. The chamber is then evacuated. The vials are then heated by adjusting the shelf temperature (*T*s) and chamber pressure (CP). The temperature and pressure setpoints during primary drying need to be set based on the properties of the formulation such that product temperature (*T*p) needs to be below collapse temperature (*T*c) by approximately 2–5 °C. The chamber pressure (CP) is typically between 100 and 200 mTorr and can be estimated for the target product temperature [10]. The end of primary drying is defined when the CP by Pirani gauge is close to the CP by capacitance manometer. This is also defined when the *T*p reaches *T*s.

Once primary drying is complete, the shelf temperature is then increased to a setpoint for secondary drying. The heating ramp rate to the temperature for secondary drying needs to be 0.1–0.4 °C/min [10]. Typically, the temperature for secondary drying can be between 25 and 40 °C with no additional change to the CP. The hold time for secondary drying is normally 3–6 h, and the time length will typically define the final moisture content in the lyophilized product. Once secondary drying is complete, the shelf is cooled to 5 °C over a period of 3–4 h and held at that temperature for a short period (2 h). The chamber is then back-filled with filtered nitrogen to a suitable chamber pressure (typically between 700 and 900 mBar). At this pressure, the shelves are collapsed and the vials are stoppered. The lyophilizer is then equilibrated to atmospheric pressure with filtered nitrogen or air. Then, the vials are unloaded for capping.

These specific parameters represent a typical lyophilization cycle from the chapter authors' perspective and are not intended as recommendation for all lyophilized products.

20.7 Risk Assessments

Guidance from the FDA on ICH Q9 provides a systematic approach to quality risk management [2]. This guidance provides principles and examples of tools for quality risk management that can be applied to different aspects of pharmaceutical quality. These aspects can be applied to the lyophilization process. Risks to quality have been assessed and managed in a variety of ways. Below is a non-exhaustive list of some of these tools:

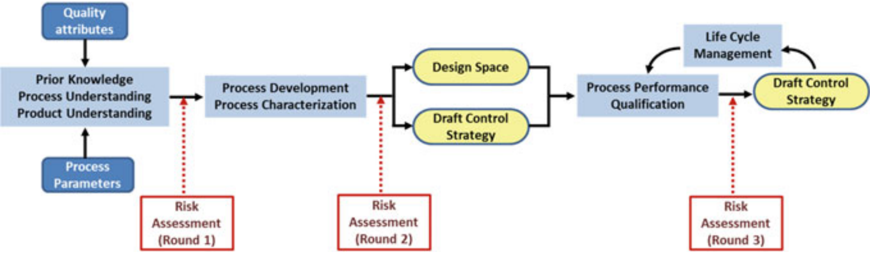


Fig. 20.3 Risk management approach

- Basic risk management facilitation methods (flowcharts, check sheets, etc.),
- Failure mode effects analysis (FMEA),
- Failure mode, effects, and criticality analysis (FMECA),
- Fault tree analysis (FTA),
- Hazard analysis and critical control points (HACCP),
- Hazard operability analysis (HAZOP),
- Preliminary hazard analysis (PHA),
- Risk ranking and filtering,
- Supporting statistical tools.

Risk assessments are performed at specific points in the product development progression toward commercialization. The typical points where these assessments are done are shown in Fig. 20.3.

20.7.1 Risk Assessment (Round 1)

This stage of risk assessment is done prior to process development and characterization. A tool that can be utilized is the cause and effect matrices (C&E) to help identify process parameters that impact quality attributes which will enable creating knowledge space.

The C&E matrix provides a mechanism to assess process parameters (inputs) against quality and process attributes (outputs) in order to prioritize parameters for experimental studies; however, it does not provide manufacturing control boundaries (process parameter ranges) to assess the potential severity impact. The goals of the C&E matrices are to capture the current and prior knowledge and the relationships between inputs and outputs, to prioritize areas for further study and experimental design, and to further understand the lyophilization process.

The key deliverable is the prioritization of potentially high-risk process parameters for designed process characterization experiments. As knowledge of the commercial manufacturing process and facility becomes available, facility control and procedural capabilities may also be evaluated, with failure modes and knowledge gaps identified. The quality attributes for the product should be

identified prior to the creation of a C&E matrix, and the criticality (based on initial information) needs to be defined based on its potential impact on product quality, safety, or efficacy. Each process parameter (input) is assessed based on the potential impact on the outputs of a particular focus area, including quality attributes or process performance attributes. The inputs (process parameters) can be parameters impacted by people, equipment, measurements, lyophilization process, materials, environment, etc., while the outputs are quality attributes such as aggregates, biopotency, endotoxins, contaminants, product degradants, step yield, etc. The objective is to establish the functional relationship between quality attribute and process parameters. The relationships that have the higher score are then assessed based on prior knowledge or new experiments.

Based on the cause and effect exercise for the lyophilization process step, the parameters and associated experiments that were considered important need to be assessed [11]. The important parameters are:

1. Chamber pressure during primary drying (Prim Drying CP),
2. Shelf temperature during primary drying (Prim Drying *T*s),
3. Hold time during primary drying (Prim Drying hold time(s)),
4. Shelf temperature during secondary drying (Sec Drying *T*s),
5. Hold time during secondary drying (Sec Drying hold time),
6. Ramp rate to secondary drying temperature setpoint (Ramp to Sec Drying),
7. Ramp rate to primary drying temperature setpoint (Ramp to Prim Drying *T*s).

20.7.2 Risk Assessment (Round 2)

The second round RA is typically conducted following process development and characterization studies and prior to process performance qualification (process validation) batch manufacture. The round 1 risk assessments need to be reassessed to ensure that the process parameter impact on quality attributes is accurate based on any new information obtained from the experiments performed. Based on manufacturing knowledge and other experiments, the typical and acceptable operating ranges are defined. Other outcomes from this round of risk assessment include process parameter risk identification/mitigation and potential parameter criticality classification. In addition, a draft control strategy is developed and evaluated during the risk assessment.

Failure mode effects analysis (FMEA) is conducted to evaluate the drug product manufacturing processes and the potential impact on process performance and product quality. FMEA is a tool for methodically evaluating, understanding, and documenting the potential risks to the process operation/consistency and product quality, i.e., “what can go wrong.” The FMEA is conducted by a multidisciplinary team comprised of process experts familiar with process development and characterization, and manufacturing site representatives with expertise in manufacturing operations, manufacturing procedures, equipment capabilities and controls.

FMEA can be employed throughout the product commercialization stages using an iterative approach such that the initial FMEA template is developed and refined with improved process knowledge and greater understanding of the manufacturing capabilities.

The first stage of the FMEA is to assign process parameter severity (*S*) scoring based on the potential impact of the parameter on quality attributes and process performance (Fig. 20.4). The scoring for occurrence (*O*) should focus on the likelihood of deviating beyond the specified typical operating range or setpoint/target for the process parameter assessed. When considering occurrence, it is important to focus on common rather than extraordinary failure events. Unexpected events (e.g., force majeure) are generally not considered. Other occurrence considerations may include prior knowledge, manufacturing history, equipment failure, and human error and should be described in the FMEA worksheet. The final stage of the FMEA is an assessment of detectability (*D*) for identifying a potential deviation beyond the specified typical operating range or setpoint/target. The scoring range is consistent with scores assigned for severity and occurrence. A final risk priority number (RPN) number is assigned based on multiplying the scores for severity, occurrence, and detection ($S \times O \times D$) with appropriate rationales for each process parameter described. During the FMEA assessment, risk control or mitigation strategies are discussed and planned for implementation where appropriate. The RPN numbers for the unit operation are reviewed collectively, and a

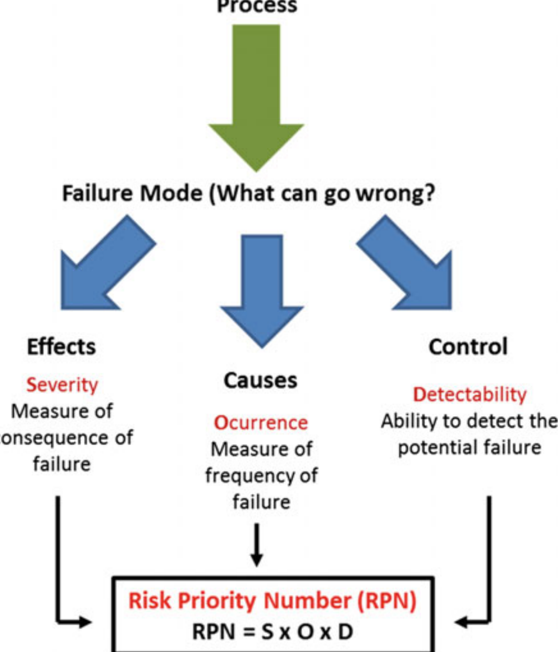


Fig. 20.4 Failure mode effects analysis

cutoff number (threshold) may be selected based on the data distribution to aid the selection of parameters for risk mitigation and/or criticality. The impact of severity on the process and product is dependent on the freeze drying that enables successful removal of moisture from the product without impacting product quality.

20.7.3 Risk Assessment (Round 3)

During this round of risk assessment, the data from the process performance qualification batches (process validation) are assessed and the C&E and FMEAs are reviewed to ensure that the process knowledge is accurately represented. Based on this understanding, the critical process parameters and critical quality attributes are confirmed and a final control strategy is defined. This round 3 of the risk assessment is performed to verify that the process understanding developed through different stages of development fully supports the progression of the process to commercial manufacturing and no severe/impactful risks are identified.

Leveraging all product and process knowledge and experience along with the output of risk assessments is critical to facilitate focus on the right parameters. It is impractical to conduct studies which account for all variations of process parameters (described in Sect. 7.1). For example, a lyophilization process risk assessment will result in multiple areas for further assessment and understanding. These are likely to include ramp rates, shelf temperature, and hold time for freezing, annealing temperature, annealing time, primary and secondary drying chamber pressure, primary and secondary drying shelf temperature, primary and secondary drying hold times, chamber pressure for stoppering, and primary and secondary drying ramp rates to shelf temperature setpoints. Assuming three levels of change (low, high, and target) of these parameters, a full factorial analysis would require 1000 or more experiments. A far more feasible approach involves application of lyophilization principles, product manufacturing experience, manufacturing knowledge of similar products, and in silico modeling to allow product understanding such that the number of experiments needed to address remaining uncertainty is manageable. It will also facilitate focus on select parameters which require manufacturing flexibility and development of the knowledge space to support that flexibility.

In silico modeling is essentially using “non-experimental models and methods” to build understanding regarding the outcome of freeze drying. One such approach utilizes mathematical-based predictions regarding the time trajectory of freeze drying, whereas the current practice is generally limited to predictions regarding the outcome of primary drying or sublimation. The concept uses a set of basic initial experiments to empirically derive measures of heat transfer characteristics of the lyophilization equipment, components, and product, and the mass transfer characteristics of the product, along with the performance limitations of the freeze dryer [12–14]. For example, the minimum controllable pressure of the equipment is determined using water-based experiments to establish the maximum water

transport through the equipment. Pilot lyophilization cycles use product temperature data profiles to ascertain understanding of cake-resistance by deriving product-specific mass transfer coefficients. Commercial-scale experiments determine heat transfer coefficients for the product-specific vials in the actual freeze drying equipment or equivalent. Then, these data are fit to heat and mass transfer equations (first-, second-, and third-order curve fitting) from basic physics principles to arrive at a mathematical relationship that equates the heat transfer to the product to the mass transfer from the product at steady state. The result is the ability to predict the time course of freeze drying according to hypothetical conditions with strong linkage to the actual experience, data, and performance of the product, the vial, and the target commercial freeze dryer. These numerical analyses then serve as a means of elaborating the design space efficiently and effectively and provide an understanding of freeze drying beyond the laboratory and commercial experiences.

Ultimately, this knowledge of quality and process criticality can be incorporated into the regulatory dossier to justify criticality of process parameters and overall control strategy in the process description to deliver consistent product quality. A number of tools are available for process monitoring which can be utilized in developing a robust process [15–17]. This knowledge will also drive a process description that delivers manufacturing and regulatory flexibility without compromising quality.

20.8 Lyophilization Process Development and Optimization

Using the GFDPs (detailed in Sect. 6.2), the lyophilization cycle needs to be developed and optimized. During this development process, some additional characterization tools can be utilized to understand the thermal behavior of the frozen liquid (e.g., freeze drying microscopy, differential scanning calorimetry). In addition, these tools can be utilized to obtain a deeper understanding of product quality attributes during cycle development. Further, these tools can be utilized to understand product quality at intended storage and accelerated stability conditions.

The optimized cycle will be defined based on experimental runs, in silico modeling, and prior knowledge. In addition, risk assessments can be performed to identify and understand the relationship and criticality of quality attributes and process parameters. For lyophilization, the process parameters of each step that can typically impact the process have been identified and are provided in Table 20.1. There are several risk assessment tools available which will facilitate the identification of relationships between quality attributes and process parameters. If uncertainty exists through this risk assessment, the next critical step is determining how to address that uncertainty, whether through assessing literature-based information or performing small-scale experiments.

Table 20.1 Process parameters for each lyophilization step

Step	Parameter
Freezing	Shelf temperature during loading
	Freezing ramp rate
	Freezing temperature
	Hold time for freezing
Primary drying	Chamber pressure during primary drying
	Ramp rate to primary drying hold
	Primary drying temperature
	Hold time for primary drying
Secondary drying	Ramp rate to secondary drying hold
	Secondary drying temperature
	Hold time for secondary drying

A description of the lyophilization cycle will be included in the regulatory dossier with appropriate operating ranges. The ranges will be defined based on the experience from optimization experiments as well as robustness experiments, performed at small scale. Once the cycle is defined and optimized, the cycle can be scaled up and tested at commercial scale.

For the purposes of the discussion below, an example optimized cycle is provided in Table 20.2. This cycle is a hypothetical example and is being used to illustrate linkage to an example of the regulatory description.

Using this optimized lyophilization cycle, each step involving parameters to be considered for quality impact on product is discussed below with guidance about data or knowledge that may be used to support the control strategy and regulatory process description.

Table 20.2 Example of an optimized lyophilization cycle

Step	Process parameters		Step time (min)	Cumulative time (h)
	Shelf temperature (ramp/hold)	Pressure (mT)		
Freezing	Load at 5 °C		n/a	–
	Hold at 5 °C		30	0.5
	Ramp to −50 °C		110	2.33
	Hold at −50 °C		120	4.33
Chamber evacuation	Hold at −50 °C	100	60	5.33
Primary drying	Ramp to +20 °C	100	140	7.66
	Hold at +20 °C	100	1440	31.66
Secondary drying	Ramp to +50 °C	100	150	34.16
	Hold at +50 °C	100	360	40.16
Post lyo	Ramp to 5 °C	100	120	42.16
	Hold at 5 °C	100	n/a	

20.8.1 Freezing

The parameters to be examined for freezing are:

(1) Load temperature,
(2) Freezing ramp,
(3) Freezing temperature,
(4) Hold time.

20.8.1.1 Load Temperature

The load temperature can be defined based on stability data. Assuming that the product has solution data available at 5, 25, and 40 °C, and these have been found to lead to acceptable quality for up to 1–2 weeks (no significant change in QA), then load temperature does not have to be defined. If the product is not stable, then the load temperature specification based on stability data may be needed. If needed, the load temperature is required in the process description. In the case considered for this document, the authors are assuming that there is sufficient appropriate stability data to not include the load temperature in the process description.

20.8.1.2 Freezing Ramp

The freezing ramp is assessed using different ramp rates: 0.1, 0.5, and 1.0 °C/min, assuming that the 0.5 °C/min ramp is likely to be executed within the base case cycle. The non-freeze-ramp portion of the process uses the baseline cycle parameters to assess product quality. Typically, the product quality attributes (QAs) evaluated are appearance, moisture, particulates, and reconstitution time. This is dependent on existing data such as freeze/thaw studies, as well as other experimental or manufacturing data.

20.8.1.3 Freezing Hold Shelf Temperature

The freezing hold temperature can be assessed with freezing setpoints at −50, −40, and −55 °C. The product from these cycles needs to be evaluated for quality attributes as mentioned above and, if needed, placed in a short-term stability study (6–9 months of accelerated stability at 40 or 50 °C).

20.8.1.4 Freezing Hold Duration

The hold time at the end of freeze can be assessed with freezing setpoint at −50 °C and 2 and 3 h duration. The freeze hold duration higher than 3 h need not be tested; if all product is frozen at 3 h, there are no additional changes expected. The product from these cycles needs to be evaluated for quality attributes as mentioned above and, if needed, placed in a short-term stability study (6–9 months of accelerated stability at 40 or 50 °C).

Note: Annealing is needed when the formulation contains excipients that need to be crystallized prior to primary drying or if the annealing step adds efficiency to the process (for instance, the ability to freeze dry under more aggressive conditions due to reduced product resistance). If annealing step is needed, then annealing temperature and time need to be assessed as a two-factor DOE study. Ramps can be defined based on the data generated for freezing ramps.

20.8.1.5 Freezing Operating Space (Proven Acceptable Range)

The above-mentioned experiments provide proven acceptable parameter ranges for freezing:

- Shelf temperature control of −40 to −55 °C,
- and freezing hold duration of no less than the highest risk (worst case) cycle, 2 h.

20.8.2 Condenser Cooling and Evacuation

Typically, condenser temperature is not considered a critical process parameter (as long as it is below −40 °C). Chamber pressure control throughout drying is typically used to verify that the refrigeration system performance is sufficient.

The condenser will be typically maintained below a nominal threshold of −40 °C throughout the drying process. This limit may be as high as −30 °C based on the freeze dryer capability (appropriate pressure control) and the process being used. The duration of condenser cooling and evacuation is not critical to the success of freeze drying, so cooling and evacuation are not generally timed steps in the control systems at commercial and pilot scale. Recipe specifications for condenser cooling or evacuation are not for determination of process success or failure (unless unusually slow cooling rates indicate insufficient refrigeration of condenser system due to leaks, etc.), are not intended for the process description in the registration, and are only used to allow the equipment to transition from the freezing to primary drying steps.

The chamber evacuation duration to reach the setpoint for pressure is based on the characteristics of the freeze dryer and the properties of the formulation filled in the container closure. In the hypothetical recipe used in this document, the chamber

pressure control point was selected to be 100 mT, a pressure control point representative of many freeze dried products. The ranges for pressure control in freeze drying will be defined in the primary drying section.

20.8.3 Primary Drying

The parameters to be examined for primary drying are:

(1) Chamber pressure,
(2) Ramp rate to primary drying hold,
(3) Primary drying temperature,
(4) Hold time for primary drying.

20.8.3.1 Primary Drying Ramp

Based on prior knowledge for similar formulations (similar thermal characteristics), fill volume and container closure, if a ramp rate can be defined that will ensure success, then additional evaluation of ramp rate is not necessary as long as the freeze dryer is capable of withstanding initially high sublimation rates without loss of pressure control. If this information is not available, the ramp rate for primary drying is assessed using different ramp rates: 0.1, 0.5, and 1.0 °C/min, assuming that the 0.5 °C/min ramp is likely to be executed within the base case cycle.

The product from these cycles needs to be evaluated for quality attributes as mentioned above and, if needed, placed in a short-term stability study (6–9 months of accelerated stability at 40 or 50 °C).

20.8.3.2 Primary Drying Process Control (Primary Drying Shelf Temperature and Chamber Pressure)

The process parameters that impact primary drying are shelf temperature and chamber pressure. To understand the impact of these parameters on product quality, the most aggressive condition would be the combination of highest chamber pressure and shelf temperature, and the least aggressive condition would be the combination of lowest chamber pressure and shelf temperature. The extent of the ranges to be studied is based on existing data and process knowledge. The ranges need to be defined such that these are broad enough to allow for process variation without negatively impacting product quality.

The impact on the primary drying process can be evaluated by using the baseline primary drying ramp rate and the range of shelf temperature +35, +20, and +5 °C (where +20 °C shelf temperature setpoint is likely to be executed with the base case cycle) and chamber pressure control setpoints at 150, 100, and 50 mT (where the

100 mT chamber pressure setpoint is likely to be executed with the base case cycle) for sufficient duration of time. The range of temperature (±15 °C) and pressure (±50 mT) needs to be defined based on prior experience or knowledge. These ranges are provided as an example and are product dependent. The cycles with the higher end of temperature and pressure are denoted as +, + cycle, and the cycles with the lower end of temperature and pressure are denoted as −, − cycle in this chapter (+, + and −, − convention are indicated in Table 20.3).

Table 20.3 Table of proposed experiments

Process step	High-risk test	Low-risk test	Comments
Base case	Experiment 1 Base case cycle		Start with optimized cycle
Freezing			
Freezing ramp	Experiment 2 High rate	Experiment 3 Low rate	Remaining process parameters as base case cycle
Freezing temperature	Experiment 4 High temp (+, +)	Experiment 5 Low Temp (−, −)	Heat removal is directly related to the difference between product temperature and shelf temperature
Freezing hold	Experiment 6 Low duration		Heat removal is directly related to the freezing hold step
Primary drying			
Primary drying ramp	Experiment 4 High rate	Experiment 5 Low rate	As part of +, + and −, − cycle
Primary drying pressure	Experiment 4 High pressure (+, +)	Experiment 5 Low pressure (−, −)	Heat transfer is directly related to the chamber pressure
Primary drying hold temperature	Experiment 4 High Temp (+, +)	Experiment 5 Low temp (−, −)	Heat transfer is directly related to the shelf temperature
Primary drying duration	See comment for this parameter		Based on cycle optimization and PAT sublimation endpoint info (Pirani)
Secondary drying			
Secondary drying ramp	Experiment 4 High rate	Experiment 5 Low rate	As part of +, + and −, − cycle
Secondary drying temperature	Experiment 4 High temp (+, +)	Experiment 5 Low temp (−, −)	Pressure will be covered though it has no impact on sec drying
Secondary drying duration	Experiment 4 High temp (+, +) Base case cycle	Experiment 5 Low temp (−, −)	Assess the moisture level to optimize hold time—define hold time with available data
Worse case moisture	Experiment 7 Base cycle without secondary drying with sampling during ramp		Worst case moisture and impact on stability

The low ramp rate for primary drying is not studied as this is not expected to impact product quality. Hence, this conservative approach has not been considered by the authors.

The duration of primary drying is determined using suitable endpoint determination techniques such as:

(1) Product temperature (when the product temperature is near or equal to shelf temperature),
(2) Comparative chamber pressure measurement using both Pirani gauge and capacitance manometer,
(3) Pressure rise test.

The duration of primary drying is described to be not less than (NLT) the duration established for the end of primary drying for the highest risk cycle (+, + cycle).

20.8.3.3 Primary Drying Operating Space (Proven Acceptable Range)

The above-mentioned experiments provide primary drying proven acceptable parameter ranges:

- Shelf temperature control of +5 to +35 °C,
- Chamber pressure control of 50 to 150 mT,
- Primary drying hold duration of no less than the highest risk cycle (+, + cycle), duration determined by end point testing.

20.8.4 Secondary Drying

The parameters to be examined for secondary drying are:

(1) Ramp rate to secondary drying hold,
(2) Secondary drying temperature,
(3) Hold time for secondary drying.

The science of freeze drying accepts the conclusion that pressure control is not critical during secondary drying (as long as pressure is controlled by nitrogen injection rather than a valve between condenser and vacuum pump). The approach recommended in these experiments is to use the chamber pressure setpoint selected in primary drying for the secondary drying unit operation for each experiment. This will give a range of pressures tested in secondary drying equivalent to the range tested in primary drying.

20.8.4.1 Secondary Drying Ramp

The ramp rate for secondary drying is assessed using different ramp rates: 0.1, 0.2, and 1.0 °C/min, assuming that the 0.2 °C/min ramp is likely to be executed within the base case cycle.

20.8.4.2 Secondary Drying Process Control (Secondary Drying Shelf Temperature and Chamber Pressure)

The process parameters that impact secondary drying are shelf temperature and time for secondary drying. To understand the impact of these parameters on product quality, the most aggressive condition would be the highest shelf temperature and the least aggressive condition would be the lowest shelf temperature. Chamber pressure does not have an impact on the secondary drying process (as long as pressure is controlled by nitrogen injection rather than a valve between condenser and vacuum pump). The duration is assessed based on the shelf temperature.

The impact on the secondary drying process can be evaluated by using the baseline secondary drying ramp rate and the range of shelf temperatures +35, +50, and +65 °C (where +50 °C shelf temperature setpoint is likely to be executed with the base case cycle) and chamber pressure control setpoints at 150, 100, and 50 mT (where the 100 mT chamber pressure setpoint is likely to be executed with the base case cycle) for sufficient duration of time. The range of temperature (±15 °C) needs to be defined based on prior experience or knowledge. The range of pressure is not expected to have an impact on the process.

The duration needs to be defined based on prior knowledge and can be as long as 6–10 h. The duration of secondary drying is determined by sampling during the secondary drying hold and assessing cake appearance and moisture. The duration is defined based on the time when acceptable cake moisture is achieved. The time can be set as no less than (NLT) the duration seen for cycle which has the highest secondary drying temperature.

In the absence of the equipment for sampling during secondary drying, 2–3 cycles can be run at baseline cycle and +, + cycle condition and cycle can be stopped at 4, 6, 8 h and assessed for cake appearance and moisture. The time can be defined based on the duration where acceptable moisture level and cake appearance are achieved.

The product from these cycles (baseline, +, + and −, − cycles) needs to evaluated for quality attributes as mentioned above. In addition, these samples need to be placed in a long-term stability study at storage conditions [alternatively, a shorter-term study at accelerated conditions (40 or 50 °C)].

20.8.4.3 Secondary Drying Operating Space (Proven Acceptable Range)

The above-mentioned experiments provide secondary drying proven acceptable parameter ranges:

- Shelf temperature control of +35 to +65 °C,
- Chamber pressure control of 50 to 150 mT,
- Secondary drying hold duration of no less than the highest risk cycle (+, + cycle) duration.

20.8.5 Other—Moisture Impact on Quality Attribute

One of the quality attributes (QA) that is known to impact product quality is cake moisture. This QA is typically included in product specifications and needs to be justified and is typical considered as a CQA. This CQA is expected to have maximum impact on other CQAs (e.g., aggregation, hydrolysis). To enable understanding with data, samples with varying levels of moisture are generated. These samples can be generated by sampling during a cycle run using the baseline cycle at end of primary drying, at two points during the ramp up to the secondary drying hold step, and at the end of the ramp to secondary drying hold. Alternately, freeze dried samples can be equilibrated to higher moisture in a dry box or dessicator containing salt solutions. The resulting moisture needs to encompass the moisture range typically seen and needed for the specification—e.g., if typical moisture levels are seen at 0.5% and the specification is NMT 2.0%, then the samples to be tested need to have moisture levels ranging from 0.5 to 2.0% and if possible to 2.5 and 3%.

The product from these different moisture levels needs to be evaluated for quality attributes as mentioned above. In addition, these samples need to be placed in a long-term stability study at storage conditions [or alternatively, a shorter-term study at accelerated conditions (+40 or +50 °C)].

20.8.6 Summary of Studies and Process Description (Table 20.3)

Based on the discussion above, the experiments that are needed to support a scientifically well-characterized lyophilization process are provided in Table 20.3.

These seven cycles can be performed, and the quality attributes of the resulting samples can be assessed to evaluate product quality. In addition, these samples can be placed in a stability study, and the product quality can be further assessed as a function of storage condition as well as accelerated conditions.

20.9 Process Understanding—Defining CPPs and CQAs and Control Strategy

ICH Q8 (R2) provides the following definition for critical process parameter (CPP):

> A process parameter whose variability has an impact on a critical quality attribute and, therefore, should be monitored or controlled to ensure the process produces the desired quality [1].

Based on the definition for CPP, a change or variability in the parameter has an impact on a CQA. While the extent of impact is not defined, CPPs should be separated from other PPs into those that have substantial impact on the CQAs. Identification of these CPPs can be performed using the risk assessment stated earlier, and sometimes repeated iterations of the risk assessments may be necessary. As knowledge increases or as improvements are made to a process throughout the product lifecycle, risks may be reduced and the level of impact for a CPP can be modified and control strategies adjusted accordingly.

The control strategy will include a combination of documentation control, process automation and control as well as using process analytical tools. Documentation control includes control of the process provided in the batch record and verification that the validated parameters have been programmed for the lyophilization cycle. Process automation will include stepping through a sequence of steps, requiring logical completion of each step endpoint in order to proceed to the next step. Additional elements for process automation include control of the process clock and execution of a hold step, control of condenser cooling and evacuation, shelf temperature and pressure control algorithm, control of temperature ramp step (temperature and timing control). Process analytical tools provide ability to confirm completion of a process step by using equipment indicators. For example, the completion of primary drying can be determined with a drop in pressure shown by the Pirani gauge and equalization to the value shown by the capacitance manometer. In addition, the primary drying endpoint can be determined by successful pressure rise test measurement. Such tools can be employed to show completion of the process step and progression to the next step in the sequence.

20.10 Benefits of Applying QbD Principles to Drug Product Lyophilization

Application of QbD principles to lyophilization process development allows enhanced process understanding and control strategy design and thus results in the manufacture of a drug product with greater quality assurance. Understanding critical quality attributes and the critical process parameters controlling those attributes during manufacture will also contribute to management of product quality through the lifecycle of the product. Additional benefits of applying QbD principles to lyophilization process development are provided below.

20.10.1 Regulatory Flexibility and Process Description

The ultimate goal of QbD application is the creation of a design space, defined in ICH Q8(R2) as a multidimensional combination and interaction of input variables and process parameters that have been demonstrated to provide assurance of quality such that working within the design space is not considered as a change. Thus, a design space if approved by the Health Authority creates regulatory flexibility by allowing changes within that design space that would otherwise result in a post-approval submission to the commercial license application.

Enhanced process understanding and control through the application of QbD principles may also lead to potential regulatory flexibility without claiming to be a design space. In the commercial application, the process description as presented in Section 3.2.P.3.3 Description of Manufacturing Process and Process Controls and critical process parameters as presented in Section 3.2.P.3.4 Controls of Critical Steps and Intermediates provide the necessary process steps and critical process parameters to manufacture quality product. Changes to the information presented in these sections will result in a post-approval supplement to the application or license requiring Health Authority review and approval which will require financial and people resources to manage this change throughout all applicable markets. Depending on the degree of change, number of markets where the application is approved and Health Authority review and approval timelines, it may take years before a change can be fully implemented globally. Even if the sponsor does not choose to claim a design space in the application, regulatory flexibility may be achieved by presenting a process description and process control strategy that is based on scientific principles, product and process knowledge and full understanding of the parameters that are critical to ensure product quality.

With the knowledge gained through application of scientific principles, risk assessments and process understanding studies (lyophilization experiments), critical process parameters, and established ranges are identified. Based on these ranges, regulatory process description for lyophilization can be provided. An example is provided below:

1. Freezing—Load the filled vials and freeze the product by adjusting the shelf temperature to not more than −40 °C for a duration not less than 2 h.
2. Cool the condenser (below −40 °C) and evacuate the chamber to not more than 150 microns throughout primary drying.
3. Primary Drying—Sublime the ice by adjusting the shelf temperature to not more than +35 °C while maintaining chamber pressure below 150 microns for a duration not less than 18 h [determined by end point detection (Sect. 20.8.3.2)].
4. Secondary Drying—Desorb bound water by adjusting the shelf temperature between +35 and +65 °C for a duration not less than 5 h [determined by sampling (Sect. 20.8.4.2)]

Table 20.4 Typical list of critical process parameters and control limits for lyophilization

Lyophilization step	Parameter	Control limits
Freezing	Freezing temperature of shelf	Not more than −40 °C
Primary drying	Chamber pressure	Below 150 microns
Primary drying	Shelf temperature	Not more than +35 °C
Primary drying	Hold time	Not less than 18 h
Secondary drying	Shelf temperature	Between 35 and 65 °C
Secondary drying	Hold time	Not less than 5 h

5. Stoppering—At the completion of drying, aerate the chamber with sterile filtered nitrogen and fully insert the stoppers into the vials by collapsing the shelves.
6. Unload the stoppered vials and move the vials to the capping area.

The critical process parameters may be presented in Section 3.2.P.3.4 as indicated in Table 20.4.

The lyophilization process description in the regulatory dossier should be supported by the development and robustness studies and presented in Section 3.2.P.2. Pharmaceutical Development. A synopsis of the above studies and data should be presented in Section 3.2.P.2. Pharmaceutical Development to underwrite the identification of critical process parameters and the control strategy of those parameters. Additionally, it is important to present information from studies justifying why a process parameter is not considered critical and thus not included in the process description and control strategy. It is recommended to use a synopsis of the above sections which presents the design, rationale, and data from these studies.

The regulatory flexibility to process controls and control strategy in the commercial application may be realized in various ways. Identifying and scientifically establishing parameters as non-critical in the lyophilization process may facilitate flexibility in what information is included in the commitment sections of the application or license, such as identifying the parameter but not the proven acceptable range. Additionally, enhanced understanding may support a wider proven acceptable range for select critical parameters. In the case of lyophilization as provided in the example above, parameters may not require a range but an upper or lower limit of that parameter to ensure robust lyophilization and product quality.

20.10.2 Regulatory Change Management Plans and Protocols

During the commercial lifecycle management of a product, some types of CMC changes will require Health Authority approval prior to implementing. These may be known as established conditions as defined in the current draft ICH Q12 guidance (Technical and Regulatory Considerations for Pharmaceutical Lifecycle

Management). As part of the regulatory assessment for a CMC change, the potential quality impact on product and how to support a CMC change to ensure product quality post-change is a critical consideration to form the basis of that assessment. Enhanced product and process understanding will offer an advantage to assessing CMC changes during the lifecycle and may facilitate the development of change management plans which identify future changes and contain protocols to support those plans.

Change protocol management is a regulatory pathway that exists in select regions (e.g., USA and European Union). This concept is also incorporated in the draft ICH Q12 guidance and builds upon the QbD principles and risk management approaches presented in ICH Q8 (Pharmaceutical Development) and ICH Q9 (Quality Risk Management) [1, 2, 18]. The intent of the protocol for a particular CMC change is to outline the studies, tests, and acceptance criteria to demonstrate lack of impact on product quality following the CMC change and gain early agreement with Health Authorities as to how to support that CMC change. The protocol may be filed in the initial commercial application or during the commercial lifecycle of the product. If the protocol is approved by the Health Authority, the data resulting from the protocol may be submitted as a lower reporting category. Filing the data as a lower category may result in shorter timelines of approval and enable faster implementation of that change for the product.

In the case of a lyophilization process, a change management protocol may be proposed to support additional lyophilizers to be used within the facility if greater manufacturing capacity is needed in the future. An enhanced understanding of the lyophilization critical parameters should aid in establishing equivalency between lyophilizers with appropriate validation and analytical studies to demonstrate acceptable process control and product quality.

20.10.3 Product Quality Management

Throughout the product lifecycle, quality must be maintained to ensure provision of a safe and efficacious drug to patients. A robust quality assurance (QA) program should be in place to ensure product quality through compliance with good manufacturing practices (GMP) and manage quality issues if they occur. When quality issues do occur, an assessment which may include an investigation or other actions will result in a decision as to whether impact on product quality also occurred. A deviation involving a temperature setpoint or time duration for an operation step may occur during the lyophilization process, and when this occurs, an enhanced understanding of the process through development studies, risk assessments, and identification of critical process parameters will enable better quality decision making about impact of that deviation to product quality.

20.11 Conclusions

Using QbD principles such as product and process knowledge, scientific principles, and risk assessments which lead to focused development and robustness studies, a robust lyophilization process can be developed with an understanding of those critical parameters that impact product quality. The lyophilization process then serves as a foundation to not only ensure consistent product quality, but also provide regulatory flexibility by presenting the lyophilization process description with the appropriate focus on critical parameters and control of those parameters in commercial applications. Other benefits are also realized with enhanced process and product knowledge which can facilitate the design of an acceptable change management plan and enhanced quality management. This may also offer regulatory flexibility by obtaining agreement on the regulatory reporting category where data is submitted based on an agreed upon change management plan.

Teams should approach lyophilization development with the following in mind:

- Are there data on the manufacturing of the lyophilized product that can be applied to support the knowledge space of the critical process parameters?
- Knowing the capabilities of lyophilizers and having detailed product and process knowledge, can transfers be accomplished independent of scale?
- Can data from other lyophilized products be leveraged to support the knowledge space of a process parameter for the molecule?
- Are there process parameters of particular interest to ensure flexibility if possible for the product? This may be based on deviation trending for commercial products or prior knowledge to drive focus on select parameters and flexibility to work within wide ranges or one-sided limits for a given parameter.

One should keep in mind that process parameters and their ranges (examples are presented in this chapter) are strictly product-specific and need to be evaluated based on product characteristics. Careful consideration of QbD concepts will allow directed lyophilization process development and optimization with approaches to studies as presented in the sections above. The results of these studies along with existing or prior knowledge will provide flexibility within the lyophilization process. This will result in regulatory flexibility without compromising product quality.

References

1. ICH Q8 (R2) Pharmaceutical development, Nov 2009.
2. ICH Q9 Quality risk management, Jun 2006.
3. ICH Q10 Pharmaceutical quality system, Apr 2009.
4. Mitchell M. Determining criticality-process parameters and quality attributes part I: criticality as a continuum. BioPharm Int. Dec 2013;26(12).
5. Mitchell M. Determining criticality—process parameters and quality attributes part II: design of experiments and data-driven criticality. BioPharm Int. Jan 2014;27(1).

6. ICH Q8, Q9, & Q10, Questions and answers, Appendix—Q&As from Training Sessions, July 2012.
7. A-Mab: a case study in bioprocess development, CMC biotech working, group, Ver 2.1, Oct 2009, http://c.ymcdn.com/sites/www.casss.org/resource/resmgr/imported/A-Mab_Case_Study_Version_2-1.pdf.
8. Nail S et al. Fundamentals of freeze-drying, pharmaceutical biotechnology. Feb 2002;14:281–360, https://doi.org/10.1007/978-1-4615-0549-5_6.
9. Pikal MJ. Freeze drying, encyclopedia of pharmaceutical technology. 2006;1:1, 1807–33, https://doi.org/10.1081/e-ept-100001712.
10. Tang X, Pikal MJ. Design of freeze-drying processes for pharmaceuticals: practical advice. Pharm Res. 2004;21(2):191–200.
11. Shah A, Patel SM, Jameel F. Application of QbD principles for Lyophilized formulation development. In: Jameel F et al. (editors), Quality by Design for biopharmaceutical drug product development, AAPS advances in the pharmaceutical sciences series 18, Berlin: Springer; 2015. https://doi.org/10.1007/978-1-4939-2316-8_8.
12. Pikal MJ. Use of laboratory data in freeze drying process design: heat and mass transfer coefficients and the computer simulation of freeze drying. J Pharm Sci Technol. 1985;39(3):115–39.
13. Gieseler H, Kramer T. Use of manometric temperature measurement (MTM) and SMART freeze dryer technology for development of an optimized freeze-drying cycle. J Pharm Sci. 2007;96(12):3402–18.
14. Milton N, Pikal MJ. Evaluation of manometric temperature measurement as a method of monitoring product temperature during lyophilization. PDA J Pharm Sci Technol. 1997;51(1):7–16.
15. Patel SM, Doen T. Determination of end point of primary drying in freeze-drying process control. AAPS PharmSciTech. 2010;11(1):73–84.
16. Gieseler H, Kessler WJ. Evaluation of tunable diode laser absorption spectroscopy for in-process water vapor mass flux measurements during freeze drying. J Pharm Sci. 2007;96(7):1776–93.
17. Nail S, et al. Recommended best practices for process monitoring instrumentation in pharmaceutical freeze drying—2017. AAPS PharmSciTech. 2017. https://doi.org/10.1208/s12249-017-0733-1.
18. Final Concept Paper—Q12: technical and regulatory considerations for pharmaceutical product lifecycle management, dated 28 July 2014.

Chapter 21
Manufacturing of Highly Potent Drug Product in a Clinical Multi-Product Aseptic Facility and Transfer of Principles to Antibiotic Drug Product

Karoline Bechtold-Peters and Silke Mohl

Abstract The manufacturing of highly potent drug products in a multi-product aseptic facility and the transfer of principles to antibiotic drug products is certainly a challenging field. The authors have successfully implemented this in a clinical manufacturing unit and have been able to explain the rationales to regulating authorities. The assumption of a physically imaginable worst-case approach considering various risk-reducing factors based on scientific considerations is key. Several examples of design as well as of operational features to avoid cross-contamination are described. The authors conclude that at least clinical facilities should be able to manufacture the increasingly higher potent drugs of the future in multi-product units. There is no scientific rationale why this should not also be amenable for commercial facilities from a product risk perspective.

Keywords High potent drugs · Aseptic drug product facility · ADC Multi-product · Cleaning validation

Terms

ADE	Acceptable daily exposure
CFU	Colony forming units
OEL	Operational exposure level
AAC	Antibody–antibiotic conjugate
ADC	Antibody–drug conjugate
ADE = PDE	Acceptable or permitted daily exposure
CIP	Cleaning in place
CIP/SIP/DIP	Cleaning in place, sterilization in place, drying in place
ELISA	Enzyme-linked immunosorbent assay

K. Bechtold-Peters (✉)
Novartis Pharma AG, Basel, Switzerland
e-mail: Karoline.Bechtold-Peters@novartis.com

S. Mohl
F. Hoffmann-La Roche Ltd, Basel, Switzerland

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*, AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_21

EU	Endotoxin unit
LTP	Liquid transfer port
MAC	Maximum allowable carryover
MBS	Minimum batch size
MDD	Maximum daily dose of the subsequent product
ppb	Parts per billion
ppm	Parts per million
RTP	Rapid transfer port
STD	Smallest therapeutic dose
TDC	Thiomab-drug conjugate
VHP	Vaporized hydrogen peroxide
WIP	Washing in place

21.1 Introduction

Dealing with **Highly Potent Drugs** is currently an important topic in the pharmaceutical industry. For a long time, highly effective chemotherapeutic agents have been part of the oncological or antiviral therapy (e.g., Cyclophosphamide, Endoxan®, or Ganciclovir, Cymevene®). Companies operating in this field provide appropriately protected manufacturing units during development and production, particularly when processing powdered material in order not to expose employees to the active ingredient. Also, hospital pharmacies mostly have available specific installations for preparation of cytotoxic drugs [1].

For some time, a trend to higher potent classed molecules is also recognizable beyond the classical chemotherapy, namely in the biopharmaceuticals field. Targeted therapies based on antibodies or parts of antibodies are a relatively new class of cancer drugs that can overcome many of the issues seen with the use of cytotoxics lacking cell specificity. As different target proteins are expressed by different cancer types, the targeted therapy drugs are used on a cancer type specific, or even on a patient-specific basis. Immune answer strengthening molecules like the Bispecific T cell enhancing antibodies BiTEs (e.g., Blinatumomab®) or drug–toxin conjugates (ADCs, TDCs, example Kadcyla®) are effective in very small doses. Furthermore, safety, health, and environment departments tend to apply more cautious considerations and sort early phase candidates without entire toxicological assessment sometimes rather into higher safety classes. High compensation punishments in the USA are a threat and an additional motivation to endanger employees as little as possible.

A similar challenge shows the production of **antibiotic drugs**, especially when dealing with sensitizing antibiotics. For a long period of time, it was little attractive for the pharmaceutical industry to invest into antibiotic research, but this is currently changing due to the strong increase in the occurrence of multi-resistant microbes. Also in this field, targeted therapies using biologic molecules can be developed [2].

