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Parag Kolhe
Mrinal Shah
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Sterile Product Development

Formulation, Process, Quality and Regulatory Considerations

AAPS Advances in the Pharmaceutical Sciences Series

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Sterile Product Development

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Preface

Sterile products represent a significant fraction of parenteral products and encompass a variety of challenging dosage forms. Development of sterile products poses multifaceted challenges which can be broadly categorized into three areas: formulation dosage form development, processing and manufacturing and assurance of purity, safety and efficacy of the manufactured product. The chapters in this book are structured along this theme and offer a useful guide for scientists and personnel working on commercialization of sterile drug products.

The first part of the book covers the formulation aspects of sterile product development including basic principles of formulation development, formulation selection via molecule and manufacturability assessments, and formulation approaches for polymer, lipid-based systems, PEGylated biotherapeutics, nasal delivery, vaccines and adjuvant systems. The second part focuses on manufacturing process, container closure, and delivery considerations. This section covers freeze-thaw processing, technology transfer of sterile products, transfer across barrier systems, in addition, it focuses on recent innovations in aseptic filling, and approaches for developing lyophilized parenterals. The part also emphasizes on recent innovations in pen and autoinjector drug delivery devices and the methods available to establish container closure integrity (CCI). The last part of this book expands on quality and regulatory aspects of sterile products including particulate issues and appearance defects in sterile products, sterile filtration, and intravenous admixture compatibility. As sterilization process is an essential component of aseptic processing, the last four chapters cover the basic principles behind commonly used sterilization techniques, associated validation strategies along with an overview of microbial measurement methods.

The book commences with an introduction to basic principles of sterile product formulation development. This chapter presents cogent approaches on development of injectable products and discusses formulation development considerations such as solubility, challenges for lipophilic formulations, nanoparticles, suspensions and dry formulations. Furthermore general considerations for compatibility with primary packaging and manufacturing are provided.

Chapter 2 discusses an approach for selection of molecules based on manufacturability assessment so that robust commercial formulation can be developed for protein products. This chapter covers aspects of protein sequence analysis from product quality standpoint and identifying "hot spots" for degradation and approaches for initial formulation screening through assessment of physical and chemical stability. This chapter dives deeper into considerations for manufacturability assessment through various process studies by subjecting molecule to stresses experienced during manufacturing process.

Chapter 3 provides a snapshot of polymer- and lipid-based drug delivery technologies. Drug delivery technologies, especially controlled drug release technologies have come a long way and their use in commercial drugs highlights the utility of such technologies. This chapter reviews the matured technologies that are being used in parenteral drug delivery and focuses on in situ forming gel depot formulations and lipid-based drug delivery technologies. Authors have also provided insights into the considerations for the development of newer technologies.

Chapter 4 touches upon an important area of PEGylated biotherapeutics where a comprehensive review of commercial products and clinical products in development has been provided for readers. Authors initiate the discussion with chemistry of PEGylation and go into the details of manufacturing and formulation aspects such as issues during reaction, process considerations and characterization for drug substance, and stability aspects to consider during formulation development followed by delivery challenges for PEGylated products due to viscosity issues.

Chapter 5 shifts the attention to nasal delivery aspects for sterile products. This chapter navigates readers through nasal physiology and mechanism of delivery, provides good review of local and systemic acting nasal products, discusses various challenges encountered during nasal drug delivery, and provides a comprehensive approach for formulation development and characterization. One of the important aspects in nasal delivery is the consideration for delivery devices. This chapter also provides an in-depth discussion on delivery devices, analytical testing, regulatory expectations, and manufacturing aspects through relevant case studies.

Chapter 6 focuses on considerations for vaccine formulation development which include antigen and adjuvant formulation development. This chapter differentiates between the protein formulation vs. vaccine formulations and guides readers to the important aspect of immune response and how it is achieved in vaccines. The chapter reviews formulation considerations and available adjuvants for vaccines. In addition, authors discuss the impact of route of delivery and challenges in stability and analytical characterization of vaccines.

Part II begins with a chapter on freeze-thaw processing of bulk protein solutions. This chapter provides excellent insight into mechanistic aspects of freezing process for protein solutions, impact of freezing on proteins through cold denaturation, ice–liquid interface, and implications of cryoconcentration effects through solute crystallization and phase separation. Furthermore considerations for formulation and protection against freezing induced stress, design of freeze-thaw process parameters, scale down studies, and container closure aspects are discussed.