21.2 Definitions

Since potency is a continuum, i.e., from very low to extremely high potency, it is under discussion whether the expression "**highly potent drug**" or "**highly potent active compound**" should be considered as obsolete and thus too simplified to group substances into two groups: potent and non-potent. Already Sebastian Hahnemann following Paracelsus' routes defined some hundreds of years ago "Dosis fiat venenum" [3], the dose makes the poison, meaning that every substance can be toxic or non-toxic, and it finally depends on the dose. Even too much sodium chloride or water can lead to death. It is only the limit value (PDE/ADE/OEL) of a substance that describes where it is located on this continuum [4]. From both an environmental health protection perspective and a cross-contamination point of view, it is not adequate to take every precaution for "the potents" and do nothing for "the non-potents." Qualitatively, the same measures must be applied for all compounds. Quantitatively, the measures differ as much as the compounds' toxicity [2].

Likewise, the term "**cytotoxic**" to describe a drug is not clear and should be abandoned. A definition that might be somehow acceptable is that a "cytotoxic" substance kills living cells of the organism to be cured at the intended therapeutic dose.

Finally, the meaning of "**sensitizing antibiotics**" needs to be defined. The next level of definition relates to "non-sensitizing" and "low-sensitizing" antibiotics versus "sensitizing" or "highly sensitizing" antibiotics. Typically, beta-lactams (Penicillins or Cephalosporins) or Sulfonamides are classified as sensitizing antibiotics [5], because a non-sensitizing dose cannot be established. As opposed to this for non- or low-sensitizing antibiotics an acceptable daily exposure (ADE) value can be defined.

21.3 "Share or not to Share" and the Regulatory Environment

In the context of the manufacture of highly potent and antibiotic drug products, the following guidelines are important (list not meant to be comprehensive):

- **EudraLex—Volume 4 Good manufacturing practice Guidelines, Part I (Basic Requirements for Medicinal Products), Chapter 3 (Equipment and Premises)** [6] says that "…Cross-contamination should be prevented for all products by appropriate design and operation of manufacturing facilities. The measures to prevent cross-contamination should be commensurate with the risks. Quality Risk Management principles should be used to assess and control the risks.

  Depending of the level of risk, it may be necessary to dedicate premises and equipment for manufacturing and/or packaging operations to control the risk presented by some medicinal products.

Dedicated facilities are required for manufacturing when a medicinal product presents a risk because:

i. the risk cannot be adequately controlled by operational and/or technical measures,
ii. scientific data from the toxicological evaluation does not support a controllable risk (e.g., allergenic potential from highly sensitizing materials such as beta-lactams) or
iii. relevant residue limits, derived from the toxicological evaluation, cannot be satisfactorily determined by a validated analytical method…"

Compared to the previous version of Chapter 3, the vague expressions "certain cytotoxics," "certain hormones," "certain highly active drugs" have been removed and also "biological preparations (e.g., from live microorganisms)" are no longer specifically mentioned. A strong focus is put on having proper risk assessments and controls, rather than requiring dedicated and self-contained facilities as a must. Most interestingly, the definition of dedication may also be interpreted as a dedication for a certain time (formerly expressed as working in campaigns) [4].

- In **EudraLex—Volume 4 Good manufacturing practice Guidelines, Part I (Basic Requirements for Medicinal Products), Chapter 5 (Production)** [7] it is further explained as regards prevention of cross-contamination in production that "…Contamination of a starting material or of a product by another material or product should be prevented. This risk of accidental cross-contamination resulting from the uncontrolled release of dust, gases, vapors, aerosols, genetic material, or organisms from active substances, other starting materials, and products in process, from residues on equipment, and from operators' clothing should be assessed. The significance of this risk varies with the nature of the contaminant and that of the product being contaminated. Products in which cross-contamination is likely to be most significant are those administered by injection and those given over a long time. However, contamination of all products poses a risk to patient safety dependent on the nature and extent of contamination…"

  In the revised Chapter 5 issued in August 2014 mentioning "highly sensitizing materials, biological preparations containing living organisms, certain hormones, cytotoxics, and other highly active materials" as most hazardous contaminants has similarly to Chapter 3 been omitted, and the application of a Quality Risk Management process is emphasized.

  The outcome of the Quality Risk Management process should be the basis for determining the extent of *technical and organizational measures* required to control risks for cross-contamination. Examples for such measures are given.

  The guidance now takes into account principles and guidance through the ICH Q9 process (Quality Risk Management). "…A Quality Risk Management process, which includes a potency and toxicological evaluation, should be used to assess and control the cross-contamination risks presented by the products

manufactured. Factors including facility/equipment design and use, personnel and material flow, microbiological controls, physicochemical characteristics of the active substance, process characteristics, cleaning processes, and analytical capabilities relative to the relevant limits established from the evaluation of the products should also be taken into account. The outcome of the Quality Risk Management process should be the basis for determining the necessity for and extent to which premises and equipment should be dedicated to a particular product or product family. This may include dedicating specific product contact parts or dedication of the entire manufacturing facility…" The latter means that those products which combine relatively high-risk pharmacological/ toxicological characteristics with high-risk physicochemical properties and process design should not be manufactured using shared facilities unless justified after following a rigorous Quality Risk Management process and taking any appropriate risk mitigating measures for part or all of the process.

- **EMA Guideline on setting health-based exposure limits for use in risk identification in the manufacture of different medicinal products in shared facilities** [8, 9] refers to Chapters 3 and 5 and gives further guidance to risk-reducing measures: "…Chapters 3 and 5 of the GMP guideline have been revised to promote a science and risk-based approach and refer to a "toxicological evaluation" for establishing threshold values for risk identification.
  Cleaning is a risk-reducing measure and carryover limits for cleaning validation studies are widely used in the pharmaceutical industry. A variety of approaches are taken in order to establish these limits and often do not take account of the available pharmacological and toxicological data. Hence, a more scientific case by case approach is warranted for risk identification and to support risk reduction measures for all classes of pharmaceutical substances. The objective of this guideline is to recommend an approach to review and evaluate pharmacological and toxicological data of individual active substances and thus enable determination of threshold levels as referred to in the GMP guideline. These levels can be used as a risk identification tool and can also be used to justify carryover limits used in cleaning validation…."
  The guideline thoroughly explains how to derive and calculate a *permitted daily exposure (PDE) value*. Very interestingly the guideline states that for therapeutic macromolecules and peptides the determination of health-based exposure limits using PDE limits of the active and intact product may not be required, since they "…are known to degrade and denature when exposed to pH extremes and/or heat and may become pharmacologically inactive. The cleaning of biopharmaceutical manufacturing equipment is typically performed under conditions which expose equipment surfaces to pH extremes and/or heat, which would lead to the degradation and inactivation of protein-based products."
  This statement, however, certainly cannot be transferred to hybrids of macromolecules or peptides with small molecular toxins, such as the ADCs.

To make the picture complete also reference is given to ANVISA and Health Canada rules:

- ANVISA requires in its **RESOLUTION—RDC Nº 17, OF 16.04.10** [10] Article 125 that "…The production of certain drugs, such as certain biological preparations (e.g., live microorganisms) and the highly sensitizing materials (e.g., Penicillins, Cephalosporins, Carbapenems, and other beta-lactam derivatives) should be segregated and have dedicated facilities in order to minimize the risk of serious damage to health due to contamination. In some cases, such as highly sensitizing materials, segregation should also occur between them…"
  According to Article 256: "… The occurrence of cross-contamination must be avoided by appropriate technical or organizational measures, such as:

  I Production in exclusive and closed areas (e.g., Penicillins, Cephalosporins, Carbapenems, the other beta-lactam derivatives, preparations with biological organisms, certain hormones, cytotoxic substances, and other highly active materials);
  II Campaign production (separation time) followed by appropriate cleaning in accordance with a validated procedure. For products listed in paragraph (a), the principle of campaign work is only applicable in exceptional cases such as accidents or emergency situations;
  III Use of antechambers, differential pressure and supply air and exhaust systems;
  IV Reducing the risk of contamination caused by recirculation or re-air of untreated or insufficiently treated;
  V Use of protective clothing where products or materials are handled;
  VI Use of validated procedures for cleaning and decontamination;
  VII Using "closed system" of production;
  VIII Testing of waste and
  IX The use of labels on equipment to indicate the state of cleanliness."

- Health Canada describes in its **Good Manufacturing Practices (GMP) Guidelines—2009 Edition, Version 2 (GUI-0001)** [11] that "Self-contained" facilities are required for:

  – certain classes of highly sensitizing drugs such as penicillins and cephalosporins.
  – other classes of highly potent drugs such as potent steroids, cytotoxics, or potentially pathogenic drugs (e.g., live vaccines), for which validated cleaning or inactivation procedures cannot be established (e.g., the acceptable level of residue is below the limit of detection by the best available analytical methods)."

Based on the review of the various guidelines, it can be concluded that current regulatory thinking allows for a risk-based approach when using multi-product/shared facilities even for highly active and antibiotic compounds. This is accepted by major authorities (EMA, FDA), if adequate countermeasures to prevent cross-contamination based on thorough risk assessments and validated cleaning procedures following scientific data can be provided. Inspection risks, however, prevails with certain authorities like ANVISA which still requires exclusive and closed areas.

21.4 Multi-Purpose Facility Versus Dedicated Facility—How to Properly Design and Run Facilities? Case Study Parenteral (Aseptic) Facility

As outlined in EudraLex—Volume 4/Part I/Chapter 5 [7], there are *technical and operational measures* to be taken to prevent cross-contamination in a multi-product facility especially in case high-risk products are handled in the same area as products of less risk. Technical measures are those that are achieved by properly designing rooms, flows, facility, and equipment while operational measures are prescribed in standard operational procedures or batch records and define the how to operate and act to further reduce any cross-contamination risk.

a. Risk analysis regarding cross-contamination risk

A careful multistage risk analysis of the production process is of course a prerequisite for proper system design (Section 4.b) and the establishment of procedural measures (Section 4.c).

In Table 21.1, possible failure modes and hazards are given as an example which could lead to release of the highly potent drug substance and to cross-contamination:

Table 21.1 Possible failure mode/hazard per process step for ADC Drug Product Manufacture

	Possible failure mode/hazard—example only
Receipt and storage of API and bulk thaw/Re-Freeze	e.g., damage of cryo vessel (dripping) due to defective sealing
Transfer of API solution from cryo vessel to mobile compounding vessel	e.g., liquid spill because compounding vessel not opened or still pressurized
Compounding	e.g., liquid spill due to leaking connection of tubing to compounding unit
Bioburden reduction filtration	e.g., liquid spill when filter is handled
Transport of vessel to filling line	e.g., cross-contamination of material locker (C to D) due to outside contamination of vessel
Filling	e.g., contamination of containment and microbiological monitoring system due to spillage of liquid
Freeze drying	e.g., contamination of freeze dryer and/or unloading part of the containment due to breakage of vials upon stopper closure
Transport processes from filling line to cleaning rooms and storage rooms	e.g., contamination of all GMP zones that were passed
IPC and batch release analysis	e.g. liquid spill or airborne contamination
Cleaning of compounding equipment, filling product path, containment, filling line or facility	e.g. manual or automated cleaning is not sufficient

b. Example for proper *design* of facilities and equipment to enable manufacture of high potent drugs or antibiotics in a shared facility

Cross-contamination can occur through mechanical transfer when a contaminant residue moves from one process or area to another one or by airborne transfer when aerosol (powder, droplets) is available in the air and contacts product or equipment. For both transfer routes is key to reduce probability of occurance: closed processes and containment with adequate pressure differential, adequate working flows of equipment and personnel, airlocks including the gowning. In the following, appropriate design features are listed as examples that render a parenteral facility able to function as a shared facility for highly potent or antibiotic drugs (unless sensitizing). GMP and SHE requirements may mix.

- Appropriate air supply for the clean rooms and the building—ideally 100% fresh air, no recirculation (Fig. 21.1).
  It is important that aerosolized material cannot be spread via aeration in rooms or compartments in which at the same time or later other products are processed. Regularly check of air filters and/or provision of preferably 100% of fresh air needs to be guaranteed.
- cRABS or isolator technology with pressure management in different compartments, pressure hold test before filling, pressure alarmed (Fig. 21.2).
  Non-RABS or open RABS design is certainly not adequate to protect the environment from contamination by highly active drugs. Closed containment RABS or isolators should have a pressure cascade from the most critical area (filling, stopper setting) to the area in which most likely the solid, aerosolizable material is produced, i.e., the unloading area of the lyophilizer. It is advantageous if the area in front of the lyophilizer can even operate during the discharge

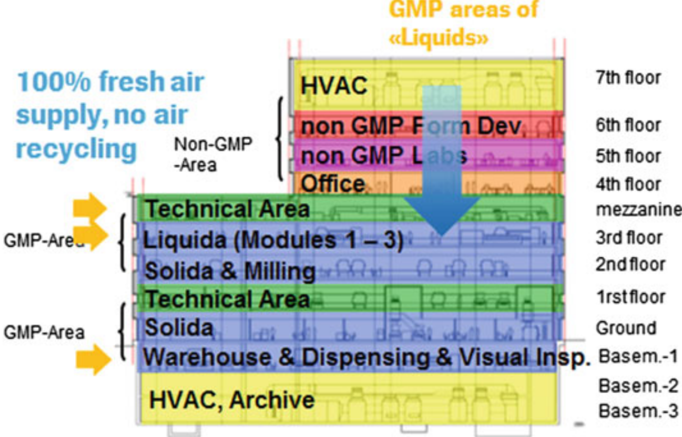


Fig. 21.1 Schematics of a TR&D building hosting GMP manufacture for highly potent solids and for highly potent parenterals in same building

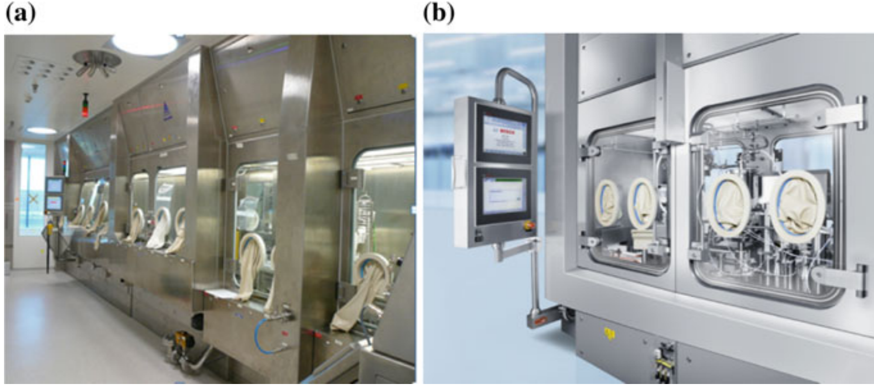
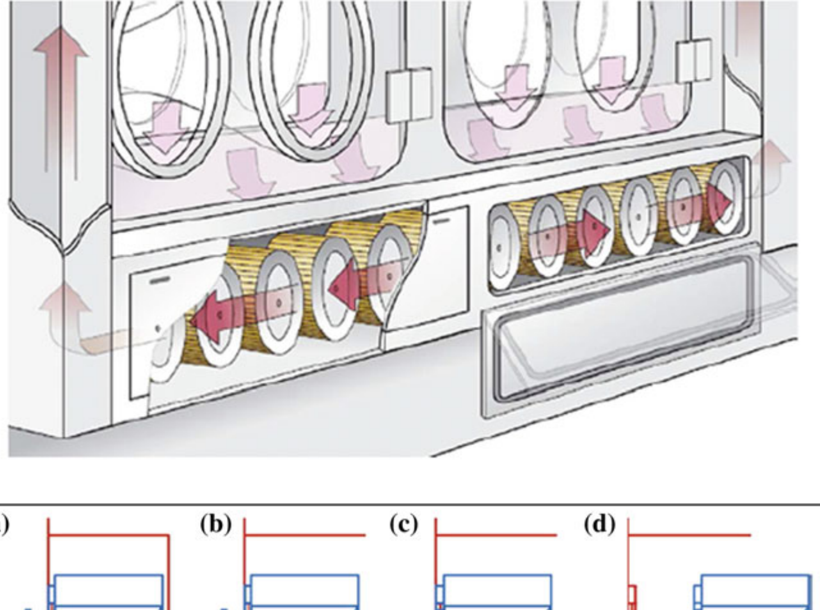


Fig. 21.2 Filling lines designed as closed RABS facilities: vials line from Groninger/Skan (2**a**, left) and prefilled syringe line from Bosch (2**b**, right), both located at F. Hoffmann-La Roche, Basel, Switzerland

mode under lower or at least equal pressure relative to the environment. Closed RABS systems or isolators are leak tested prior to use and are pressure alarmed during operation. For aseptic cRABS or isolator compartments, the special feature is that they are generally operated in positive pressure relative to the environment to meet primarily the microbiological protection requirements. The surrounding clean room thus acts as a sink area. Therefore, there are facilities that provide an additional sink lock for the personnel in which the clean room clothing remains.

- Save change filters to protect ductwork and the exhaust air handling system, returning air H14 filtered before reentering the containment (Fig. 21.3). To avoid the distribution of aerosolized material within a cRABS system or in the environment, exhaust and circulating air must be suitably filtered. Save change filters [12] also prevent contamination of the area during removal and transport of potentially contaminated filters.
- In case of multiple lines, access by separate personnel locks. When in the same area two or more filling lines operate, in which highly potent substances are processed, separate personnel, or material locks into the class B zone should be provided.
- Automated check weighing: Ideally, during the processing of highly actives an open and destructive IPC of fill weight or deliverable volume is avoided as well as the need for manual intervention, e.g., conducting any sample draws. Through automated tare net weighing both SHE and cross-contamination issues can be addressed.
- Outside washing machine to decontaminate the vials from the outside (preferentially automated) (Fig. 21.4). It is primarily a SHE request that vials with highly active ingredients needs to be cleaned at the outside after the filling in order not expose, e.g., the staff in the visual inspection. On the other hand, to not



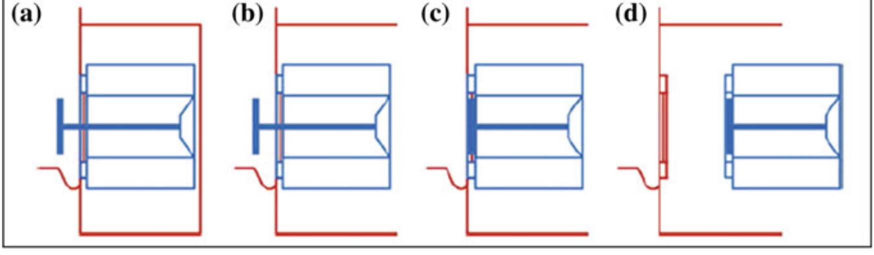


Fig. 21.3 Skan save change filter system **a** Position during manufacturing, **b** Opening of the return air duct, **c** Locking of the cap with a tool, **d** Remove of the closed filter unit [12]

jeopardize the product, the outside cleaning should not go beyond the shoulder portion of the vial.

- Liquid transfer ports (LTPs) represent a very good means to perform aseptic and contamination-free connection of the hold vessel with the bulk solution to the filling line. LTPs are now also available as a disposable material.

c. Additional *operational* measures

Only examples can be given. The individual measures must be derived from a thorough risk analysis of the processes (see Section 4.a).

- CIP of lyophilizer and other not easy to access areas, WIP for easy to access areas (Fig. 21.5). In order to be able to change the format parts after the filling process, it must be ensured that before the opening of the containment everything is cleaned so far that neither man nor B-space are at risk from contamination. Inaccessible areas should be cleaned more automated (CIP), accessible sites can be cleaned, for example by spray guns (WIP).

Fig. 21.4 Validated outside washing of vials after crimping in the containment, drying of the vials without heat-exposition; equipment located at F. Hoffmann-La Roche, Basel, Switzerland

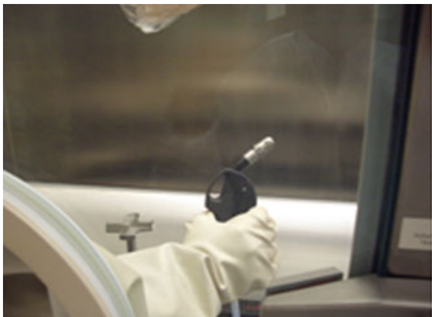


Fig. 21.5 Automated CIP of lyophilizer loading/unloading zone (right) and manual wash-in-place of filling compartment (WIP, left); equipment located at F. Hoffmann-La Roche, Basel, Switzerland

- First rinse of containment and molten ice of condenser discarded into special wastewater system. It is clear that at least the first rinse water should be collected separately and special treatment has to be supplied. Through online measurement methods or previous generic cleaning studies, the amounts of wash water to be disposed in an expensive manner can be reduced. If automated or manual spray-cleaning procedures are employed, a drain for the wash water is needed. The stands in contradiction to the EU GMP Guide, Annex 1 [13], which does not allow drains in the A/B zones ("50. Sinks and drains should be prohibited in grade A/B areas used for aseptic manufacture."). Therefore, such a drain should be completely sealed in the functional state clean room class A/B and used only after the filling under non-A/B conditions.
- Use of endless bag system to remove solid waste during/after filling (Fig. 21.6). One of the marketed bag systems can be purchased from the company Lugaia which is, for example, employed in the Clinical Manufacturing area at F. Hoffmann-La Roche in Basel.

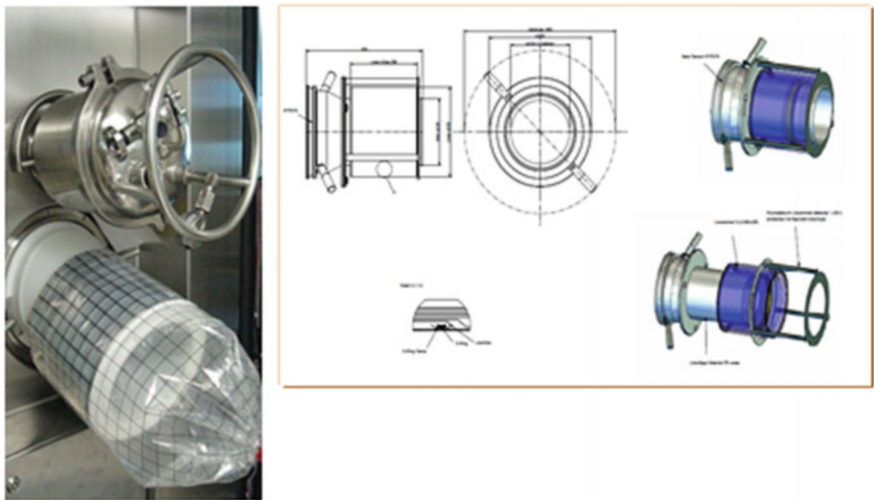


Fig. 21.6 Waste removal though Lugaia port/endless bag system; equipment located at F. Hoffmann-La Roche, Basel, Switzerland

- Opening of RABS during filling not allowed and blocked by the special filling recipe, i.e., when switching to the recipe "High Potent" the compartment doors can be opened only with a very high administrator level.
- Consequent use of manufacturing execution systems (MES) to track material and equipment in order to render the use of the multi-use or dedicated equipment secure and reduce the possibilities of operator errors.
- Check of compartment HEPA filter integrity after each high potent or antibiotic batch or provision of studies demonstrating no aerosolization risk. If the ventilation of the cRABS or isolator includes a recirculation of air, it must be considered whether the returning air can be contaminated by the aerosolized drug/device combination products. Here it may be separated between liquid and solid active ingredients. Drug/device combination products distributed in the filling compartment in the liquid state (dripping needle, needle splatters) is dried and even denatured in the case of therapeutic proteins/antibodies thereby. An entry of active ingredient into the air circulation is very unlikely. With solid product such as lyophilized material, however, a drug/device combination products release upon vial breakage could result during discharge from the freeze/thaw dryer in the distribution of micro-fine dust. The testing of the efficiency of the air filtration of the exhausted or recirculated air (testing for integrity of the exhaust filter or of the supply air filter after manufacture of high potent batches) or the counter-evidence that de facto no micro-fine dust can develop seem necessary.
- Protection of microbial sampling plates by special (clean) zip bags (Fig. 21.7). In order to be able to exit the plates safely and quickly without further surface treatment by surface cleaning.

Fig. 21.7 Zipper bags firmly holding together the microbial plates while removal through RTPs; equipment located at F. Hoffmann-La Roche, Basel, Switzerland

- Specific gowning procedures when handling open material. This could be e.g., the use of disposable clothing and double gloves in the compounding area when openly dealing with highly active ingredients. The disposable clothes should be discarded when leaving the area and where possible applying the “onion methods”. i.e. undressing of potentially contaminated clothing layer by layer.

d. Cleaning and cleaning verification/validation concept including definition of a “reasonable worst case”

The “rinsability” or ease of removal of product may be evaluated in a small-scale cleaning study or rinsability study, where the product material or inactivated or fragmented product (dependent on the cleaning procedure) is spiked onto representative coupons and exposed to a worst-case (e.g., no impingement, lower flow rate, etc.) water rinse in comparison to full scale cleaning cycles [14]. If the worst-case rinse removes the product spike from the coupon, it demonstrates that the product fragments are not a carryover concern. Regarding ADCs the cleaning procedure should not cleave off the toxin as it may be less water-soluble than the full-size conjugate and might also be more toxic.

Cleaning verification/validation must be performed in order to confirm cleanliness after filling and subsequent cleaning [15]. Cleaning limits are to be established prior to introduction of any high potent drug. Ideally high potent drug product batches are manufactured in a multi-product facility campaign-wise (“time-wise segregation”), and cleaning verification is only performed after completion of the last batch of a campaign. Three successful cleaning events using a representative manufacturing process are required for cleaning validation.

Product-specific cleanliness limits are defined for shared equipment which consider the acceptable daily exposure or permitted daily exposure (ADE = PDE) value established for a specific product. Here the sampling method is typically by swabbing an appropriate area at the worst-case and representative locations. The analytical method used to assess cleanliness must be sensitive enough to match with

the low ADE value in case of high potent drugs. For biologics like Antibody–Drug conjugates, this means mostly performing an ELISA as protein-specific test.

Cleaning limits can be assigned based on, e.g.,

- the minimum batch size (MBS) in units, i.e., the minimum allowable batch size to be produced of the subsequent product using the same product train. The MBS is calculated from the maximum daily dose (MDD) of the subsequent product. This minimum allowable batch size of the subsequent product is affected by a potential carryover from the previous batch, and
- the maximum allowable carryover (MAC) outlining the maximum amount of product residue that is allowed based on the drug's acceptable daily exposure (ADE)

How difficult it is to fulfill the cleanliness limit depends on the ADE value, the area accessible to swabbing and the limit of detection by ELISA.

ADE or PDE Value

The acceptable or permitted daily exposure (ADE or PDE) or acceptable daily intake (ADI) is according to the EMA Guideline on setting health-based exposure limits for use in risk identification in the manufacture of different medicinal products in shared facilities defined as follows [8].

$$\mathbf{PDE(mg/day)} = \frac{\mathbf{NOAEL \times Weight\,Adjustment}}{\mathbf{F1 \times F2 \times F3 \times F4 \times F5}}$$

F1 = Factor for extrapolation between species
F2 = Factor for variability between individuals
F3 = Factor for repeat-dose toxicity studies of short duration (<4 weeks)
F4 = Factor for severe toxicity (e.g., non-genotoxic carcinogenicity, neuro-toxicity, teratogenicity)
F5 = Factor if no NOAEL is available, if only LOAEL is available
where
NOAEL = no-observed-adverse-effect level
LOAEL = lowest-observed-adverse-effect level
Note: the PDE must be determined by Toxicology/Safety Assessment

In case of ADCs based on vcMMAE, the PDE values may be as low as 0.5 μg/day for the complete conjugate, 0.1 μg/day for the toxin molecule still retaining the linker/spacer and 0.05 μg/day for the cleaved MMAE molecule.

Minimum Batch Size (MBS)

Batch sizes must be set at or above the minimum batch size in order to ensure that active ingredient carryover from a former product does not compromise the next product. No more than a specific fraction of the former product may be carried over into the largest single dose of the next product manufactured in the same

equipment. If a PDE value is determined, this value is to be used, in other cases 1/1000th of the smallest therapeutic dose of the former product can be used, whatever is more strict.

Determination of Maximum Allowable Carryover (MAC)
The maximum allowable carryover from the previous product into the next product is determined by the PDE value, by the minimum batch size and the Maximum Daily Dose of the subsequent product according to

$$\mathbf{MAC(mg)} = \frac{\mathbf{PDE(mg/day)} \times \mathbf{MBS(g)}}{\mathbf{MDD(g/day)}}$$

where
PDE = Acceptable daily exposure from previous product
MBS = Minimum batch size of subsequent product
MDD = Maximum daily dose of subsequent product

Maximum allowable residue (MAR) for equipment parts not in contact with product (e.g., lyophilizer, area in front of the lyophilizer, filling area) based on a reasonable worst-case scenario

Equipment parts for use in high potent drug manufacturing in contact with the product should be either disposable, i.e., are discarded after each use, or used dedicated for the specific product, unless efficient cleaning can be demonstrated which might be difficult in case of low PDE values. Those equipment parts are not considered when calculating the maximum allowable residue, since they are not used for multi-product purposes and do not contribute to a potential cross-contamination into another product. These parts could be, for example

- Cryovessel (dedicated)
- Tubing used for cryovessel recirculation (disposable)
- Hold tank (dedicated)
- Surge tank (dedicated)
- Silicone tubing for transfer of API from vessel to vessel and from hold tank to nozzle (disposable)
- Filter housing (dedicated)
- 0.2 μm or sterile filters (disposable)
- Needles (dedicated)
- LTP adapters (dedicated)
- Connecting and distributing parts (dedicated)

Normally only cleaning procedures for product contact surfaces of the equipment need to be verified or validated. But also consideration should be given to non-contact parts into which product may migrate. For example, seals, flanges, mixing shaft, fans of ovens, heating elements, etc. [14].

For those parts not in direct contact with the product, a risk-based approach is used which is described in a technical report of PDA [16]. In TR No. 29 of PDA, 2012, it is stated that there are "non-product contact surfaces which may contact the product *indirectly* such as by a vector or by an airborne route. These are sometimes called "indirect product contact surfaces." Examples of these types of surfaces might include, e.g., lyophilizers. These indirect product contact surfaces should be included in the cleaning validation program. However, because of the limited impact of these product contact surfaces, requirements for cleaning validation, such as limits, may be different from cleaning validation for *direct* product contact surfaces. A risk assessment should be utilized to define the requirements, which will depend on the specifics of the manufacturing situation." Also the Baseline Guide: Risk-Based Manufacture of Pharmaceutical Products (Risk-MaPP) of ISPE, 2010, provides a scientific risk-based approach, following ICH Q9 Quality Risk Management, to manage the risk of cross-contamination in order to achieve and maintain an appropriate balance between product quality and operator safety and mentions a risk evaluation for residues on non-product contact surfaces [17].

Therefore, with the area contribution and the probability of cross-contamination being taken into consideration a maximally allowable residue (MAR) value can be calculated as follows which includes a scientifically derived Risk Reduction Factor, RRF:

$$\mathbf{MAR_{equipment\ part}} = \mathbf{MAC \times area\ contribution\ of\ equipment\ part / RRF_{related\ to\ equipment\ part}}$$

with
RRF = risk reduction factor considering cross-contamination probability

Specific thoughts must be given in case of freeze-dried products to the lyophilizer, since it cannot be used dedicated in a shared facility: The only way material is transferred as cross-contaminant from the lyo chamber surfaces into vials of the subsequent product is by entrainment of material (from spillage when vials have fallen over, have broken or have boil over, or from sublimation or aerosolization of material together with water during the freeze-drying process) when flushing the chamber with nitrogen at the end of the process (Fig. 21.8). Due to the vacuum in the lyo chamber and the vials, a distribution of the material

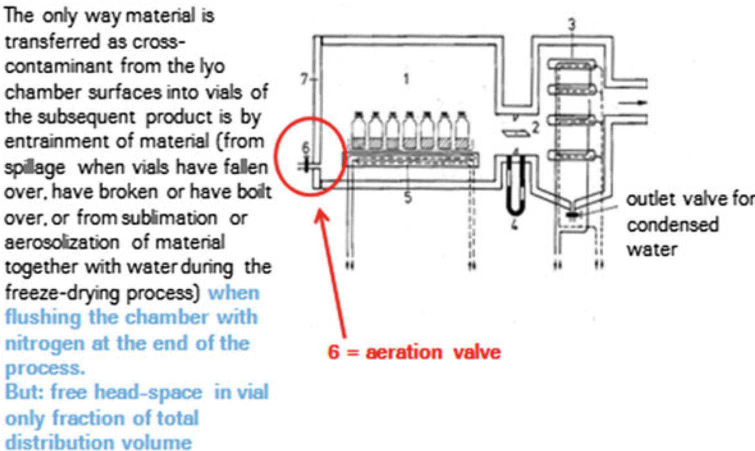


Fig. 21.8 Potential contamination mechanism during lyophilization

according to the respective volume ratio of lyo chamber and vial open space can be assumed. These ratios together with the assumption that the material remaining on the lyophilizer surface after the preceding CIP/SIP process is fixed to the surface and does not readily enter the air can be used to calculate a lyophilizer-specific risk reduction factor based on a "reasonable worst case."

A summary of the whole concept is depicted in Fig. 21.9.

Since the maximum allowable residue, exterior, MAR_{ext}, on the outside of the vials after passing the exterior vial washing machine is not a GMP topic, but relevant for SHE aspects (safety of operators during visual control, safety of clinical personnel during preparation and application), a respective SHE rational might be compiled outside of GMP documentation.

e. Use of disposable materials

The use of disposable materials in the DP processing of highly active ingredients reduces the costs of cleaning and cleaning verification significantly. A mix up of dedicated equipment, e.g., due to an operator error is impossible. In addition to replacing compounding/mixing and holding vessels by 2D and 3D bags, disposable filter cartridges, tubings, filling needles, connectors, ports, and much more disposables can be employed. For an overview see Fig. 21.10.

However, also the limits and drawbacks when using disposable material should not be forgotten. Especially the increased risk of a leak leading to the uncontrolled release of the highly active agent must be considered. In particular, in the frozen state regularly damages of bag systems are reported, e.g., during transportation [18].

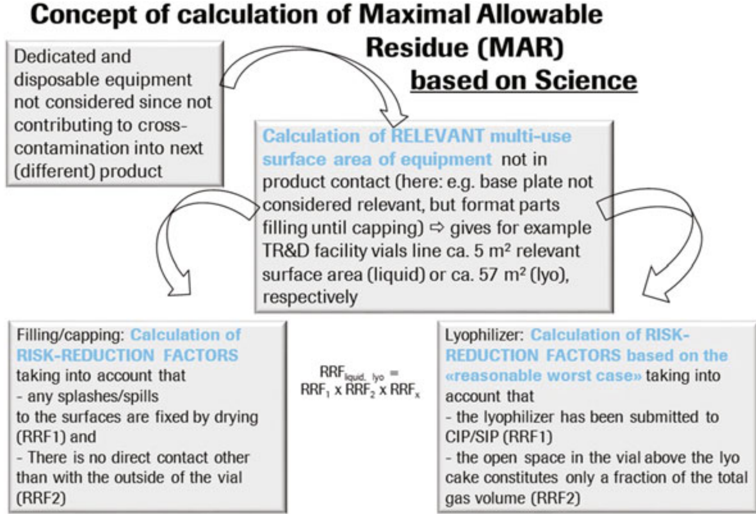


Fig. 21.9 Concept to transfer an ADE value into MAR values using a risk-based approach

Fig. 21.10 Potential application of disposable materials for manufacture of sterile drug product

Table 21.2 Pros and cons (specifically for material in contact with product)

Advantages	Disadvantages

• Reduced risk of cross-contamination when dealing with high potent drugs manufacture
• Equipment, i.e., disposable are quasi-dedicated
• No cleaning validation or verification needed
• Increased sterility assurance (closed system)
• Reduced burden to quality/calibrate/store/ purchase and use of stainless steel equipment
    • Savings of maintenance costs
    • Savings of CIP/SIP (re-)validation costs
    • Energy savings of WFI and clean steam for CIP/SIP
    • Reduction of capital bound as CAPEX
• Higher production flexibility, e.g., because various sizes available
	• Potential of damage during transportation and handling (increased concern in case of high potent drugs)
	• Potential for leachables; need to generate product-specific data during development (phase-appropriate)
	• Adsorption/permeation of active pharmaceutical ingredient or of excipients
	• Absence of standardization
	• Dependency on supplier and their quality systems (changes, consistency of manufacturing process, sterilization)
	• Long-term costs?
	• Waste management (volume)
	• Interchangeability discussion
	• <Compatibility> (interconnection) of various systems?

Advantages and disadvantages of single-use materials are summarized in Table 21.2.

21.5 Transfer of Principles to Antibiotic Drug Product

In principle, the design and procedural measures to avoid cross-contamination when aseptically filling highly potent drugs can be transferred to low to moderately sensitizing antibiotics. As stated in Sect. 21.2, this does not apply to antibiotics with high sensitizing potential.

However, another consideration must be made in the case of antibiotics for processing in aseptic areas: The microbiological monitoring might no longer be reliable and meaningful in the presence of the antibiotic agent due to splashes during filling. To prove this, the placement of additional settling plates might be sensible that are tested after filling for growth promoting features with the microbe most sensitive to the antibiotic.

The presence of the antibiotic may also jeopardize validation of the bioburden test before sterile filtration as well as complicate validation of the test for sterility and should be taken into account in the lead time calculation for the manufacture of a clinical product.

21.6 Differences Between Clinical and Commercial Facilities

Clinical and commercial aseptic manufacturing areas differ mostly not only by size and output per time (e.g., 1 or 2 needle filling machine vs 5 or 10 needles) or by shift model (1 shift/5 days vs 2 to 3 shifts/6 to 7 days), but also regarding the multitude of products that are manufactured annually. In clinical supply units only few, sometimes only one batch of a product is produced per year. Single-product long-lasting campaigns are rarely feasible—also regarding highly potent or antibiotic drugs. Due to the basic determination of clinical supply units to deliver numerous, various products, flexibility, and short change-over times are important KPIs. A time-wise segregation in the same unit between high and non-high potent/ antibiotic projects can and must be assessed via a risk analysis as of Section 4a and thus the acceptance as multi-purpose facility is obtained.

A further difference constitutes the authority surveillance: While commercial units expect, e.g., a Pre-Approval Inspection of the FDA prior marketing, the new uptake of products in a clinical facility is the rule and is not assured by an individual inspection. Start-off inspections prior to first use of the unit or after major changes as well as regular GMP inspections are performed.

Most companies have, finally, global quality guidance's for which some exemptions are allowed regarding IMPs. Beyond this, it is out of any doubt that there is no "GMP light" and that compliance with US FDA 21 CFR Parts, 11, 210 and 211, 820, Rule 4, EU GMP EudraLex Volume 4 and applicable annexes, applicable ICH Guidance's, and USP/EP/JP is mandatory.

21.7 Conclusion and Summary

The manufacturing of highly potent drug products in a multi-product aseptic facility and the transfer of principles to antibiotic drug products is certainly a challenging field. The authors have successfully implemented this in a clinical manufacturing unit and have been able to explain the rationales to regulating authorities.

The assumption of a physically imaginable worst-case approach considering various risk-reducing factors based on scientific considerations was key. Furthermore, several examples of design as well as of operational features to avoid cross-contamination were described.

Thus, at least clinical facilities should be able to manufacture the increasingly higher potent drugs of the future in multi-product units. And there is no scientific rationale why this should not also be amenable for commercial facilities from a product risk perspective.

References

1. Griffiths W, et al Standard Practices to ensure safe cytotoxic drug reconstitution in hospital pharmacies: an international review. Poster at EAHP Vienna 2002.
2. Lehar SM, et al. Novel antibody–antibiotic conjugate eliminates intracellular S. aureus. Nature. 2015; 527:323–328. https://doi.org/10.1038/nature16057.
3. Kobert R. Lehrbuch der Pharmakotherapie. Stuttgart: Ferdinand Enke Verlag; 1908.
4. Presentation of Andreas Flückiger, F. Hoffmann-La Roche, on "International Corporate Standards for Handling ("Highly Potent") Pharma Products", APV Seminar—Highly Potent Products in the Pharmaceutical Industry, November 22/23, 2015, Berlin.
5. Sebastian K, et al. Evaluation of the sensitizing potential of antibiotics in vitro using the human cell lines THP-1 and MUTZ-LC and primary monocyte-derived dendritic cells. Toxicol Appl Pharmacol. 2012;262(3):283–92.
6. EudraLex. The rules governing medicinal products in the European Union, vol. 4. EU Guidelines for Good Manufacturing Practice for Medicinal Products for Human and Veterinary Use, Part 1, Chapter 3: Premises and Equipment, Brussels, 13 August 2014.
7. EudraLex. The rules governing medicinal products in the European Union vol 4. EU Guidelines for Good Manufacturing Practice for Medicinal Products for Human and Veterinary Use, Part 1, Chapter 5: Production, Brussels, 13 August 2014.
8. Guideline on setting health based exposure limits for use in risk identification in the manufacture of different medicinal products in shared facilities, EMA/CHMP/ CVMP/ SWP/ 169430/2012, 20 November 2014.
9. Presentation of Rainer Gnibl. Government of Upper Bavaria, on "Essentials from EU-Guidelines on cross-Contamination in shared Facilities". APV Seminar—Highly Potent Products in the Pharmaceutical Industry, November 22/23, 2015, Berlin.
10. ANVISA Resolution—RDC № 17, of 16.04.2010, English translation.
11. Health Canada, Good Manufacturing Practices (GMP) Guidelines—2009 Edition, Version 2 (GUI-0001), 2011.
12. Bässler HJ, Lehmann F. Containment technology, chapter 4, Springer-Verlag Berlin Heidelberg 2013. https://doi.org/10.1007/978-3-642-39292-4_4.
13. EudraLex. EU GMP Guidelines, Volume 4, Annex 1 Manufacture of Sterile Medicinal Products, 2008.
14. PIC/S, PI 006-3, 25 September 2007, Validation mater plan installation and operational qualification non-sterile process validation cleaning validation.
15. Mott A, et al. Methodology for assessing product inactivation during cleaning part ii: setting acceptance limits of biopharmaceutical product carryover for equipment cleaning. 2013. http://www.ivtnetwork.com/article/methodology-assessing-product-inactivation-during-cleaning-part-ii-setting-acceptance-limits.
16. PDA TR29, Technical Report No. 29 (Revised 2012) Points to Consider for Cleaning Validation.
17. ISPE Baseline Guide: Risk-Based Manufacture of Pharmaceutical Products (Risk-MaPP) 2010.
18. Goldstein A, et al. Disposable Freeze Systems in the Pharmaceutical Industry, American Pharmaceutical Review, posted December 10, 2012.

Part VI
Novel Constructs

Chapter 22
Introduction into Novel Constructs

Susanne Joerg, Kapil Gupta and Margarida Rodrigues

Abstract New biologic molecules—such as mAb-derived formats, nanobodies, bispecifics, and fusion proteins—are quite diverse and come along with new formulation and process development challenges. The properties are very dependent on the molecule and difficult to generalize. Hence, only by selection of optimized formulation conditions it is ensured to keep challenging molecule properties under control. In addition, a variety of analytical methods needs to be applied to thoroughly investigate all critical quality attributes. Finally with regard to compatibility, appropriate experimental design that mimics clinical administration procedure is essential (including product in-use stability and stability during administration conditions) and creative solutions might be required to ensure clinical dosing. Thorough pharmaceutical development is key to enable integrity and stability of novel biologics compounds upon storage and administration.