Chapter 8 is a comprehensive collection of case studies by the author on best practices for technology transfer of sterile products. This chapter provides outstanding overview of requirements for material release testing for API and excipients with underlying case studies. The author discusses production aspect preparations, compounding operation, lyophilization, and sterilization elucidated with case studies. Product release testing approach is explained through visual inspection, particulate matter testing, and sterility testing.

Chapter 9 discusses transfer of material across barrier systems in aseptic fill finish operations. It outlines topics such as requirements based on type of material being transferred, methods of transfer for solids, liquids, and suspensions. A comprehensive discussion on selection of appropriate transfer process is presented with illustrating case studies with isolator for potent compounds, biotech products in prefilled syringes, and for sterile suspensions for vaccines.

Challenges and recent innovations in aseptic filling technology are covered in Chap. 10. Aseptic filling requirements for clean room classifications, environmental monitoring, operator training, and gowning requirements are explained. An in-depth discussion on barrier system such as restricted access barriers (RABS) and isolators has been provided. Various filling containers and available filling processes are discussed which include ampoules, vials, prefilled syringes, cartridges, and Blow Fill Seal (BFS). This chapter concludes with case study on closed vial technology.

Because not all products have adequate stability in solution state, manufacturers rely on the lyophilization process to manufacture formulations, both biologics and small molecules, in freeze-dried state. Chapter 11 provides a detail overview of the scientific and technological advancements in the field of lyophilization. The chapter is an ideal resource for scientists involved with process design and qualification of freeze-dried products. Specific guidance on critical process parameters, critical quality attributes, and design space principles are covered that will help one to design process characterization studies. The author has also provided pertinent discussion on application of recent FDA guidance on process validation for this unit operation.

Assurance of CCI is critical to ensure safety of the final drug product presentation for the end user. Process development scientists should have a general understanding of the CCI testing and associated technical challenges in order to develop a robust drug product presentation. Chapter 12 provides an overview of regulatory expectations and Industry trend in CCI testing. A variety of CCI test methods are discussed along with considerations for method selection, development, and validation.

The ease and flexibility associated with the self-injection of parenteral drugs has also led to the increase in use of pens and autoinjectors as delivery devices. Chapter 13 provides an overview of the different types of injection devices as well as what the development of such a device entails. Regulatory requirements applicable to device development are discussed and examples are included to describe various steps associated with the injection device development and commercialization.

Part III brings the focus on regulatory and quality aspects of sterile product development. Chapter 14 reviews our understanding of the particulate matter as a critical quality attribute and the related concerns that impact product safety. The

origin of these particles as well as the available enumeration techniques are discussed in detail. The chapter also provides an overview of the pharmacopeial requirements and guidance on addressing regulatory queries related to subvisible particles in biopharmaceuticals. The discussion on product appearance is continued in Chap. 15. This chapter deals with the visual inspection of drug products with a focus on defects that are visible to eye—both cosmetic and functional. The chapter covers inspection attributes, regulatory expectations for manual and automated visual inspections, and related case studies.

The next chapter focuses on sterile filtration, a critical step in the manufacturing process needed to ensure sterility of the final product. The chapter covers the performance requirements for sterile filtration and membrane properties including pore size and material of construction that can affect the filtration process. Basic filtration concepts such as filter selection and filter sizing are included. Operational practices including installation, sterilization, flushing, and integrity testing are also covered, as well as the main components of filter validation including retention studies and filter integrity testing.

Intravenous (IV) admixture studies are an integral part of developing a safe and efficacious sterile drug product intended for IV administration. Chapter 17 discusses requirements and challenges associated with conducting IV admixture studies and the related regulatory guidance. A pharmaceutical admixture consists of a drug product mixed with an appropriate diluent in a suitable dosing/delivery device for the purpose of parenteral infusion to the patient. The discussion presented in the chapter will help researchers identify critical admixture issues for their products and gain insights into addressing those issues while meeting regulatory expectations.

The last four chapters of the book focus on sterilization process, microbiological methods, and the associated validation challenges. Chapter 18 deals with basics of sterilization methods commonly employed for sterile products. This chapter discusses requirements for sterilization methods that are subject to review by regulatory agencies when a sterile product is filed for approval. The author provides a summary of requirements for sterilization process, sterilization method details, and considerations for validation of sterilization methods including heat sterilization, radiation sterilization, and ethylene oxide sterilization. In addition the author discusses aseptic processing requirements which include filters, clean room consideration, personnel qualification, and aseptic process validation.

Chapter 19 discusses the common errors made during investigation of microbial contamination events. Several thought patterns and behavior that prevents one from finding the source of contamination and the associated root cause are covered. Multiple case studies are provided to aid the reader in conducting the best possible root cause investigation for a sterility assurance failure. Microbiological testing used to assess product sterility as well as microbiological quality of components, ingredients, environment and utilities, is foundational to the operation of pharmaceutical facilities. Given the slow turnaround time associated with the conventional methods, there has been an increasing interest in the use of rapid microbiological methods (RMMs). Chapter 20 discusses different types of available RMMs and provides guidance on the validation of such RMMs. A detailed

discussion of the validation strategy is provided including the user requirements, vendor assessments, documentation, and other qualification activities needed to meet regulatory expectations.

Chapter 21 describes the current expectations for validation of dry and moist heat sterilization cycles. The chapter explains the basic concepts behind dry and moist heat sterilization including the mechanism of sterilization, determination of worst case conditions, and the relevant loading configurations. References to key literature from Parenteral Drug Association and the International Organization for Standardization are provided to the reader for further details. The chapter also covers assessment of biological and physical aspects of sterilization process and provides guidance on strategy for validation of sterilization cycles.

Overall this book covers essential aspects of sterile product development with excellent contributions, including several case studies, made by key experts in the field. We trust that this book serves as reference guide for researchers, process engineers, pharmaceutical and biotechnology scientists as well as academic students.

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Part I
Formulation Approaches for Sterile Products

Chapter 1
Basic Principles of Sterile Product Formulation Development

Martin A. Joyce and Leonore C. Witchey-Lakshmanan

Abstract Sterile product formulation development is more than just deciding which excipients to use with the given drug substance. The development of a sterile product requires that specific critical quality attributes be considered and evaluated, regardless of the route of delivery or the type of registration application. The chapter begins with an overview of ICH Q8 *Pharmaceutical Development* requirements and Health Authority Quality by Design expectations with regard to raw materials, packaging, and manufacturing process. Each of the various stages of formulation and product development is then explored, from API characterization, formulation identification and development, stability and compatibility, process requirements, to patient in-use studies and other human factor considerations. Thus, the chapter offers the formulator an overview of the foundational principles associated with formulation development of sterile products, from preformulation to commercialization.

1.1 Introduction

This chapter presents an overview of the basic approach to formulation development of sterile products. Later chapters will present more detailed consideration of the concepts introduced in this chapter. In general, the development of a sterile product requires that certain studies be carried out regardless of the type of product, be it an intravenous, intramuscular, subcutaneous, intrathecal, intra-articular, ophthalmic, or inhalation product. The required studies are virtually the same whether

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the product is registered as a new chemical entity (NCE) filed as a 505(b)(1) new drug application (NDA), a new formulation of an older drug substance filed as a 505(b)(2) NDA, or a generic filed as a 505(j) abbreviated NDA (ANDA). The required studies are also the same for veterinary drugs registered as new entities filed as new animal drug applications (NADA) or as generics filed as abbreviated new animal drug applications (ANADA).

1.1.1 ICH and the Q8 Guidance Document

The parameters required by the Health Authorities for ensuring quality during the registration of the product are outlined in the International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use guidance Q8 *Pharmaceutical Development* (FDA Guidance for Industry 2009b).

The stated objective of the Q8 guidance follows.

> This guidance describes the suggested contents for the 3.2.P.2 (Pharmaceutical Development) section of a regulatory submission in the ICH M4 common technical document (CTD) format.

The Q8 guidance *Pharmaceutical Development* document provides an outline for the presentation of "the knowledge gained through the application of scientific approaches and quality risk management (as defined in ICH Q9 *Quality Risk Management*) to the development of a product and its manufacturing process." The section is "first produced for the original marketing application and can be updated to support new knowledge gained over the lifecycle of a product. The Pharmaceutical Development section should provide a comprehensive understanding of the product and manufacturing process for reviewers and inspectors."

The Q8 guidance "also indicates areas where the demonstration of greater understanding of pharmaceutical and manufacturing sciences can create a basis for flexible regulatory approaches." According to the Q8 guideline, "The degree of regulatory flexibility is predicated on the level of relevant scientific knowledge provided."

While, technically, the guidance does not apply to early preclinical and clinical work, the knowledge gained in preparing materials for these studies can provide a valuable resource for future development.