Keywords ADC · Bispecifics · Fusion proteins · Developability
Formulation

22.1 Introduction

a. Change of biotechnology market from monoclonals to novel formats

In 1986—almost thirty years ago—the first recombinant monoclonal antibody Orthoclone® OKT 3 (Muromonab-CD3) was approved by the US Food and Drug

S. Joerg (✉) · M. Rodrigues
Biologics Process Research and Development, Early Phase Development, Pharmaceutical Development, Novartis Pharma AG, Basel, Switzerland
e-mail: Susanne.joerg@lonza.com

K. Gupta
Biologics Process Research and Development, Integrated Biologics Profiling, Novartis Pharma AG, Basel, Switzerland

S. Joerg
Pharma and Biotech Drug Product Services, Lonza AG, Basel, Switzerland

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*, AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_22

Administration (FDA) for the prophylaxis of acute kidney transplant rejections (www.orthobiotech.com/history.html). Since then, the steady progress of the market of therapeutic biologicals was inexorable, and nowadays, the segment of monoclonal antibodies (mAbs) is set as a numerically small, but fast growing therapeutic sector.

Recombinant antibodies are used in a wide area of disease conditions, which are mainly focused on oncology as well as arthritis, inflammation, and immune disorders [1]. In 2011, over 1000 clinical trials were reported using mAbs either as active drug or comparator [1]. Antibody sales (based on 25 marketed mAbs) were US$ 41bn in 2010 and were forecasted to increase to US$ 63bn in 2015 [1], with a further encouraging outlook for the future years.

Current marketed antibody formats differ with regard to the level of humanness; these include murine (no human sequences), chimeric (about two-thirds human sequences), humanized (typically >95% human sequences) or fully human mAbs. Modifications of recombinant monoclonal antibodies can influence the mechanism of action, side effects, and efficacy, and hence, current antibody design and engineering efforts target both the variable antigen-binding Fv region and the constant Fc regions. The Fc region is known to be critical for IgG effector functions, as complement activation and binding to Fc-gamma receptors, as well as for the control of IgG serum half-life, due to the Fc-dependent binding of IgG to the human neonatal FcRn.

Nevertheless, the market of recombinant antibody therapeutics is still moving. Advances in molecular biological techniques have led to development of an increasing number of bio-therapeutics that differ significantly from the traditional monoclonal antibody format. Novel constructs could comprise either antibody derivatives or de novo designs of therapeutic proteins or protein fusions that do not occur in nature, but are artificially generated by joining two or more genes by genetic engineering. These novel molecular formats offer superior biological properties; e.g., bispecifics can bind multitargets, ADC can deliver cytotoxic payload to target cells (ADC), and glycol engineered antibodies have increased ADCC activity through Fc-gamma interaction.

Consequently, many biopharmaceutical companies are developing these novel format molecules over antibodies for providing better drug candidates. Currently, only a few of these novel concepts and scaffolds are on the market, but they are definitively expected to rise in importance, as many are currently undergoing clinical testing. At the time of this review, clinical trials.gov listed multiple clinical trials that are either in recruitment of patients or active for a variety of disease indications including novel constructs like antibody-derived fragments (100 ongoing or completed clinical studies), nanobodies (5), fusion proteins (427), bispecific antibodies (67), and antibody drug conjugates (126).

b. Overview of novel constructs (antibody-derived fragments, fusion proteins, pegylated molecules, ADC, bispecific molecules)

Novel antibody formats can be clustered in antibody-derived fragments, fusion proteins, antibody drug conjugates, and bispecific or multispecific antibodies possessing multiple antigen-binding sites (Table 22.1).

Table 22.1 Overview on novel constructs [1]

Forms	Construct	Pipeline/marketed examples
Antibody fragments	Fab and (Fab)2	Ranibizumab (Lucentis)
	Single-chain Fv (scFv)	
	Single domain antibodies (sdAbs)	TNFR1 Dab (Cephalon, GSK)
	Nanobodies	Anti-vWF (von Willebrand factor) Nanobody, Anti-IL-17A/F Nanobody, Anti-IL6R Nanobody
	scFab, scFv-Fc, Fv-CH, sc scFv-zipper	
Fusion proteins	Fc fusion protein	Etanercept (Enbrel) Abatacept (Orencia) Romiplostim (Nplate)
	Albumin fusion protein	Dulaglutide (TrulicityTM) Albiglutide (TanzeumTM) Aflibercept (EYLEA)
Antibody drug conjugates	Antibody linked with cytotoxic payload	Brentuximab vedotin (Adcetris) Trastuzumab emtansine (Kadcyla)
Multifunctional antibodies	Bispecific mAbs (2 homodimers)	AFM13 (CD30/CD16) Affimed Therapeutics
	Bispecific mAb-scFv	
	Diabody: two mAbs, each separately mutated in the CH_3 domain	Genmab
	Bispecific T-cell Engager (BiTe)®two linked scFv units	Blinatumomab anti-CD3/CD19 (Micromet)
	Dual-variable domain immunoglobulin (DVD-Ig)	

In general, novel formats are more complex than an antibody, e.g., different antigen-binding domain in a same molecule, different molecular domains linked through flexible linkers, heterogeneous product through conjugation of cytotoxic drug to amino acids, and their physical–chemical attributes, manufacturability, stability, and in vivo properties have to be carefully assessed to select candidates

going from discovery to development stage. Very often, formulation challenges are more pronounced due to overall decrease of conformational stability, decreased solubility and/or precipitation, increased aggregation, higher levels of protein degradation and chemical instability, viscosity challenges and potential issues with material incompatibility and adsorption [2, 3].

Antibody Fragments

Since for some diseases the longer serum half-life and/or cell-killing mechanisms of mAbs are not required, antibody-derived fragments, skipping the Fc (fragment crystallizable) part, are rising in importance. The most prominent antibody-derived scaffold is the fragment antibody binding (Fab) part; additional potential formats of antibody fragments known are single-chain scFv, nanobodies, and single domain antibodies (sdAbs). Whereas sdAbs as smallest portion derived from native antibodies are consisting of either a single stable heavy chain or light chain variable domain (VH or VL), only, scFv is made of one VH linked through a flexible hydrophilic spacer to one light chain variable domain (VL). Very similar to sdAbs are nanobodies as single variable domains (called VHH). They can be derived from llama IgG2 and IgG3 whole antibodies that do contain only two heavy chains and do not exhibit the classically associated light chains [4]. All, sdAbs, scFvs and nanobodies can be further engineered to generate more complex heavy multiunit formats such as bispecific antibodies (see section below on multifunctional antibodies).

All antibody-derived fragments contain the variable domains responsible for binding to specific antigenic epitopes, which are known to be quite susceptible to unfolding and aggregation when present in antibody fragments or single domain antibodies as compared to intact antibodies. The better stabilization of these variable domains is assumed to be derived from multiple effects of various non-covalent interactions between complementary domains in a full-length antibody, such as sugar interaction and covalent disulfide bonds. This complementary stabilization remains preserved, and the more domains of the native antibody are present, resulting in a relatively higher stability of Fab fragments as compared to Fv fragments which show elevated tendencies to unfold and aggregate [5].

Fusion Proteins

Fc fusion proteins are considered a major class of novel constructs, comprising a protein, peptide, or receptor exodomain fused to the Fc portion of an IgG molecule. In these IgG-like molecules, the Fc portion typically contains the hinge region usually along with the conserved N-glycosylation site in the CH_2 domain. Thus, half-life extension of the attached smaller proteins or receptors is achieved by the Fc portion, without much affecting their biological activity. A similar effect is known by classical albumin fusions.

The first of these new drugs to be approved was etanercept (Enbrel), a fusion portion containing a section of the tumor necrosis factor (TNF) receptor fused to the Fc portion of human IgG1. Abatacept (Orencia) is another fusion protein that is constructed of a modified IgG Fc domain and the soluble part of the T-cell receptor CTLA-4.

There are, however, intrinsic challenges that need to be overcome, such as immunogenicity that may occur due to the formation of novel epitopes at the junction between the fusion partners even if fully human proteins are connected. In contrast to native IgGs or naturally occurring multidomain proteins, genetically engineered fusion proteins often lack mutual inter-domain stabilization. Thus, the conformational stability of the least stable domain was observed to trigger the aggregation propensity of the fusion molecule abatacept and to support identification of most favorable formulation conditions to reduce aggregate formation [6]. Kleemann et al. reported the susceptibility of the peptide moiety for posttranslational and chemical modifications due to the lack of structural preservation. Potential degradation reactions may be oxidation, deamidation, isomerization, disulfide scrambling, and clipping [7]. Add link to other book chapter.

Antibody Drug Conjugates

Therapeutic antibodies are employed as delivery agents being conjugated with chemotherapeutic drugs, immunotoxins, radioisotopes, or cytokines to deliver cytotoxic payloads to tumor cells [1]. The antibody drug conjugates (ADCs) link the cytotoxic drugs chemically through a stable or specifically cleavable linker to either Lys residues or Cys residues, which allows release of the toxin from the antibody after cellular uptake of the ADC by the tumor cells. Beyond the stability of the antibody, formulation development needs to consider as well any stability issues of the attached agent and stability of the linker used to join the attached agent to the antibody. The pharmaceutical development of ADCs is covered in chapter xxx of this book.

Multifunctional Antibodies

Bispecific and multispecific antibodies contain two or more variable domains with specific affinity to bind different antigens. The first bispecific antibody to reach the market in 2009 was catumaxomab (RemovabTM), with each one variable domain targeting both EpCAM and CD3. Nowadays, the manifold of existing different formats of bispecific antibodies comprises IgG-like and Fab fragment-based constructs, which may have additional antigen-binding domains attached, as well as tandem to multiple single-chain Fv or diabody fusion proteins.

Multifunctional antibodies can be generated by a variety of different protein engineering approaches, such as DuoBody, dual-variable domain immunoglobulins (DVD-Ig), dual affinity retargeting technology (DART), bispecific T-cell Engager (BiTE). DuoBody is formed by combining two antibodies, each separately mutated in the CH3 domain, resulting in a more favorable recombination over formation of the parental antibodies when reduced and re-oxidized. DVD-Igs carry four variable-binding domains, since each heavy and light chain contains two variable-fused domains. A bispecific, monovalent scaffold, based on two scFvs, being associated with a disulfide bridge, is obtained by DART technology. The VH and VL of one given variable domain are located on each side of the two different polypeptide chains. Bispecific T-cell Engager (BiTE) as a monovalent diabody scaffold is also formed by two scFvs, however, being connected by flexible linkers rich in glycin and serine residues. For the tandem antibody (TandAb), the two scFv

fragments are being associated with non-covalent interactions in a head–tail orientation to form a bispecific and tetravalent molecule [4].

IgG-like bispecific antibodies are either asymmetric or symmetric. Asymmetric bispecific antibodies bear the inherent problem of heavy and light chain mispairing.

22.2 Profiling of Novel Construct Molecules

In past, selection of bio-therapeutics candidates transitioning from discovery into clinical development has been mainly focused on biological properties pertaining to target binding and activity profiling. This approach can lead to selection of some poorly behaved molecules that present severe challenges during drug substance and/or drug product production. Significant resources need to be incurred to overcome these technical challenges, and in some cases a patient convenient drug product may not be feasible due to stability issues. This has highlighted the need for developability assessment to ascertain manufacturing feasibility, ease of formulation, in vivo compatibility and immunogenicity in discovery phase. This approach has now been widely adopted in biopharmaceutical industry to select molecules with good biological and developability properties.

Developability assessment is even more important for novel formats as contrary to antibodies most organizations have limited experience in development and commercialization of these molecules. Assessment of molecular properties during early phases of drug development is critical to flag potential challenges that might lead to technical failure. This section will outline a generalized approach to developability assessment for candidate selection with focus on molecular liabilities pertaining to non-antibody formats.

Developability Profile of a Candidate

From development perspective, an ideal candidate can be produced with high yields and quality in a standard manufacturing process. It can be formulated in a liquid formulation at high concentration to enable a self-administration drug product presentation if desired. It is stable under physiological conditions and does not have atypical pharmacokinetics properties. Additionally, it does not present immunogenicity risk associated with the format or sequence. Many novel format candidates do not exhibit these desired properties, and early profiling can support deselection of poorly behaved molecules or recommend protein engineering approach for mitigating developability challenges. Table 22.2 describes a detailed list of developability parameters for mAb candidates with additional considerations for novel format molecules. These can serve as an initial guidance for drug development that should be adapted based on intended target product profile (TPP). For instance, high-concentration formulation is not a major consideration for potent molecules that are efficacious at a low therapeutic dose. Similarly, self-administered drug

Table 22.2 Developability benchmark for candidate selection

Category	Attribute	Criteria for mAb like molecules	Considerations for novel format molecules
Manufacturing	Expression system	High titers >1 g/L Control of glycoforms	Variable titers dependent on expression system Control of proteolytic clipping
	Purification process	2–3 chromatographic step process with standard resins High overall yield >50%	Nonstandard resins with multiple process steps Yields variable, generally lower for molecules with clipping products
Product quality and stability	Aggregation	<5% HMW species over product shelf life (1)	Higher aggregation propensity for some molecular formats, e.g., bispecifics, ADCs
	Clipping	<5% fragmentation over shelf life (1)	Clipping at linker regions for novel formats
	Posttranslational modifications	No hot-spots for deamidation, isomerization, oxidation, and glycation in CDR region No abnormal glycosylation sites in the sequence	Hot-spots difficult to reengineer for native protein, e.g., Fc-fusion molecule O-linked glycosylation sites for some formats
Formulation	Solubility	>100 mg/mL to support high dose No precipitation in physiological conditions	Solubility limitation due to hydrophobicity, e.g., ADC, Fc-fusion proteins
	Viscosity	<= 20cP for high-concentration products	High viscosity for some bispecific formats
	Dosage form	Liquid in a vial or prefilled syringe	Liquid formulation challenging due to stability
Pharmacokinetics	FcRn binding	Binding at low pH and minimal binding at high pH	Only relevant for Fc fusion formats
	Half-life	Typically 10–14d (2)	Half-life variable-based on format. ADCs and Fc-fusion typically have shorter half-life than mAbs
	Serum stability	No significant loss of binding and activity after storage over half-life No significant degradation (cleavage) over half-life	Proteolytic clipping consideration in serum for fusion molecules Hydrolysis of linkers leading to drug loss for ADC

product might not be an important criterion for oncology indication products that are administered in clinical setting.

Candidate Selection Process: Merging Biology and Developability

1. Steps in molecule generation from protein engineering to target validation and integration of developability

The process of generating candidate molecules starts with immunization with a target or screening against an in vitro phage or hybridoma library to yield initial set of binders. In vitro, library screening approach can typically generate an initial hit of candidates that are on the order of 10^2. These candidates are tested in binding and activity assays and molecules with poor potency are deselected. Site-directed mutagenesis of CDR might be performed to increase binding affinity if the potency is too low. This process typically reduces the set of candidates to the order of 10^1 that are further characterized for potency in cellular functional assays (3). Humanization or germ-lining might be needed to minimize immunogenicity risks for candidates that are derived from non-human species. The lead molecules from the pool of candidates are then studied for efficacy, tissue cross-reactivity, and preliminary toxicity in animal model to select the final candidate to move into clinical development.

Integrating developability assessment in the screening funnel enables consideration of both biological and manufacturability properties during candidate selection. Similar to biological assessment, developability assessment is performed in multiple stages starting with high-throughput analysis using selected assays to screen large number of molecules followed by detailed characterization of top molecules to select a lead candidate.

The candidate selection process described below is mainly established for mAbs where large number of candidates is generally available in discovery phase (3,4). Similar concept is applied for novel format candidate, but the number of candidates varies based on the format. Bispecific molecules can have large number of candidates from CDR diversity, while fusion constructs typically have limited candidates from half-life extension (albumin or Fc fusion) or linker length variants.

2. Multistage drug product developability process

Figure 22.1 describes a generalized two-staged approach for developability assessment that is performed in parallel to functional activity testing to select a lead candidate. At stage 1, some selected biophysical and product quality attributes are tested on a large number of candidates with limited material availability. Molecules are typically produced using discovery stage process at this stage which is often not representative of clinical process; therefore, measurement of product quality attributes such as charge variants, glycosylation is not relevant to test at this stage. Melting temperature, hydrophobicity, and isoelectric point (pI) are some of the key biophysical properties that can be tested as taken together they can provide an estimate on overall stability of molecule. These tests can be performed with a small amount of material in a high-throughput setup which is an important consideration at stage 1 due to material availability.

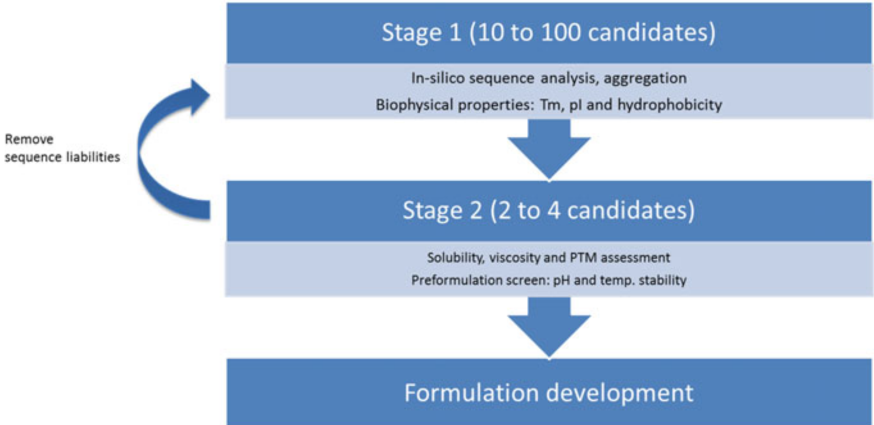


Fig. 22.1 .

Differential scanning calorimetry (DSC) or differential scanning fluorimeter (DSF) technique can be used for measuring melting temperature. These techniques are shown to correlate well for determining major unfolding transition of a molecule (5). DSC usually provides a better resolution for low endothermic transitions and for novel formats like fusion proteins; it might be better technique for determining the melting transition of a small fusion partner. It is useful to study the change in melting temperature in different buffers as for some novel formats it can vary significantly across a pH range. This data is useful for designing the formulation studies in later stages. If material is limited for an extensive pH screening, melting temperature can be measured at two pH conditions, low pH away from pI representative of formulation buffer and at physiological pH representative of in vivo environment. The molecular design of a novel format can have a significant impact on conformational stability. Molecules constructed by fusing different domains by linkers can be destabilized due to inter-domain interactions. Figure 22.2 shows the melting temperature profile of a multivalent antibody fragment molecule constructed by fusing three V_{HH} domains using flexible—GS—linkers. The melting

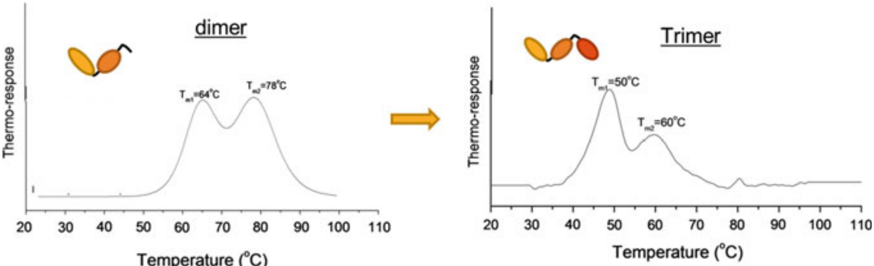


Fig. 22.2 Melting transition of multivalent V_{HH} domain protein

transition of the dimer is lowered by almost 10 °C by fusion of the third V_{HH} domain. Molecule construct might be optimized by changing the fusion construct from N to C terminus or by altering linker length to minimize inter-domain interactions.

Hydrophobicity is a general measure of exposed hydrophobic patches in a molecule. Highly hydrophobic molecules pose development challenges due to aggregation, solubility, or non-specific binding. Common techniques used to measure average hydrophobicity of a molecule include hydrophobic interaction chromatography (HIC), reverse phase chromatography (RP-HPLC), or fluorescence spectroscopy using hydrophobic dyes (6). Any of these methods can be applied to rank molecules based on hydrophobicity. Isoelectric point (pI) and molecular charge can be calculated from the amino acid sequence, and for antibody formats it generally correlates well with measured pI. However for some novel formats like fusion proteins, there can be significant differences between calculated and measured pI and actual measurement might be necessary.

Among product quality attributes, soluble aggregates by size exclusion chromatography (SEC) and higher order aggregates by light scattering are important attribute to be measured at stage 1 of developability assessment. A higher threshold should be used for flagging aggregation risk at this stage if molecules are not produced in a representative process and/or partially purified. As candidates are generally tested at a low concentration at this stage, significant aggregation (>10%) finding indicates a high propensity of aggregation and should be used as a criteria for deselecting a molecule. Sequence analysis can be performed at this stage to identify potential hot-spots for posttranslational modifications, e.g., deamidation, isomerization, and oxidation, particularly in the binding region. Advanced sequence analysis using homology models and aggregation "hot-spot" identification techniques like spatial aggregation propensity (SAP) can be applied to flag liable molecules (7). For novel formats, particularly fusion proteins, assessment of molecule integrity, i.e., clipping, can be done at this stage using a high-throughput method like chip-based gel electrophoresis (Caliper technology) if molecules are produced in an expression system similar to clinical development.

The combined assessment of biological properties and developability parameters is done to narrow down the pool of candidates at stage 1 and select the top candidates for further evaluation. From developability perspective, while all parameters should be taken into consideration, aggregation, sequence hot-spots, and clipping (for novel constructs) can serve as primary criteria for ranking and deselection.

The candidate number in stage 2 can vary from 2 to 4 candidates depending on available molecules that still possess the desired biological properties. If multiple candidates are selected, it is important to factor in sequence diversity to ensure all molecules do not carry similar liability. The extent of molecular assessment is dependent on the availability of material at this stage. If candidate number is limited, several grams level of material can be produced through a representative process allowing for detailed preformulation characterization at high concentration. Alternatively, if the amount of material is limited to hundred milligram level, then high-concentration properties need to be tested using surrogate assays.

Solubility and self-interaction propensity are important developability parameters to test at this stage of developability to assess risk for developing high-concentration products. Solubility can be measured by different techniques including concentrating the protein solution using a membrane-based ultrafiltration approach. Although a very straightforward approach, it is often not practical to apply this at early stage of developability assessment due to high material need and low throughput. PEG precipitation is a useful alternative technique that can be applied to measure solubility in multiple solution conditions with a few mg of protein in a high-throughput manner. It has been shown that a log-linear relationship exists between protein solubility and weight percent of poly ethylene glycol (PEG) in the solution. Molecules with high solubility require higher weight percent of PEG to precipitate, and comparative assessment of solubility can be easily performed. However, extrapolation at zero PEG concentration overpredicts the maximum solubility as non-idealities present in high protein concentration solutions are not accounted. Therefore, this technique is mainly useful for comparative ranking purposes and confirmation of protein solubility by other techniques is needed to covert PEG concentration to actual solubility range. Mechanism of PEG precipitation is based on depletion of hydration layer around a protein molecule; therefore, different molecular formats show differences in this assay due to changes in size and conformation. Threshold needs to be established for each molecular format individually, and criteria established for mAbs might not be directly applicable to other formats.

Self-interaction propensity is another important parameter for developability of high-concentration liquid formulation products. Molecules with attractive self-interaction parameter tend to show viscosity or self-association at high concentration (4,8). Self-interaction chromatography (SIC) and diffusional interaction parameter (k_D) are some of the techniques established to measure self-interaction parameter for developability assessment. Both SIC and k_D measure different parameters that can be correlated to second virial coefficient (B_{22}) that provides a measure of colloidal stability of a solution. Diffusional interaction parameter (k_D) is particularly suitable for developability assessment as it requires very small amount of material and can be performed in plate-based high-throughput format using a dynamic light scattering (DLS) instrument. It has been established that molecules with negative k_D demonstrate attractive self-interactions at high concentrations leading to high viscosity. Diffusional interaction is an important developability parameter to test at stage 2 of developability assessment as a predictor for viscosity. Threshold criteria should be established for assessing viscosity risk from k_D values by testing a set of molecules. Correlation of k_D with viscosity has not been established for novel format molecules, and it might be different from mAbs. However in general, positive k_D values are an indicator for repulsive interactions between molecules and can be used as a criterion for ranking candidates when tested in similar conditions.

Molecules flagged with PTM “hot-spots” in CDR or binding region should be tested for susceptibility of modification. These PTMs include deamidation, isomerization, oxidation, glycation, and undesired glycosylation. Intact or reduced

mass spectrometry measurements can be performed to measure oxidation, glycation, and glycosylation variants (9-11). For novel format molecules, characterization should be done to check for the presence of o-linked oligosaccharide moieties in linkers or other regions of the molecule. These modifications can have an impact on activity or immunogenicity of the molecule and bring additional challenges for analytical characterization. Enzymatic digestion followed by analysis by LC-MS is needed to test for PTMs like deamidation, isomerization, and glycation in a molecule. Wherever possible, stress test should be performed to accelerate the rate of modification by incubating molecules in extreme pH buffers at high temperature. Short incubation at 37–40 °C at low pH (pH < 5) can accelerate aspartate isomerization reaction, while high pH (pH > 7) promotes asparagine deamidation. Samples from stress test should be assessed for loss in biological activity by testing them in binding or activity assays together with control samples. Molecules that show drop in potency from modifications at PTM site should be considered high risk for developability. They are either mutated to remove the susceptible site or deselected in favor of other candidates. Even if a PTM site does not result in a loss in activity, a susceptible site introduces additional heterogeneity in a molecule that needs to be further characterized later in development to assess if it is a critical quality attribute (CQA). This additional characterization effort should be avoided by mutation of the site if possible or ranking the molecules lower in favor of molecules with no PTMs.

Molecules are ranked for developability by comparing solubility, self-interaction propensity, PTM liabilities, and biophysical properties to recommend the top candidate from technical development considerations. If additional material is available, then preformulation assessment at high concentration can be performed to further differentiate these candidates. This includes confirmation of actual solubility and viscosity of candidates and studying short-term stability of molecules in different formulation conditions to support formulation development. High-concentration formulations (>100 mg/mL) are needed for molecules that require high doses (>1 mg/kg) for efficacy. Solubility should be tested at different pH and buffer conditions at high concentrations ($\geq$ 100 mg/mL) by incubating protein in different buffers and monitoring loss in concentration due to precipitation. Solubility is influenced by multiple parameters, e.g., pH, ionic strength, and temperature. and if material allows, candidates should be compared across a range of pH and ionic strength conditions at different temperatures. Molecules that are soluble across a broad range of solution conditions provide a large design space for formulation development and are preferred from development perspective. It is important to include buffers that mimic physiological condition in the solubility screen to ascertain any potential precipitation challenge upon administration of the drug by intravenous or subcutaneous routes. Table 22.3 provides a general setup for solubility assessment that can be performed for developability assessment. Some non-mAb molecules can show significant solubility challenges typically not expected based on the pI of the molecule. Figure 22.3 shows the solubility data of an Fc-fusion molecule in different pH conditions ranging from acidic to neutral with or without salt. This molecule shows solubility in very narrow pH range and precipitates in the presence of low concentration of salt. The results are unexpected

Table 22.3 Parameters to be studied during solubility assessment

Protein concentration	pH	Ionic strength	Temperature
50–150 mg/mL	4–8	0–300 mM	5 and 25 °C

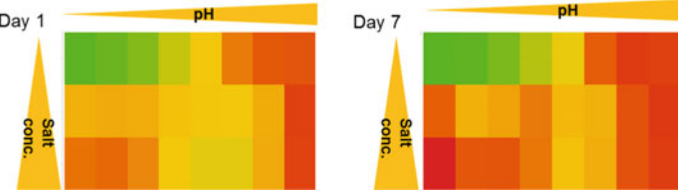


Fig. 22.3 Solubility of a fusion protein at different pH and salt concentrations

as this molecule has a theoretical pI of ~8 but shows precipitation at 3–4 pH units away from the pI where it is expected to be highly positively charged. This highlights the need for assessing solubility behavior early in development to develop strategies for overcoming these technical challenges.

Viscosity is another critical parameter to be confirmed at this stage of developability. It should be assessed as a function of protein concentration in a relevant formulation buffer for comparison. Molecules with high viscosity pose manufacturing challenge during ultrafiltration/diafiltration step, filtration and filling operations. Moreover, viscous proteins bring drug delivery challenges due to high injection forces that might fall out of the range for a self-administration. This can significantly impact the commercialization potential of a molecule, and therefore viscosity of candidates should be measured as a function of shear rate using a rheometer and compared as a critical parameter for developability assessment.

Long-term stability of high-concentration liquid formulations can be challenging due to degradation mechanisms such as aggregation, particle formation, and chemical modification. It is important to assess the risk for long-term stability of liquid formulation for different candidates during developability assessment. Preformulation assessment can be performed to study accelerated stability of molecules in representative formulation conditions. The goal of the assessment is not to define a formulation composition for clinical development but instead to identify challenges in developing one. A small set of formulation composition spanning a range of pH conditions and excipient types that have been shown to stabilize most molecules can be included in this screen. In some organizations, a formulation platform might have been established for early stage clinical stage molecules. Preformulation assessment can include pH robustness around a platform formulation to check if a molecule is fit for the liquid formulation platform. The assessment includes short-term liquid stability (4 weeks) at accelerated storage temperatures of 25 and 40 °C. Molecules are tested for aggregation, clipping, and charge variants by SEC, CE-SDS, and ion exchange chromatography or capillary zone electrophoresis, respectively. Studies can be performed in plate-based setup to minimize material consumption. In addition to temperature stress, molecules should

also be tested for stability against freeze/thaw and agitation stresses that are typically encountered during manufacturing and shipping. The analytical results from all assays are summarized, and molecules are rated based on fit to a formulation platform or ease of developing a high-concentration liquid formulation. A similar setup can be performed for novel format molecules; however, formulation conditions should be adapted based on the biophysical properties of the molecule. Formulation platform that is usually established for mAbs might not be suitable for novel formats. It is advisable to test stability across broad pH ranges and with multiple excipient types to understand the degradation pathway of these molecules. It is also useful to study stability of molecules at multiple product concentrations in preformulation screen to support later stage formulation development. Accelerated temperature studies should be setup carefully considering the onset of melting transition for some novel format molecules. For some molecules, the onset of unfolding can be close to accelerated temperature stress resulting in significant degradation that might not be representative of stability at storage temperature. Table 22.4a, b describes a typical preformulation screen and stress conditions that can be included in developability assessment. These screens are amenable to plate-based setup and can be easily adapted to work with automated setup using a liquid handling system to increase throughput.

The results from biophysical assessment, high-concentration screens, and preformulation screen should be summarized to perform a detailed developability risk assessment on the available candidates. Molecule with favorable properties in most assays is recommended as lead candidate from technical development, and selection of these candidates should be pursued.

Table 22.4 **(a)** Preformulation setup for high-concentration products. **(b)** Accelerated stability and stress conditions

(a)

Protein concentration	pH	Excipient/s	Surfactant
50–150 mg/mL	4–8	Sugars and amino acids	PS-20 or PS-80

(b)

Stress	Time points/cycles
40 and 25 °C	0, 2w and 4w
Freeze/thaw (−70 °C freezing/25 °C thawing)	3 or 5 cycles
Agitation stress	2–5 days

22.3 Formulation Strategies for Novel Construct Molecules

a. Target product profile: dose/concentration and application route; dosage form: lyo versus liquid

In addition to the current and future improvements coming along with protein and recombinant antibody therapeutic drug design leading to additional novel constructs, formulation scientists are making several efforts in order to cope with the requirements for customer-tailored biological drug products [2].

While developing a formulated product, the formulation scientist must consider several aspects. Hence, formulation strategy should be established by addressing a host of questions that gives guidance on what to develop and on the definition of the target product profile (TPP). The following table presents examples of what needs to be considered for each type of evidence [8] and shows examples of the TPP for three marketed products—one Fab fragment, one fusion protein, and one ADC (ref). Even though the development of drug product formulation requires certain studies to be carried out regardless of the type of product, the final target product profile will set the basis on which studies will be performed first (Table 22.5).

In general, the activities necessary to develop a parenteral product can be divided into the following three broad areas: preformulation, formulation, and scale-up.

Molecular profiling and developability assessment including biophysical criteria, as described in previous section, act as starting point for preformulation activities. Preformulation includes the general characterization of the bulk drug such as the determination of degradation routes and the common physicochemical factors (structure of protein drug, isoelectric point (pI), molecular weight, amino acid composition, disulfide bonds, spectral properties hydrophobicity, glycosylation). Additionally, susceptibility of the protein candidate to a variety of pharmaceutically relevant stress conditions should be evaluated, thereby establishing stability-indicating assays and identifying the major formulation challenges [9].

A thorough formulation development program focuses on the choice of the appropriate dosage form and the optimization of several factors contributing to protein stability such as the most powerful variable of pH value, the necessary excipients with appropriate concentrations, the container closure system, and the storage conditions [8, 10]. Basically, a stable formulation of a therapeutic biologic planned for market use—either being liquid or lyophilized—should reveal a shelf life of minimum eighteen months, at least if stored under refrigerated conditions, and meet the common requirements of parenteral formulations including the need for sterility, isotonicity, and being near-neutral pH [10].

Further obligatory studies address protein stability during processing, storage, transportation, handling, and delivery and confirm the exclusion of critical in vivo biological properties of the protein formulation observable in preclinical and clinical studies [8]. Finally, scale-up activities aid in moving the product to a manufacturing

Table 22.5 Required information for designing formulation studies of protein pharmaceuticals. Adapted from [8] and product prescribing information

Information	Examples	Marketed products target product profile		
		Lucentis® (ranibizumab)	Orencia® (abatacept)	Kadcyla® (ado-trastuzumab emtansine)
	Class	Fab fragment	Fusion protein	ADC
Clinical indication	Site of treatment (self-administration, office visit, hospital), methods of delivery, concomitant medication, competition	Wet age-related macular degeneration (wAMD), macular edema following retinal vein occlusion (RVO), diabetic macular edema (DME) Hospital administration.	Rheumatoid arthritis (RA)	Metastatic breast cancer (mBC) Hospital administration Concomitant medication
Patient population	Age, strength, tolerability, capability to manipulate devices, sensitivity to excipient	Geriatric population	Population with inadequate response to anti-TNFα therapy	Oncology patients
Typical routes of delivery	Injectables (intravenous (IV), subcutaneous (SC), intra-articular (IA), intravitreal (IVT), intramuscular (IM), intrathecal (IT), topical, inhalation, nasal, oral, etc.	Intravitreal injection (IVT) – 0.05 mL	Intravenous infusion (IV) or subcutaneous injection (SC)	Intravenous infusion (IV)
Dose requirement/ concentration	Pharmacokinetics (PK) profile, frequency of dosing, variable vs. fixed dose, single dose/multidose	Administration once a month. Fixed dose Single dose container	After initial dose, administration at 2 and 4w, then every 4w Single dose container	3.6 mg/kg given as an iv infusion every 3 weeks. Single dose container
Drug interaction	Co-administration with other drug, dilution or reconstitution with other solution; the presence of undesirable compounds, e.g., reducing sugars, preservatives	Not applicable	Insufficient experience to assess safety and efficacy of Orencia administered concurrently with other biologic RA therapy	No formal drug–drug interaction studies have been conducted

(continued)

Table 22.5 (continued)

Typical dosage forms	Liquid, lyophilized, spray-dried aerosol by liquid or powder, other novel carrier; stability, physical properties, reconstitution approach	Liquid in vial at 10 mg/mL solution (0.5 mg) and 6 mg/mL solution (0.3 mg) in a single dose	Lyophilized powder in vials containing 250 mg per vial. Liquid in prefilled syringe at 125 mg/mL	Lyophilized powder in single-use vials containing 100 mg per vial or 160 mg per vial
Container/closure	Vial/stoppers, prefilled syringes, prefilled cartridges, dual chamber, cartridges, product contact, material, leachables, breakage, light sensitivity, moisture penetration	Glass Vial/stopper combination	Glass Vial/stopper combination. Prefilled syringe	Glass Vial/stopper combination
Delivery device	Syringes, prefilled syringes, pen injectors, auto-injectors	Not applicable Administration using a 5-micron, 19G filter needle, a 1-cc tuberculin syringe, and a sterile 30G x ½” needle for the IVT injection	Prefilled syringes	Not applicable Administration using sodium chloride infusion bags and a 0.2 or 0.22 mm in-line polyethersulfone (PES) filter. Do not administer as iv push or bolus

site. Critical process parameters such as in-process changes in pH, storage and handling conditions, compounding sequence, material compatibility, agitation, filtration, filling, and lyophilization cycles should be addressed in the design and evaluation of the manufacturing process and fill and finishing [11].

With regard to the definition of dosage form, despite the fact that lyophilization is often used to preserve biopharmaceuticals and the first therapeutic proteins were primarily formulated in freeze-dried form, it is desirable and convenient to develop biological therapeutics as ready-to-use, aqueous formulations. The rationale behind this suggestion is among other things the potential reduction of protein stability during both the freezing and drying process, the implications of an improperly performed re-dissolution step for patient safety and the workload of preparation for administration. Besides that, liquid dosage forms are cheaper to manufacture. Indeed, newly available proteins and recombinant monoclonal antibodies are increasingly launched as liquid formulations posing a variety of physical and chemical degradation pathways and this is the trend for novel formats as well [12, 36].

Further production technology and formulation challenges are evolving, particularly with the need for remarkably stable or soluble high-concentration protein formulations and the preference to administer those by subcutaneous (s.c.) injection in view of self-administration [13, 36]. For novel biologics formats, the definition of the intended dosage form as an aqueous solution versus a dry formulation is therefore primarily triggered by the solubility and stability of the molecule, posing an additional challenge. The early definition of dose requirement/concentration is thus of increasing importance in these cases [3].

b. Formulation screening: broader screening required than for mAbs; pH-optimum and buffer choice, addition of functional excipients, e.g., antioxidants

For novel biologics molecules, a more tailored, customized formulation development with broader preformulation and formulation screenings is required to ensure molecule stabilization. Formulation screenings may include an initial solubility screening evaluating broad protein concentration and broad pH range based on molecule solubility, to obtain a range of optimum pH and selection of buffers suitable for that target pH. Other parameter that should be considered at this stage is viscosity, evaluating of the impact of protein concentration. Afterward, a formulation screening should be performed (in the liquid and/or lyophilized state) with focus on evaluation of excipients (stabilizers, functional excipients, surfactants) and optimization of excipient concentrations. These formulations should not only be evaluated upon thermal stress but also after mechanical stress as freeze/thaw and agitation/shaking studies [14]. Optimization studies might be performed if needed, looking at optimum excipient concentrations. If a lyophilized drug product is being developed, additional lyophilization screening studies could be required.

The most widely used protein-stabilizing excipients that may be used in formulation studies of novel constructs can be broadly divided into categories: buffering agents, sugars and polyols, amino acids, salts, surfactants, and antioxidants (Table 22.6) [15].

Table 22.6 Common excipients used in protein formulations (according to [15, 37])

Excipient class	Examples	Effects
Stabilizing buffer	• Acetate, citrate, phosphate • Glycinate, histidine, maleate, succinate, tartrate, tris	• Prevent small changes in pH • Additional stabilizing effects through buffer–ion-specific interactions with protein
• Sugars and polyols	• Sucrose, maltose, trehalose • Glycerol, mannitol, sorbitol, xylitol	• Non-specific protein stabilizers (preferential exclusion) in liquid and lyophilized states • Tonicifying agents • Protect from protein oxidation • Cryo- and lyoprotectants
• Salts	• Sodium, potassium, magnesium chloride • Ammonium, sodium sulfate	• Non-specific protein stabilizers (preferential exclusion) or destabilizing effects on proteins, especially with anions (Hofmeister salt series) • Electrostatic shielding • Tonicifying agents • Cryoprotectants
• Amino acids	• Arginine, glycine, histidine, lysine, proline	• Non-specific protein stabilizers (preferential exclusion) • Buffering and tonicity agents • Cryo- and lyoprotectants
• Surfactants	• Polysorbates (20, 80) poloxamer, pluronic	• Prevention of protein surface adsorption, denaturation and aggregation
• Antioxidants	• Ascorbic acid Sulphurous acid salts Cysteine, methionine	• Inhibit oxidation

A suitable buffer system should reveal a good buffering capacity at the optimal pH range (considering the buffer pKa), providing acceptable molecule stability and solubility, and be stable to temperature changes or freezing stress [8]. For a novel format with a pI >8, it may be required to evaluate different buffers (e.g., succinate, citrate, acetate, maleate, glycine, histidine), a broad pH range (pH 4.0–6.0) and wide-ranging protein concentration to find a condition with suitable solubility and viscosity (Fig. 22.4).

In this case, a formulation containing succinate is the one with best read out offering better buffering capacity toward lower pH and significant improvement over higher pH in precipitation behavior. In addition, lower protein concentration leads to lower precipitation and lower viscosity.

During a formulation stability study for a single-chain monoclonal antibody fragment, turbidity stays rather constant for high-concentration formulations upon thermal stress. However, buffer 2-based formulations show clear increased turbidity when compared to buffer 1-based formulations (Fig. 22.5a). The same trend is visible for aggregation product levels (Fig. 22.5b).

If aggregates or precipitates are detected during formulation development of a protein pharmaceutical, surfactants have been found to be effective stabilizers [16].

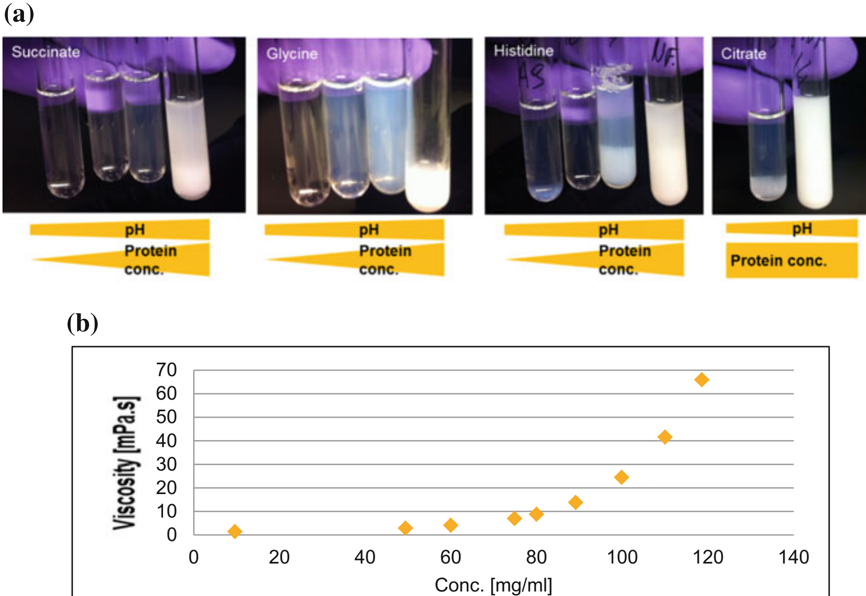


Fig. 22.4 Solubility screen for a fusion protein with dependence of pH, buffer system and protein concentration (**a**) and viscosity concentration profile (**b**)

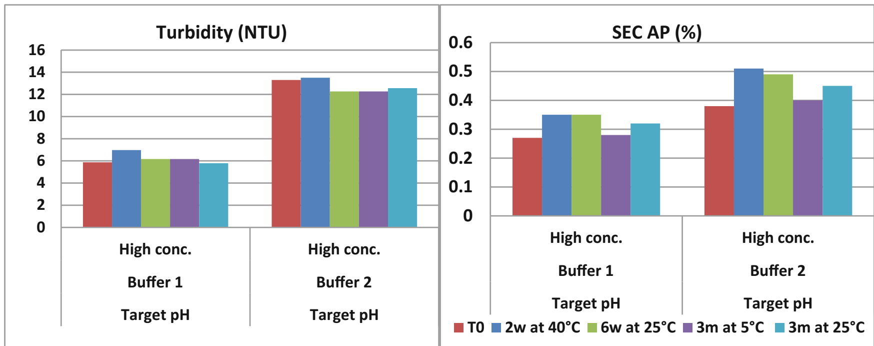


Fig. 22.5 Formulation screening stability study: turbidity (**a**) and aggregation products by SEC-HLPLC (**b**) results for a single-chain monoclonal antibody fragment

As ionic surfactants tend to denature proteins, low concentrations of nonionic surfactants, e.g., polysorbate 20 or 80, are generally preferred. However, the contamination of nonionic surfactants with peroxides and its implications on protein chemical stability should be taken into account. Various hypotheses exist for the stabilization of proteins against aggregation by surfactants [17].

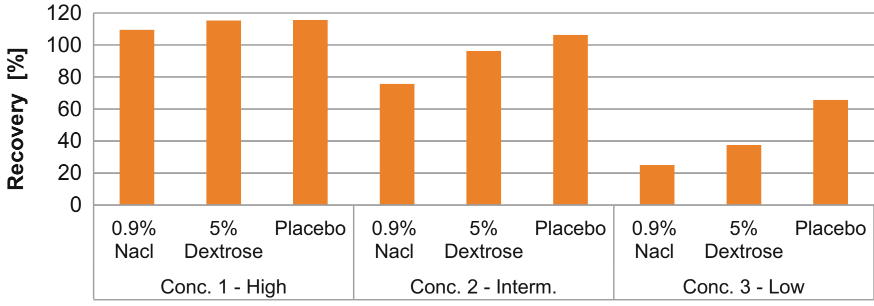


Fig. 22.6 Recovery obtained during compatibility feasibility testing for a single-chain monoclonal antibody fragment when diluted with 0.9% normal saline, 5% dextrose, or matching placebo to three different protein concentrations

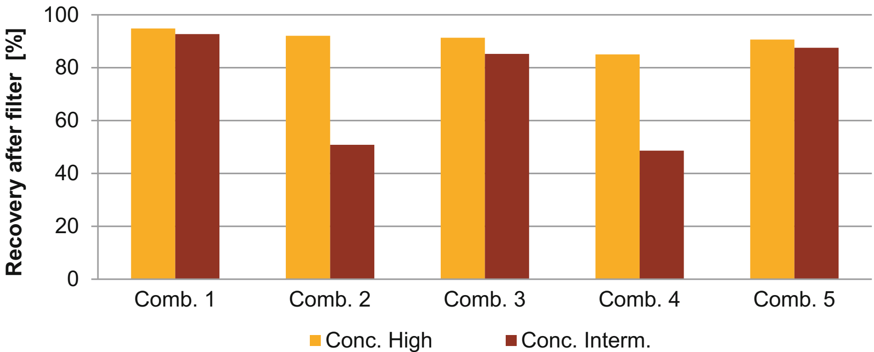


Fig. 22.7 Recovery obtained during compatibility feasibility testing for a single-chain monoclonal antibody fragment after simulated infusion through different in-line filters. The in-line filter product contact material for the different combinations is: #1 Polyamide+, #2 Neutral PES, #3 PES +, #4 Neutral PES, and #5 Polyamide+

The formulation components or excipients should be compatible with their intended use, which are preferably of pharmacopoeial grade and acceptable in the major pharmaceutical markets. This is of particularly importance if a formulation is intended for parenteral use (e.g., intravenous infusion, subcutaneous, ophthalmic, or intra-articular injection) since excipients should be non-toxic and physiologically harmless at the amounts administered to the patient, and the formulation should be sterile and exhibit acceptable viscosity and tonicity [10, 18].

c. Process development: freeze/thaw, filter, pump, lyo cycle robustness: high-low energy; safety factors for transfer

This section is intended to outline the general strategy of process development studies applicable to all novel formats, considering the need of flexibility to accommodate specific requirements of an individual molecule and/or process. Once

the preliminary formulation has been determined, but prior to the drug product manufacture at larger scale (e.g., to support toxicology studies or clinical studies), it is important to understand the processing conditions the molecule will undergo during the different unit operations in the manufacturing process [19]. Hence, the impact of the manufacturing process and fill and finishing operations on drug product stability may be monitored by simulating the critical process steps during manufacturing, in order to mitigate any potential risk for drug product production [11]. The ultimate goal of any process development activity is the production of a viable commercial product. Thus, a robust process that can be smoothly scaled up to commercial scale and executed reproducibly is a necessity.

In the past decades, the extensive development of monoclonal antibodies leads to the definition of platform approaches in drug product manufacturing by which the development timelines have been shortened. For novel formats, it is intended to consider these learning. Consequently, process development studies have the intention of either confirming that the manufacturing procedure primarily selected is applicable to the molecule being developed or, if using platform processes (with common process elements), to verify its suitability and identify early on potentially critical processing steps for which selected process parameters may require adaptation. Furthermore, process development studies should aim at identifying provisional in-process controls and corresponding requirements and to further help on definition of safety factors for the transfer to the final production site. It should also cover availability of physicochemical data to support process hold times [20].

Critical process parameters such as in-process changes in pH, storage and handling conditions, compounding sequence, material compatibility, agitation, filtration, filling, and lyophilization cycles should therefore be addressed in the design and evaluation of the manufacturing process. Table 22.7 presents the series of unit operations that should be evaluated during early phase drug product manufacturing development and associated stress factors that can impact final product quality [21]. The processing considerations are further covered in chapter xxx of this book.

Drug substance thawing: The freeze and thaw process may impact protein stability due to heterogeneous protein and solute distribution along with potential of aggregate formation [22]. After selection of the storage container, being the most typical plastic bottles or single-use plastic bags, thawing of drug substance should be done under defined conditions of temperature, agitation, thawing rate, and time. In addition, interim storage of bulk dug substance prior to pooling should be performed to investigate physicochemical interim storage stability.

Formulation compounding and mixing: During this step, the protein is exposed to different contact surfaces in combination with stirring and shaking stresses and its stability may therefore be impacted. There are also several air–liquid interfaces that can induce protein denaturation [23]. Hence, one of the parameters to be investigated during drug substance pooling and manufacturing of bulk drug product solution that may include dilution of that purified substance to a target concentration is homogenization, as controlled by the stirring time and speed. The pH adjustment strategy both in the buffer solution and in the final bulk drug product solution needs also to be evaluated.

Table 22.7 Process and performance parameters for various drug product processing operations [11]

Unit operation	Process parameters (examples)	Effects
Drug substance Freeze/thaw	• Temperature • Agitation during thaw • Freeze and thaw rates and time • Container	• Freeze concentration • Agitation • Ice–water interface
Formulation compounding and mixing	• Stirring time • Stirring speed • Batch size • Vessel type (e.g., stainless steel) • pH adjustment strategy	• Shear/cavitation • Air–liquid interface • Inhomogeneity
Filtration	• Capacity • Filtrate flow rate • Prerun and pressure • Vmax	• Shear • Adsorption • Dilution • Compatibility • Leachables and extractables
Filling	• Pump type • Interruptions • Fill weight and speed • Tolerances • Container closure integrity (CCI)	• Shear/cavitation • Inhomogeneity • Leachables and extractables
Lyophilization	• Shelf temperature • Chamber pressure • Freezing rate • Primary drying temperature • Ramping rate between primary and secondary drying • Drying time • Stopper moisture content	• Freeze concentration • Ice–water interface • Thermal history • Cake properties: collapse • Inhomogeneity • Residual moisture

Filtration: To achieve a sterile product, drug product fill–finish operations include one or more filtration steps. During this process step, the protein may be adsorbed on the membrane surface and this can lead to protein loss or misfolding on the membrane surface. This is of special importance for products with low concentration or in case the batch size is small and if the bulk is not being pooled after filtration, prior to filling [24]. Hence, evaluation of this process step should encompass filter selection as well as scale-up at the potential final manufacturing settings. Confirmation of suitability of selected membrane material/type (e.g., PVDF vs. PES; single layer vs. bilayer filter) should be performed evaluating the compatibility of the molecule with the filter after repeated filtration steps, mimicking a potentially necessary re-filtration in case of unexpected process deviations. Other process parameters to be monitored during filtration include the effect of differential pressure on bulk drug product stability.

Filling/dosing system selection: The pumping process may create air–liquid interfaces that can induce protein denaturation. Additional effects on product quality to consider when studying the filling system are related to the fact that the protein solution may experience high shear force as well as interaction with different

contact surfaces corresponding to containers (vials, syringes) or components (stoppers, plungers) [25]. Foreign particles can also be introduced during this process which may lead to protein aggregation that may be associated with immunogenicity risks. Hence, for dosing system selection investigations, the drug product solution should be continuously filled with different pump types (e.g., peristaltic, piston) at a speed covering worst case conditions, thereby investigating the general susceptibility of the molecule to pumping induced aggregation and degradation and overall bulk drug product quality. This also allows testing the compatibility of bulk drug product with the tubing material using during filling operations.

Lyophilization: Freeze-drying studies are an additional requirement in case a solid dosage form is being developed. Such investigations should include the characterization of bulk drug product solution and final lyophilisate thermal properties, i.e., the glass transition temperature of the maximally freeze-concentrated solution Tg' and the collapse temperature Tc to allow for a scientific-based decision regarding the freezing temperature and the maximum allowable product temperature during primary drying. Then, confirmation of freeze-drying conditions at full freeze-dryer load and robustness testing such as low and high energy runs with associated different freeze-drying parameters should also be performed [26]. Process analytical technology (PAT)-based automation controls may be used during these investigations. The optimized process should be robust and up-scalable and yield a comparable drug product.

As an example, Table 22.8 shows the thermal properties obtained for three formulations of a single-chain monoclonal antibody fragment with different target protein concentrations. The glass transition temperature Tg' decreases with decreasing protein concentration (lower protein to excipient ratio) whereas the residual moisture increases. In addition, Tg is much lower for the formulation with higher residual moisture which could lead to collapse of the lyo cake upon long-term storage. Hence, there is a need for lyophilization cycle development for low protein concentration formulation and this could be done by, e.g., lowering freezing temperature and changing primary drying conditions from a ramp to a step. With this lyo lyophilization cycle optimization, Tg increases from approximately 20–51 °C and the residual moisture is reduced to 0.9%.

Table 22.8 Thermal properties of three formulations with different target protein concentrations for a single-chain monoclonal antibody fragment obtained by DSC. (Tg' and Tg glass transition temperature)

Form. #	Cycle	Protein conc. (mg/ml)	Tg' (°C) during first heating (−50 °C → 20 °C)	Tg' (°C) during second heating (−50 °C → 50 °C)	Tg (°C) (−50 °C → 200 °C)	Residual moisture—H_2O per cake (%)
F1	1	20.0	−29.82	−29.65	19.3	2.9
F2	1	25.0	−27.73	−27.66	36.6	0.5
F3	1	33.3	−25.49	−25.48	48.1	0.2
F1	2	20.0	–	–	51.2	0.9

During fill and finish operations, the main stability concern would be physical stability of the protein. Nevertheless, the physical and covalent stability of the molecule should be determined using orthogonal assays in order to assess the overall stability of the molecule to various bioprocessing stresses. Hence, during process development studies, analytics should include, among others, particle analytics to monitor particle formation (e.g., visual, turbidity, sub-visible particles analysis), analytics to monitor aggregation and degradation propensity, and assay to quantify adsorption to surfaces.

d. Compatibility testing

The preparation of parenteral drugs is accompanied by the risk of incompatibilities, being this an undesirable physicochemical reaction that occurs between the drug and the solution, container or another drug and where the resulting product can affect the efficacy and safety of the therapy [27]. The main causes of incompatibilities in standard parenteral therapies are: (1) incompatible drug formulation (due to preservative, buffer, stabilizer, or solvent); (2) non-appropriate solution used as diluent; (3) incompatible material of administration containers or medical devices, which can concern the nature of the material used and/or reactions at the inner surface (e.g., adsorption); and finally (4) mixture of incompatible drugs (drug–drug incompatibility), e.g., within the same infusion line (simultaneous infusion) and/or intravenous container or when administered one after the other, but within the same infusion line [27, 28].

Physical incompatibilities may lead the drugs to become immobilized at the inner surface of administration containers or lines (adsorption) and so lowers the concentration, drastically decreasing the quantity of the drug administered to a patient and that can lead to a therapeutic failure. Furthermore, aggregation and/or particle formation may also be associated with risk of immunogenicity [29, 30].

Physical and chemical stability can be difficult to maintain over extended storage, especially in the case of intravenous administration since the formulation components are diluted within the intravenous bag contents and under these diluted concentrations the excipients may not be effective in stabilizing the proteins [31]. Upon diluting the drug product into the infusion bags, besides the decrease of the surfactant and other excipient concentrations also the increase of the air–water and liquid–surface interface has to be considered. Especially with regard to surfactants, the lower surfactant to protein ratio may affect its ability to protect the protein against physical stresses (agitation and/or air–water interface stress) and prevent adsorption to the plastic components [32].

In this section, the focus will be on compatibility testing of protein pharmaceuticals with administration materials with associated strategic considerations. As stated in the ICH Guideline—Pharmaceutical Development Q8 [33], the compatibility of the drug product should be addressed to provide appropriate and supportive information for the labeling. It is further detailed to the point that the testing should not only cover the recommended in-use shelf life and storage conditions, but also the conditions of admixture or dilution of products prior to administration (e.g.,

when the product added to large volume infusion containers). Therefore, prior to use of any materials for the administration of drug products in clinical trials (and later on in the market), physicochemical compatibility of the active ingredient with the administration materials should be assessed.

Commonly, physicochemical compatibility testing of protein pharmaceuticals is focused on the evaluation of the interactions between the drug and the different components of the administration kit (e.g., syringes, needles, infusion bags, lines and in-line filters). The testing itself can be performed on a kit-specific basis, meaning that it is done for specific combinations of administration components (e.g., infusion bag, line, in-line filter, and pump) and this specific combination is identified by its supplier's reference number and material description. The specific administration kits are only allowed as such for use in clinical studies. Consequently, considerable time and efforts have to be invested in order to succeed with specific testing. Another possibility is to follow a material-based testing approach, namely testing of administration materials based on investigations done for most common materials being in contact with the drug product allowing those to be used in clinical trials. This approach provides flexibility and choices to the teams with broad use of most common contact materials of administration without limitation on brand/suppliers.

There are several prerequisites necessary prior starting compatibility testing: Information is needed with regard to the intended clinical dose range, in which administration route will be considered, details on administration mode (e.g., duration of infusion, infusion bag size, injection volume, maximum/minimum patient weight). Then, the materials intended to be used for the administration of the drug at the site must be available for testing. It is helpful to determine the availability of various administration devices in the geographic area of interest well in advance. When all this information is compiled, a decision has to be made on which testing approach is applied (material-based approach or basic testing approach). During compatibility testing itself, the physicochemical protein stability as well as the biological protein activity in the admixture solution in contact with administration components is evaluated. Two main objectives should be considered at this stage: (1) assessment of protein adsorption to the administration components and (2) assessment of protein stability in the presence of the administration components. Once these assessments have been performed, the materials tested are allowed for administration at the clinical sites.

Most novel biopharmaceuticals constructs have associated potential stability issues related to higher aggregation propensity, sub-visible particles formation, or susceptibility of adsorption to surfaces. In order to gain knowledge on the behavior of these molecules prior the setup of the compatibility study itself, it is proposed to implement a pretest, which provides evidence on which compatibility testing strategy to be applied (material based vs. basic testing approach). The concept of the pretest is to characterize the protein molecule, focusing on compatibility of drug product formulation with dilution fluids (if needed) and administration materials in order to define the compatibility testing strategy.

Therefore, in the first step compatibility studies generally evaluate compatibility of the drug with the diluent if needed (e.g., placebo, normal saline ,or 5% dextrose) and in a second instance compatibility with the fluid-path contact material. At the early development stages, the definitive administration settings are usually not yet known, and hence worst case settings may be considered (e.g., minimum/maximum patient weight, dose range, administration volumes, and times). The selection of which administration materials will be tested should be based on a broad evaluation of available administration materials from major global suppliers and from different markets. Then, the compatibility study should be designed to evaluate the concentration ranges that provide the desired dose levels in the clinics. With the aim of efficiently designing the experiment, a bracketing approach is typically used, covering the lowest and highest dose levels intended to be tested in the clinics and corresponding concentrations. Other considerations in the study design may include the clinical dosing strategy, the administration material availability, hold times, and storage conditions. The physicochemical hold times, transport, and storage conditions of the solution may vary depending on the sensitivity of the product. For many clinical studies, clinicians generally request hold times and conditions that provide them with the greatest flexibility in terms of product storage, stability, and administration materials [34].

The data generated to make an assessment of protein adsorption to the administration components or on the protein stability in the presence of the administration components is generally focused on product quality attributes of the active species. Therefore, testing is focused on physicochemical protein stability as well as the biological activity of the protein in the admixture solution in contact with these materials. Typical analytical assays to monitor the points above include: concentration (to determine recovery of drug after incubation in the administration component and simulated dosing), turbidity, particulate matter, aggregation levels, and bioactivity. Significant changes in these assays may indicate incompatibility of the admixture solution with the diluent or the contact material.

From the testing, there are three possible outcome scenarios: (1) no compatibility issues observed; (2) failure of single materials and/or suppliers; or (3) severe compatibility issues identified. If severe compatibility issues are encountered during testing, one needs to elaborate on possible mitigation options. Mitigation options may be grouped in three categories as alternative administration mode, addition of excipients or supply of specific materials to the site. The assessment of the technical feasibility as well as implementation of any mitigation option in the clinics are crucial to define the probability of success of each option [35].

For instance, Fig. 22.6 shows the recovery values obtained during compatibility feasibility testing for a single-chain monoclonal antibody fragment when diluted with 0.9% normal saline, 5% dextrose, or matching placebo down to three different protein concentrations. Once diluted to an intermediate concentration, adsorption is observed for the dilution in normal saline whereas for the lowest concentration evaluated strong adsorption observed in all cases with recovery below the requirements. For the same molecule, different degrees of adsorption to PES membranes from different suppliers were observed: Adsorption strongly increased

for "neutral" PES filters, while positively charged filters showed little protein adsorption (as shown in Fig. 22.7). For that reason, it can be concluded that minor material differences may have tremendous impact on compatibility.

22.4 Conclusion

New biologic molecules—such as mAb-derived formats, nanobodies, bispecifics, and fusion proteins—are quite diverse and come along with new formulation and process development challenges. The properties are very dependent on the molecule and difficult to generalize. Hence, only by selection of optimized formulation conditions it is ensured to keep challenging molecule properties under control. In addition, a variety of analytical methods needs to be applied to thoroughly investigate all critical quality attributes. Finally with regard to compatibility, appropriate experimental design that mimics clinical administration procedure is essential (including product in-use stability and stability during administration conditions) and creative solutions might be required to ensure clinical dosing. Thorough pharmaceutical development is key to enable integrity and stability of novel biologics compounds upon storage and administration.

References

1. Elvin, et al. Therapeutic antibodies: market considerations, disease targets and bioprocessing. Int. J. Pharm. 2013; 440(1, 2):83–98. http://www.sciencedirect.com/science/article/pii/S0378517311011690.
2. Carter PJ. Introduction to current and future protein therapeutics: A protein engineering perspective. Exp. Cell Res. 2011;317(9):1261–9.
3. Plitnick L, Herzyk D.. Nonclinical Development of Novel Biologics, Biosimilars, Vaccines and Specialty Biologics. Elsevier Science;2013.
4. Wurch T, Pierré A, Depil S. Novel protein scaffolds as emerging therapeutic proteins: from discovery to clinical proof-of-concept. Trends Biotechnol. 2012;30(11):575–582. http://www.sciencedirect.com/science/article/pii/S0167779912001163.
5. Gil D, Schrum AG. Strategies to stabilize compact folding and minimize aggregation of antibody-based fragments. Adv. Biosci. Biotechnol. 2013;4:73–84. http://dx.doi.org/10.4236/abb.2013.44A011.
6. Fast JL, Cordes AA, Carpenter JF, Randolph W, Physical Instability of a Therapeutic Fc Fusion Protein: domain contributions to conformational and colloidal stability. Biochemistry. 2009;48:11724–11736. http://pubs.acs.org/doi/pdf/10.1021/bi900853v.
7. Kleemann GR, Beierle J, Nichols AC, Dillon TM, Pipes GD, Bondarenko PV. Characterization of IgG1 Immunoglobulins and Peptide-Fc Fusion Proteins by Limited Proteolysis in Conjunction with LC-MS. Anal. Chem. 2008;80:2001–2009. http://pubs.acs.org/doi/pdf/10.1021/ac701629v.
8. Chang B, Hershenson S. Practical approaches to protein formulation development. In: Carpenter J, Manning M, editors. Rational design of stable protein formulations. USA: Springer;2002. p. 1–25.

9. Gibson M. Pharmaceutical preformulation and formulation: a practical guide from candidate drug selection to commercial dosage form. CRC Press;2009.
10. Akers MJ. Excipient–drug interactions in parenteral formulations. J Pharmaceutical Sci. 2002;91(11):2283–2300.
11. Rathore N, Rajan RS, Current perspectives on stability of protein drug products during formulation, fill and finish operations. Biotechnol. Progress. 2008;24(3):504–514.
12. Walsh G. Biopharmaceutical benchmarks 2014. Nat. Biotech. 2014;32(10):992–1000.
13. Harris RJ, Shire SJ, Winter C. Commercial manufacturing scale formulation and analytical characterization of therapeutic recombinant antibodies. Drug Develop. Res. 2004;61(3):137–154.
14. Kiese S, Papppenberger A, Friess W, Mahler HC. Shaken, not stirred: mechanical stress testing of an IgG1 antibody. J. Pharm. Sci. 2008;97(10):4347–4366.
15. Ohtake S, Kita Y, Arakawa T. Interactions of formulation excipients with proteins in solution and in the dried state. Adv. Drug Delivery Rev. 2011;63(13):1053–1073.
16. Chang BS, Kendrick BS, Carpenter JF. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. J Pharmaceutical Sci. 1996;85(12):1325–1330.
17. Randolph T, Jones L. Surfactant-Protein Interactions. In: Carpenter J, Manning M. editors Rational design of stable protein formulations. USA: Springer;2002. p. 159–175.
18. Wang W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. Int. J. Pharm. 1999;185(2):129–188.
19. Gad SC. Pharmaceutical manufacturing handbook: production and processes. Wiley;2008.
20. Rathore N, Rajan RS, Freund E. Impact of manufacturing processes on drug product stability and quality. In: Formulation and process development strategies for manufacturing biopharmaceuticals. Wiley;2010. p. 917–940.
21. Patro SY, Freund E, Chang BS. Protein formulation and fill-finish operations. Biotechnol. Ann. Rev. Elsevier. 2002;8:55–84.
22. Kolhe P, Badkar A. Protein and solute distribution in drug substance containers during frozen storage and post-thawing: a tool to understand and define freezing–thawing parameters in biotechnology process development. Biotechnol. Progress 2011;27(2):494–504.
23. Thomas CR, Geer D. Effects of shear on proteins in solution. Biotechnol. Lett. 2011;33 (3):443–456.
24. Lutz H. Rationally defined safety factors for filter sizing. J. Membrane Sci. 2009;341(1–2):268–278.
25. Biddlecombe JG, Craig AV, Zhang H, Uddin S, Mulot S, Fish BC, Bracewell DG. Determining antibody stability: creation of solid-liquid interfacial effects within a high shear environment. Biotechnol. Prog. 2007;23(5):1218–22.
26. Pikal M. Freeze-Drying of Proteins. Formulation and delivery of proteins and peptides. Am. Chem. Soc. 1994;567:120–133.
27. Newton DW. Drug incompatibility chemistry. Am. J. Health Syst. Pharm. 2009;66(4):348–357.
28. Gikic M, Di Paolo E, Pannatier A, Cotting J. Evaluation of physicochemical incompatibilities during parenteral drug administration in a paediatric intensive care unit. Pharmacy World Sci. 2000;22(3):88–91.
29. den Engelsman J, Garidel P, Smulders R, Koll H, Smith B, Bassarab S, Seidl A, Hainzl O, Jiskoot W. Strategies for the assessment of protein aggregates in pharmaceutical biotech product development. Pharmaceutical Res. 2011;28(4):920–933.
30. Ratanji KD, Derrick JP, Dearman RJ, Kimber I. Immunogenicity of therapeutic proteins: influence of aggregation. J. Immunotoxicology. 2014;11(2):99–109.
31. Sreedhara A, Glover ZK, Piros N, Xiao N, Patel A, Kabakoff B. Stability of IgG1 monoclonal antibodies in intravenous infusion bags under clinical in-use conditions. J. Pharm. Sci. 2012;101(1):21–30.
32. Kerwin BA. Polysorbates 20 and 80 used in the formulation of protein biotherapeutics: structure and degradation pathways. J. Pharm. Sci. 2008;97(8):2924–2935.

33. ICH. Final guideline on Pharmaceutical Development Q8. International conference on harmonization of technical requirements for registration of pharmaceuticals for human use. I. I. C. o. Harmonization. 2008.
34. Bardin C A, Astier A, Vulto G, Sewell J, Vigneron R, Trittler M, Daouphars M, Paul M, Trojniak F, Pinguet P, French Society of Oncology. Guidelines for the practical stability studies of anticancer drugs: a European consensus conference. Ann Pharm Fr. 2011;69 (4):221–31.
35. Sharma M, Cheung J, Dabbara A, Petersen J. Intravenous admixture compatibility for sterile products: challenges and regulatory guidance. In: Kolhe P, Shah M, Rathore N, editors. Sterile product development. New York: Springer;2013. 6:461–474.
36. Bye J, Platts L, Falconer R. Biopharmaceutical liquid formulation: a review of the science of protein stability and solubility in aqueous environments. Biotechnol. Lett. 2014;36(5): 869–75.
37. Kamerzell TJ, Esfandiary R, Joshi SB, Middaugh CR, Volkin DB. Protein–excipient interactions: Mechanisms and biophysical characterization applied to protein formulation development. Adv Drug Delivery Rev. 2011;63(13):1118–1159.

Chapter 23
Novel Constructs—Half-Life Extensions

Jeonghoon Sun and Mark Michaels

Abstract A dearth of biologics have been developed and used as therapeutics for numerous disease indications, including IgG monoclonal antibodies, non-IgG recombinant proteins, bi- and multi-specific antibodies, and antibody drug conjugates. A remarkable portion of these biologic constructs exhibits a short plasma half-life that results in a significant reduction in therapeutic efficacy. Frequently, biologic drugs need to be designed to maintain the effective concentration range during the therapeutic window with an extended serum half-life. Provided here is a comprehensive overview of various half-life extension methods involving FcRn engagement, chemical and genetic fusion, post-translational modifications and formulation.

Keywords Half-life extension · FcRn · PEGylation · Human serum albumin Fc loop

Diverse forms of therapeutic biologics such as monoclonal antibodies, hormones, growth factors, cytokines, coagulation factors, enzymes, receptors, and fusion proteins have been developed and used as therapeutics for numerous disease indications [1, 2]. In addition to conventional monoclonal antibodies and recombinant proteins, more complex forms of biologics such as bi- and multi-specific antibodies and antibody drug conjugate constructs have become an important part of current biologic portfolios. Many of these biologic constructs exhibit a short plasma half-life that significantly reduces the therapeutic efficacy, which requires frequent and high doses for therapeutic activity while others that have long half-lives can benefit by extending PK further to increase patient convenience and compliance and gain a competitive advantage in a challenging marketplace.

J. Sun (✉)
Department of Biotherapeutics, Celgene Corporation, 4242 Campus Point Court, Suite 300, San Diego, CA 92121, USA
e-mail: jesun@celgene.com

M. Michaels
Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA 92320, USA

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*, AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_23

The most important objective in pharmaceutical design of therapeutic constructs is to maintain the effective concentration range of a drug during the therapeutic window. Drug concentrations above the effective concentration range can cause excessive toxicity, while those below the lower limit can lead to insufficient or lack of efficacy. Biotherapeutic drugs with a longer serum half-life have flatter pharmacokinetic profiles and stay in the therapeutic window longer with improved efficacy and safety.

Biotherapeutics are increasing in number and complexity, which creates more challenges in the development of half-life extension (HLE) constructs not only in optimizing their biochemical and biophysical properties but also in dealing with regulatory concerns. Provided is an overview of half-life extension methods that are in use or in development within the biotechnology industry.

d. Half-life extension

Plasma half-life is a crucial pharmacokinetic parameter that strongly influences the efficacy of therapeutic peptides and proteins. A number of therapeutic peptides and proteins are rapidly removed from circulation in serum through receptor-mediated clearance, renal filtration, and/or metabolic degradation. Frequently, therapeutic applications of these proteins require maintaining an extended half-life. Current half-life extension (HLE) technologies include increasing serum residence time by associating serum proteins with prolonged half-lives, inhibiting receptor-mediated clearance and avoiding renal clearance by increasing hydrodynamic volume of a therapeutic protein.

Facilitating the neonatal Fc receptor (FcRn)-mediated recycling and inhibiting clearance can elongate the half-lives of the molecules that bind to FcRn. Immunoglobulin G (IgG) and serum albumin are abundantly present in plasma and have an elongated half-life of 2–4 weeks, which makes IgG, serum albumin and their fusion proteins distinctive from other plasma proteins with similar hydrodynamic volumes [3]. The relatively long in vivo half-lives of IgG molecules are due to recycling mediated by FcRn [4, 5]. Recently, key mechanistic steps involved in FcRn-mediated IgG recycling were identified [6]. Upon internalization by macropinocytosis in endothelial cells, IgG and albumin undergo binding to the FcRn in the acidic early endosome (pH 6). This binding salvages IgG and albumin from degradation in the lysosome and redirects them at the plasma membrane to release them back into the blood plasma at slightly alkaline conditions (pH 7.4) [6]. The FcRn-mediated Fc or albumin recycling mechanism can be used in native form to increase the half-lives of proteins or can be engineered to increase half-life even further. For example, fusing or tagging a recombinant protein or peptide to the Fc-region of IgG or albumin elongates the half-life. Fc or albumin constructs that associate with FcRn with a high affinity in acidic pH and dissociate from FcRn with a low affinity give rise to an extended half-life. Substitution mutations on Fc or albumin for HLE have been isolated [6] by rational design or combinatorial display. Restrained random loop library insertion into Fc-regions followed by phage or yeast display screening has efficiently identified novel HLE constructs [7].

Certain extracellular receptors actively internalize bound antibodies and proteins. Once bound to these receptors, the antibodies and proteins are subjected to

lysosomal degradation. Customized protein engineering to reduce the receptor-mediated clearance has been showed to help with HLE of G-CSF and an anti-IL6 receptor antibody [8, 9].

Biologic drugs are cleared from circulation through multiple mechanisms including renal filtration and hepatic degradation, peripheral blood-mediated metabolic degradation via proteolysis and receptor-mediated endocytosis [10]. Smaller-sized molecules that are less than 50 kDa molecular mass are rapidly cleared by filtration through the glomerular filtration barrier (GBM) in kidneys [11]. The size of a protein is proportional to its hydrodynamic radius. Therefore, increasing a hydrodynamic volume of the molecule hampers renal clearance. Increasing the hydrodynamic volume can be accomplished by fusing the molecule to proteins such as Fc domain of IgG, human serum albumin (HSA) or its domain and other non-immunogenic plasma proteins. Chemical conjugation of poly-ethylene glycol (PEG), genetic fusion of non-structured repetitive amino acids or post-translational modification (PTM) including glycosylation or sialylation can increase the hydrodynamic volume. The fusion and PTM also protect the naked antibodies and proteins from proteolytic degradation and chemical decomposition. Formulation with liposomes or nanoparticles with chemical conjugation may give rise to more options for elongating the in vivo plasma half-life of the therapeutic biologics. In addition, optimization of physicochemical properties such as charge distribution may help delay the clearance.

WHO, FDA, EMEA, and other safety authorities offer guidelines regarding regulatory expectations and risk assessment for biotherapeutic products [12, 13]. The regulatory considerations for HLE constructs should cover all the safety aspects for given antibodies or recombinant proteins. These include primary, secondary, and higher order structure, size and charge variations, glycosylation and other post-translational modification, antigen binding and resulting biological activity.

In summary, strategies for HLE are grouped into three categories: (1) utilizing FcRn-mediated recycling, (2) modulating stability of antibody/protein-receptor complexes in the sorting endosome, and (3) increasing the hydrodynamic volume. HLE strategies should be carefully selected depending on the given therapeutic needs. HLE can be achieved more effectively with synergistic combination of strategies.

23.1 Fc Associated HLE by Modulating FcRn-Mediated Recycling

23.1.1 FcRn–Fc Interaction

The neonatal Fc receptor (FcRn) is a heterodimeric integral membrane protein consisting of an MHC-class-I-like α chain and a β2-microglobulin (β2m) chain [6]. FcRn was first discovered in rodents transporting IgG from the mother's milk

across the epithelium of the newborn rodent's gut into its bloodstream [11]. In humans, FcRn transfers IgG from the mother to her fetus across the placenta and the proximal small intestine [6, 14]. Residing in the early acidic endosomes, FcRn captures endocytosed IgG, rescues bound IgG from degradation in the lysosomal compartment, transports the IgG to the cell surface, and releases the IgG at neutral extracellular pHs. Through this FcRn-mediated salvage mechanism, IgG maintains a long serum half-life. FcRn also plays an important role in serum albumin homeostasis by a similar transporting mode for IgG [15–17]. These properties are critical for maximizing the bioavailable pharmacokinetics of IgG and Fc or albumin fused biotherapeutics [15, 18, 19].

FcRn–IgG interactions have been well characterized (Fig. 23.1) [17]. FcRn binds to the CH2–CH3 hinge region of the Fc of IgG1, IgG2, and IgG4 subclasses in a pH-dependent manner. At physiological pH 7.4, FcRn does not bind IgG. However, at the acidic pH of the early endosome (pH 6–6.5), FcRn has approximately a 100 nM K_D affinity for IgG. Interestingly, FcRn does not undergo a significant conformational change [20] because the pH dependency is mediated by a general acid–base titration of histidine, which has a pKa of approximately 6.5. Below pH 6.5, protonated histidine residues on Fc interact with either glutamate or aspartate residues on the surface of FcRn.

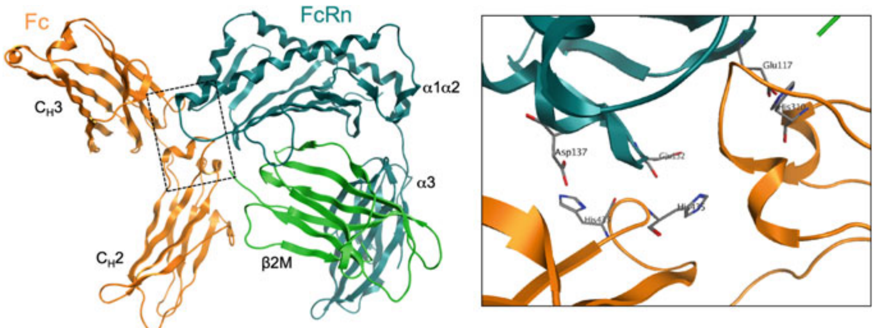


Fig. 23.1 FcRn–Fc interaction. FcRn–Fc interaction has a central role in regulating serum IgG levels. Coordinates of a rat FcRn with a human Fc complex (PDB ID 1FRT, Burmeister et al. [17], Nature 372: 379–383) were utilized to create the figures. On the left: CH2–CH3 hinge region of IgG binds to FcRn. The box on the left includes key residues that are involved in the Fc–FcRn interaction. These residues are shown in the box on the right. Simple acid–base chemistry roles in the Fc–FcRn interaction FcRn binds to Fc in a pH-dependent manner. No to minimal binding affinity between Fc and FcRn at pH 7–7.5 is detected while moderate binding (KD of 0.1 LM) at pH 6–6.5 is detected. Structurally, FcRn does not undergo conformational changes upon binding to Fc, which demonstrates that the pH dependence of Fc–FcRn interaction is mediated by histidines on Fc and acidic residues on FcRn by acid–base titration

23.1.2 Fc-Fusion Constructs

Many recombinant proteins including peptides, ligands, and ecto-domains of receptors have short half-lives. Fc fusion can extend the half-life through FcRn-mediated recycling and delayed renal clearance by increasing the molecular mass and the hydrodynamic radius of the fusion protein. Since the Fc is a homodimer, Fc-fusion constructs are bivalent, which can be advantageous in improving the efficacy by avidity effects. Depending on the therapeutic indication and needs or concerns for ADCC or CDC either IgG1, 2 or 4 Fc can be selected. The fusion sites are usually at the N-terminus or C-terminus of Fc or even at both ends. However, peptides or recombinant proteins can be inserted into Fc regions, in particular, the loop regions [21, 22]. Recently with the emergence of bi-specific antibody technology such as CH3-CH3 heterodimerization, many creative forms of Fc fusion have become available.

A number of Fc-fusion proteins have been developed for therapeutic applications. These include trap molecules constructed by fusing Fc to cytokines, extracellular receptor domains, antibody domains, peptides, and other recombinant proteins [23, 24]. A correlation between affinity for FcRn and half-life was established for conventional monoclonal IgG antibodies and Fc-fusion proteins [25]. Interestingly, several Fc-fusion proteins including etanercept, abatacept, and alefacept exhibited a twofold–threefold lower affinity for FcRn and shorter half-lives compared to IgG antibodies. This might be due to a structural perturbation by the fused receptor domains [24].

23.1.3 Engineered Fc Constructs—Substitution and Insertion Mutants

Improving the affinity of the FcRn–Fc interaction at an acidic pH can result in Fc HLE. Various combinatorial and computational methods have identified a number of substitution mutations at the FcRn–Fc interface as well as allosteric mutations. Fc residues involved in the FcRn–Fc interaction were site-specifically or combinatorially mutated to obtain HLE [6]. Many point or combination mutations including primate-specific ones at T250, M252, I253, S254, T256, T307, E380, M428, H433, and N434 improve the pH-dependent binding of human Fc to FcRn [26–33]. For example, Xencor's Xtend Fc with mutations, M428L and N434S, led to a 11-fold increase in affinity for FcRn at pH 6 and resulted in extension of the serum half-life of bevacizumab from 9.7 to 31.1 days. The half-life of cetuximab was also extended from 2.9 to 13.9 days in cynomolgus monkeys [33]. Meanwhile, Fc variants with mutations that abolish FcRn–Fc interaction and reduce the serum half-life in vivo were identified [26, 27, 34, 35, 36, 37]. These Fc variants are useful for an acute therapeutic effect with minimal toxicity [38] or for prognostic imaging of IgG distribution [39].

Recently, a novel loop insertion approach identified a number of HLE constructs [7]. The loop insertion sites were selected near or apart from the CH2–CH3 hinge region. Instead of targeting natural FcRn-binding residues found on Fc with substitution mutations, restrained small hexa-amino acid combinatorial loop libraries were inserted. Initially, these libraries were panned against human FcRn using phage or yeast display at pH 5.5. The high-affinity human FcRn binders at pH 5.5, with no or moderate binding at pH 7.4, were moved forward. The most interesting set of HLE variants was obtained from the library that was inserted in the CH3 region, which is remote from the conventional FcRn-binding residues. Noteworthy is that some of the new HLE constructs do not contain additional histidine residues. Unlike some conventional substitution HLE variants, CH2 destabilization effects on these Fc loop constructs were not detected and protein expression was not compromised. Many Fc loop constructs revealed significant HLE effect in cynomolgus monkey pharmacokinetic studies.

The pH-dependent binding of IgGs to FcRn can be a very important parameter for the in vivo homeostasis of IgGs if the K_D at pH 7.4 is very low [40–43]. For example, the half-lives of two human IgG1 molecules with the same affinity for murine FcRn at pH 6.0 and tenfold difference at near neutral pH were compared, and the affinity increased construct at near neutral pH reduces persistence [44]. Another study reported a PK effect of N434A and N434W IgG1 variants. Specifically, N434A exhibited a fourfold affinity improvement at pH 6 with a moderate affinity improvement at pH 7.4 to primate and non-primate FcRn. N434 W exhibited a striking 80-fold improvement in affinity at pH 6 while retaining a high affinity to FcRn at pH 7.4. The PK of N434W was not improved while N434A exhibited an approximately twofold decrease in clearance in cynomolgus monkey [33]. This supports the notion that modest to no increases in pH 7.4 FcRn affinity is a critical parameter for improved pharmacokinetics [45]. FcRn affinity with pH differentiation is one of the most important factors that affect HLE. Although minor, there are other factors that affect HLE including antibody stability, disease microenvironment, disease propagation rate, FcRn expression pattern, and specific clearance mechanisms such as antigen-dependent clearance [46].

23.2 Non-Fc HLE by Modulating FcRn-Mediated Recycling

23.2.1 HLE via Albumin Fusions and Conjugates

Composed mostly of α helices and loops, human serum albumin (HSA) is a very stable, monomeric protein with a molecular mass of 67 kDa. HSA is the most abundant plasma protein with a serum concentration of approximately 35–50 mg/mL [47]. HSA exhibits a long half-life of 19 days in humans. This long half-life, like Fc, utilizes pH-dependent, FcRn-mediated recycling but the binding site of

albumin on FcRn does not overlap with the Fc-binding site [48]. Unlike Fc, HSA does not bind to Fcγ receptors or complement system. Therefore, HSA fusion provides an excellent alternative for therapeutic applications where Fc-mediated effector functions are not required or desired due to safety issues.

HSA has been fused to therapeutic proteins including hormones, cytokines [49], coagulation factors [50], domain antibodies [51], and other recombinant proteins [52]. Many cytokines have therapeutic potential including immunomodulatory, antiproliferative, or antiviral effects. However, their therapeutic use is limited due to poor PK and stability properties including very short half-lives. Interferon α is currently used to treat hepatitis B and C. The half-life of interferon α is less than 3 h in the human body. An HSA fused interferon α, albinterferon α has an extended half-life of 6 days [53]. This reduces the dose frequency to once biweekly, which is the same as the recommended administration frequency as the PEGylated form of interferon α (PEGASYS®), thus providing an alternative option with a more homogeneous molecular entity [54]. PK studies of HSA fusion with factor IX, scFv, TNFα, IL-2, and other recombinant proteins revealed significant HLE and efficacy with fewer infusions and lower doses [55–59].

The N-terminus, C-terminus, or both ends of albumin can be utilized in the fusion of peptides and proteins including scFvs [57–59]. In addition to the ends of HSA, there are several internal loops and turns that can be used for internal insertion sites. Among the three domains in HSA including DI, DII, and DIII, DIII is a fusion partner in which the FcRn-binding residues reside. For example, a DIII-fused diabody leads to a significantly extended half-life. The molecular weight of DIII is approximately 22 kDa which is smaller than the molecular weight of Fc or HSA. Thus, HSA domain fusion provides a means to finely adjust HLE which is dependent on therapeutics needs [60].