1.1.2 The ICH Q8 Guidance and Quality by Design

The outline given in the Q8 guidance document presents the various sections that are required for the 3.2.P.2 Pharmaceutical Development report needed for the CTD required for the registration of a product. Each section of the outline is based on fundamental aspects of good science that a formulation development specialist

normally addresses. In addition, Q8 provides a recommended plan for assessing risk by designing studies aimed at identifying critical process parameters (CPPs) and implementing appropriate controls to ensure consistent product quality.

The guidance comes from the position that quality cannot be tested into a product; quality must be designed in. The term often used is "Quality by Design" (QbD). The QbD philosophy implies an expectation that the development scientist will design studies and report data for all aspects of development unique to the product that ensure a quality finished product.

1.1.2.1 Design Space

One aspect of QbD is developing a "design space" for the product. Developing design space means assuring consistent product quality by establishing ranges for formulation component levels and ranges for process parameters. Design space includes the assessment of the raw materials (active pharmaceutical ingredient (API) and excipients), container/closure systems, and the manufacturing process.

Raw Materials

In assessing the API, understanding the physicochemical properties of the API is important. This understanding includes descriptions of the primary structure of the molecule, solubility, water content, impurity types, impurity levels, and crystal structure, as well as the secondary/tertiary/quaternary structures for biotechnology products. The ICH Q6A guidance *Specifications*: *Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products*: *Chemical Substances* (FDA Guidance for Industry 2000) provides decision trees that give guidance on the type of testing that might need to be established.

Example: Most small molecule products are solutions, but crystal structure of the sourced API can impact solubility. Using the wrong polymorph could result in prolonged mixing times or other process deviations. For a suspension, where solubility directly impacts bioavailability, erratic dosing and efficacy could result. Other physical properties, such as particle size distribution, may also be considered critical characteristics that need to be controlled.

Excipient selection and characterization are also of concern. Compatibility with the API must be demonstrated as part of the justification for use. In addition, excipients must be chosen to fulfill a specific need. They cannot be added unnecessarily. The most common excipients are buffers, tonicity adjusters, stabilizers, bulking agents, antimicrobials, and antioxidants. However, the formulator sometimes needs to control viscosity or other attributes. For instance, the formulator may need to stabilize particles in a suspension or an emulsion.

Compatibility studies, in which only one or two excipients are compounded with the drug substance, can provide excellent information concerning the interaction of the excipients with the API. The studies can then be extended to multiple

ingredients to assure compatibility with the API and each of the excipients. Excipient levels should be kept to within a functional concentration, reflecting the levels at which they will be used in a formulation. Later studies are then performed to demonstrate that the final level chosen is appropriate by exploring concentrations above and below the proposed concentration.

Container/Closure Systems

The container/closure system can also impact stability. The primary packaging components that are most likely to impact product stability are a rubber stopper or a polymeric container. Polymers have materials that can leach into the formulation causing degradation or precipitation. The oxygen and moisture permeability of these materials can also impact stability. Degradation of oxygen-sensitive compounds will lead to decreased shelf life. Moisture permeation can impact dry products such as lyophilized powders. Not even the glass of a vial or ampoule can be ignored. Glass can delaminate under certain conditions, such as high pH. In addition, ions can be extracted by strongly chelating drugs such as pamidronate disodium.

Manufacturing Process

Once the preliminary formulation has been determined, the overall manufacturing process must be defined. The definition could be as simple as describing the order of addition of the ingredients and the required mixing speeds. The definition could also be a description of extremely complex procedures, such as those at times employed in the production of liposomes.

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. A process that is not robust will give rise to process deviations and out of specification test results (i.e., rejected batches). Even worse, a poorly developed process can lead to recalls putting patients unnecessarily at risk.

Thus, both the formulator and process engineer must always focus on the end product being delivered to the patient. The formulator and process engineer must conscientiously compile comprehensive developmental data. The need to properly document all activities and data is critical throughout the entire development program both for the safety of the patient and in preparation for regulatory inspection. The proper maintenance, qualification, and calibration of development equipment must always be considered. In the case of NCEs, where early candidates may be used in human phase I studies, all the work supporting the product *must* be conducted in a compliant manner. Even preformulation work should be conducted using good scientific practices such as equipment IQ/OQ, formal calibration, maintenance, and good documentation.