HSA can be fused to peptides and proteins not only recombinantly but also chemically via site-specific conjugation. Chemical conjugation is specifically useful to extend the half-lives of very small peptides that are difficult to produce recombinantly. It is also very useful to fuse albumin and other protein molecules to multiple sites on proteins outside of the N-terminus or C-terminus. Typically, the cysteine side chain is activated by maleimide chemistry to create a thioether bond between albumin and the peptide. For example, a 34 amino acid anti-HIV peptide with HSA chemical fusion has a ninefold increase in HLE [61].

23.2.2 HLE via Interaction with Serum Albumin

Serum albumin circulates in serum at a very high concentration range and has a long serum half-life. These two features create another opportunity for HLE by non-covalent binding to albumin instead of engaging recombinant fusion or chemical conjugation. The molecular entities for non-covalent albumin tagging have been engineered for several desired features including high affinity,

cross-reactivity, high stability, site-specificity on albumin-binding sites and no or minimal immunogenicity [3, 62].

Interestingly, streptococcal protein G contains albumin-binding domains (ABDs). The ABDs are small three helix bundle proteins composed of about 50 amino acids with a molecular mass of 6 kDa. Domain antibodies were fused to an ABD and exhibited remarkable HLE on par with PEG conjugation as seen in animal studies [63–65]. The ABDs facilitated superior tissue penetration compared to the PEG conjugated domain antibodies. HSA-binding sites on ABDs were analyzed and high-affinity deimmunized ABD affibodies were engineered [66, 67]

Small domain antibodies, antibody fragments, and peptides were engineered to bind albumin and resulted in HLE [68–70]. For example, a trivalent nanobody composed of two domains that bind to EGFR and one domain that bind to mouse albumin has been engineered and the engineered nanobody exhibited HLE from 1 to 44 h and significant efficacy improvement in tumors with EGFR overexpression in an in vivo murine xenograft model [69, 70]. AlbudAbs™ is another albumin-binding entity that was engineered by phage display from a human domain antibody. The half-life of the human domain antibody is approximately 45 min. Remarkably, AlbudAbs with a molecular mass of approximately 12 kDa exhibit an HLE comparable to serum albumin with a molecular mass of 67 kDa. AlbudAb technology has been applied to inhibiting IL-1 signaling by a soluble IL-1 receptor (IL-1r), which is an effective modality for the treatment of rheumatoid arthritis. However, IL-1r has a very short half-life; therefore, AlbudAbs were fused to IL-1r. Although the AlbudAb/IL-1r fusion has a similar in vitro potency, its in vivo efficacy was dramatically improved due to its HLE [71]. Smaller peptides can extend half-life like AlbudAbs. Using peptide phage display, peptides that bind serum albumin from multiple species with high affinity were identified. The half-life of a protein fused with a albumin-binding peptide was extended up to 26–37 times in animal models [72].

HLE via tagging albumin can be accomplished through small molecules as well. Various ligands and metabolites with high affinity to HSA have been identified [73, 74]. The primary ligands for serum albumin are fatty acids that can be conjugated to peptides and proteins for HLE by HSA. One of the most prominent examples for fatty acid conjugation is with insulin. Insulin is a small protein composed of two peptide chains, A and B, with a molecular mass of approximately 5.8 kDa and has a half-life of less than 2 h [75]. Through the years, long-acting insulin analogs with protracted absorption from the subcutaneous tissue have been desired. One of the two approved long-acting insulin variants is insulin detemir (Levemir®, Novo Nordisk). The threonine at position B30 of the C-terminus of the insulin B chain was removed and the C_{14} myristoyl fatty acid acetylated to the lysine residue at position B29. The half-life and bioavailability of insulin detemir were improved significantly [76].

Conjugating proteins for HLE via tagging albumin are expanded to other small molecules. Various chemical molecules that bind to serum albumin were identified from chemical combinatorial libraries and used for HLE of small antibody fragments such as scFvs and disbodies [77, 78]. For example, a hexanoic acid derivative

selected for albumin-binding activity from a DNA-encoded chemical library was coupled to an anti-fibronectin scFv fragment containing an additional C-terminal cysteine. Half-life of this scFv was significantly improved from 30 min to 40 h in mice [78].

23.3 HLE by Chemical and Genetic Fusion

23.3.1 PEGylation

Polyethylene glycol (PEG) is a flexible, inert, non-immunogenic, non-antigenic, heterogeneous, and highly hydrophilic molecule. PEGylation is a process by which PEG chains are covalently coupled to other molecular entities including proteins and peptides. PEGylation improves major PK parameters of biologic molecules by increasing molecular mass and shielding effects [79, 80]. Increasing the molecular mass results in increased hydrodynamic volume, which delays renal clearance. The shielding effects help to protect PEGylated proteins from metabolic degradation, such as proteolysis, enzymatic metabolization, and chemical decomposition, such as oxidation of susceptible exposed methionine and tryptophan residues. Therefore, PEGylation often results in drugs that are more effective and safer with significant HLE.

In the last two decades, ten PEGylated biologic drugs have been approved, including enzymes, hormones, and an antibody fragment [79, 80]. Moreover, there are numerous PEGylated biologic drugs under development in preclinical and clinical stages, including engineered nanobodies and alternative protein scaffolds like lipocalins, fibronectin III, thioredoxin and Kunitz domain. These molecular entities are stable, foldable, soluble, monomeric, and manufacturable. However, most of them have very short half-lives that negatively affect the in vivo efficacy.

PEG reagents are commercially available with a molecular mass range of 5–40 kDa. PEGylation can occur via random conjugation or site-specific coupling reactions. Primary and secondary amine, carboxyl, hydroxyl, sulfur-hydroxyl, and disulfide moieties of the target proteins can be chemically activated for the random coupling reaction with the PEG moiety [81, 82]. However, the most frequently used functional group is the ε-amino group of the exposed lysine residue. Several biologics with random PEGylation that demonstrate a substantial HLE are available on the market including Mircera®, Pegasys® and PegIntron® [83–85].

Random PEGylation may interfere with the interaction between the biologic and the target molecule, resulting in a reduction or abrogation of the activity of the therapeutic protein [87]. A site-specific mono-PEGylation is accomplished by reductive alkylation on the amide group at the N-terminus of a protein. Neulasta®, an N-terminally mono-PEGylated G-CSF with a 20 kDa PEG, was generated by reductive alkylation. When one or more site-specific PEGylation reactions are needed, thiol groups in natural or engineered solvent-exposed cysteines can be

activated by maleimide PEG reagents, creating stable protein-PEG conjugates. The free-thiol-mediated PEGylation has been applied to create HLE constructs of interferon-α, Factor VIII and an antibody fragment PEG analogs [86–88]. Certolizumab pegol (Cimzia®) is a bacterially produced anti-tumor necrosis factor (TNF) Fab' fragment. A 40 kDa PEG chain was conjugated to the free cysteine at the C-terminus of the heavy chain, far away from the antigen-binding site.

There are several disadvantages of PEGylation. First, even the site-specific PEGylation reduces the functional activity of a PEGylated protein. However, this can be offset by the HLE effect, resulting in better efficacy in vivo. Second, although the PEGylation of proteins for HLE, in general, is safe and a well-tolerated method [89], renal tubular or CNS vacuolization can occur because of the accumulation of the non-degradable or less-degraded PEG fragments in the kidney or brain [80]. Third, the viscosity of the product can cause complications in production and administration.

23.3.2 Recombinant PEG Mimetics

PEGylation by chemical conjugation imposes several disadvantages [90, 91]. First, PEGylation requires additional conjugation and purification steps in manufacturing. Second, protein-PEG conjugates are a heterogeneous mixture of polymeric molecules. Third, branched PEG moieties are not completely degradable causing vacuolar accumulation. In order to overcome the disadvantages of the chemical PEGylation methods, recently, alternative technologies to the chemical PEGylation have been invented. The basis of these technologies is substituting PEG with a flexible, non-structured, hydrophilic, and non-immunogenic polypeptide chain by recombinant genetic engineering methods. In this way, all of the above-mentioned disadvantages can be overcome. In addition, production yield can increase resulting in reduction of the production cost.

There are a handful of recombinant PEG mimetic technologies involving the substitution of PEG with well-behaving recombinant polypeptides [92, 93]. However, these technologies differ in composition of amino acid building blocks in the PEG mimicking polypeptides and combination of the building blocks. One of the most representative examples is Amunix's XTEN® polypeptides that comprise an unstructured non-repetitive amino acid polymer with the residues P, E, S, T, A, and G [92, 93]. Interestingly, there are no positively charged amino acids in the polymer building blocks. Systematic combinations of the six amino acids were screened for manufacturability avoiding hydrophobic residues causing aggregation and T-cell-dependent HLA/MHC-II immune response. Avoiding a B cell-dependent immune response can be rationalized by the non-structured flexible nature of XTEN® polymers. Studies based on animal models demonstrate that the XTEN® polymers which are composed of 288 non-repetitive amino acids fused to glucagon are safe and minimally immunogenic. Most importantly, the half-life of glucagon fused to the XTEN® polymers has been increased from 10 min to over 8 h for the treatment of nocturnal hypoglycemia [93].

23.4 HLE by Post-Translational Modifications

23.4.1 Glycosylation

Glycosylation is one of the most prevalent post-translational modifications that occur on proteins. The two most predominant carbohydrate chains of glycosylated proteins are N-linked and O-linked oligosaccharides. The mechanism of N-linked glycosylation is better characterized than the mechanism of O-linked glycosylation, including glycosylation consensus sequences. The N-linked protein glycosylation pathways were originally discovered in bacteria [94]. Native proteins are often found to be glycosylated, and the glycosylated proteins have extended half-lives [95]. Glycosylation can extend the half-life by increasing hydrodynamic radius and protecting the protein from metabolic degradation of the proteins. The N-linked glycosylation of oligosaccharides is triggered in the lumen of the endoplasmic reticulum (ER) at an amide side chain of asparagine of proteins in the consensus sequences of N-X-S/T where X is any amino acid but proline.

Enhancement of epoetin in vivo activity was achieved through glycoengineering to create darbepoetin alfa (Aranesp), a hyperglycosylated form of EPO. Glycosylation analogs with new N-linked glycosylation consensus sequences introduced into epoetin were screened for additional N-linked carbohydrates and retention of activity. Suitable consensus sequences were combined in one molecule, resulting in two additional N-glycosylation analogs which substantially increased in vivo activity with HLE [96].

N-linked glycoengineering has been applied to HLE for a single-chain diabody bi-specific antibody with a very short terminal half-life in mice of 5–6 h [97]. Multiple N-glycosylation sites were introduced systemically into the bi-specific diabody resulting in N-glycosylated molecules with increased hydrodynamic radii and approximately twofold–threefold of HLE [97].

Although less characterized compared to the N-glycosylation, some biotherapeutics molecules have been introduced with additional O-glycosylation sites, resulting in HLE. Native O-glycosylation sites are often found in small peptide molecules. For example, the human chorionic gonadotropin beta (hCGβ) subunit contains a distinct 28-amino acid C-terminal peptide (CTP) bearing four O-linked glycosyl groups covalently linked to the serine side chains. Fusion of one or multiple copies of the CTP to EPO and HGH, respectively, resulted in O-glycosylated derivatives with improved in vivo potency and half-life [24, 25]. Bioactivities of hFSHβ and hTSHβ were increased by HLE via engineering O-linked glycosyl CTP moieties to these molecules [98, 99].

23.4.2 Conjugation of Carbohydrates

Chemical conjugation of carbohydrates opens up a way to covalently link diverse inert carbohydrates to proteins. Often, addition of these carbohydrates is not possible by post-translational modification. Hydroxyethyl starch (HES), which is a

branched amylopectin chemically modified from maize starch, is a safe non-immunogenic moiety that increases the plasma volume of proteins [100]. Since the first HES product, Hespan®, was launched in the US in the 1970s, many different types of HES have been available. HESylation was applied to improve the pharmacokinetic and pharmacodynamic properties of therapeutic proteins. Erythropoietin (EPO) was derivatized by HESylation and the HESylated EPO achieved significantly improved HLE compared to Aranesp® [101].

Polysialic acid (PSA) is another naturally occurring carbohydrate polymer that has been applied to protein conjugates, in particular, as an alternative to PEG with improved biocompatibility and biodegradability. Site-specific conjugation of PSA has been successfully applied for HLE to enzymes, hormones and antibody fragments. Currently, there are several preclinical and clinical stage PSA conjugated protein therapeutics [102].

23.5 HLE by Formulation and Nanotechnology

23.5.1 Liposomes and Nanoparticles

Liposomes are colloidal vesicle particles that are used as a drug delivery system. Liposomes are composed of bilayer membranes of self-assembled lipid amphiphiles surrounding discrete aqueous compartments [103]. Liposomes differ in particle size, lamellae number, and lipid composition. Unilamellar phospholipid vesicles of 100-nm diameter are the most common liposomes for formulations. The aqueous interior of the liposomes can accommodate hydrophilic molecules, and the lipid bilayer region can incorporate hydrophobic molecules. A wide range of chemical and physical properties of liposomes can be modified for suitable PK and PD properties of the molecules to be possibly designed. However, the mononuclear phagocyte system clears liposomes rapidly from the circulation, which results in a very short half-life (<2 h) [104]. Half-lives of liposomes can be extended by coupling flexible, bulky, and hydrophilic molecular entities, such as PEG, with the phospholipids on the surface of the liposomes [105]. The half-life of PEGylated liposomes is approximately 20 h in mice and 45 h in human [106]. In addition to the PEG incorporation, active-targeting moieties that can direct liposomes to certain cells or tissue sites can be conjugated to liposomes. These moieties can be peptides, proteins, ligands, soluble receptors, antibodies, or antibody fragments that recognize the target molecules. Formulation of therapeutic proteins in PEGylated liposomes were applied to the delivery of cytokines and coagulation factors, resulting in significant HLE in animal models [107]. Liposome formulations are being tested in preclinical and clinical experiments and have yet to be proven and approved for therapeutic applications.

Liposome formulations tend to be unstable in serum due to their low critical micelle concentration. This can be overcome by either crosslinking the lipids in the

liposomes or engaging other more robust delivery vehicle entities such as polymeric nanoparticles [108]. Generating polymeric nanoparticles requires several steps. The nanoparticles are obtained by radical polymerization and functionalization followed by polyreactions in small emulsified droplets [109]. Various hydrophilic and hydrophobic molecular entities can be encapsulated inside the nanoparticles or conjugated to the nanoparticles and used for therapeutic and diagnostic applications. Significant disadvantages of nanoparticles exist, including limitation to only few polymers that be used (e.g., PLGA), burst release effects, lack of loading efficacy of the active drug and manufacturing and administration challenges. Also, their fate as future technology in the marketplace remains to be seen.

23.6 Conclusion

HLE platforms have diversified and improved over the last two decades. The majority of traditional HLE construct strategies involve increasing the hydrodynamic radius of a protein and engaging the FcRn-mediated recycling mechanism. New HLE construct strategies have been developed by mimicking and improving the traditional strategies or by introducing new technologies. For example, traditional chemical PEGylation which has more than 20 years of history inspired new approaches such as genetic fusion of hydrophilic, non-immunogenic, and flexible polypeptides. These new strategies are intended to mimic the beneficial properties of PEG while incorporating other advantages such as biodegradability, lack of immunogenicity, and improved manufacturability. Furthermore, a better understanding of the molecular mechanisms underlying the HLE of serum albumin and IgGs has led to novel strategies involving FcRn- or other receptor-mediated HLE. These strategies involve recombinant fusion or non-covalent binding to albumin, fusion to the Fc region of IgG or enhancing the binding of IgGs with FcRn. Many structural, computational, and combinatorial methodologies were developed independently or together to create new HLE constructs. In addition to various substitution mutations in Fc, combinatorial Fc loop strategies were generated for improved FcRn binding, resulting in HLE. Most of these novel half-life extension strategies, including liposome or nanoparticle formulations are still at the early preclinical stage and need to be verified for safety. However, it is expected that many of these new technologies will be evaluated in clinical trials and become approved methods for HLE in the future. Optimized HLE constructs should not only benefit patients by increasing convenience and compliance but also should benefit companies by reducing protein production costs and by gaining market share. In an increasingly competitive marketplace, the development of optimized HLE constructs for sophisticated forms of biotherapeutic molecules, including bi-specific, multi-specific, ADC, bi- or multi-specific ADC, and other protein fusion constructs seem essential.

References

1. Walsh G. Biopharmaceutical benchmarks. Nat Biotechnol. 2010;28:917–24.
2. Scott AM, Wolchok JD, Old LJ. Antibody therapy of cancer. Nat Rev Cancer. 2012;12: 278–87.
3. Kontermann RE. Strategies for extended serum half-life of protein therapeutics. Curr Opin Biotech. 2011;22:868–76.
4. Ghetie V, Hubbard JG, Kim JK, Tsen MF, Lee Y, Ward ES. Abnormally short serum half-lives of IgG in β2-microglobulin-deficient mice. Eur J Immunol. 1996;26:690–6.
5. Tesar DB, Bjorkman PJ. An intracellular traffic jam: Fc receptor-mediated transport of Immunoglobulin G. Curr Opin Struct Biol. 2010;20:226–33.
6. Roopenian DC, Akilesh S. FcRn the neonatal Fc receptor comes of age. Nat Rev Immunol. 2007;7:715–25.
7. Sun J, Han SJ, Harris SM, Ketchem RR, Lu J, Michaels ML, Retter MW, Tsai MM. Variant fc-polypeptides with enhanced binding to the neonatal fc receptor. WO2013096221 A1, PCT/US2012/070146;2012.
8. Sarkar CS, Lowenhaupt K, Horan T, Boone TC, Tidor B, Lauffenburger DA. Rational cytokine design for increased lifetime and enhanced potentcy using pH-activated histidine switching. Nat Biotechnol. 2002;20:908–13.
9. Igawa T, Ishii S, Tachibana T, Maeda A, Higuchi, Shimaoka S, Moriyama C, Watanabe T, Takubo R, Doi Y et al. Antibody recycling by engineered pH-dependent antigen binding improves the duration of antigen neutralization. Nat Biotechnol. 2010;28:1203–1208.
10. Tang L, Persky AM, Hochhaus G, Meibohm B. Pharmacokinetic aspects of biotechnology products. J Pharmaceut Sci. 2004;93:2184–204.
11. Tryggvason K, Wartiovaara K. How does the kidney filter plasma? Physiology. 2005;20: 96–101.
12. Gramppa G, Kozakb RW, Schreitmuellerc T. Policy considerations for originator and similar biotherapeutic products. Pharm Policy Law. 2016;18:121–39.
13. World Heath Organization. Regulatory expectations and risk assessment for biotherapeutic products. WHO/RRA BT_DRAFT/24; January 2014.
14. Kristoffersen EK. Human placental Fcγ-binding proteins in the maternofetal transfer of IgG. APMIS Suppl. 1996;64:5–36.
15. Simister NE, Mostov KE. An Fc receptor structurally related to MHC class I antigens. Nature. 1989;337:184–7.
16. Ober RJ, Martinez C, Lai X, Zhou J, Ward ES. Exocytosis of IgG as mediated by the receptor, FcRn: an analysis at the single-molecule level. Proc Natl Acad Sci USA. 2004;11076–11081.
17. Burmeister WP, Gastinel LN, Simister NE, Blum ML, Bjorkman PJ. Crystal structure at 2.2 Å resolution of the MHC-related neonatal Fc receptor. Nature. 1994;372:336–43.
18. Ahouse JJ, et al. Mouse MHC class I-like Fc receptor encoded outside the MHC. J Immunol. 1993;151:6076–88.
19. West AP, Herr AB, Bjorkman PJ. The chicken yolk sac IgY receptor, a functional equivalent of the mammalian MHC-related Fc receptor, is a phospholipase A2 receptor homolog. Immunity. 2004;20:601–10.
20. Vaughn DE, Bjorkman PJ. Structural basis of pH-dependent antibody binding by the neonatal Fc receptor. Structure. 1998;6:63–73.
21. Strohl WR. Fusion proteins for half-life extension of biologics as a strategy to make biobetters. BioDrugs. 2015;29:215–39.
22. Qiu Y, Lv W, Xu M, Xu Y. Single chain antibody fragments with pH dependent binding to FcRn enabled prolonged circulation of therapeutic peptide in vivo. J Control Release. 2016;229:37–47.
23. Huang C. Receptor-Fc fusion therapeutics, traps, and MIMETIBODY technology. Curr Opin Biotechnol. 2009;20:692–9.

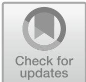
24. Kontermann RE. Therapeutic proteins: strategies to extend plasma half-lives of recombinant antibodies. Wiley-VCH;2012. p. 158–62.
25. Suzuki T, Watabe AI, Tada M, Kobayashi T, Kanayasu-Toyoda T, Kawanishi T, Yamaguchi T. Importance of neonatal FcR in regulating the serum half-life of therapeutic proteins containing the Fc domain of human IgG1: a comparative study of the affinity of monoclonal antibodies and Fc-fusion proteins to human neonatal FcR. J Immunol. 2010;184:1968–76.
26. Shields RL, et al. High resolution mapping of the binding site on human IgG1 for FcγRI, FcγRII, FcγRIII, and FcRn and design of IgG1 variants with improved binding to the FcγR. J Biol Chem. 2001;276:6591–604.
27. Petkova SB, et al. Enhanced half-life of genetically engineered human IgG1 antibodies in a humanized FcRn mouse model: potential application in humorally mediated autoimmune disease. Int Immunol. 2006;18:1759–69.
28. Hinton PR, et al. Engineered human IgG antibodies with longer serum half-lives in primates. J Biol Chem. 2004;279:6213–6.
29. Kamei DT, et al. Quantitative methods for developing Fc mutants with extended half-lives. Biotechnol Bioeng. 2005;92:748–60.
30. Vaccaro C, Zhou J, Ober RJ, Ward ES. Engineering the Fc region of immunoglobulin G to modulate in vivo antibody levels. Nat Biotechnol. 2005;23:1283–8.
31. Dall'Acqua WF, Kiener PA, Wu H. Properties of human IgG1 s engineered for enhanced binding to the neonatal Fc receptor (FcRn). J Biol Chem. 2006;281:23514–24.
32. Hinton PR, et al. An engineered human IgG1 antibody with longer serum half-life. J Immunol. 2006;176:346–56.
33. Yeung YA, et al. Engineering Human IgG1 Affinity to Human Neonatal Fc Receptor: Impact of Affinity Improvement on Pharmacokinetics in Primates. J Immunol. 2009;182:7663–71.
34. Raghavan M, et al. Analysis of the pH dependence of the neonatal Fc receptor/ immunoglobulin G interaction using antibody and receptor variants. Biochemistry. 1995;34:14649–57.
35. Kim JK, et al. Mapping the site on human IgG for binding of the MHC class I-related receptor, FcRn. Eur J Immunol. 1999;29:2819–25.
36. Firan M, et al. The MHC class I-related receptor, FcRn, plays an essential role in the maternofetal transfer of -globulin in humans. Int Immunol. 2001;13:993–1002.
37. Kamei DT, et al. Quantitative methods for developing Fc mutants with extended half-lives. Bioeng. 2005;92:748–60.
38. Stern M, Herrmann R. Overview of monoclonal antibodies in cancer therapy: present and promise. Crit Rev Oncol Hematol. 2005;54:11–29.
39. Kenanova V, et al. Tailoring the pharmacokineticsand positron emission tomography imaging properties of anti-carcinoembryonic antigen single-chain Fv-Fc antibody fragments. Cancer Res. 2005;65:622–31.
40. Datta-Mannan A, et al. Humanized IgG1 variants with differential binding properties to the neonatal Fc receptor: relationship to pharmacokinetics in mice and primates. Drug Metab Dispos. 2007;35:86–94.
41. Gurubaxani BM, et al. Analysis of a family of antibodies with different half-lives in mice fails to find a correlation between affinity for FcRn and serum half-life. Mol Immunol. 2006;43:1462–73.
42. Gurubaxani BM, et al. Development of new models for the analysis of Fc–FcRn interactions. Mol Immunol. 2006;43:1379–89.
43. Borrok MJ, et al. pH-dependent binding engineering reveals an fcrn affinity threshold that governs IgG recycling. J Biol Chem. 2015;290:4282–90.
44. Ward ES1, Ober RJ. Multitasking by exploitation of intracellular transport functions the many faces of FcRn. Adv Immunol. 2009;103:77–115.
45. Dall'Acqua WF, et al. Increasing the affinity of a human IgG1 for the neonatal Fc receptor: biological consequences. J Immunol. 169:5171–80.

46. Yeung YA, et al. A therapeutic anti-VEGF antibody with increased potency independent of pharmacokinetic half-life. Cancer Res. 2010;70:3269–77.
47. Andersen JT, Sandlie I. The versatile MHC class I-related FcRn protects IgG and albumin from degradation: implications for development of new diagnostics and therapeutics. Drug Metab Pharmacokinet. 2009;24:318–32.
48. Chaudhury C, et al. Albumin binding to FcRn: distinct from the FcRn-Igg interaction. 2006. Biochemistry. 2006;45:4983–90.
49. Duttaroy A, et al. Development of a long-acting insulin analog using albumin fusion technology. Diabetes. 2005;54:251–8.
50. Weimer T, et al. Prolonged in-vivo half-life of factor VIIa by fusion to albumin. Thromb Haemost. 2008;99:659–67.
51. Müller Dafne, et al. Improved pharmacokinetics of recombinant bispecific antibody molecules by fusion to human serum albumin. J Biol Chem. 2007;282:12650–60.
52. Chuang VTG, Kragh-Hansen U, Otagiri M. Pharmaceutical strategies utilizing recombinant human serum albumin. Pharm Res. 2002;19:569–77.
53. Rustgi VK, et al. Albinterferon alf-2b, a novel fusion protein of human albumin and human interferon alf-2b, for chronic hepatitis C. Curr Med Res Opin. 2009;25:991–1002.
54. Nelson DR, et al. Albinterferon alfa-2b was not inferior to PEGylated interferon-a in a randomized trial of patients with chronic hepatitis C virus genotype 2 or 3. Gastroenterology. 2010;139:1267–76.
55. Schulte S, et al. Half-life extension through albumin fusion technologies. Thromb Res. 2009;124:S6–8.
56. Metzner HJ, et al. Genetic fusion to albumin improves the pharmacokinetic properties of factor IX. Thromb Haemost. 2009;102:634–44.
57. Müller D, et al. Improved pharmacokinetics of recombinant bispecific antibody molecules by fusion to human serum albumin. J Biol Chem. 2007;282:12650–60.
58. Yazaki PJ, et al. Biodistribution and tumor imaging of an anti-CEA single-chain antibody-albumin fusion protein. Nucl Med Biol. 2008;35:151–8.
59. Evans L, et al. The production, characterisation and enhanced pharmacokinetics of scFv-albumin fusions expressed in Saccharomyces cerevisiae. Protein Expr. 2010;73:113–24.
60. Kenanova VE, et al. Tuning the serum persistence of human serum albumin domain III: diabody fusion proteins. Protein Eng Des Sel. 2010;23:789–98.
61. Xie D, et al. An albumin-conjugated peptide exhibits potent anti-HIV activity and long in vivo half-life. Antimicrob Agents Chemother. 2010;54:191–6.
62. Kontermann RE. Therapeutic proteins: Strategies to Extend Plasma Half-Lives of Recombinant Antibodies. Wiley-VCH;2012. p. 223–242.
63. Stork R, Müller D, Kontermann RE. A novel tri-functional antibody fusion protein with improved pharmacokinetic properties generated by fusing a bispecific single-chain diabody with an albumin-binding domain from streptococcal protein G. Protein Eng Des Sel. 2007;20:569–76.
64. Hopp J, et al. The effects of affinity and valency of an albumin-binding domain (ABD) on the half-life of a single- chain diabody-ABD fusion protein. 2010. Protein Eng Des Sel. 2010;23:827–34.
65. Stork R, et al. Biodistribution of a bispecific single-chain diabody and its half-life extended derivatives. J Biol Chem. 2009;284:25612–9.
66. Sand KMK, et al. Dissection of the neonatal Fc receptor (FcRn)-albumin interface using mutagenesis and anti-fcrn albumin-blocking antibodies. J Biol Chem. 2014;289:17228–39.
67. Löfbloma J, et al. Affibody molecules: Engineered proteins for therapeutic, diagnostic and biotechnological applications. FEBS Lett. 2010;584(12):2670–2680.
68. Roovers RC, et al. Efficient inhibition of EGFR signalling and of tumour growth by antagonistic anti-EGFR nanobodies. Cancer Immunol Immunother. 2007;56:303–17.
69. Tijink BM, et al. Improved tumor targeting of anti-epidermal growth factor receptor nanobodies through albumin binding: taking advantage of modular nanobody technology. Mol Cancer Ther. 2008;7:2288–97.

70. Holt LJ, et al. Anti-serum albumin domain antibodies for extending the half-lives of short lived drugs. Protein Eng Des Sel. 2008;21:283–8.
71. Walker A, et al. Anti-serum albumin domain antibodies in the development of highly potent, efficacious and long-acting interferon. Protein Eng Des Sel. 2010;23:271–8.
72. Dennis MS, et al. Albumin Binding as a General Strategy for Improving the Pharmacokinetics. J Biol Chem. 2002;277:35035–43.
73. Petersen CE, et al. A Dynamic Model for Bilirubin Binding to Human Serum Albumin. J Biol Chem. 2000;275:20985–95.
74. Kontermann RE. Therapeutic proteins: Strategies to Extend Plasma Half-Lives of Recombinant Antibodies. Wiley-VCH;2012. p. 269–294.
75. Havelund S, et al. The mechanism of protraction of insulin detemir, a long-acting, acylated analog of human insulin. Pharm Res. 2004;21:1498–504.
76. Owens DR et al. Pharmacokinetics of 125I-labeled insulin glargine (HOE 901) in healthy men: comparison with NPH insulin and the influence of different subcutaneous injection sites. Diabetes Care. June 23 2000;813–819.
77. Dumelin CE, et al. A portable albumin binder from a DNA-encoded chemical library. Angew Chem Int Ed. 2008;47:3196–201.
78. Trussel S, et al. New strategy for the extension of the serum half-life of antibody fragments. Bioconjug Chem. 2009;20:2286–92.
79. Brocchini S, et al. Disulfide bridge based PEGylation of proteins. Adv Drug Deliv Rev. 2008;60:3–12.
80. Gaberc-Porekar V, et al. Obstacles and pitfalls in the PEGylation of therapeutic proteins. Curr Opin Drug Discov. 2008;11:242–50.
81. Jevsevar S1, Kunstelj M, Porekar VG. PEGylation of therapeutic proteins. Biotechnol. J. 2010;5:113–28.
82. Turecek PL, Bossard MJ, Schoetens F, Ivens IA. PEGylation of biopharmaceuticals: a review of chemistry and nonclinical safety information of approved drugs. J Pharm Sci. 2016;105:460–75.
83. El-Komy MH1, Widness JA, Veng-Pedersen P. Pharmacokinetic analysis of continuous erythropoietin receptor activator disposition in adult sheep using a target-mediated, physiologic recirculation model and a tracer interaction methodology. Drug Metab Dispos. 2011;39:603–9.
84. Pegasys—European Medicines Agency—Europa. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR__Product_Information/human/000395/WC500039195.pdf.
85. PegIntron—European Medicines Agency—Europa. http://www.ema.europa.eu/docs/en_GB/document_library/EPARProduct_Information/human/000280/WC500039388.pdf.
86. Kinstler O, Molineux G, Treuheit M, Ladd D, Gegg C. Mono-N-terminal poly (ethylene glycol)-protein conjugates. Adv Drug Deliv Rev. 2002;54:477–85.
87. Molineux G. The design and development of PEGfilgrastim (PEG-rmetHuG-CSF, Neulasta). Curr Pharm Des. 2004;10:1235–44.
88. Means GE, Feeney RE. Reductive alkylation proteins. Anal Biochem. 1995;224:1–16.
89. Veronese FM, Mero A. The impact of PEGylation on biological therapies. BioDrugs. 2008;22:315–29.
90. Veronese FM. Peptide and protein PEGylation: a review of problems and solutions. Biomaterials. 2001;22:405–17.
91. Roberts MJ, Bentley MD, Harris JM. Chemistry for peptide and protein PEGylation. Adv Drug Deliv Rev. 2002;54:459–76.
92. Schellenberger V, et al. A recombinant polypeptide extends the in vivo half-life of peptides and proteins in a tunable manner. Nat Biotechnol. 2009;27:1186–90.
93. Geething NC, et al. Gcg-XTEN: an improved glucagon capable of preventing hypoglycemia without increasing baseline blood glucose. PLoS ONE 2010;5:e10175.
94. Baker JL, et al. Expanding the glycoengineering toolbox: the rise of bacterial N-linked protein glycosylation. Trends Biotechnol. 2013;31:313–23.

95. Sola RJ, Griebenow K. Glycosylation of therapeutic proteins: an effective strategy to optimize efficacy. BioDrugs. 2010;24:9–21.
96. Elliott S, et al. Enhancement of therapeutic protein in vivo activities through glycoengineering. Nat Biotechnol. 2003;21:414–21.
97. Stork R, et al. N-glycosylation as novel strategy to improve pharmacokinetic properties of bispecific single-chain diabodies. J Biol Chem. 2008;283:7804–12.
98. Fares FA, et al. Design of a long-acting follitropin agonist by fusing the C-terminal sequence of the chorionic gonadotropin beta subunit to the follitropin beta subunit. Proc Natl Acad Sci U S A. 1992;89:4304–8.
99. LaPolt PS, et al. Enhanced stimulation of follicle maturation and ovulatory potential by long acting follicle-stimulating hormone agonists with extended carboxyl-terminal peptides. Endocrinology. 1992;131:2514–20.
100. Agreda-Vasquez GP, et al. Starch and albumin mixture as replacement fluid in therapeutic plasma exchange is safe and effective. J Clin Apher. 2008;23:163–7.
101. Elliott S, et al. Control of rHuEPO biological activity: The role of carbohydrate. Exp Hematol. 2004;32:1146–55.
102. Constantinou A, et al. Site-specific polysialylation of an antitumor single-chain Fv fragment. Bioconjug Chem. 2009;20:924–31.
103. Akbarzadeh A, et al. Liposome: classification, preparation, and applications. Nanoscale Res Lett. 2013;8:102–10.
104. Immordino ML, Dosio F, Cattel L. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. Int J Nanomedicine. 2006;1:297–315.
105. Nag OK, Awasthi V. Surface engineering of liposomes for stealth behavior. Pharmaceutics. 2013;5:542–69.
106. Yatuv R, Robinson M, Dayan I, Baru M. The use of PEGylated liposomes in the development of drug delivery applications for the treatment of hemophilia. Int J Nanomedicine. 2010;5:581–91.
107. Yatuv R, Robinson M, Dayan I, Baru M. Enhancement of the efficacy of therapeutic proteins by formulation with PEGylated liposomes; a case of FVIII, FVIIa and G-CSF. Expert Opin Drug Deliv. 2010;7:187–201.
108. Talelli M, et al. Core-crosslinked polymeric micelles: principles, preparation, biomedical applications and clinical translation. Nano Today. 2015;10:93–117.
109. Allouche J. Chapter 2: synthesis of organic and bioorganic nanoparticles: an overview of the preparation methods. In: Brayner R, et al., editors. Nanomaterials: a danger or a promise? London:Springer-Verlag;2013.

Chapter 24
"Fc Fusion Proteins"



Carole Heath and Dean Pettit

Abstract IgG-based therapeutics has become an increasingly important category of the over two hundred biopharmaceutical products approved in the USA and the EU by late 2014. While a large percentage of this consists of monoclonal antibodies, Fc fusion proteins make up an important class of IgG-based biotechnology drugs. This chapter reviews the rationale for creating Fc fusion proteins, describes challenges, regulatory considerations, and improvements that have been made with this important class of therapeutics.

Keywords Fc fusion · Peptibody · Mimetibody · Expression · Half-life
Effector function

IgG-based therapeutics has become an increasingly important category of the over two hundred biopharmaceutical products approved in the USA and the EU by late 2014. While a large percentage of this consists of monoclonal antibodies, Fc fusion proteins make up an important class of IgG-based biotechnology drugs. Linking a ligand, peptide, enzyme, or extracellular domain of a receptor to the Fc domain of IgG can have a significant impact on its clinical potential. Addition of the Fc domain prolongs the serum half-life of proteins, and thus its exposure in the target tissue primarily by the pH-dependent binding of the neonatal Fc receptor (FcRn), which prevents the protein from being degraded in endosomes. Because the fusion protein is markedly larger in size than it was without the Fc, renal clearance is slowed, which also contributes to the longer half-life. In addition, improved potency can be achieved by the doubled valency with the same ligand fused to the hinge on each of the two Fc arms. Fusion to the Fc domain can also add effector

C. Heath
Process Development, Amgen, 1 Amgen Center Dr, Thousand Oaks, CA 91320, USA

D. Pettit (✉)
Just Biotherapeutics, 401 Terry Ave N, Seattle, WA 98109, USA
e-mail: dean.pettit@just.bio

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_24

functions by conferring the ability to interact with Fcγ receptors on immune cells. In this chapter, we describe the advantages and disadvantages of using Fc fusion as biotherapeutics as well as advances in improving their efficacy for human use.

24.1 Background

Fc fusion proteins can be constructed in different formats such as homodimers, heterodimers, monomeric Fc fusions, peptibodies, and others depending on the intended use and desired molecular properties (Fig. 24.1). Generally speaking, the C-terminus of the receptor or other domain is genetically fused to the N-terminus of the Fc domain (hinge, CH2, and CH3) of IgG1 because it binds relatively strongly

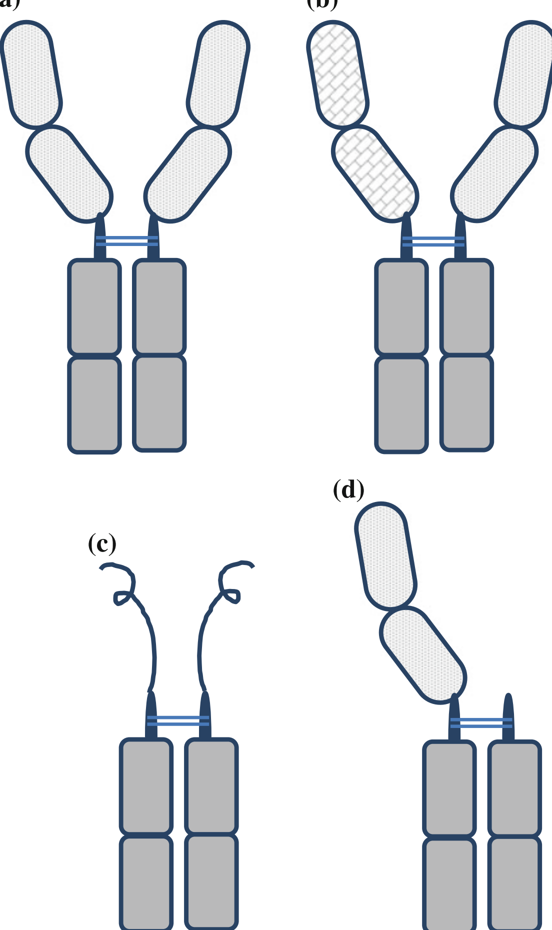


Fig. 24.1 Cartoon structures of selected Fc fusion proteins. **a** The homodimer structure consists of the same protein fused to each N-terminus of the Fc domain hinge region; **b** the heterodimer structure consists of different proteins fused to each N-terminus of the Fc domain hinge region; **c** peptibodies or peptide-Fc fusions are active peptides fused to the Fc domain; **d** monomeric Fc fusions are smaller and consist of only one protein fused to the Fc domain

to all FcγRs [1]. Because the CH3 domains interact strongly with each other, the molecule exists as a homodimer. The hinge regions provide flexibility to the two arms, which link to the same or different ligands, and molecular stability via the disulfide bonding between the two chains. An Fc fusion with only one copy of the ligand or receptor, i.e., a monomeric Fc fusion, is smaller and may result in increased tissue penetration but may also possess lower potency. Molecules comprised of heterodimeric subunits or multi-subunit complexes with enhanced pharmacokinetics (PK) have also been constructed.

The Fc fusion approach for biologics has been successful both clinically and commercially. Genentech was the first to publish the use of an Fc fusion protein in 1989 by linking the ligand-binding domains of CD4 to the IgG1 Fc domain [2]. Now, there are several Fc fusion proteins on the market (Table 24.1), more in clinical trials (Table 24.2) and others in development. Fc fusion proteins on the market include etanercept (Enbrel®), abatacept (Orencia®), aflibercept (Eylea®), rilonacept (Arcalyst®), romiplostim (Nplate®), belatacept (Nulojix®), factor VIII Fc (Eloctate®), and factor IX Fc (Alprolix®). In 2012, US sales of Fc fusion proteins reached $5.8 billion dollars, up by 35.3% over the previous year total, which was the highest growth rate among all biologics [3]. The highest portion of

Table 24.1 Approved therapeutic Fc fusion proteins

Drug	Fusion protein	Type	Trade name	Company	Year approved
Abatacept	CTLA-4/ Fc-IgG_1	Homodimer	Orencia®	Bristol Meyer Squibb	2005 (US) 2007 (EU)
Aflibercept	VEGFR 1 and 2/ Fc-IgG_1	Trap	Elyea®	Regeneron	2011 (US) 2012 (EU)
Alefacept	LFA-3/ Fc-IgG_1	Homodimer	Amevive®	Astellas	2003 (US)[a]
Belatacept	CTLA-4/ Fc-IgG_1	Homodimer	Nulojix®	Bristol Meyer Squibb	2011 (US and EU)
Etanercept	TNFR/ Fc-IgG_1	Homodimer	Enbrel®	Amgen	1998 (US) 2000 (EU)
Factor IX-Fc	FIX/Fc-IgG1	Monomeric Fc	Alprolix®	Biogen	2014 (US)
Factor VIII Fc	FVIII/Fc IgG_1	Monomeric Fc	Eloctate®	Biogen	2014 (US)
Rilonacept	IL-1R/ Fc-IgG_1	Trap	Arcalyst®	Regeneron	2008 (US) 2009 (EU)[b]
Romiplostim	TPO/Fc-IgG_1	Peptibody	Nplate®	Amgen	2008 (US) 2009 (EU)

[a]Withdrawn in 2011
[b]Withdrawn from the EU in 2012

Table 24.2 Fc fusion proteins in recent/current clinical trials

Drug	Fusion protein	Type	Company
Trebananib	ANG-1/-2/Fc-IgG1	Peptibody	Amgen
Pinta 745	MSTN/Fc-IgG1	Peptibody	Atara Biotherapeutics
STM 434	ACTR2B/Fc-IgG2	Homodimer	Atara Biotherapeutics
Dalantercept	ALK1/Fc-IgG1	Homodimer	Acceleron
Dulaglutide	GLP-1/Fc-IgG4	Homodimer	Eli Lilly
Conbercept	VEGFr/Fc-IgG1	Homodimer	Chengdu Kanghong Biotech
Sotatercept	ACTR2A/Fc-IgG1	Homodimer	Acceleron
APG101	CD95/Fc-IgG1	Homodimer	Apogenix

those sales ($3.9 of $5.8 billion) went to Amgen's Enbrel® (etanercept), which is the most commercially successful Fc fusion protein to date. Enbrel® is a fusion of the soluble extracellular domain of human p75 TNFα receptor and Fc from human IgG1 and was approved in 1998 for the treatment of moderate to severe rheumatoid arthritis. Enbrel® works by neutralizing both the soluble and, to a lesser extent, the membrane-bound forms of TNFα resulting in the reduction of concentrations of serum inflammatory cytokines [4]. Enbrel® is now also approved for treatment of psoriatic arthritis, ankylosing spondylitis, moderate to severe polyarticular juvenile ideopathic arthritis, and moderate to severe plaque psoriasis.

Approved in November 2011, the fusion protein aflibercept (Eylea®, which contains portions of the VEGF receptors 1 and 2 linked to Fc from IgG1), a treatment for age-related macular degeneration, reached nearly $1 billion in sales only a year after its approval [3]. Alefacept (Amevive®) approved for treatment of plaque psoriasis but discontinued in 2011, worked by binding to CD2 on T cells, reducing their interaction with antigen-presenting cells and thereby limiting T cell activation. Abatacept, first approved for rheumatoid arthritis, works in a similar manner to prevent interactions between T cells and antigen-presenting cells. Belatacept differs from abatacept by only two amino acids but has a slower dissociation rate and a higher potency.

Recently approved fusion proteins include recombinant factor IX Fc [5] and recombinant factor VIII Fc [6] from Biogen. Recombinant factor VIII Fc (Eloctate®) and recombinant factor IX Fc (Alprolix®) fusion proteins are long-acting coagulation factors for the treatment of hemophilia A and B, respectively [7]. These long-acting proteins have the advantage of reduced injection frequency that simplifies treatment and increases patient compliance. Both of these clotting factors were produced as monomeric Fc fusions because the homodimeric forms were not as effective [8].

Peptide-Fc fusions or peptibodies, developed by Amgen, are chimeric proteins generated by linking the Fc domain of IgG to one or more copies of a bioactive peptide, combining the biologic activity of the peptide with the stability of a monoclonal antibody. There are a considerable number of peptibodies in preclinical and clinical development [9]. With two peptides in each peptibody, the molecule

can have increased avidity for its target. Orientation is important as certain peptides are more active when fused with the carboxy terminus of the Fc domain [9]. With a typical total molecular weight of less than 69 kDa, peptibodies have a lower potential to develop anti-drug antibodies (ADA) than their larger counterparts such as monoclonal antibodies or larger fusion proteins, which is an advantage from a clinical immunology perspective [10, 11]. The affinity of the peptibody for its target can be increased by addition of flanking residues, spacers, or linker sequences [12]. Romiplostim (marketed under the trade name Nplate®), the first peptide-Fc fusion developed as a biologic, is a peptide mimetic of thrombopoietin fused with the Fc domain of IgG1 and was approved by the FDA for treatment of chronic immune thrombocytopenia purpura [13]. Glycine linkers were added to increase the flexibility of the peptide moiety [14]. There are several other peptibodies, including trebananib, which is a peptibody antagonist to angiopoietins 1 and 2 for use in oncology settings, and PINTA 745, which is an anti-myostatin peptibody for use in end-stage renal disease and is in clinical trials (https://clinicaltrials.gov/ct2/show/NCT01958970?term=pinta+745&rank=1. Accessed April 10, 2015).

A similar approach is used with Mimetibody™ technology, developed by Centocor, where the bioactive peptide is a mimetic identified, for example, from phage libraries and is then linked to the Fc. The first Mimetibody™ successful in phase I clinical study, i.e., CNTO 528, contained a 20 amino acid erythropoietin mimetic peptide [15]. New generation Mimetibody™ proteins have been developed by rational protein engineering to alleviate the proteolytic degradation and aggregation inherent in the earlier version. For example, adding carbohydrates to the flanking region of the peptide reduces the tendency to form inter-chain disulfide bonds [15].

In order to achieve high-affinity binding to some receptors, two or more distinct receptor molecules may be needed. Regeneron developed a "trap" approach in which the ligand-binding domains from different receptors were sequentially fused to the Fc [15]. An engineered protein called a Trap-Fc, which contains the ligand-binding domains of EGFR and ErbB-4 fused to an Fc domain, binds to and neutralizes both ligands. The TRAP-Fc has high binding affinity, inhibits metastatic phenotypes in vitro, and performs better than using a combination of the two individual antibodies in mouse tumor models [16]. Using the same technology, a ligand trap was also created by linking the second and third domains of VEGFR1 and VEGFR2 that is reportedly more efficacious at inhibiting angiogenesis than VEGFR2 antibody alone [17, 18]. This molecule has been approved by the FDA for treatment of age-related macular degeneration (aflibercept, Eylea®). Rilonacept (Arcalyst®) is a Trap-Fc fusion protein and consists of the C-terminus of the IL-1RAcP ligand-binding region fused to the N-terminus of the IL-1RI extracellular region, which is then fused to the Fc domain of human IgG1. This fusion strategy results in a highly potent, long half-life therapeutic [19]. The Trap-Fc approach is also a highly effective method for treatment with cytokines, which typically act as multi-component systems to mediate desired immunological responses. Fc fusion proteins with two independent and potentially synergistic binding domains may

result in effective therapeutics for treating excessive cytokine release [20]. Cytokine traps consisting of the extracellular domains of two different cytokine receptor components fused to human Fc as a heterodimer are potent blockers of cytokine activity [21].

24.2 Advantages of Fc Fusion Proteins

The primary advantages to developing biotechnology drugs as Fc fusions are increased efficacy via half-life extension, increased potency (via valency), and addition of effector functions. Half-life extension results in increased target exposure leading to lower or less frequent dosing, which can reduce costs and improve patient compliance. The addition of effector functions can be particularly useful in therapeutic areas such as oncology where cell killing is desired [22].

A clear demonstration of some of these effects is the early study showing that the dimeric sTNFR: Fc molecule was a more potent inhibitor of TNF than the monomeric sTNFR by 50–100X as assessed both in vitro by inhibition of TNF binding or bioactivity and in vivo with mice injected with a lethal dose of LPS [4]. The increased efficacy is believed to have resulted both from the higher affinity of TNF for sTNFR:Fc than sTNFR [4] and from the fivefold increase in serum half-life with TNFR:Fc after intravenous injection compared with the soluble receptor alone [23]. In another example, fusion of the anti-angiogenesis factor apolipoprotein(a) kringle V to the Fc domain of IgG1 significantly increased both the half-life and bioavailability without affecting activity in mice [24]. Similarly, fusion of recombinant human lactoferrin with Fc resulted in a greater than ninefold increase in serum half-life in rats compared to lactoferrin alone, and a longer half-life than that of the PEGylated version of lactoferrin [25].

The increase in serum half-life attained by fusion to the Fc domain may be even more significant for cytokines, which are relatively small and cleared rapidly from the body. Because increased size, however, slows diffusion through mucus, only a single cytokine molecule is typically fused to the Fc domain forming a monomeric Fc. This compromise results in improved pharmacokinetics without significant reduction in mucus diffusion [26].

In addition to the increased half-life, Fc fusions have been explored to provide a noninvasive route of administration, namely through the pulmonary mucosa as an aerosolized therapeutic. Because FcRn, which is responsible for IgG transport, is expressed in human epithelial cells of the lung, conjugation of small proteins to the Fc domain of IgG allows transport of biologically active molecules to the bloodstream [20, 27]. This format confers noninvasive delivery and less frequent administration, which would be more convenient for patients particularly those with chronic conditions. While dimeric Fc fusions of human erythropoietin were shown to be effective in non-human primates and human volunteers, second-generation Fc fusion molecules have been developed as monomers with respect to the therapeutic protein but dimeric with respect to the Fc [20]. The monomers have improved

transport efficiency, increasing the bioavailability, and a longer serum half-life compared to dimeric Fc fusions; some of the monomeric fusions also have greater biological activity. Factor IX and erythropoietin have been developed as monomeric Fc fusion proteins specifically with the intent of noninvasive pulmonary delivery [20, 27]. Similar results were observed with monomeric Fc fusions of IFNα and IFNβ, which both had higher efficacy than their dimeric counterparts [20].

In some circumstances, use of an Fc fusion protein may result in improved safety outcomes for the patient population compared to mAbs. The British Society for Rheumatology Biologics Register reported that use of anti-TNF monoclonal antibodies (adalimumab and infliximab) resulted in a higher rate of tuberculosis reactivation than the Fc fusion protein (etanercept) [28]. A similar result, that is a higher risk of TB with anti-TNF mAb therapy than with sTNFR therapy, was found by the French registry [29]. Their conclusion was that these differences in TB recurrence could be supported by how the mAb and the fusion protein separately interact with membrane-bound TNF, leading to a differential effect on effector T cells and Treg cells. Hunt and Emery suggest that etanercept’s structure may offer some potential benefits over mAbs because only the fusion part of etanercept contains potential immunogenic epitopes leaving the TNF-binding portion unaffected, whereas an antibody response can be directed to several epitopes within the variable region of mAbs [30]. Going even further is the idea that the Fc domain may confer immunomodulatory properties to the molecule by inducing antigen-specific tolerance with the use of specific epitopes and/or carbohydrate side chain modifications [8].

An added benefit of Fc fusions is that the proteins can be easier to manufacture than the smaller ligand or receptor. Linking the ligand or receptor to an Fc domain often results in higher expression and secretion and easier purification by Protein A chromatography, which was the case for TNFr-Fc compared to its soluble receptor [31]. Difficult to produce molecules can sometimes be expressed more effectively as an Fc fusion by conferring greater stability to the molecule [32]. The solubility and stability of fusion proteins can also be improved with protein engineering, aiding the handling of these proteins during manufacturing and long-term storage [15, 31, 33]. Because of their structural similarity to a portion of an antibody, production of Fc fusion proteins is accomplished using existing antibody manufacturing platforms. Fc fusion proteins can be expressed in a variety of hosts although mammalian cells; e.g., Chinese hamster ovary (CHO) and human embryonic kidney (HEK), are typically preferred because they provide the necessary folding and post-translational modifications for human use. Some fusion proteins, however, may be more effectively produced in bacteria; this is the case for peptibodies. Romiplostim is produced as a single chain polypeptide in *E. coli*, using a process that involves fermentation and downstream processing and includes refolding and multiple column chromatographic steps. In addition to the high expression level and the faster manufacturing process compared with mammalian cell culture systems, the homogeneity of the final product and the absence of glycosylated forms are important advantages of production in *E. coli* [12]. Because romiplostim has an aglycosylated Fc moiety, it is partially Fc-silenced [34].

24.3 Challenges Associated with Fc Fusion Proteins

The Fc fusion format can also have its challenges, many of which are shared by traditional monoclonal antibodies. These include safety concerns (immune and non-immune), unwanted effector function, and manufacturing issues.

Although relatively rare, adverse (on- and off-target) reactions to mAbs and Fc fusion proteins alike include serious infections, malignancy, cytokine release syndrome, anaphylaxis, hypersensitivity, and immunogenicity [35]. Excessive triggering of Fc-mediated effector functions can induce cytokine release syndrome as has been observed with the monoclonal antibody Rituximab [35]. The suppression of T lymphocytes by the fusion protein belatacept is associated with an increased risk of post-transplant lymphoproliferative disease [36], resulting in boxed warnings and a monitoring plan to protect patients. While etanercept also has boxed warnings for serious infections and malignancy, other immunomodulatory Fc fusions such as abatacept, alefacept, and rilonacept do not have similar warnings [35]. Generally speaking, all biologic therapeutics can result in some level of immunogenicity, which is characterized by either neutralizing or non-neutralizing anti-drug antibodies. Because the composite fusion proteins are constructed from two or more proteins, each of which may or may not be modified, they can be non-native to the body and therefore are potentially immunogenic. The presence of anti-drug antibodies can affect the pharmacokinetic profile, result in loss of tissue penetration, and/or reduce the biological activity of the drug [37].

While Fc fusion proteins are typically easier to produce and purify than their original receptor or ligand, there can still exist issues with manufacturing; production and/or purification difficulties can vary on a molecule by molecule basis. Manufacturing of Fc fusion proteins can result in product variants, aggregates, and clipped species. This product heterogeneity may stem from disulfide isoforms, oxidation, and inconsistent glycosylation. These and other variants can complicate product purification and reduce the overall yield of the process.

24.4 Improving Fc Fusion Proteins

As with other IgG-based therapeutics, Fc fusions have recently been and will continue to be extensively engineered in the Fc domain and/or in the fusion partner to improve efficacy, safety, and manufacturability. This section will focus on engineering the Fc because of its commonality to all of the Fc fusion proteins. Fc engineering, whether for antibodies or Fc fusion proteins, can be used to increase or decrease antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and/or complement-dependent cytotoxicity (CDC). There are multiple antibodies and Fc fusion proteins already approved, in clinical trials, and in development in which the Fc has been modified without compromising the specificity of the therapeutic moiety [38].

Fc Engineering: Increased Effector Function Benefits of enhancing effector functions include greater efficacy, lower or less frequent dosing and decreased cost-of-goods [33]. Most prior Fc engineering efforts have focused on the IgG hinge/CH2 region, which can engage Fcγ receptor binding to recruit immune effector functions. Where effector function increase is desired, such as in oncology applications where destruction of the target cell is the goal, the protein sequence has been affinity-optimized for FcγRs by substitutions or by glycoform engineering [39]. Fc glycosylation is necessary for fusion proteins to elicit effector functions as the glycans attached to asparagine at the 297 position in the CH2 domain are critical for complement activation and FcγR binding [39]. While sialylated Fc has the advantage of anti-inflammatory properties, non-sialylated antibodies may bind with increased affinity to FcγR for enhanced receptor-mediated cytotoxicity [40]. Enhanced affinity of mAbs for Fcγ RIIIA, by modifying the amino acid sequence in the Fc domain or by defucosylating the N-linked oligosaccharides on the Fc region, has been shown to increase tumor killing through ADCC [41]. These same modifications were successful for Fc fusion proteins derived from TNFRII and LFA-3 where there was significantly higher Fc receptor γIIIa binding and increased Fc-mediated cytotoxicity on target cells but binding activity was unchanged [42]. This correlation was demonstrated further in another study where binding of IgGs to FcγRIIIa was enhanced at least tenfold by glycans that lack the core fucosyl moiety [43]. In contrast to the known correlation between fucose content and ADCC activity, the impact of other sugar residues in the Fc-bound glycan structure on immune effector functions is not well understood [44]. The effect of a terminal galactose on Fc function, for example, is not fully defined and appears to be antibody specific [1]. A recent study, however, showed that terminal galactose does affect FcγR binding [45]. Clearly, more studies are needed to fully understand the impact of Fc glycoforms on immune effector function. Changes to Fc glycosylation will most certainly also affect PK and PD, which will need to be evaluated on a per molecule basis [1].

Another approach to achieve increased effector function is to use tandem Fc repeated homodimers. A TNFRII-Fc-Fc fusion showed augmented binding to FcγRs and complement, leading to enhanced ADCC and CDC without affecting its binding to TNFα or its neutralizing activity [46]. Typically, these constructs are bigger causing some concern about tissue penetration, but there may be advantages to slower penetration and accumulation in certain therapeutic situations.

Fc Engineering: Increased Half-life Fc engineering efforts have also been aimed at further extending half-life by increased Fc—FcRn binding. Mutations have been identified in the CH2-CH3 region, where IgG interacts with FcRn, that increase the binding affinity at acidic but not neutral pH, ensuring that the molecule can still be released into the circulation [47]. Incorporating these mutations into mAbs has been shown to increase half-life and improve tumor killing [48]. These same mutations might be equally or more effective in Fc fusion proteins, which typically have shorter half-lives than intact mAbs, [49].

Fc Engineering: Decreased Effector Function For applications where effector functions are undesirable, FcγR affinity can by eliminated by using aglycosylated Fc and/or amino acid substitutions in the hinge/CH2 region. Modifications to the Fc portion through mutations in specific amino acid residues can prevent the interaction of the Fc domain with other Fc receptors and complement components, and thus reduce or eliminate unwanted effects [35]. Fc functionality has been silenced for antibodies or Fc fusion proteins that target receptor or cell-surface proteins especially on immune cells to minimize the safety risk. An example of this is Orencia® where the C1q- and FcγR-binding sequences of the hinge region were modified to greatly reduce CDC and ADCC [38]. Scallon et al. [50] recently showed that sialylation decreased ADCC of three different human antibodies, both by reducing the affinity of the antibodies to FcγRIIIa and to their target. With abatacept, a series of directed, select mutations in the hinge region were introduced to both improve protein production and reduce Fc-mediated binding to limit the effects normally associated with binding of Fc such as CDC and ADCC [51]. Using results from in vitro binding studies, abatacept therapeutic activity is thus primarily a result of receptor binding rather than activities mediated by the modified Fc domain [51]. The authors hypothesized that this could result from steric hindrance (conformational change resulting from deletion of hinge cysteines) or altered glycosylation (introduction of serines, one of which was glycosylated).

While many of the fusion proteins reaching the clinic consist of the Fc domain from IgG1, effector function decrease can be achieved by using the Fc domain from other IgG subclasses, such as IgG2 or IgG4, which have relative lower affinity to FcγRs and complement receptors [52]. One example of the intentional use of the Fc domain from another IgG subclass is development of a long-acting glucagon-like peptide 1 (GLP-1), which has potential as a treatment for diabetes [53]. Of all the IgG isotypes, the Fc domain of IgG2 was chosen as the fusion partner for GLP-1 because it has the lowest affinity for FcγRI, a high-affinity Fc receptor that can induce ADCC [52]. In addition, the Fc domain from IgG2 does bind to FcγRIIB, an inhibitory receptor on some immune cells, reducing the likelihood of Fc-induced immunity [52]. This Fc fusion was shown to have superior therapeutic and pharmacologic properties compared to native GLP-1 in a mouse model of Type 1 diabetes with a diminished concern for immunogenicity [53]. More recently, an Fc variant of IgG2 was engineered with multiple substitutions to eliminate affinity for Fcγ receptors and C1q complement protein and, as a result, immune effector functions [54]. This type of an approach makes sense for therapeutic blocking antibodies where immunostimulatory functions conferred by Fc domains are unwanted and Fc interaction with FcRn to extend serum half-life is retained [54]. Another approach is to fuse the protein to a hybrid Fc (hyFc) containing the CH2 and CH3 regions of IgG4 and the highly flexible hinge region of IgD, both with low ADCC or CDC potential, which has been done with TNF receptor [55]. Using an in vitro bioassay, the neutralizing efficacy of TNFR-hyFc was about 1.5-fold higher than that of etanercept, perhaps because of the improved hinge flexibility [55].

Fc Engineering: Monomeric Fc There is circumstances where the bivalency of IgG is not desired and can cause unwanted effects. One example is when the target is a multimeric soluble molecule where use of a bivalent Fc fusion protein could result in unwanted crosslinking. Another example is antagonism of a cell-surface target where use of a homodimer Fc fusion protein could result in excessive agonist activity. Forming a cross-linked network in plasma or unwanted agonist activity can be avoided with the one-armed format. N-glycosylation engineering to disrupt the CH3–CH3 interface and mask the exposed hydrophobic surface of CH3 domains enables the formation of Fc fusion proteins that are monomeric with respect to the Fc region [56]. Use of this double glycosylation variant improved the properties of the monomeric Fc domain while maintaining the pH-dependent binding to FcRn. The monomeric Fc was highly soluble and remained monomeric up to 200 mg/L. Although the serum half-life was shorter than for a control antibody, tandem repeats of the monomeric Fc domain increased the avidity and extended half-life [56]. Recent studies have shown that the monomeric IgG1 CH3 domain may also serve as an effective fusion partner, conferring increased tissue penetration, access to restricted binding sites and increased therapeutic efficacy, while maintaining long serum half-life, compared to a typical Fc fusion because of the former's smaller size. The CH3 domain, even after engineering in an additional disulfide bond to increase thermal stability, maintains binding to FcRn keeping the long half-life associated with those interactions [57]. An additional advantage of the monomeric CH3 domain as a fusion partner is that the product can be produced at a high level in *E. coli* and can bind to both proteins A and G although with lower affinity than a dimeric Fc.

24.5 Regulatory Considerations

The primary concern of regulatory agencies regarding fusion proteins as biotherapeutics is their potential for eliciting an immune response. Regulatory agencies expect more complete characterization of the anti-drug antibody (ADA) response with fusion proteins because they consist of both a foreign protein (Fc-hinge) and an endogenous protein. This is especially true if one or both is engineered. Sequence modification, codon optimization, charge pair mutations, disulfide bridge insertion, and linker shortening or removal, while often improving expression, protein folding, or stability, can also have unwanted effects such as altered protein–protein interactions, aggregation, and immunogenicity [19]. The foreign sequence could initiate a cell-mediated antibody response that can sometimes also induce an immune reaction to the fused native protein [19]. Depending on the type and extent of the immune response, ADA could neutralize the intended effect of the therapeutic or, even worse, could cross-react with autologous proteins resulting in an adverse event for the patient. The FDA recommends that the ADA response to fusion molecules, especially those that are engineered, be assessed using assays that can determine reactivity not only to the complete molecule but also to its individual

components [58]. This approach will help to determine if the immune response is directed at one or more of the individual components or novel epitopes in the fusion region.

24.6 Conclusions

Fc fusion proteins continue to provide great clinical promise for treating a variety of diseases especially as molecular understanding of the associated physiology is improved. Given the availability of sophisticated engineering knowledge and tools, the application of IgG-based therapeutics is rapidly evolving to include a new generation of engineered Fc fusion proteins with potentially improved efficacy and safety. These advances are leading to “fit-for-purpose” Fc fusion proteins with precise targeting, rationally designed effector functions and optimized half-lives.

References

1. Jiang X-R, et al. Advances in the assessment and control of the effector functions of therapeutic antibodies. Nat Rev Drug Disc. 2011;10:101–10.
2. Capon D. Designing CD4 immunoadhesins for AIDS therapy. Nature. 1989;525–531.
3. Aggarwal S. What’s fueling the biotech engine—2012–2013. Nat Biotechnol. 2014;32(1): 32–9.
4. Mohler K, et al. Soluble tumor necrosis factor (TNF) receptors are effective therapeutic agents in lethal endotoxemial and function simultaneously as both TNF carriers and TNF agonists. J Immunol. 1993;151(3):1548–61.
5. Ducore JM, Miguelino MG, Powell JS. Alprolix (recombinant factor IX Fc fusion protein): extended half-life product for the prophylaxis and treatment of hemophilia B. Expert Re Hematology. 2014;7(5):559–71.
6. Powell JS, et al. Safety and prolonged activity of recombinant factor VIII Fc fusion protein in hemophilia A patients. Blood. 2012;119(13):3031–7.
7. Shapiro A. Development of long-acting recombinant FVIII and FIX Fc fusion proteins for the management of hemophilia. Expert Opin Biol Ther. 2013;13(9):1287–97.
8. Rath T, et al. Fc-fusion proteins and FcRn: structural insights for longer-lasting and more effective therapeutics. Crit Rev Biotechnol. 2013.
9. Wu B, Sun Y-N. Pharmacokinetics of peptide-Fc fusion proteins. J Pharm Sci. 2014;103:53–64.
10. Hermeling S, Crommelin D, Schellekens H, Jiskoot W. Structure-immunogenicity relationships of therapeutic proteins. Pharm Res. 2004;22(6):897–903.
11. Dintzis H, Dintzis R, Vogelstein B. Moleculare determinants of immunogenicity: the immunon model of immune response. Proc Natl Acad Sci USA. 1976;73(10):3671–5.
12. Shimamoto G, Gegg C, Boone T, Queva C. Peptibodies: a flexible alternative format to antibodies. mAbs. 2012;4(5):586–591.
13. Cines D, Yasothan U, Kirkpatrick P. Romiplostim. Nat Rev Drug Discov. 2008;7:887–8.
14. Molineux G, Newland A. Development of romiplostim for the treatment of patients with chronic immune thrombocytopenia: From bench to bedside. Br J Haematol. 2010;150(1): 9–20.

15. Huang C. Receptor-Fc fusion therapeutics, traps, and MIMETIBODY technology. Curr Opin Biotechnol. 2009;20:692–9.
16. Lindzen M, et al. A recombinant decoy comprising EGFR and ErbB-4 inhibits tumor growth and metastasis. Oncogene. 2012;31:3505–15.
17. Holash J, et al. VEGF-Trap: a VEGF blocker with potent antitumor effects. PNAS. 2002;99:11393–8.
18. Kim ES, et al. Potent VEGF blockade causes regression of coopted vessels in a model of neuroblastoma. PNAS. 2002;99:11399–404.
19. Kimchi-Sarfaty C, et al. Building better drugs: developing and regulating engineered therapeutic proteins. Cell. 2013;34(10):534–48.
20. Dumont J, Low S, Bitonti A. Monomeric Fc fusions: Impact on pharmacokinetic and biological activity of protein therapeutics. Biodrugs. 2006;20(3):151–60.
21. Economides A, Carpenter L. Cytokine traps: multi-component, high-affinity blockers of cytokine action. Nat Med. 2003;9:47–52.
22. Nimmerjahn F, Ravetch J. Fcgamma receptors as regulators of immune responses. Nat Rev Immunol. 2008;8:34–47.
23. Jacobs C, et al. Pharmacokinetic parameters and biodistribution of soluble cytokine receptors. Int Rev Exp Pathol. 1992;34B:123.
24. Yu H-K, et al. Immunoglobulin Fc domain fusion to apolipoprotein(a) kringle V significantly prolongs plasma half-life without affecting its anti-angiogenic activity. Protein Eng Des Sel. 2013;26(6):425–32.
25. Shiga Y, et al. Recombinant human lactoferrin-Fc fusion with an improved plasma half-life. Eur J Pharm Sci. 2015;67:136–43.
26. Jazayeri JA, Carroll GJ. Fc-based cytokines. Biodrugs. 2008;22(1):11–26.
27. Valee S, et al. Pulmonary administration of interferon beta-1a-fc fusion protein in non-human primates using an immunoglobulin transport pathway. J Interferon Cytokine Res. 2012;32 (4):178–84.
28. Dixon W, et al. Drug-specific risk of tuberculosis in patients with rheumatoid arthritis treated witn anti-TNF therapy: results from the British Society for Rheumatology Biologics Register (BSRBR). Ann Rheum Dis. 2010;69(3):522–8.
29. Tubach F, et al. Risk of tuberculosis is higher with anti-tumor necrosis factor monoclonal antibody therapy than with soluble tumor necrosis factor receptor therapy: the three-year prospective French Research Axed on Tolerance of Biotherapies registry. Arthritis Rheum. 2009;60(7):1884–94.
30. Hunt L, Emery P. Etanercept in the treatment of rheumatoid arthritis. Expert Opin Biol Ther. 2013;13(10):1441–50.
31. Peppel K, Crawford D, Beutler B. A tumor necrosis factor (TNF) receptor-IgG heavy chain chimeric protein as a bivalent antagonist of TNF activity. J Exp Med. 1991;174:1483–9.
32. Zhang J, et al. Fusion partners as a tool for the expression of difficult proteins in mammalian cells. Curr Pharm Biotechnol. 2010;11(3):241–5.
33. Carter P. Introduction to current and future protein therapeutics: a protein engineering perspective. Exptl Cell Res. 2011;317:1261–9.
34. Kumagai Y, et al. Pharmacodynamics and pharmacokinetics of AMG 531, a thrombopoiesis-stimulating peptibody, in healthy Japanese subjects; a randomized, placebo-controlled study. J Clin Pharmacol. 2007;47(12):1489–97.
35. Sathish J, et al. Challenges and approaches for the development of safer immunomodulatory biologics. Nat Rev Drug Disc. 2013;12:306–24.
36. Grinyo J. An integrated safety profile analysis of belatacept in kidney transplant recipients. Transplantation. 2010;90:1521–7.
37. Chen X, Zaro J, Shen W-C. Pharmacokinetics of recombinant bifunctional fusion proteins. Expert Opin Drug Metab Toxicol. 2012;8(5):581–95.
38. Strohl W. Optimization of Fc-mediated effector functions of monoclonal antibodies. Curr Opin Biotechnol. 2009;20:685–91.

39. Jefferis R. Glycosylation as a strategy to improve antibody-based therapeutics. Nat Rev Drug Discov. 2009;8:226–34.
40. Kaneko Y, Nimmerjahn F, Ravetch J. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. Science. 2006;313:670–3.
41. Stavenhagen J, et al. Enhancing the potency of therapeutic monoclonal antibodies via Fc optimization. Adv Enzyme Regul. 2008;48:152–64.
42. Shoji-Hosaka E, et al. Enhanced Fc-dependent cellular cytotoxicity of Fc fusion proteins derived from TNF receptor II and LFC-3 by fucose removal from Asn-linked oligosaccharides. J Biochem. 2006;140:777–83.
43. Matsuda K, et al. Enhanced binding affinity for FcgammaRIIIa of fucose-negative antibody is sufficient to induce maximal antibody-dependent cellular cytotoxicity. Mol Immunol. 2007;44 (12):3122–31.
44. Kellner C, Derer S, Valerius T, Peipp M. Boosting ADCC and CDC activity by Fc engineering and evaluation of antibody effector functions. Methods. 2014;65:105–13.
45. Houde D, Peng Y, Berkowitz S, Engen J. Post-translational modifications differentially affect IgG1 conformation and receptor binding. Mol Cell Proteomics. 2010;9:1716–28.
46. Nagashima H, et al. TNF receptor II fusion protein with tandemly repeated Fc domains. J Biochem. 2011;149(3):337–46.
47. Yeung Y, et al. Engineering human IgG1 affinity to human neonatal Fc receptor: impact of affinity improvement on pharmacokinetics in primates. J Immunol. 2009;182:7663–71.
48. Zalevsky J, et al. Enhanced antibody half-life improves in vivo activity. Nat Biotechnol. 2010;28(2):157–9.
49. Czajkowsky D, Hu J, Shao Z, Pleass R. Fc-fusion proteins: new developments and future perspectives. EMBO Mol Med. 2012;4:1015–28.
50. Scallon B, et al. Higher levels of sialylated Fc glycans in immunoglobulin G molecules can adversely impact functionality. Mol Immunol. 2007;44(7):1524–34.
51. Davis P, et al. Abatacept binds to the Fc receptor CD64 but does not mediate complement-dependent cytotoxicity or antibody-dependent cellular cytotoxicity. J Rheum. 2007;34(11):2204–10.
52. Bruhns P, et al. Specificity and affinity of human Fcgamma receptors and their polymorphic variants for human IgG subclasses. Blood. 2009;113:3716–25.
53. Wang Q, et al. Novel GLP-1 fusion chimera as potent long acting GLP-1 receptor agonist. PLoS ONE. 2010;5:e12734.
54. Vafa O, et al. An engineered Fc variant of an IgG eliminates all immune effector functions via structural perturbations. Methods. 2014;65:114–26.
55. Lee J-H, et al. Biochemical characterization of a new recombinant TNF receptor-hyFc fusion protein expressed in CHO cells. Protein Expr Purif. 2013;87:17–26.
56. Ishino T, et al. Engineering a monomeric Fc domain modality by N-glycosylation for the half-life extension of biotherapeutics. J Biol Chem. 2013;288(23):16529–37.
57. Ying T, et al. Engineered soluble monomeric IgG1 CH3 domain: generation, mechanisms of function, and implications for design of biological therapeutics. J Biol Chem. 2013;288 (35):25154–64.
58. FDAGov. Guidance for industry: Immunogenicity assessment for therapeutic protein products. [Online] Available at: http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm338856.pdf. Accessed 24 Mar 2015.

Part VII
Lifecycle Management

Chapter 25
Lifecycle Management of Biotherapeutic Dosage Forms

Nicholas Warne, Bryan Balthazor and William Parr

Abstract Lifecycle management of biotherapeutic dosage forms has become an increasingly important strategy for providing patients with enhanced convenience during storage and administration as well as providing companies with an opportunity to compete in an increasingly crowded marketplace. Often, lifecycle management is first considered after a product becomes available for commercial distribution. This approach can cause delays in innovation and result in a loss of opportunity to serve patients and remain competitive. A thoughtful approach to lifecycle management of dosage forms should begin in early product design, providing a framework for continuous improvement and the ability to take advantage of cumulative learning of both the specific compound as well as the therapeutic area and patients' needs.

Keywords Lifecycle management · Dosage forms · Drug product profile

25.1 Introduction and Scope

In its narrowest application, any change to an existing licensed manufacturing process or product can be considered lifecycle management (LCM). In the context of biotherapeutic manufacturing, lifecycle management for a given product while retaining the same mode of action can take many forms: process improvements to

N. Warne (✉)
Biotherapeutic Pharmaceutical Research and Development, Pharmaceutical R&D, Pfizer Inc., One Burtt Road, Andover, MA 01810, USA

B. Balthazor
BioTherapeutics Pharmaceutical Sciences, Pharmaceutical R&D, Pfizer Inc., 700 Chesterfield Parkway West, Chesterfield, MO 63017, USA
e-mail: Bryan.balthazor@pfizer.com

W. Parr
Global Marketing, Pfizer Innovative Health, Pfizer Inc., 500 Arcola Rd, Collegeville, PA 19426, USA

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*, AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_25

increase productivity during cell culture, modifications to harvest or purification steps to increase yield or purity, or new dosage forms to provide a more competitive product or one that is more patient friendly.

While these are acceptable applications of lifecycle management, they are, indeed, too narrow. By focusing almost exclusively on the traditional product lifecycle, which starts at drug product launch, this concept of lifecycle management ignores the significant amount of work that brackets a product's development through years of clinical research, commercial process and presentation development, and eventual post-launch optimization. A comprehensive approach to product lifecycle management begins early in development when the compound is initially defined, the preliminary (toxicology or Phase 1) manufacturing process is defined, and the first-in-human dosage form is developed. It is critical to consider LCM early in development because the initial process and products are often, appropriately, not suitable for long-term commercialization. A clinical dosage form, for example, is intended to provide clinicians with flexibility in dose, route of administration, and frequency of dose. This level of flexibility is problematic for commercial application. Having a strategy that encompasses a holistic LCM approach starting from early Phase 1 process and product development enables a project team to intentionally and strategically plan for process and product enhancements. The outcome of planning that encompasses multiple phases of product development and a thorough control strategy with continuous process verification and compliance with ICH Guideline Q8–Q11 [1–4] at its core allows for a thoughtful approach suitable for clinical and commercial development.

For the purpose of this chapter, the authors will focus on lifecycle management as focused on dosage form design. Specifically, we will review the rationale and strategies for design and implementation of new dosage forms, post-launch, for biotherapeutic products. Changes may include new dosage strengths to enable more convenient dose preparation and administration, new formulations such as substituting a liquid dosage form for a lyophilized powder and the introduction of convenience devices such as auto-injectors, dual-chambered syringes, and reconstitution aids. Out of scope of this chapter are modifications to the active ingredient itself (for example, PEGylation to extend circulating half-life) as well as changes to the route of administration. In effect, we wish to assess strategies and examples in which products have evolved to fulfill customer or patient needs.

25.2 Lifecycle Management and Product Development

In creating a thoughtful dosage form lifecycle management strategy, the project team must take into account the entire life of a product, rather than simply the product's intended commercial lifecycle on the market. From product concept and early research to marketing and dosage form differentiation, the project team must strive to recognize and accommodate the evolving needs of the customer. The "customer" term encompasses a number of diverse groups: clinical research, the commercial

Table 25.1 Stage-specific considerations of dosage form lifecycle management

Stage of development	Important considerations	Product options
Phase 1 to 2a (safety and dose finding)	Clinical research is the key customer	Early stage dosage forms must provide flexibility across a wide dosing range, often 100x. Need to determine whether flat or weight-based dosing
	Doses are unknown and could span a wide range	
	Route of administration often unknown (IV, SC are typical; IM for vaccines)	Stability of clinical supplies is important to avoid clinical trial interruptions and reduce cost of resupply
	Frequency of administration unknown	Masking of clinical trial materials may be required
Phase 2b to 3 (registration trials)	Clinical research has significant input	Dosage forms and launch supplies (kits) should be aligned in preparation for registration
	Commercial team must help to define drug product (kit) launch configuration	For combination products (e.g. prefilled syringes), human factors studies should be assessed using “representative” supplies and instructions
	Phase 3 trials should be performed with representative commercial supplies	Masking of clinical trial materials, or comparators, may be required
Initial registration and launch	Dosage forms must be suitable for launch	Proposed dosage forms should be biochemically comparable to, or representative of, clinical trial materials
	Depending on timing of program and registration, may not be optimal dosage form for market	
Post-launch	Post-launch, optimized dosage forms may be required for product differentiation based on customer or patient needs	New dosage forms must leverage previous experience with product
	Cost management may drive product and process improvements	Must add sufficient value in order to justify product enhancements

marketing team, payors, and patients all exert some degree of influence on the product. Moving through the product’s lifecycle, priorities must necessarily change to accommodate the current customer. For this reason, a consciously developed strategy for lifecycle management that maintains awareness of the different groups involved enables smoother transitions throughout the stages of a product’s maturity. Table 25.1 illustrates the challenges and considerations of dosage form design.

During the early stages of development, biotherapeutics dosage forms must be designed to accommodate the needs of the clinical research team. In this early stage of development, much is unknown including dose (may vary from 0.1 to 10 mg/kg for a typical monoclonal antibody, a 100x range), route of administration (typically subcutaneous injection or intravenous infusion depending on the needs of the

patient community and administration setting; vaccines are often intramuscular), and frequency of administration (may vary depending on allometric scaling and pharmacokinetic data from pre-clinical studies). These significant unknowns require a heightened level of flexibility. Further, given that the probability of early candidate compounds reaching registration is approximately 10% [5] at an estimated cost of $2.6B [6] in which one seeks to invest as late as feasible in a disciplined, milestone-driven manner, the formulator may wish to avoid prematurely optimizing the dosage form for competitive purposes and utilize a standardized or platform formulation [7] to reduce development time and resources while maintaining a high-quality product. This delay in product optimization is often due to a lack of understanding regarding dose level and frequency, market demands, and an overall commitment to further product development which are not clarified until the end of Phase 2a or 2b clinical trials.

During Phase 1–2a clinical trials, the primary customer is clinical research as they assess safety, activity, and dose finding. The need for convenience is less relevant, as during early clinical trials the doses are often prepared by highly trained clinical pharmacists. The stability of early clinical supplies must be intentionally assessed depending on the product's development strategy, timeline, and tolerance for risk. Utilizing a more stable dosage form (lyophilized powder or frozen liquids for example) is often less desirable from a dosage preparation and commercial perspective, but at this stage of development, the use of a more stable dosage form does accommodate a reduced number of manufacturing batches so that a single batch of drug product can be utilized through Phase 2a clinical trials. This strategy helps to reduce incremental development costs (resupply manufacturing, release, and perhaps stability testing), as well as decreases concerns related to stability failures possibly resulting in clinical delays, additional resupply batches, and quality concerns.

If suitably stable, a liquid dosage form could be utilized for early clinical trials, which is more convenient and more versatile if product differentiation is judged to be an important attribute in a given patient population. Understanding the dynamics of a disease, how it is managed by patients and healthcare providers and the dosage form requirements is critical to defining a dosage form strategy and managing expectations within a project team. Biotechnology products in oncology, for example, are often delivered via IV infusion; therefore, dosage form convenience is less critical. For diseases in which self-administration is a key success factor (diabetes for example), the need for convenient dosage forms and devices is critical to product advancement. While the shelf-life may be somewhat compromised for a liquid dosage form in comparison to a lyophilized powder, a liquid product may be more representative in terms of dosage form and quality attributes of the product to be developed for late-stage trials. In either case, the formulation and dosage form should be designed to accommodate the needs of clinical research as well as provide a foundation for product enhancements later in the product lifecycle. It is important that the formulator be able to link the dosage form development and manufacture of early clinical supplies to eventual launch supplies to ensure that they are representative of each other, especially regarding the critical quality

attributes of the product [8]. For example, if a product evolves from a more stable lyophilized powder in early clinical trials to a somewhat less stable liquid dosage form in later trials, then the safety of the new dosage form will need to be evaluated to exclude the possibility that novel degradants may be present in liquid clinical supplies that were absent or at reduced levels in the initial safety trial.

In late clinical development, for example Phase 2b and Phase 3 pivotal trials, the dosage forms must be representative of the proposed launch supplies. The use of the term "representative" is open to interpretation but can be assessed as meeting a defined set of specifications or critical quality attributes as well as utilizing a manufacturing process that are consistent regardless of scale or site of production and are representative of the eventual commercial supplies. At this stage of development, the dose should be known as well as the route and frequency of administration. While the final dose may not be available during Phase 2b trials, possibly requiring the development of dosage strengths that may not be registered, it is important that Phase 2b trials use dosage forms representative of the clinical trial materials that have already been tested in patients as well as are representative of the proposed dosage forms. Depending on the indication, patient population and clinical trials design, a well-matched placebo may be required, further complicating product development. While the placebo is of little commercial concern, it is often critical to a successful late-stage clinical trial and should not be underestimated. Finally, if the product is considered a combination product [9], then the dosage form and instructions for use should be assessed by risk assessment and human factors' studies to ensure compliance and a mature understanding of how the product is to be used.

The transition from early- to late-stage development is a critical point in dosage form development. The late-stage product must be representative, if not identical, to the proposed launch product. Further, the commercial team must be fully engaged in the design of the late-stage dosage form since it must accommodate their need for meeting requirements of the patients or healthcare provider, product differentiation, maintain an acceptable quality profile, provide flexibility throughout the supply chain, and be cost effective in manufacture. In order to understand the commercial drivers, a series of discussions to identify the drug product profile (DPP) should be held prior to initiation of late-stage dosage form development to ensure that the product being developed by the pharmaceutical scientist meets the needs of both the clinical research and commercial groups. Aspects of the DPP are discussed later in this chapter.

Post-launch dosage form development must be driven by clear commercial and medical needs. Table 25.2 provides guidance regarding potential commercial and medical drivers for post-launch dosage form enhancements.

Post-launch dosage form development must be justified by demonstrating that the benefits of the introduction of enhanced dosage forms outweigh the investment in time and resources required. This calculation can take many forms including a projected return on investment (ROI), calculation of net present value (NPV), or internal rate of return (IRR) [10]. There are strengths and weaknesses associated with each of these tools, but some quantitative projection must be made in order to

Table 25.2 Drivers for post-launch dosage form enhancements

Driver	Options
Ease of storage	Switch from refrigerated storage to room temperature; important for self-administration
Ease of dosage preparation	Switch from lyophilized powder to ready-to-use formulation (liquid, prefilled syringe)
	Switch from lyophilized powder in vial to lyophilized powder in dual-chambered syringe
	For lyophilized powders, introduce reconstitution aid
	Switch from single-use vials to multiple-use cartridges
	Develop more dosage strengths to accommodate patient dose and mass
	For large doses, where multiple vials are required, provide larger dosage strengths to accommodate one vial per dose
Ease of administration	Switch from vialed product to prefilled syringe
	Switch from prefilled syringe to auto-injector
	Reduce injection volume
	Switch from intravenous infusion to subcutaneous injection

justify the expense as well as compare competing proposals. Review of an individual LCM proposal requires a disciplined tool for creating a financially and medically driven decision. When comparing across proposals, utilizing the same calculation with similar assumptions is vital to trusting the output. One aspect of each of these assessments is that the enhancement must provide a tangible benefit to the patient or customer. By making the product easier to prepare or administer, the patient may become increasingly compliant, possibly reducing the incidence of missed doses due to inconvenience of administration [11]. In addition, having the ability to better customize doses will reduce waste as well as diminish the possibility of sub-optimal patient outcomes resulting from over- or under-dosing. Finally, it must be recognized that patient desires, such as ease of administration, can play a role in the success of a particular product.

25.3 Drug Product Profile

As described earlier, understanding the clinical and commercial needs of the patient population is critical to designing the appropriate product. To improve success with the target patient population, it is important to discuss the drug product profile (DPP) with the integrated project team including clinical and commercial colleagues. This discussion should occur no later than Phase 2a, after certain clinical aspects are known (dose, route of administration, frequency, etc.), and prior to initiating development of the commercial dosage forms and manufacturing process. The DPP differs from the target product profile (TPP) in that typically the TPP accounts for a broader perspective on the product including target indication(s),

Table 25.3 Points to consider during DPP development

Question	Information useful in product design
Who will administer this compound?	Self-administration, healthcare professional, family member, or friend
Where will the dose be administered?	In home, physician's office, pharmacy, infusion center, etc.
What is the frequency of administration?	Daily, weekly, biweekly, monthly, etc.
Who will manage the patient's drug supplies and how will they obtain them?	Storage at home, storage at pharmacy, home delivery
What is the competitive landscape in this therapeutic area?	Novel product and mode of action; product will be one of several available
Are there any indication-specific considerations?	Patient with severe rheumatoid arthritis may be dexterity challenged
	Patient population has no history with injectables

safety profile, expected medical outcomes, and, to some extent, cost of treatment [12]. What the TPP lacks is sufficient detail regarding the *desired* dosage form, a crucial aspect of marketing.

Before defining the commercial dosage form, several aspects of the marketplace should be well understood. This information can be gathered by market research, which may be important depending on the indication, regional, and competitive landscape, or it may be based on experience with comparable products. Table 25.3 presents several examples of questions which should be addressed prior to defining the commercial dosage form.

Understanding the responses to questions presented in Table 25.3 allows the integrated project team to characterize the need for specific dosage forms. An example of how this is applied could be the development of an oncology product which requires intravenous administration in a clinical setting. Given this profile, there is little need to develop a ready-to-use prefilled syringe and a vialed product may be suitable. In contrast, if one is developing a product for rheumatoid arthritis that is to be self-administered, then the team must consider development of a prefilled syringe as well as auto-injector dosage forms in order to compete in a marketplace that has established an expectation for these types of dosage forms. Injection experience has been cited as a reason for patient discontinuation of a specific therapy [13].

Finally, one must have a mature understanding of both the DPP and TPP prior to establishing the Phase 3 dosage form, Quality Target Product Profile (QTPP). The QTPP, as described in ICH Q8, integrates what is critical to the patient in terms of therapeutic outcomes with product design, control, and specifications. Generation of a thoughtful TPP and DPP is prerequisites in understanding why a specific dosage form is being developed. The QTPP subsequently characterizes how one wishes to control the manufacture of the product in order to satisfy the TPP.

25.4 Case Studies

There are several examples of successful lifecycle strategies of biotherapeutic dosage forms. Because it is challenging to document the many dosage forms utilized during the clinical development of a compound, we will focus on post-launch LCM strategies using publicly available information. The two examples we have selected are both injectable drugs, but their dosage form development was influenced by competitive pressures unique to the product area. Human growth hormone, administered daily via subcutaneous injection, has been commercially available for 30 years by a number of firms. Recombinant factor VIII, for the treatment and management of hemophilia A, is administered by intravenous infusion and has been commercially available for over 20 years. Both compounds are often self-administered or often administered by a family member in the home, so convenience plays an important factor in product choice. Both compounds are for chronic conditions; therefore, training of patients and caregivers is important to ensure good injection practices. Both of these products are in highly competitive therapeutic areas, but their markets have evolved differently as the examples will present.

Human growth hormone, manufactured by several firms, has evolved significantly since it was introduced into the marketplace, and is a good example of dosage form differentiation via convenience, specificity, and a desire for continuous innovation as a means of promulgating brand loyalty. The example of recombinant FVIII clotting factor demonstrates how several companies will differentiate their products on the basis of formulation, safety, and dosage forms, seeking to compete with an assurance of quality and convenience.

25.5 Growth Hormone

Human growth hormone (rhGH), also called somatotropin, is a peptide hormone used to treat patients who have a human growth hormone deficiency resulting in growth failure and short stature [14]. rhGH is often prescribed for children but can be used by adults. rhGH is produced using recombinant techniques [15] and was one of the first biotechnologically derived products.

All of the companies below share similarities in their dosage form trajectory for growth hormone, from lyophilized vials to cartridges to injection pens, making for an informative discussion of which factors determine market success. Initially approved in 1985, rhGH was modified from naturally occurring human growth hormone by including an additional N-terminal methionine (192 amino acids, somatrem) due to the expression system, and was provided as a lyophilized powder in a vial. Since that approval, numerous improvements have been made to the recombinant growth hormone dosage form including approval of the 191 amino acid growth hormone (somatropin), introduction of a liquid formulation that does

not require reconstitution, multiple dosage strengths, and the use of many different devices for the reconstitution and delivery of recombinant growth hormone. Competition to maintain market position and growth is the driver of these improvements. The improvements themselves are identified through innovative enhancements that provide convenience and flexibility to the patient.

The first recombinant growth hormone (somatrem) was licensed as Protropin® by Genentech (part of the Roche group). The pioneering product was provided as a lyophilized powder in a vial and was available in a single dosage strength (5 mg, somatrem). In 1987, Eli Lilly introduced Humatrope®, the fully human, 191 amino acid, recombinant growth hormone (somatropin) available in a Redi-Vial, a dual-chambered vial containing the lyophilized powder and diluent. Genentech and Novo both had approval for somatropin in 1987, but were unable to market until 1993 due to Lilly's orphan drug protection for Humatrope®. Genentech made few changes to Protropin® other than the addition of a 10 mg dosage form in 1989. They continued to market Protropin® until 2004 when it was removed from the market; however, the primary focus of the recombinant growth hormone market shifted to somatropin due to the higher risk of serious side effects associated with the extra methionine of Protropin®.

The appearance of growth hormone on the market was followed by efforts to make the dosage form more convenient and user friendly. In 1999, Eli Lilly introduced the first pen-type device for human growth hormone called HumatroPen®. This device uses cartridges (6 mg, 12 mg, or 24 mg) that are reconstituted with a prefilled syringe before inserting the cartridge into the pen, resulting in multiple reconstitution, assembly, and dosing steps. The reusable pen has a dose selection knob to control the proper dose, and after initial release, a hidden needle cover became available for use with HumatroPen®. After reconstitution, the cartridges are stable for up to 28 days when refrigerated.

In 1993, Genentech began to market Nutropin® (somatropin) before the expiration of Humatrope®'s orphan exclusivity by obtaining approval to treat chronic renal insufficiency rather than human growth hormone deficiency. The original dosage form of Nutropin® was a lyophilized powder supplied as 5 mg or 10 mg vials. After Humatrope®'s exclusivity expired in 1994, Nutropin® was approved for treatment of human growth hormone deficiency.

In December 1995, Genentech's liquid version of somatropin (Nutropin AQ®) was approved. This was the first liquid formulation of human growth hormone available in the USA and greatly simplified the dosing process by removing reconstitution. Nutropin AQ® was originally available as ready-to-use, liquid 10 mg vials. In 2002, in association with Ipsen, Genentech introduced a solution containing cartridge (Nutropin AQ Pen®) in a reusable injection pen (10 mg). Later, in 2008, they released a 20 mg cartridge for use in the Nutropin AQ Pen® and introduced the NuSpin® multidose disposable injection pen in three strengths (5, 10, and 20 mg). The NuSpin® comes prefilled and requires refrigeration (maximum of 28 days after initial use). This evolution of dosage forms, in retrospect, reflects the emerging market needs of patients, their families, and a competitive environment.

In 1996, Pharmacia (Pfizer) launched Genotropin® (somatropin) dispensed from Intra-Mix (now called Genotropin Mixer), a two-chambered cartridge container containing either 1.5 mg or 5.8 mg of somatropin and 1.1 mL of water for injection. The reconstitution involved turning part of the two-chambered cartridge to mix the lyophilized powder with the diluent, removing the overpressure with a needle, and withdrawing the product from the cartridge with an additional needle and syringe. This innovative dosage form resulted in enhanced convenience for patients and a competitive advantage when compared to other growth hormone products available as lyophilized powder within a vial. In 2000, Pfizer introduced MiniQuick, containing lyophilized somatropin. The MiniQuick is a single-dose dual-chambered syringe that is available in 10 dosage strengths. It is a single-dose, disposable syringe that is small and portable. Additionally, it is able to be stored at room temperature for up to 3 months making it much more convenient for patients. Pfizer subsequently released the Genotropin® Pen, which is a multidose, reusable device and uses dual-chambered cartridges. Since its release, Pfizer has made multiple improvements to the device including a hidden needle option, a digital display, and customizable caps and covers.

In 1996, after Genotropin® was released, Serono become the fourth company to sell somatropin in the USA when they released Saizen®, available as a lyophilized powder in 5 mg or 6 mg vials. In 2000, they introduced a convenience device combining a diluent containing prefilled syringe, adapter, and lyophilized powder in a vial as a single device (click.easy). Serono followed the device with a wave of several innovative injector systems incorporating a wealth of features and options for the user. The one.click reusable cartridge-based auto-injector was introduced in 2001 which uses the click.easy cartridges. A needle-free injector system was introduced in 2006 (cool.click) which combined a reconstituted vial or cartridge of growth hormone with a needle-free device. cool.click 2 was released in 2010 which incorporated an improved nozzle, a digital display, less noise, and a more ergonomic design. In 2008, Merck Serono introduced the easypod system, an electronic injector, which provides an onscreen display of dosing information and a hidden needle feature utilizing the click easy cartridges. Benefits of the electronic injector include customizable injection depth and speed, preset dosing by a HCP, multiple languages, and injection schedule review by the HCP.

Novo Nordisk entered the global market with Norditropin® in 1988; however, it was unable to sell to American market, for legal reasons, until 1997. The original dosage form of Norditropin® was a lyophilized powder supplied as 4 mg or 8 mg vials. In 2000, Novo Nordisk introduced their first injector pen for Norditropin®, NordiPen, a multiuse pen with a liquid formulation of human growth hormone. In 2010, they released the current generation of the Norditropin® device, the FlexPro. The FlexPro is a prefilled, disposable use pen designed to fit more comfortably in small hands with an easy to push button. It must be refrigerated prior to the first use, but can then be stored at room temperature for use within 3 weeks. Also available with the FlexPro is NovoFine, a 32 gauge, 6 mm needle and Autocover, which hides the needle before, during, and after injections. With the FlexPro, Novo

Nordisk has become the market leader for growth hormone, demonstrating commitment to continuous innovation.

Additionally, in 2006 Sandoz Sandoz began selling their recombinant growth hormone, Omnitrope. It was originally available as a lyophilized powder supplied as 5.8 mg vials. However, in 2008 Sandoz introduced Omnitrope Pen, using 5 mg liquid cartridges featuring a dose dialing knob. They subsequently released a 10 mg pen, in 2010.

Figure 25.1 presents recent global sales figures for the firms discussed above. The sales figures are collected from publicly available resources, primarily corporate annual reports, and regulatory filings [16].

The sales figures illustrate that the competitive growth hormone market is dynamic and can be driven by innovation and listening to the customer. During the past decade, Novo Nordisk has emerged as the market leader for growth hormone therapies, based on sales. The other four firms, included in this figure, have shown modest growth (Merck Serono) or slow decline. As the market for growth hormone has grown globally, due to increased access and patient mass, the increase in sales has not been shared equally by these five firms. This example allows us make several observations:

1. **A successful approach must be coupled with a consistent commitment to innovation and product enhancements to maintain market growth**: Novo Nordisk has consistently introduced innovation through improved dosage delivery systems, convenience and flexibility for the customer, and a generally pain-free injection experience with the use of 32-gauge needles. Their use of liquid-filled cartridges, similar to other products in their portfolio, makes the

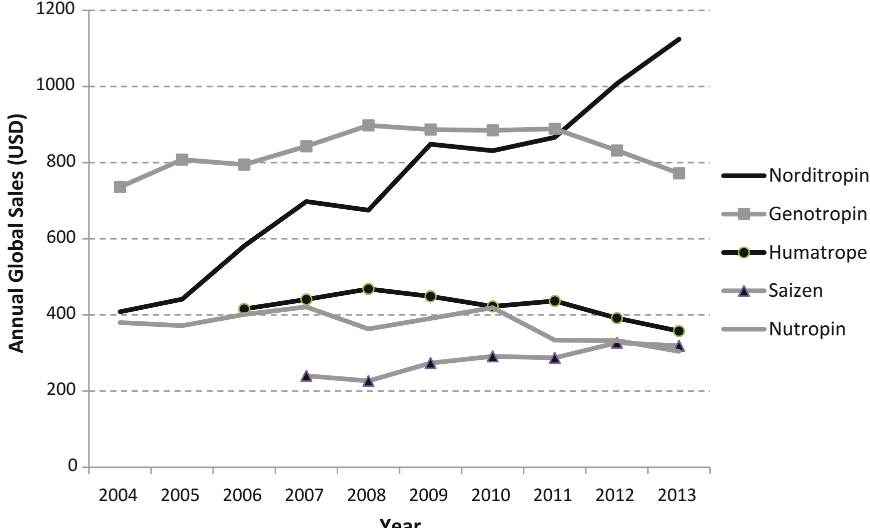


Fig. 25.1 Annual global sales of select growth hormone products

manufacture and control of the active product predictable and provides patients with confidence that the growth hormone will be consistent. This is important because with chronic diseases patients and healthcare providers are reluctant to switch between products due to risk of dosing errors and immunogenicity.

2. **Knowing patient preference matters**: As discussed above, liquid-filled cartridges have been a successful platform for Novo Nordisk. That said, Genentech (with Roche and Ipsen) and Merck Serono (click.easy) introduced a liquid-filled cartridge within 2 years of the Norditropin® launch. The principal difference between the two products is due to the pen design, dosing options, and needle selection. Novo Nordisk has utilized a continuous improvement approach to enhance the patient experience and increase market share.
3. **Convenience is key**: While two product lines utilize liquid-filled cartridges, the other products use lyophilization as the stabilization process. This results in the need for reconstitution of the product prior to administration. Regardless of how simple this can be made with the use of dual-chambered cartridges or vial adapters, it results in additional steps for the patient or healthcare provider and therefore less convenience. The most extreme example of this is the HumatroPen device which must utilize a separate reconstitution kit prior to administration and the cool click device which requires multiple reconstitution and loading steps prior to use. Both of these devices have a built-in disadvantage relative to the convenience of a liquid-filled 3 mL cartridge.
4. **Innovation is not always beneficial for all customers**: Growth hormone, like insulin, has received tremendous attention in terms of alternate delivery systems to reduce the frequency of administration as well as provide less invasive administration. Some of these systems, while technically effective, have not been found to appeal to customers. Nutropin® depot PLGA microspheres (Genentech/ Alkermes) was introduced to the market in 1999 and subsequently withdrawn in 2004 due to poor sales. Likewise, Pfizer introduced a needle-free system (ZipTip) in 2002 and the product was withdrawn due to poor sales. Finally, Merck Serono has maintained their needle-free device (Cool Click) and kept it on the market for patients who have concerns about the use of needles for daily injections. While these innovative approaches have enjoyed technical success, they have not made significant progress in terms of market share relative to the convenient liquid-filled cartridges and pens of their competitors.

25.6 Recombinant Factor VIII

Factor VIII therapies have evolved from initial treatments purified from fractionated plasma to recombinant therapies introduced in the 1990s [17]. After the introduction of recombinant FVIII, several advances were made to reduce the potential risk of viral infection by eliminating non-recombinant albumin from the process and formulation. Recombinant factor VIII (Recombinate) was licensed by Baxter (now

Baxalta) in 1992. In 2003, an albumin-free formulation was made available under the trade name Advate. Since that time, the principal advance in dosage forms has been the introduction of additional strengths, reconstitution aids, and the expansion of room temperature storage periods. In spite of these advances, Recombinate still enjoys commercial success due, in part, to patient's reluctance to switch across brands. In 2002, the Baxject vial adapter was released, and in 2008, an improved version (Baxject II) became available. In 2014, Baxject III was released which greatly simplified reconstitution with an all-in-one reconstitution system containing the active vial, diluent vial, and transfer device. In November 2015, Baxalta, with Nektar, introduced a PEGylated version of FVIII to reduce the frequency of administration (Adynovate).

Bayer introduced Kogenate recombinant factor VIII (also licensed as Helixate by CSL Behring) in 1993. In 2000, the second-generation products manufactured in the absence of albumin, Kogenate FS and Helixate FS, were licensed and eventually replaced Kogenate and Helixate. As with other recombinant FVIII products, it is provided as a lyophilized powder which must be reconstituted prior to administration. Besides the introduction of additional strengths, the primary change has been the introduction of vial adapters and diluent containing prefilled syringe ease of use. In 2005, Bayer released the use of BioSET which includes a prefilled syringe of WFI and a needle-free reconstitution system. In the same year, CSL Behring released the Mix2Vial needle-free transfer device for use with Helixate FS.

Wyeth (now Pfizer) introduced ReFacto, a second-generation B-domain deleted recombinant factor VIII, in 2000, and replaced it with the third-generation albumin-free version, ReFacto/Xyntha, in 2008. Besides developing additional dosage strengths, in 2004 Pfizer introduced a needle-free vial adapter and diluent containing prefilled syringe kit for patient convenience (R2 Kit). In 2010, the company also introduced a dual-chambered syringe (SoloFuse®/FuseNGO®), simplifying the drug preparation process and reducing the number of components required to reconstitute and administer the product from three (prefilled syringe, vial, vial adapter) to one (prefilled, dual-chambered syringe). This is particularly helpful since factor VIII therapies are commonly self-administered.

In 2015 Novo Nordisk released NovoEight®, a third-generation recombinant FVIII available as a lyophilized powder in a vial with a vial adapter and prefilled syringe.

Figure 25.2 presents sales figures for the four leading brands of recombinant factor VIII clotting factor [18]. In contrast to the human growth hormone examples, there has been a consistent upward trend in sales for the four products. Additionally, one must note that there has been little change in market share since 2004, which may indicate stability and product loyalty from patients, their families, and healthcare providers. Unlike growth hormone, there is limited opportunity to innovate via dosage form design and convenience, presumably due to the need for intravenous injection. With the addition of convenience devices (vial adapters, diluent containing prefilled syringes, and dual-chambered syringes), the primary innovations during the past 20 years have been the elimination of human serum albumin from the process and product and the recent emergence of extended half-life versions of FVIII with PEGylation (Baxter's Adynovate) or Fc Fusion proteins (Biogen's Eloctate).

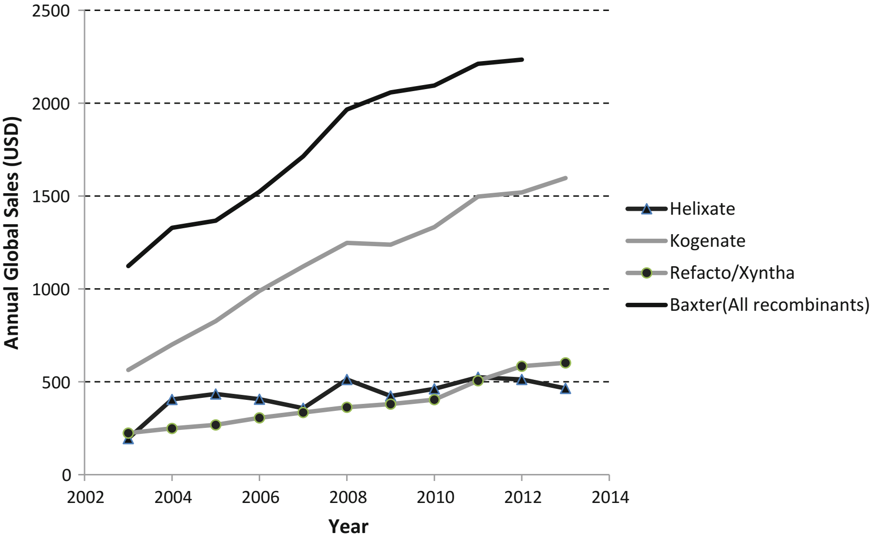


Fig. 25.2 Annual growth sales of select factor VIII products

25.7 Keys to Success

After review of the above case studies for growth hormone factor and FVIII clotting factor, we have identified four keys to a successful LCM program.

25.7.1 The Company Must Be Dedicated to Brand and Therapeutic Area

All of the companies discussed above have made long-term commitments to a specific therapeutic area. Furthermore, all of them have made substantial investments in the innovative science that served to either develop the products initially or enter the market with a significant market enhancement that increases convenience to the patient or caregiver. In growth hormone therapy, four of the firms have had a substantial presence in this area for over 25 years. Additionally, while a relative newcomer to the field in 1997, Novo Nordisk has provided innovative solutions that serve patients' needs and is currently the market leader. A similar situation exists for recombinant factor VIII in which the four companies (Pfizer via Genetics Institute) have served the hemophilia community for over 20 years. Especially crucial in rare diseases, this commitment to patients and healthcare providers enables companies to establish brand loyalty by maintaining those critical relationships with the patient base.

One benefit to sustained presence in a therapeutic area is consistent financial performance (consider the consistent sales growth for Norditropin (12% average growth per year for 10 years), Kogenate (13% per year), and Baxter's recombinant FVIII family (9% per year)). Additionally, this sustained commitment to rare disease helps drive further investment in similar therapeutic areas, solidifying the company's identity and corporate brand.

25.7.2 Innovation Requires a Competitive Environment in Which Continuous Improvement Is Expected

If a product is in a competitive field, then the drive for continuous innovation is clear. The human growth hormone example illustrates the ongoing commitment to enhanced auto-injector design, ease of dose preparation and administration, and increased flexibility for patients to manage their disease. The result of this competitive environment, and the changing menu of options for patients, is a dynamic landscape of products and market share. Novo Nordisk's disproportionate gain of new patients is perhaps the result of patients changing their treatments due to increased convenience. As patients become more aware of the variety of choices in their treatment, Novo Nordisk's commitment to finding innovative options results in market leadership.

The recombinant factor VIII field is also highly competitive, but the diversity of options for development is more limited. The dosage forms cannot change radically due to the product's requirement of lyophilization for stabilization and intravenous administration. Innovative solutions such as a dual-chambered syringe allow a company to compete, but against a backdrop of brand loyalty due to concerns about immunogenicity and patients who are reluctant to switch, it is challenging to alter the status quo. The recent introduction of extended half-life clotting factors will be a significant breakthrough to this therapeutic area if the patients and healthcare providers see real value and the reduced frequency of administration provides measurable benefit.

25.7.3 An Experienced Interdisciplinary Team Is Required to Ensure Development of the Best Product for the Market

As mentioned above, product development is tied to the stage of development, therapeutic need, market dynamics, and competition. During the clinical stage of development, cost is less critical than flexibility to enable a wide range of doses in various indications possibly with multiple routes of administration. For late-stage development, resulting in a license application, it is critical to bring forward the

most competitive product at the time of launch. The reasons for getting it right, and not simply getting to market first, are that competition will eventually arrive and may have a superior dosage form in terms of convenience and market acceptance. Recent product launches of injectable PCSK-9 inhibitors for lipid lowering [19] offer convenient dose preparation and administration in order to stand out in a highly competitive field dominated by solid oral products. Additionally, the costs associated with development of alternate dosage forms, including clinical evaluation, can be prohibitive. It is better to absorb these costs in the development of the product for launch than to accrue additional costs after launch.

Development of a commercial biotechnology product requires a mature product development team with intimate knowledge of the biochemistry and process limitations of the product. It also requires a knowledgeable commercial organization and clinical team, with a vision for the marketplace and what the customer (patient, healthcare provider, payor) wants. The challenge that the team faces is that the Phase 3 and commercial dosage form decisions must often be made well in advance of an understanding of the efficacious dose of the product. This increases the cost and risks of development associated with developing the correct product in time for launch.

25.7.4 Loss of Exclusivity (LOE) Is not the End of Innovation

Review of the two case studies highlights that the patent expiry, for a biotechnology product like growth hormone or recombinant factor VIII, is not a critical barrier to enhancing the value of the product. While the number of biosimilars products is increasing globally [20], there is still value in providing innovative solutions to customers' needs. Many of the advances in biotechnology dosage form development were made on compounds *after* their initial patents expired and the innovator companies needed to compete in order to retain a market presence. Unlike traditional pharma products (small molecules), biotechnology products have maintained much of their commercial value after LOE. The benefit to remaining in a market is clear, especially once a company has made a substantial investment in product development, manufacturing, and growing a well-trained sales team.

25.8 Summary

Lifecycle management of biotechnology dosage forms begins in early clinical development and continues until a company decides to withdraw a product. This "cradle-to-grave" approach requires a mature understanding of the market's needs, the product's limitations, and a company's commitment to a specific therapeutic

area and patient population. Several tools exist which enable creation of this lifecycle management strategy which are outlined in this chapter including a target product profile, drug product profile, and ultimately a QTPP. These must work in harmony to ensure that a company is developing the right dosage form for the market while ensuring safety and manufacturing quality.

The development of convenient, cost-effective dosage forms, and the advancement of product-specific technologies, is often driven by competition. Competition drives dosage form innovation and provides options to patients and healthcare providers. After laying the foundation for success with a well-designed product, a company must consistently innovate to maintain market growth via new dosage forms, improved product administration, and increased patient convenience. However, the innovation must be intelligently directed, which requires an understanding of the target audience's needs and preferences. By maintaining these critical relationships with patients and providers, the company contributes to its reputation in a specific therapeutic area and promotes brand loyalty. This ultimately leads to a deeper investment in the company's area of focus and drives consistent sales growth.

Experience with lifecycle management enables a company to view the stages of product life from early development to commercialization to loss of exclusivity as a continuum. By considering LCM early, the project team can take advantage of experience with similar products and design a thoughtful plan to minimize wasted resources during development by building dosage differentiation into the product strategy during development.

References

1. ICH Harmonised Tripartite Guideline—Pharmaceutical Development Q8(R2); Step 4 Version August 2009.
2. Guidance for Industry—Q9 Quality Risk management; United States FDA June 2006 ICH.
3. Guidance for Industry—Q10 Pharmaceutical Quality System; United States FDA April 2009 ICH.
4. ICH Harmonised Tripartite Guideline—Development and Manufacture of Drug Substances (Chemical Entities and Biotechnological/Biological Entities) Q11; Step 4 Version 01 May 2012.
5. Clinical Development Success Rates 2006–2015—BIO, Biomedtracker, Amplion (2016).
6. Evens RP. Pharma success in product development—does biotechnology change the paradigm in product development and attrition. AAPS J. 2016;18:281–5.
7. Warne NW. Development of high concentration protein biopharmaceuticals: the use of platform approaches in formulation development. Eur J Pharm Biopharm. 2011;78:208–12.
8. Rathore AS, Reason AJ, Weiskopf A. Defining critical quality attributes for monoclonal antibody therapeutic products. BioPharm Int. 2014; 27.
9. Guidance for Industry and FDA Staff: Current Good Manufacturing Practice Requirements for Combination Products—Draft Guidance published January 2015.
10. Gallo A. A refresher on net present value. Harvard Bus Rev. 2014.
11. Jorgensen JT. Improvement of patient convenience in treatment with growth hormone. J Pediatr Endocrinol. 1994;7:175–80.

12. Guidance for Industry and FDA Review Staff: Target Product Profile—A Strategic Development Process Tool—Draft Guidance published March 2007.
13. Bolge SC, Goren A, Tandon N. Reasons for discontinuation of subcutaneous biologic therapy in the treatment of rheumatoid arthritis: a patient perspective. Patient Prefer Adherence. 2015;9:121–31.
14. Mehta A, Hindmarsh PC. The use of somatropin (recombinant growth hormone) in children of short stature. Pediatr Drugs. 2002;4:37–47.
15. Goeddel DV, et al. Direct expression in *Escherichia coli* of a DNA sequence coding for human growth hormone. Nature. 1979;281:544–8.
16. Product sales for hGH and FVIII products were obtained from annual financial reports and converted to USD using the yearly average exchange rate.
17. Hemophilia: From Plasma to Recombinant Factors. American Society of Hematology. In: 50 years in hematology: research that revolutionized patient care, www.hematology.org. 2008.
18. Product histories were obtained from product websites, prescribing information, press releases, the U.S. Food and Drug Administration website, and Pharma Intelligence.
19. PCSK9 Inhibitors: A Brief Primer. Merle Myerson; Medscape 28 March 2016.
20. Delivering on the Potential of Biosimilar Medicines; IMS Institute for Healthcare Informatics March 2016.

Chapter 26
Switching from an IV to an SC Formulation—Considerations for Formulation Development and Formulation Bridging

Claudia Mueller and Michael Adler

Abstract Subcutaneous (SC) administration is one of the most common application routes for biologics besides intravenous infusion (IV) and represents a beneficial alternative to IV, as it may represent an increased patient convenience. However, several considerations need to be taken into account for successful development of SC products as will be outlined in the following chapter.

Keywords Formulation bridging · Intravenous · Subcutaneous
Therapeutic proteins · Development · Liquid · Lyo · Biologics

26.1 General Considerations

26.1.1 Background

Subcutaneous (SC) injection is one of the most common routes of administration for biologics besides intravenous infusion [1]. Various therapeutic proteins are approved for SC administration, such as insulin, growth hormone, interferon-β, interferon-α and several monoclonal antibodies [2]. SC administration represents an alternative which offers benefits over IV administration. It reduces the administration times and has additionally been shown to reduce health care costs relative to IV administration [3, 4]. SC formulations may be more convenient for patients, offering potential improvements in quality of life and treatment adherence [5, 6]. In

C. Mueller (✉)
Pharmaceutical Development and Supplies, PTD Biologics Europe (PTDE-P),
F. Hoffmann-La Roche Ltd., Bldg. 72/347a, 4070 Basel, Switzerland
e-mail: Claudia.Mueller.cm8@roche.com

M. Adler
Pharmaceutical Development and Supplies, PTD Biologics Europe (PTDE-P),
F. Hoffmann-La Roche Ltd., Bldg. 72/318, 4070 Basel, Switzerland
e-mail: Michael.Adler@roche.com

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*,
AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4_26

the case of alemtuzumab, SC administration was better tolerated than IV administration [4, 7]. Since more gradual absorption of the therapeutic from the SC space occurs as compared to the IV route, administration-associated reactions may be less severe than infusion-related reactions [8].

Several considerations need to be taken into account for development of SC products. In general, the bioavailability of the therapeutic is reduced compared to the IV route [2]. If a rapid mode of action of the active compound is required, the slower absorption of the therapeutic from the SC space may be suboptimal. Local reactions may be observed due to the longer presence of the therapeutic at the injection site [9]. Also, greater variability in between different individuals might be observed and a potentially increased immunogenicity may be given after SC administration [10].

SC formulations can be introduced at various time points within a development program: (i) directly for early clinical studies, (ii) for late clinical studies or (iii) post-launch. Drugs for which a switch from an IV to a SC formulation has been realized are, e.g. Trastuzumab, Rituximab, Tocilizumab, Abatacept.

For scenario (i), no switch is required, whereas for the latter two scenarios a switch from IV to SC formulation is required. Scenario (ii) (testing of IV formulations in early clinical studies and introduction of the SC formulation for late clinical studies) might be triggered for various reasons, which may be on the one hand limited active pharmaceutical ingredient (API) availability in early development, and/or fast timelines for early-in-human/clinical trials and on the other hand, a typically more challenging development of an SC formulation in terms of stability and/or viscosity (refer to section SC formulation development and administration challenges). Additionally, SC formulations can also be introduced post-launch as lifecycle management (scenario iii) for the reasons given above, e.g. to reduce the time in hospital, or to enable self and/or home administration. Switching from an IV to a SC formulation post-launch has the advantage that efficacy and safety of a drug have already been established and clinical studies require less patients and time if health authorities accept, e.g. a pharmacokinetic (PK) non-inferiority study design (refer to section Clinical testing).

From a presentation point of view, three switching scenarios are possible: (1) from liquid IV to lyo SC, (2) from liquid IV to liquid SC and (3) from lyo to liquid SC. The least preferred option is a lyo to liquid switch as the stability of an liquid SC formulation is typically worse due to the higher protein concentration leading to higher levels of degradation products, e.g. aggregates or chemical degradation products or potentially new degradation products. The acceptability of higher levels of degradation products depends on the amount covered in preclinical and clinical studies. Qualification of any new degradation product may require additional preclinical studies or even clinical studies.

26.1.2 SC Formulation Development and Administration Challenges

Many biologics, especially monoclonal antibodies, require high doses in the mg/kg range [11, 12]. Furthermore, the bioavailability (BA) of biologics after SC injection is highly dependent on the molecule and species and is typically less than 100% [2]. For monoclonal antibodies, bioavailability ranges between 24 and 95% were reported [2]. Administration of high doses via the SC route can either be achieved by injecting high concentration protein formulations and/or by injecting large volumes (Fig. 26.1).

26.1.2.1 High Concentration Protein Formulations

It is well known that elevated protein concentrations may result into increased protein aggregation, decreased solubility and/or increased viscosity as the system deviates more from ideal solution conditions enabling increased protein–protein interactions due to molecular crowding phenomena [12–14]. Elevated viscosities of a formulation may result into problems during manufacture (e.g. ultrafiltration/diafiltration, sterile filtration, filling) as well as administration (syringeability, injection time) [12, 13, 15, 16]. Increased protein aggregation may result into long-term stability issues and in turn into an insufficient drug product shelf life. Protein instability can be mitigated via lyophilized formulations as proteins are typically more stable in the dry state. However, lyophilized protein formulations with a high protein content can lead to long freeze-drying cycles and long reconstitution times [12].

26.1.2.2 Large Injection Volumes

In case sufficiently high doses using high concentration protein formulations cannot be achieved due to stability and/or viscosity issues, it is also possible to inject larger volumes in the SC tissue. In general, larger volumes can either directly delivered

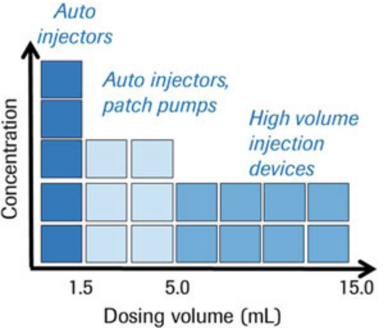


Fig. 26.1 Administration of high-dose protein formulations

via a disposable syringe or administration can be facilitated by an infusion pump or patch injector [17–19].

For example, in the case of bortezomib (Velcade) 3-mL SC injections were well tolerated [20]. Based on their product monographs, new drugs such as degarelix (Firmagon) and azacitidine (Vidaza) have been approved for SC administration in volumes of 3–4-mL per injection site, respectively; however, information on injection site reaction details remains limited [21, 22].

The dispersion of even larger volumes can be facilitated by co-injection or co-administration with rHuPH20, recombinant human hyaluronidase, [5, 23]. However, the addition of the enzyme poses additional challenges for formulation and process development.

26.1.3 Formulation Bridging Program

The following activities can generally be considered as part of a formulation bridging program from an IV to an SC formulation:

1. Analytical testing (comparability);
2. Preclinical testing;
3. Clinical testing.

The extent/scope for the bridging program is determined by the stage of development as well as the extent of the overall formulation change.

26.1.4 Analytical Testing

Release and stability data at relevant conditions of pre- and post-change formulations need to be compared and evaluated, including the amount and quantitative level of degradation products. This comparison might require extended characterization beyond the standard control system. In case higher levels of degradation products are observed, it needs to be assessed whether those are covered by already existing preclinical studies. New degradations products need to be analytically characterized and potentially evaluated in preclinical studies.

26.1.5 Preclinical Testing

When switching from an IV to a SC administration, the PK profile is likely to change due to the absorption process that occurs from the SC compartment to the systemic circulation which will result in changes in both the rate and extent of absorption. A change in PK may have an impact on efficacy and safety, especially if these effects are driven by peak concentrations (C_{max}) rather than overall exposure

over a dosing interval (Area under the Curve). The activities required to address this potential impact will depend on the development phase in which the switch is being introduced. The following preclinical studies need to be considered:

- For new molecular entities, a complete preclinical safety program based on SC administration in case the SC formulation is already tested during Phase 1, e.g. in the MAD arm.
- Preclinical SC local tolerance study.
- Preclinical PK study in a suitable model.

While the PK properties of monoclonal antibodies following IV administration are well predicted by studies in non-human primates, a reliable animal model to predict the PK of monoclonal antibodies following SC administration has not been clearly established [9]. Recently published data by Zheng et al. support the minipig as an alternative, less expensive and sufficiently predictive model over other commonly used species (e.g. cynomolgus monkeys) for evaluation of linear clearance rates and SC bioavailability of monoclonal antibodies [24].

26.1.6 Clinical Testing

In general, the clinical bridging program depends on the considerations whether the specific change is likely to have an impact on efficacy (e.g. if a likely change of the PK profile has a potential impact on efficacy) and/or safety (e.g. if a likely change of the PK profile or of the formulation has a potential impact on safety) and whether biomarker or surrogate endpoints and an established model to describe the relationship between PK and PD (biomarker, surrogate, clinical endpoint) are available.

For changes prior Phase 2, the human dose may be estimated based on preclinical PK data or adaptive study designs might be applied.

For post-launch changes where efficacious blood levels are well established, a PK non-inferiority design (IV versus SC) might be acceptable to demonstrate efficacy.

During clinical studies, immunogenicity needs to be monitored, according to the best practice in the development phase. Upon changes in the administration route from IV to SC higher levels of immunogenicity may be expected [10].

26.2 Case Study—Post-approval Switch from an IV to a SC Formulation of a Monoclonal Antibody

26.2.1 Background

Rituximab has become a standard regimen in the treatment of various types of B-cell malignancies, such as follicular lymphoma (FL), non-Hodgkin's lymphomas

(NHLs) diffuse large B-cell lymphoma (DLBCL) and chronic lymphocytic leukaemia (CLL) [25, 26]. The administration of rituximab occurs via the intravenous (IV) route. Typically, dosing in oncology patients occurs based upon mg/m^2 body surface area (BSA). Different dosing regimens of rituximab have been approved for the different malignancies [27].

The first IV infusion of rituximab in general takes 4–6 h, but may endure up to 12 h, whereas subsequent administrations may last between 1.5 up to 4 h [25]. In general, the IV administration is considered being invasive and can be painful [8, 26]. Further challenges exist with regard to the preparation of the infusion bags: trained health care professionals need to calculate the required dose based upon the patients' body surface area before the IV bag can be prepared with the required dose as well as infusion volume. The preparation needs to be performed in an aseptic manner to prevent from microbial contamination. For the administration to the patient, again trained medical personnel is needed to place the IV administration set or even permanent IV lines. Thus, these time-consuming procedures often happen in dedicated infusion centres. The additionally long infusion times and subsequent patient observation after infusion result into an overall slow workflow and require a considerable amount of resources.

Thus, the possibility to administer rituximab subcutaneously offers a valuable and convenient alternative to its IV administration.

26.2.2 SC Formulation Development

Rituximab for IV administration is available as single-use vials in two strengths, 100 and 500 mg/50 mL. The marketed IV formulation is a concentrate for solution for infusion and is identical for both strengths (10-mg/mL rituximab, sodium chloride, sodium citrate, polysorbate 80 and water for injection) [27]. Once the solution for infusion is prepared, it is administered as an IV infusion. For the NHL indication the dose to be administered is 375 mg/m^2 BSA per infusion, whereas for the CLL indication the dose consists of 375 mg/m^2 BSA on day 0 of the first treatment cycle and 500 mg/m^2 BSA on day 1 for the following cycles [26, 27].

The SC injection volumes needed to achieve the required doses using the rituximab for IV administration would have been too large, e.g. a 375 mg/m^2 dose for a 70 kg patient requires approximately 70 mL. In order to enable SC injection, an increased protein concentration was needed for rituximab for subcutaneous administration to reduce the administration volume. Rituximab has been concentrated 12-fold within the SC formulation as compared to rituximab for IV administration, i.e. 120 mg/mL of the active ingredient. As this protein concentration still resulted into administration volumes larger than 1.5–2 mL, Roche decided to co-formulate recombinant human hyaluronidase (rHuPH20), an enzyme expressed in Chinese hamster ovary cells, within rituximab for subcutaneous administration drug product to facilitate the SC administration. Upon injection of the drug product, rHuPh20 locally and transiently degrades hyaluronan, which is a major part of the

SC tissue. It thus reversibly opens the subcutaneous space and facilitates SC administration of volumes larger than 2 mL. Additionally, rHuPH20 facilitates the dispersion and absorption of the drug product and acts as a permeation enhancer. Thus, the injection of the 11.7 mL of rituximab SC formulation takes only ca. 5–8 min for administration as compared to several hours for the IV infusion [5, 23].

The challenges for the formulation development of rituximab SC were manifold: (1) achieve a high concentration liquid formulation of rituximab (120 mg/mL) with a rituximab stability comparable to the IV formulation (10 mg/mL), (2) identify a formulation which provides stability for two different proteins, namely rituximab and rHuPH20, with different properties, and routes of degradation (3) avoid interactions between rituximab and rHuPH20 despite differences in isoelectric points. The formulation screen resulted into a formulation containing 120 mg/mL rituximab, histidine/histidine hydrochloride, trehalose, polysorbate 80, L-methionine and 2000 U/mL rHuPH20. A change of the formulation buffer and excipients was necessary as amongst others the clarity/opalescence of the formulation would have been relatively high in the IV formulation buffer (Fig. 26.2).

Additionally, for optimization of the stability and activity of both, rituximab and rHuPH20, the excipients used in the rituximab SC drug product differ from those used in the rituximab IV drug product (Table 26.1).

Additionally, Roche decided to change from a dose based upon mg/m^2 body surface area to a fixed dose for administration of rituximab SC irrespective of the actual body surface area since the therapeutic range of rituximab was considered wide enough based upon clinical data (refer to section Preclinical and clinical testing). Thus, one vial of rituximab 1400-mg solution for subcutaneous injection contains 11.7 mL of the drug product solution at 120-mg/mL rituximab for single use indicated for the treatment of non-Hodgkin's lymphoma.

For the line extension of rituximab for SC administration, previous experience and knowledge obtained within technical development of rituximab IV was leveraged whenever possible, e.g. the cell culture and purification processes of the drug substance are identical for the IV and SC drug substance. Only the final ultrafiltration/diafiltration (UF/DF) process step has been adapted for the SC drug substance to account for the increased protein content of 120 mg/mL and different

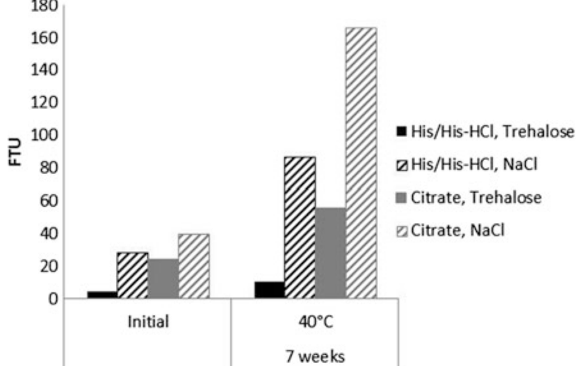


Fig. 26.2 Clarity/opalescence of 120-mg/mL rituximab in citrate and histidine buffer

Table 26.1 Comparative formulation composition or rituximab IV and rituximab SC

Ingredient	rituximab IV	rituximab SC
rituximab	10 mg/mL	120 mg/mL
Recombinant human hyaluronidase (rHuPH20)	–	2000 U/mL
L-histidine/L-Histidine hydrochloride monohydrate	–	✓
Sodium citrate dihydrate	✓	–
Sodium chloride	✓	–
Trehalose dihydrate	–	✓
L-methionine	–	✓
Polysorbate 80	✓	✓
Hydrochloric acid	✓	–
Sodium Hydroxide	✓	–
Water for injection	q.s.[a]	q.s.[a]

[a]q.s. quantum satis

formulation buffer as compared to IV drug substance. As the drug substance manufacturing process mainly remained identical, also the control of critical steps and intermediates remained the same and no further critical process parameters were identified for the SC drug substance UF/DF process step as compared to the IV process.

26.2.3 *Formulation Bridging Program*

Annex I of the Variations Regulation of the European Medicines Agency (EMA) defines that a change in the route of administration and/or the dosage strength can have a fundamental impact with regard to safety and efficacy as compared to the initial market authorization [28, 29]. Thus, for rituximab for subcutaneous administration an extension application to the existing market authorization was required. The submitted application was composed of information on administration, quality, preclinical as well as clinical data. The subsequent sections provide a high-level overview on the analytical, preclinical and clinical evaluations that were performed within the development of rituximab for subcutaneous administration. Rituximab 1400-mg solution for subcutaneous injection obtained market authorization in 2014 in the European Union and gained since then approval in more than 30 other countries worldwide.

26.2.4 *Analytical Testing*

Another example, where prior knowledge gained from rituximab IV was leveraged, is the specifications, methods, as well as the release specifications of the SC drug substance and drug product. Those have been based as far as possible upon

rituximab for IV administration. However, certain limits needed adjustment due to the previously described changes in the formulation, such as protein content, color, clarity, pH, polysorbate content.

Analytical comparability was performed between the SC as well as IV drug substance using release and extended characterization assays and it has been demonstrated that both materials are highly comparable. Additional studies performed on the drug product level using standard release and extended characterization methods have shown that the presence of rHuPH20 in the selected co-formulation has no impact on the quality of the monoclonal antibody rituximab. Additionally, the stability data from rituximab SC clinical and commercial batches were comparable and an overall shelf life of 30 months at 2–8 °C was established.

26.2.5 Preclinical and Clinical Testing

The preclinical pharmacology and pharmacokinetic studies addressed on the one hand the absorption and elimination of rituximab and on the other hand the preclinical efficacy at comparable serum concentrations after IV and SC administration [25, 26]. The studies were set up to find a dose of rituximab SC resulting into serum trough concentrations (C_{trough}) at least as high as those obtained after IV formulation injection without changes in the dosing frequency. In brief, the anti-tumour efficacy of rituximab IV and SC formulations was evaluated in a Z138 human mantle cell lymphoma cell line xenograft SCID mouse model. The pharmacokinetics of the rituximab SC formulation has been studied in mice, minipigs as well as Cynomolgus monkeys. Repeat-dose toxicity of the rituximab SC formulation was evaluated in an 8-weeks study in Cynomolgus monkeys. The latter study and an additional dedicated study in rabbits also served for the evaluation of the local tolerance of the rituximab SC formulation. More detailed information on the non-clinical program have already been described elsewhere [25, 26, 30].

Based upon the results from the preclinical studies, the clinical studies were designed. The early clinical trials were set up as a two-staged study to select a dose of rituximab SC resulting into C_{trough} of rituximab at least as high as those obtained after IV formulation injection and to investigate the pharmacokinetics, safety and tolerability of rituximab SC as part of maintenance treatment in patients with follicular lymphoma. In the first stage of the phase Ib clinical trial, the focus was set on dose finding, whereas the stage 2 addressed the dose confirmation by demonstration of pharmacokinetic non-inferiority of the selected rituximab SC dose compared to the established rituximab IV dosing [25, 26, 31]. Based upon these results, the fixed dose of 1400 mg of rituximab solution for subcutaneous injection was selected for the subsequent two-staged phase III study, which was set up as randomized, open-label study to investigate the pharmacokinetics, efficacy and safety of rituximab SC in combination with cyclophosphamide, vincristine, doxorubicin and prednisone (CHOP) or cyclophosphamide, vincristine and prednisone (CVP) versus rituximab IV in combination with CHOP or CVP in patients with previously

untreated follicular lymphoma followed by maintenance treatment with either rituximab SC or rituximab IV [25, 26, 32]. The data for the stage 1 of the phase III clinical trial showed that the pharmacokinetic profile of MabThera SC was non-inferior to rituximab IV. Those in vivo data support that the overall bridging concept applied for rituximab SC and rituximab IV is valid.

Acknowledgements We would like to thank Silke Mohl, Axel Boehnke, Sebastien Denis and Thomas Klein for providing helpful input during the review.

References

1. Patel A, Cholkar K, Mitra AK. Recent developments in protein and peptide parenteral delivery approaches. Ther Deliv. 2014;5(3):337–65.
2. Richter WF, Bhansali SG, Morris ME. Mechanistic determinants of biotherapeutics absorption following SC administration. AAPS J. 2012;14(3):559–70.
3. Lundin J, Kimby E, Björkholm M, Broliden PA, Celsing F, Hjalmar V, Möllgård L, Rebello P, Hale G, Waldmann H, Mellstedt H, Osterborg A. Phase II trial of subcutaneous anti-CD52 monoclonal antibody alemtuzumab (Campath-1H) as first-line treatment for patients with B-cell chronic lymphocytic leukemia (B-CLL). Blood. 2002;100(3):768–73.
4. Stilgenbauer S, Zenz T, Winkler D, Bühler A, Schlenk RF, Groner S, Busch R, Hensel M, Dührsen U, Finke J, Dreger P, Jäger U, Lengfelder E, Hohloch K, Söling U, Schlag R, Kneba M, Hallek M, Döhner H. German Chronic Lymphocytic Leukemia Study Group. Subcutaneous alemtuzumab in fludarabine-refractory chronic lymphocytic leukemia: clinical results and prognostic marker analyses from the CLL2H study of the German Chronic Lymphocytic Leukemia Study Group. J Clin Oncol. 2009;27(24):3994–4001.
5. Bookbinder LH, Hofer A, Haller MF, Zepeda ML, Keller GA, Lim JE, Edgington TS, Shepard HM, Patton JS, Frost GI. A recombinant human enzyme for enhanced interstitial transport of therapeutics. J Control Release. 2006;114(2):230–41.
6. Haller M. Converting intravenous dosing to subcutaneous dosing with recombinant human hyaluronidase. 2007. Available from http://www.pharmtech.com. Accessed 04 July 2015.
7. Hale G, Rebello P, Brettman LR, Fegan C, Kennedy B, Kimby E, Leach M, Lundin J, Mellstedt H, Moreton P, Rawstron AC, Waldmann H, Osterborg A, Hillmen P. Blood concentrations of alemtuzumab and antiglobulin responses in patients with chronic lymphocytic leukemia following intravenous or subcutaneous routes of administration. Blood. 2004;104(4):948–55.
8. Shpilberg O, Jackisch C. Subcutaneous administration of rituximab (MabThera) and trastuzumab (Herceptin) using hyaluronidase. Br J Cancer. 2013;109(6):1556–61.
9. McDonald TA, Zepeda ML, Tomlinson MJ, Bee WH, Ivens IA. Subcutaneous administration of biotherapeutics: current experience in animal models. Curr Opin Mol Ther. 2010;12 (4):461–70.
10. Büttel IC, Chamberlain P, Chowers Y, Ehmann F, Greinacher A, Jefferis R, Kramer D, Kropshofer H, Lloyd P, Lubiniecki A, Krause R, Mire-Sluis A, Platts-Mills T, Ragheb JA, Reipert BM, Schellekens H, Seitz R, Stas P, Subramanyam M, Thorpe R, Trouvin JH, Weise M, Windisch J, Schneider CK. Taking immunogenicity assessment of therapeutic proteins to the next level. Biologicals. 2011;39(2):100–9.
11. Wang W, Singh S, Zeng DL, King K, Nema S. Antibody structure, instability, and formulation. J Pharm Sci. 2007;96(1):1–26.
12. Shire SJ, Shahrokh Z, Liu J. Challenges in the development of high protein concentration formulations. J Pharm Sci. 2004;93(6):1390–402.

13. Shire SJ. Formulation and manufacturability of biologics. Curr Opin Biotechnol. 2009;20 (6):708–14.
14. Adler M. Challenges in the development of pre-filled syringes for biologics from a formulation scientist's point of view. 2012. Available from http://www.americanpharmaceuticalreview.com/. Accessed 04 July 2015.
15. Li L, Kumar S, Buck PM, Burns C, Lavoie J, Singh SK, Warne NW, Nichols P, Luksha N, Boardman D. Concentration dependent viscosity of monoclonal antibody solutions: explaining experimental behavior in terms of molecular properties. Pharm Res. 2014;31 (11):3161–78.
16. Warne NW. Development of high concentration protein biopharmaceuticals: the use of platform approaches in formulation development. Eur J Pharm Biopharm. 2011;72(2):208–12.
17. Mueller C, Dietel E, Heynen SR, Nalenz H, Goldbach P, Mahler HC, Schmidt J, Grauschopf U, Schoenhamnmer K. Physico-chemical stability of Mabthera drug-product solution for subcutaneous injection under in-use conditions with different administration materials. Int J Pharm Compd. 2015;19(3):261–7.
18. Hizentra (human normal immunoglobulin (SCIg)) Summary of Product Characteristics 2015. Available from http://www.ema.europa.eu/ema/. Accessed 12 Feb 2016.
19. Step-by-Step Instructions for Self-Administering Hizentra. Available from http://www.hizentra.com/infusion-administration/guidance-tips-instructions.aspx. Accessed 12 Feb 2016.
20. Ng P, Incekol D, Lee R, Paisley E, Dara C, Brandle I, Kaufman M, Chen C, Trudel S, Tiedemann R, Reece D, Kukreti V. Tolerability of Velcade (Bortezomib) subcutaneous administration using a maximum volume of 3 mL per injection site. J Oncol Pharm Pract. 2015;21(4):285–92.
21. Product Monograph Firmagon. Available from www.drugs@FDA.gov. Accessed 12 Feb 2016.
22. Product Monograph Vidaza. Available from www.drugs@FDA.gov. Accessed 12 Feb 2016.
23. Pirrello RD, Ting Chen C, Thomas SH. Initial experiences with subcutaneous recombinant human hyaluronidase. J Palliat Med. 2007;10(4):861–4.
24. Zheng Y, Tesar DB, Benincosa L, Birnböck H, Boswell CA, Bumbaca D, Cowan KJ, Danilenko DM, Daugherty AL, Fielder PJ, Grimm HP, Joshi A, Justies N, Kolaitis G, Lewin-Koh N, Li J, McVay S, O'Mahony J, Otteneder M, Pantze M, Putnam WS, Qiu ZJ, Ruppel J, Singer T, Stauch O, Theil FP, Visich J, Yang J, Ying Y, Khawli LA, Richter WF. Minipig as a potential translatable model for monoclonal antibody pharmacokinetics after intravenous and subcutaneous administration. MAbs. 2012;4(2):243–55.
25. Solal-Celigny P. Rituximab by subcutaneous route. Expert Rev Hematol. 2015;8(2):147–53.
26. Bittner B, Richter WF, Hourcade-Potelleret F, Herting F, Schmidt J. Non-clinical pharmacokinetic/pharmacodynamic and early clinical studies supporting development of a novel subcutaneous formulation for the monoclonal antibody rituximab. Drug Res (Stuttg). 2014;64(11):569–75.
27. Mabthera (rituximab) Summary of Product Characteristics 2009. Available from http://www.ema.europa.eu/ema/. Accessed 06 Feb 2016.
28. Commission Regulation (EC) No 1234/2008 (OJ L334 of 12 December 2008). Available from http://ec.europa.eu/health/documents/eudralex/vol-1/index_en.htm. Accessed 06 Feb 2016.
29. Commission Regulation (EU) No 712/2012 amending Regulation (EC) No 1234/2008 concerning the examination of variations to the terms of marketing authorisations for medicinal products for human use and veterinary medicinal products. Available from www.ema.europa.eu. Accessed 06 Feb 2016.
30. Mao CP, Brovarney MR, Dabbagh K, Birnböck HF, Richter WF, Del Nagro CJ. Subcutaneous versus intravenous administration of rituximab: pharmacokinetics, CD20 target coverage and B-cell depletion in cynomolgus monkeys. PLoS One. 2013;8(11): e80533. eCollection 2013.

31. Salar A, Avivi I, Bittner B, Bouabdallah R, Brewster M, Catalani O, Follows G, Haynes A, Hourcade-Potelleret F, Janikova A, Larouche JF, McIntyre C, Pedersen M, Pereira J, Sayyed P, Shpilberg O, Tumyan G. Comparison of subcutaneous versus intravenous administration of rituximab as maintenance treatment for follicular lymphoma: results from a two-stage, phase IB study. J Clin Oncol. 2014;32(17):1782–91.
32. Davies A, Merli F, Mihaljevic B, Siritanaratkul N, Solal-Céligny P, Barrett M, Berge C, Bittner B, Boehnke A, McIntyre C, Macdonald D. Pharmacokinetics and safety of subcutaneous rituximab in follicular lymphoma (SABRINA): stage 1 analysis of a randomised phase 3 study. Lancet Oncol. 2014;15(3):343–52.

Index

A
Accesorized prefilled syringe components, 206, 207
Active pharmaceutical ingredient, 8
Additional operational measures, 482
ADC, 473, 479, 498, 501, 503, 511, 513, 539
Administration, 5–7, 9, 10, 13, 14, 19, 26, 28, 87, 88, 101, 105–108, 113–115, 117, 125, 131, 132, 134, 135, 164–167, 173, 175–177, 182, 192, 193, 198, 199, 204, 207–209, 211, 214, 218, 225, 226, 235–237, 241–246, 248, 250, 251, 253, 255, 258, 261, 262, 338–345, 347, 349, 350, 443, 497, 498, 502, 508, 509, 512–514, 521–524, 533, 536, 539, 550, 561–567, 572, 573, 575–577, 579–587
ADE or PDE value, 486
Aggregates, 16, 36, 37, 65, 111–113, 117, 125, 126, 128, 129, 133, 145, 146, 152, 172, 210–212, 220, 320, 325, 330, 444, 454, 506, 515, 552, 580
Aggregation & particles, 132
Aggregation (soluble and insoluble aggregates), 144
Aggregation tendency, 112
Alternative viscosity methods, 151
Ammonium sulfate treatment, 298
Analytical characterization, 14, 25, 41, 47, 139, 152, 225, 302, 508
Analytical Evaluation Threshold (AET), 339, 344
Analytical methods, 47, 116, 125, 131, 139, 149, 286, 346, 348, 478, 497, 524
  choice of, 346
  selection of, 7
Analytical testing (comparability), 582
Annealing, 414
Antibiotic drug product
  transfer of principles to, 491
Antibody
  drug conjugates, 501
  fragments, 500
  therapeutics, 100
Anti-counterfeiting, 194
Aseptic drug product facility, 473, 479, 481, 492
Automated Visual Inspection (AVI), 174

B
Bacterial retention, 364
Balloon, 252
Basic requirements, 238
Bioburden reduction filters, 362
Biologics, 5, 7, 8, 36, 99, 100, 105, 125, 133, 191, 204–206, 208, 211–213, 215, 216, 220, 221, 237, 254, 258, 260–262, 273, 277, 314–316, 318, 323, 339, 353, 361, 382, 486, 497, 514, 524, 527, 529, 535, 547, 551, 579, 581
  compatibility of biologics with PFS, 211
  mini pumps, 237
    boundary conditions of, 237
Biopharmaceuticals, 26, 194, 474, 514, 522
Biotechnology market
  changes of, 497
  from monoclonals to novel formats, 497
Bio-therapeutics, 28
Bispecifics, 497, 498, 503, 524
Bolus injector, 241, 243
Bulk drug substance storage, 170

C
Candidate selection process
  merging biology and developability, 504

© American Association of Pharmaceutical Scientists 2018

N. W. Warne and H.-C. Mahler (eds.), *Challenges in Protein Product Development*, AAPS Advances in the Pharmaceutical Sciences Series 38,
https://doi.org/10.1007/978-3-319-90603-4

Case studies, 568
  post-approval switch, 583
Celsius Pak®system, 322
Chemical
  durability of glass, 292
  hydrolysis, 46
  properties, 75
  structure, synthesis and composition, 27
Chemical durability, 195, 292
Chemical instability, 9, 500
Cleanability, 174
Cleaning validation, 477, 485, 488
Cleaning verification/validation concept, 485
Clinical and commercial facilities, 492
Clinical testing, 583
Coatings, 297
Color, 17, 84, 85, 125, 126, 129–131, 134, 135, 196, 268, 271, 587
Coloration of solution, 129
Combination products, 179, 193, 194, 198, 204, 207, 216, 221, 254, 255, 563
Components of a protein formulation, 8
Concentrated protein formulations
  analytical challenges of, 143
    characterization of, 143
Condenser cooling and evacuation, 460
Condenser cooling step, 447
Conjugation of carbohydrates, 537
Container closure integrity, 192, 210, 230, 258–263, 288, 305
Container closure, 13, 191, 194, 207, 225, 230, 258, 260–265, 276, 314, 323, 344, 446, 451, 460, 461
Container closure integrity test
  attributes of, 263
  background on, 260
  for parental products, 259
  for primary container, 268
  purpose of, 258
  regulatory requirements on, 263
Container closure systems
  for parental pharmaceuticals, 194
Container size, 297
Containment, 192
Contents inside primary container, 266
Control strategy, 55, 302, 314, 340, 353, 378, 379, 441–443, 454, 456–458, 466–468, 562
Control, high dose accuracy and communication, 241
Convenience, 572
Convenient application, 193
CPPs and CQAs-control strategy, 466
Crimp caps, 198
Critical quality attributes, 444
Cryoconcentration, 78, 170, 314, 316, 320, 321, 326–328, 330, 331, 333
Cryoconcentration effects
  in large scale systems, 326
Cryovessel system, 322

D

Data handling and analysis, 19
Delamination, 293–302, 349
  additional factors influencing risk for, 296
  background/definition of, 293
  root cause of, 294
Delivery considerations, 175
Description, 243–248, 250, 251
Design and risk management-quality, 430
Design validation and human factors, 219
Destructive, 153, 155, 259, 260, 264, 266, 269, 273, 276, 281, 283, 288, 369, 420, 421, 481
Destructive versus nondestructive methods, 266
Detection and analysis, 300
Developability, 140, 502–504, 506–511
Developability profile of a candidate, 502
Development, 5–17, 25, 37, 38, 47, 83, 88, 100, 105, 107, 113, 115–117, 128, 129, 131, 132, 134, 135, 139, 140, 143, 146, 148, 149, 151, 163, 167, 169, 170, 172, 174, 176, 179, 181, 182, 191–193, 198, 199, 204, 206–213, 216–218, 220, 221, 225, 226, 228–232, 240, 250, 252–255, 263, 267, 271, 273, 278, 284–288, 302, 314, 315, 318, 324, 325, 339–341, 343, 350, 354, 360, 363, 364, 375, 379, 387–389, 396, 409, 412, 413, 418, 421, 423, 429, 431, 441–443, 445, 446, 451, 453, 456, 457, 468–470, 474, 497, 498, 500–502, 504, 506, 508–511, 514, 515, 518, 520, 521, 523, 524, 528, 535, 539, 547, 548, 552, 554, 562–568, 575–577, 579, 580, 582, 583, 585, 586
  qualification activities of
    of a prefilled syringe presentation, 207
    of new analytical methodologies, 116
    of PFS presentation for biologics, 207
Device compatibility, 163, 181, 182
Device-tailored primary packaging concepts, 253
Diffusion coefficient, 38, 66, 77, 142, 151, 316
Dilution strategy, 355
Dosage forms, 85, 230, 231, 513, 514, 561–569, 573, 575–577
Dose accuracy, 241

Drive concepts
driven by
electromechanical actuator, 247
mechanical actuator, 246
user-applied force, 250
for mini-pumps, 246
other types, 252
Drug/device combination products, 7, 10, 11, 13, 19, 27, 36, 37, 47, 55, 100, 101, 105, 107, 108, 113–115, 125, 127, 130, 133–135, 140, 145, 146, 164, 166–170, 172–175, 181, 182, 191, 193, 195, 197, 198, 206–208, 210, 211, 214, 216–219, 225, 232, 235–248, 250–255, 258, 260–262, 264, 266, 267, 273, 276, 277, 282, 283, 292–294, 296, 300–302, 306, 313–315, 317, 318, 321, 333, 338–340, 343, 348, 353–358, 361–366, 375, 379, 380, 382, 385–393, 396, 398, 400, 401, 405, 408, 441–444, 451, 454, 466, 469, 473–475, 479, 485–487, 490, 492, 497–499, 501–503, 508, 509, 511, 512, 514, 518–523, 527, 528, 538, 539, 547–549, 552, 555, 562–567, 569, 573, 577, 580, 581, 584–587
Drug, 484
Drug administration, 115
Drug-delivery device, 194, 236, 237, 253, 255
Drug product fill/Finish unit operations, 171
Drug product lyophilization
benefits of applying QbD, 466
Drug product profile, 565, 566, 577
Drug requirement, 253
Drug Substance (DS) manufacturing
leaching during the time of, 348
DS container
amount and quality of, 15
choice of, 323
compatability of, 324
practical aspects of, 321
storage considerations of, 314
DS shipping
and its temperature, 332
considerations of, 332
DS to pH shift
sensitivity of, 324

E

E/L compounds above AET, 347
Early-stage formulation development, 13
Effector function, 552–554
Electrical conductivity leak test, 275
Endpoint of primary drying, 420
Engineered Fc constructs
substitution and insertion mutants, 531
Equipment characterization and modeling, 422
Equipment validation, 284
Ergonomics and design, 241
Evacuation step, 448
Excipients, 7–12, 14–16, 18, 19, 26, 41, 49, 63, 69, 80, 87–89, 112–114, 116, 128, 131, 134, 156, 170, 175, 181, 208, 209, 211, 292, 293, 315, 317, 321, 323, 338, 340, 347, 356, 358, 360, 365, 378, 389, 392, 408–410, 414, 415, 446, 452, 460, 511, 514, 515, 517, 521, 523, 585
Expression, 100, 206, 475, 503, 506, 532, 551, 555, 568
Extended characterization, 152
Extractables/Leachables(E/L) assessment, 340
Extractables, 192, 197, 339, 340, 343, 344, 346–348, 360, 362, 364–366, 374, 375, 519
Extractables testing, 365

F

Falling ball and capillary viscometers, 149
Fc associated HLE, 529
Fc engineering
decreased effector function, 554
increased half-life, 553
monomeric Fc, 555
Fc fusion, 499, 500, 503, 504, 531, 545–556, 573
Fc-fusion constructs, 531
Fc-fusion proteins
advantages of, 550
and improvements, 552
and its constructs, 531
challenges associated with, 552
Fc loop, 532, 539
Fc methionine oxidation
in monoclonal antibodies
extent of, 390
FcRn, 36, 498, 503, 527–533, 539, 545, 550, 553–555
FcRn–Fc interaction, 529
FcRn mediated recycling, 529
Filling (pump considerations), 173
Filling, stoppering and capping, 367
Filling, 18, 19, 82, 108, 114, 132, 146, 165–168, 172–176, 180, 181, 198, 206, 210, 219, 237, 252, 264, 279, 281, 298–300, 307, 340, 353, 354, 362,

367–374, 379, 381, 386–388, 393, 394, 396, 399–401, 404, 405, 479–485, 487, 489, 491, 492, 509, 514, 518–520, 581
Filling mechanism, 371
Fill weight tolerance, 368
Filter Integrity Testing (FIT), 363
Filter requirements for early stage processes, 362
Filter sterilization, 367
Filtration, 172
Flow rate and viscosity tolerability, 240
Flush volume determination, 366
Fogging, 305–308
Formulation, 5–19, 36, 37, 41, 42, 47, 54, 55, 63, 69, 75, 89, 90, 106, 113, 114, 116, 125, 127, 128, 131, 135, 144–147, 151–154, 156, 157, 163–165, 168–170, 172, 174, 179–182, 192, 196, 197, 207–209, 211–216, 219, 240, 292–295, 297–302, 306, 308, 315–320, 323, 324, 330, 338–340, 342, 343, 345–347, 349, 350, 358–362, 365, 374, 377, 381, 388, 389, 391, 392, 394, 402, 404, 405, 408–411, 413, 414, 416, 423–428, 431, 446, 452, 460, 497, 500–503, 505, 507–512, 514–522, 524, 527, 529, 538, 564, 566, 568–570, 572, 573, 580–587
  and stabilization mechanisms, 76
  bridging program, 582
  composition, 298
Formulation bridging, 582
Formulation development, 6
  and commercialization, 14
  challenges during, 15
  for novel construct molecules, 511
  strategies and approaches of, 5
Freeze/thaw, 10, 17, 18, 26, 27, 68, 72, 73, 76, 78, 79, 192, 197, 208–210, 239, 305, 349, 355, 380, 407, 411, 441, 442, 447–451, 456, 457, 459–461, 463, 465, 467, 479, 488, 510, 514, 517–520
Freeze, 484
Freeze-drying, 71, 74, 82, 108, 197, 407, 408, 412, 451, 488, 520, 581
  success in, 451
Freeze–Thaw, 314, 316, 320–322, 324–326, 329–333
Freeze–Thaw systems
  and containers, 321
  availability of, 321
  contrallable, 321
  practical aspects of, 321
  uncontrollable, 321
Freezing
  hold duration, 460
  hold shelf temperature, 459
  operating space (proven acceptable range), 460
  Ramp, 459
  temperature, 413
  /thawing rates and robustness, 324
Freezing and thawing process parameters
  design space considerations for, 324
Frozen state, 78
Functionality, 47, 164, 174, 176–179, 208–213, 215, 221, 225, 226, 232, 338, 350, 554
Fusion proteins, 497–501, 503, 505, 506, 524, 527, 528, 531, 545–556, 573
Future directions, 115

G

Gas-driven, 251
General considerations, 579
Glass, 6, 28, 68, 71, 73, 74, 78, 80, 82, 150, 166, 178, 192–196, 198, 199, 210–214, 216, 218, 221, 226, 231, 259, 264, 267, 273, 275, 281, 283, 292–299, 301, 306–308, 316, 326, 338, 341, 349, 370, 411, 415, 447, 513
Glass composition, 296
Glass corrosion and ion leaching, 292
Glass delamination, 299, 349
Glass dynamics, 81, 82
Glass transition, 63, 81, 316, 318, 409, 415
Glass transition temperature, 10, 65, 66, 68, 72, 80, 81, 316, 410, 411, 414, 415, 417, 418, 427, 429, 452, 520
Glycosylation, 537
Grades of polysorbate, 37
Growth hormone, 568

H

Half-life, 117, 498, 500, 503, 527–538, 545, 549–551, 553–555, 562, 573, 575
Half-life extension, 500, 504, 527, 528, 539, 550
Headspace gas analyzer, 282
Health authority expectations, 339
High concentration formulation, 502

High-concentration products
 challenges in developing of, 108
High-concentration protein formulation, 175, 581
 physical properties of
  influencing factors of, 110
 need for, 105
High dose requirement, 107
High potent drugs, 473, 474, 478, 480, 486, 491, 492
HLE
 by chemical and genetic fusion, 535
 by formulation and nanotechnology, 538
 by post-translational modifications, 537
 via albumin fusions and conjugates, 532
 via interaction with serum albumin, 533
Hold times, 17, 340, 373, 374, 378, 380, 381, 456, 518, 523
Human factor studies, 206, 220
Human serum albumin, 25, 31, 529, 532, 573
Hydrogen peroxide reacting with methionine
 kinetics of, 389
Hydrophobic, 297

I
Industrial design, 253
Injectability, 132, 163, 164, 175, 176, 178, 181, 182
Injection time and tolerance, 240
Inline manufacturing monitor versus offline laboratory test, 267
Innovation, 572
Inspection, 373
Instrument, 284
Intravenous, 6, 87–89, 105, 204, 207, 235, 237, 250, 508, 512, 517, 521, 550, 563, 566, 579, 584
In-vivo performance, 117

K
Key to success, 574
Knowing patient preference matters, 572

L
Laboratory E/L testing, 344
Lab scale mixing studies, 360
Large injection volumes, 581
Late-stage formulation development, 14
Leachables, 6, 87, 179, 192, 193, 195, 197, 199, 211, 231, 293, 301, 324, 338–340, 342–350, 360, 362, 364–366, 374, 513, 519
Leachables & extractables, 87, 197, 230, 337, 362, 519
Leachables during drug substance (DS) manufacturing, 348
Leachables testing, 366
Leachables with process constituents
 interaction of, 349
Leak, 192, 193, 230, 259, 260, 264, 265, 267–273, 275–279, 281–288, 481, 489
Leak test method
 selection criteria of, 264
Lifecycle management, 442, 468, 469, 561–563, 576, 577
Liposomes and nanoparticles, 538
Liquid, 5–8, 10, 15, 18, 19, 26, 33, 35, 36, 75–77, 87, 90, 101–105, 113, 125, 126, 129, 133, 135, 140, 141, 143, 149, 153, 154, 167–169, 171, 179, 180, 195, 198, 208, 210, 225, 226, 238, 267, 270, 271, 273, 276, 284, 292, 296, 297, 299, 305–308, 314, 315, 318, 321, 325, 333, 338, 339, 342, 343, 348, 354, 361, 365, 388, 389, 391, 394, 397, 398, 400, 401, 408, 409, 411, 457, 479, 482, 484, 502, 503, 507, 509–511, 513–515, 518, 519, 521, 562, 564–566, 568–572, 580, 585
Liquid state, 76
Liquid tracer leak test, 270
Load temperature, 459
Long-term drug administration, 107
Loss of Exclusivity (LOE), 576
Lyo, 63, 89, 196, 458, 488, 489, 511, 517, 520, 580
Lyophilization, 5, 7, 9, 13, 18, 26, 75, 83, 87, 168, 195–197, 305, 354, 391, 407, 408, 410–413, 415, 419, 420, 422, 424, 425, 427, 429–431, 441–443, 446–448, 450–454, 456–458, 465–470, 489, 572, 575
 and drug products, 391
 and its state, 79
 equipment qualification, 450
 process knowledge, 446
Lyophilization process development and/or optimization, 457
 application of quality by design, 443
 based on good freeze drying principles, 451

M
Manufacturability and formulability, 18
Manufacturing cost, 108
Manufacturing unit operations, 168

Mapping of the process, 340
Marangoni effect, 36, 306
Marketed biotherapeutics
concentration ranges of, 100
Material risk evaluation, 342
Maximum Allowable Carryover (MAC)
determination of, 487
Membrane compatibility, 365
Method development, qualification and validation, 284
Microbial challenge test, 268
Micro-dosing pump, 245
Micro-dosing pumps for long-term use, 245
Micro-pump engines, 250
Mimic solutions, 174
Minimum Batch Size (MBS), 486
Mini-pump, 237–242, 247, 248, 252
new trends in, 253
regulatory consideration for, 254
types of, 242
Mixing, 171
Multifunctional antibodies, 501
Multi-product, 473, 478, 479, 485, 492
Multipurpose facility versus dedicated facility, 479
Multi-stage drug product developability process, 504

N
Non-Fc HLE, 532
Non-ideality, 81, 146, 153
Nonionic surfactants with proteins
interaction of, 29
Novel construct molecules
profiling of, 502

O
Opalescence, 84, 85, 87, 113, 125–129, 134, 135, 140, 141, 143, 585
Overview
of recombinant therapeutic proteins, 99
of viscosity measurement techniques, 165

P
Parenteral container closure system, 191
Particles, 5, 16, 17, 19, 37, 47–50, 53, 112, 125, 127–129, 133, 134, 144, 146, 151, 171–174, 193, 209–213, 227–229, 231, 286, 293, 300, 301, 372, 381, 520, 538
Patch injector, 244, 245, 582
PEGylation, 6, 535, 536, 539, 562, 573
Pen injector, 243, 244
Peptibody, 547–549
Peristaltic pumps, 248
PFS design controls and risk management, 217
PFS presentation for biologics
advantages of, 205
Pharmacopoeia requirements, 37
Physical basics, 237
Physical instability, 144
Physical properties, 64
Piezo actuators, 252
Platforms and costs, 253
Pooling and DS dilution, 355
Potential mitigation, 181
Potential needle clogging, 179
Preclinical testing, 582
Predictive tools
and early stage development, 140
for the clinical lead selection, 140
Prefillable syringes, 35, 107, 175, 193, 194, 198, 199, 204, 205, 212–214, 225, 231
Pre-filled syringe, 105, 107, 178, 197, 248, 349, 353, 566, 567
and autoinjectors, 175
emerging technologies for, 220
Preformulation, 7, 10, 11
Preparation of E/L samples, 345
Presentation and identification, 193
Primary container
material of construction, 267
system for parenteral products, 258
Primary drying
and its ramp, 461
and operating space (proven acceptable range), 463
and process control, 461
Primary packaging, 7, 10, 13, 26, 192, 196, 197, 237, 250, 253, 292, 297, 299, 300, 302, 385, 388, 401, 404, 405
Primary packaging containers, 297, 385
Primary packaging material, 10
Primary packaging preparation, 299
Process
development and transfer, 412
performance qualification/process validation, 429
qualification considerations, 379
robustness, 423
Process Analytical Technologies (PAT), 419
Process development, 19, 82, 113, 114, 174, 217, 314, 333, 340, 354, 409, 412, 441, 442, 444, 446, 453, 454, 466, 497, 517, 518, 521, 524, 582
Process parameter, 324, 357, 376, 378, 430, 453–455, 460, 466, 468, 470
Product impact, 424
Product moisture monitoring, 420

Product quality management, 469
Product specific H_2O_2 sensitivity assessment (spiking studies), 388
Product temperature, 326, 355, 374, 411, 412, 416–420, 422–425, 428, 429, 448, 452, 457, 463, 520
Product temperature monitoring, 419
Protection and compatibility, 192
Protein aggregation, 26, 37, 108, 109, 112, 113, 115, 116, 128, 129, 143, 144, 146, 171, 226, 231, 317, 349, 520, 581
Protein aggregation and sub-visible particles, 226
Protein conformation, 111
Protein formulation, 5, 7, 10, 11, 19, 26, 32, 107, 126, 130, 149, 172, 179, 389, 391, 511
  appearance of protein formulation, 126
  beyond stabilization, 5
Protein-protein interaction, 29, 108–114, 116, 117, 140, 143, 153, 154, 156, 169, 181, 555, 581
Protein–protein interactions, factors influencing of, 112
Protein stability, 29, 31, 82, 87, 126, 133, 318, 319, 374, 511, 514, 518, 522, 523
  impact of freezing stress, 318
Protein–surfactant mixtures at interfaces
  interactions of, 32

Q
Qualification, 143, 174, 230, 284, 286–288, 353, 357, 360, 362, 364, 367, 378–381, 409, 422, 429, 454, 456, 580
Quality attributes (product knowledge)
  other-moisture impact on, 444
Quality by Design (QbD), 232, 379, 441–444, 466, 467, 469, 470
Quality control, 129, 131, 134, 135, 227, 229, 260, 266, 442
Quality target product profile, 443

R
Ramp rates, 413
Recombinant Factor VIII, 572
Recombinant PEG mimetics, 536
Reconstitution, 239
Regulatory and safety aspects, 83
Regulatory change management plans and protocols, 468
Regulatory consideration
  for mini-pumps, 254
  in PFS development—design controls, 216
Regulatory environment, 475
Regulatory flexibility, 442, 443, 457, 467, 468, 470
Regulatory flexibility and process description, 467
Reliability, 242, 264–267, 285, 287
Reliability or precision, 265
Requirement
  of controlled F/T of a parental CCS, 323
Residual moisture, 299, 412, 417, 420–422, 427, 429, 444, 445, 519, 520
Reusability, 242
Rheology, 31, 37, 166, 167
Risk analysis
  and cross-contamination risk, 479
Risk assessment, 133, 219, 297, 300, 364, 375–378, 380, 430, 431, 445, 453, 454, 456, 457, 466, 488, 510, 529, 565
  Round 1, 453
  Round 2, 454
  Round 3, 456
Risk evaluation, 340–344, 348, 350, 488
Risk management, 217–219, 254, 353, 354, 364, 375, 378, 379, 382, 430, 431, 442, 443, 445, 452, 453, 469, 475–477, 488
  and quality by design, 375
  in the design control process, 219
Rotational rheometers, 150
Routes of administration, 105
Rubber stopper, 196

S
Safety aspects, 87
SC formulation development
  and administration challenges, 581
Secondary drying
  Process Control, 464
  Ramp, 464
Secondary drying operating space (proven acceptable range), 465
Secondary packaging materials, adhesives and printing, 350
Second viral coefficient (B22), the, 141
Second virial coefficient, 109, 128, 139–142, 154, 157, 507
Sensitivity, 11, 13, 43, 151, 182, 209–213, 231, 264, 265, 268, 269, 271, 273, 276, 281, 286–288, 317, 355, 373, 376, 386, 388, 393, 394, 405, 413, 512, 513, 523
Sensitivity assessment, 385, 388
Sensitivity or detection limit, 264
Shape memory alloy, 252
“Share or not to share”, 475
Shear thinning, 115, 148, 163, 167, 172, 173, 176, 177, 182

Shear thinning (*cont.*)
complex behavior under, 165
practical significance of, 165
Shipping, 108, 199, 210, 230, 279, 314, 332, 355, 356, 510
Silicone oil, 6, 28, 32, 35, 178, 193, 199, 209–214, 220, 221, 225–228, 259, 349, 416
Silicon oil, 35, 193
Simple and safe handling, 242
SiO_2, 297
Size of the DS/DP batch, 323
Small-angle scattering techniques, 139, 153, 155, 157
Solid state properties, 69
Solubility, 6, 28, 47, 63, 68, 69, 74, 78, 79, 84, 85, 87, 89, 90, 109, 113, 125, 126, 142, 143, 169, 181, 397, 500, 503, 506–509, 514–516, 551, 581
Solution state properties, 65
Solution viscosity, 110
Special considerations, 348
Spray-drying, 72, 73, 168
Stability, 5–7, 9–19, 28–30, 35–38, 41, 42, 47, 55, 76–78, 80, 82, 84, 87, 107, 109, 114, 116, 129, 131, 163–165, 169, 171, 192, 209–211, 229, 231, 241, 250, 258–260, 262–265, 267, 286, 296, 300, 302, 314–320, 323–325, 332, 339, 350, 360, 373, 374, 380, 381, 388, 389, 405, 408, 409, 413, 419, 424, 427, 429, 446, 450, 457, 459–462, 464, 465, 497, 499–505, 507–511, 513–516, 518, 519, 521–524, 529, 532–534, 547, 548, 551, 555, 563, 564, 573, 580–582, 585, 587
Stability and degradation in formulations, 41
Stability testing and forced-degradation studies, 17
Stabilizers, 76, 77, 79, 316, 321, 514, 515
Sterile drugs
outside the U.S., 262
regulations on, 261
Sterile filtration, 172, 192, 353, 359, 361, 362, 364, 378, 491, 581
Sterile filtration validation, 364
Sterility, 192
Stoppering and capping, 372
Stoppering and unloading, 418
Storage, 241
Stress conditions and its selection, 16
Structure heterogeneity in PS20 and PS80, 27
Subcutaneous, 5, 6, 105, 107, 115, 117, 134, 164, 178, 179, 204, 207, 208, 214, 220, 221, 237, 240, 255, 508, 512, 514, 517, 534, 579, 584–587
Subcutaneous tissue
back pressure on injectability, 178
back pressure on injectability
impact of, 178
Submicron particles, 134
Sucrose, 9, 63–84, 87–90, 111, 113, 154, 317, 320, 321, 392, 408, 410, 411, 515
Surface properties, 35, 172
Surfactants, 9, 16, 25–28, 30–32, 35–37, 55, 134, 306, 318, 338, 342, 356, 360, 365, 514–516, 521
Sustainability, 253
Syringe ability/injectability, 175
Syringeability, 115, 164, 175, 176, 178, 199, 581
Syringe functionality considerations, 213
Syringe pumps, 248
Syringe siliconization
potential effect of, 178
System suitability for test method, 285

T
Tangential Flow Filtration (TFF), 168
Temperature of storage, 323
Test method, 149, 193, 210, 230, 264–268, 271, 272, 276, 284, 285, 287, 288, 362
development and qualificationvalidation, 285
Test on container
over product lifecycle, 263
with product content, 263
Theoretical considerations, 108
Therapeutic proteins, 5, 26, 31, 32, 35, 42, 63, 89, 99, 105, 131, 192, 220, 484, 498, 514, 533, 538, 579
Tracer gas leak test, 279
Transport and thaw, 355
Transportation, 300
Trehalose, 9, 63–70, 72–90, 113, 171, 315–317, 323, 327, 392, 408, 410, 515, 585, 586
Tungsten, 193, 199, 210, 212, 213, 220, 225, 231, 349
Turbidity, 13, 125, 127, 128, 135, 143, 211, 268, 359, 515, 516, 521, 523

U
US Federal regulations on sterile drugs, 261

V
Vacuum decay leak test, 272

Validation, 191, 218–220, 253, 260, 264, 284, 285, 287, 288, 322, 339, 357, 358, 361, 363, 364, 373, 379, 381, 382, 387, 405, 423, 442, 454, 456, 469, 485, 491, 504
Vapor Phase Hydrogen Peroxide (VPHP), 385–388, 393–405
Vapor phase hydrogen peroxide
absorption testing, 396
absorption testing
in a scale down containment, 399
decontamination with, 386
Vials, 7, 107, 192–198, 205, 206, 211, 225, 226, 230, 237, 281–283, 293, 294, 296–298, 301, 302, 306, 308, 340, 346, 349, 354, 367, 368, 370, 372, 388, 397, 412, 414–416, 418, 419, 422, 423, 428, 446, 447, 449, 451, 452, 457, 467, 468, 479, 481, 483, 488, 489, 513, 520, 566, 568–571, 584
Viscosity/pressure, 240
Viscosity, 9, 12, 18, 36, 40, 66–69, 78, 81, 90, 107, 109–112, 114–117, 125, 126, 131, 132, 134, 139–144, 146–154, 156, 157, 163–170, 172–179, 181, 182, 199, 208, 209, 214, 215, 225, 232, 236, 238, 240, 244, 246–248, 251, 255, 316, 317, 371, 374, 411, 447, 500, 503, 507–509, 514–517, 536, 580, 581
analytical methods for measuring, 149
of concentrated protein solutions, 147
impact of shear, 147
influence of temperature, 148
technical considerations to measure, 167
Viscosity and protein aggregation
minimization of, 116
Visible particles, 17, 125, 134, 135, 144, 164, 227–229, 300, 302, 349, 412, 521, 522
VPHP-containment
after aeration of, 393
determination of, 393
VPHP quantification, 385
VPHP uptake study, 385, 388, 396–401, 403–405

W

Water replacement, 78–81

Z

Zeta FreezeSystem®, 322, 408