1.1.3 Pharmaceutical Development Report

The information from the summary reports generated at the end of the development studies justifying the API characterization, excipients, closure system, manufacturing process, manufacturing specifications, and critical quality attributes (CQAs) is compiled into the 3.2.P.2 Pharmaceutical Development report, as outlined in the ICH Q8 guidance discussed above. Submission of the applications to the Health Authorities, however, is not the end of a true Quality by Design program. New experiences gained with a product over the course of all aspects of the development cycle, as well as commercial production, should also be systematically added to the product knowledge database.

This chapter provides an overview of the various aspects of the formulation development of a sterile dosage form. The chapter begins with the choice and characterization of the API. Drug product formulation, identification, development, stability, compatibility, processability, and scalability are also addressed. Other considerations such as the use of various types of dosage forms are touched on; however, the details of each of these topic areas will be addressed in later chapters.

1.2 Active Pharmaceutical Ingredient

The selection of the specific API for a formulation is typically dictated to the formulator by the sponsoring company. In most large pharmaceutical companies, the individual formulator is assigned an NCE and requested to create the appropriate formulation. In some smaller companies, it is the charge of the formulator to identify old drug products and reformulate them in a way that overcomes long-standing issues with old formulations. In other companies, the formulator may be assigned an old drug product and asked to reproduce the innovator company's preparation with an aim toward the development of a generic product.

Regardless of the path, confirming the quality of a source of an API can be a time-consuming effort whether the product is synthesized in-house, as with an NCE, or sourced externally, as with a generic API. For small molecules, the salt form, polymorph/solvate, and impurity profile are among the first properties examined. The first properties considered for biotechnology compounds include a confirmation of the amino acid primary structure sequence and any secondary, tertiary, quaternary structures as well as labile linkages, etc. structures.

As noted earlier in this chapter, the salt and crystal form of the small molecule drug often dictates its solubility. The solubility, in turn, may rule the formulation approach, which is discussed in the next section. The solubility will also impact the overall process used for the product. Therefore, ensuring an API source exhibiting reproducible solubility is a key starting point in formulation development.

The impurity profile of the API must also be attended to. In addition to the Q6A guidance, ICH guidances Q3A (FDA Guidance for Industry 2008b), Q3B (FDA

Guidance for Industry 2006a), and Q3D (FDA Guidance for Industry 2009a) provide direction in this area. Generally, impurities over 0.1 % must be dealt with. Actions range from identification to toxicological qualification, depending on how high the impurity level is. In addition, ICH Q3C (FDA Guidance for Industry 2012) provides guidance for acceptable levels of residual solvents in the drug substance, as does USP <467>.

Sourcing a generic API offers additional challenges in that the generic development path requires the compilation of an impurity profile that reflects the allowable parameters for the innovator product. In particular, the impurity levels must be compared to the brand product in order to assure that no new impurities are introduced. Therefore, samples from multiple vendors must be obtained and analyzed in order to determine which vendors of API are able to deliver the drug substance that can meet the previously established impurity profile observed in the innovator product.

Once the API source has been confirmed, more routine preformulation can begin in order to move toward the characterization of the physicochemical properties of the drug substance. These studies include expanding the database of solubility in various solvent systems as well as at various pH levels, depending on the ionic properties of the drug substance. The molecule's tendency toward hydrolysis at various pH levels, thermal degradation, light instability, oxidation, and reduction are also examined. In addition, for biological molecules, preformulation also includes various studies such as amino acid sequencing, alpha-helix analysis, beta-sheet content analysis, sulfide linkage identification, glycosylation pattern characterization, and other evaluations that may affect formulation approaches and overall stability. The data generated by the API analyses serve as the foundation for the drug formulator in identifying and developing the final formulation.

1.3 Drug Product Formulation

Once the API is well understood, progress can be made toward formulation development. In order to generate a fully developed final formulation, a number of critical questions must be answered. This section provides an overview of questions that need to be addressed to identify the set of characteristics required of the final formulation. It also presents general considerations for commercial formulation of several types.

1.3.1 Formulation Identification

While developing a formulated product, the formulation scientist must begin with the end in mind. The formulation strategy is established by addressing a host of

questions. The following list presents a general set of the types of questions that must be answered:

- Based on the expected pharmacokinetics and pharmacodynamics, what is the intended use of the drug?
  - Is the drug intended for bolus delivery or infusion?
  - Is the drug intended for intramuscular or subcutaneous injection?
  - Is the drug intended for ophthalmic, inhaled, or an alternative route of delivery?
- What is the intended setting of delivery for the drug product?
  - Is the drug intended for hospital or clinic use only?
  - Is the drug intended for home delivery?
- What other important considerations are associated with the use of the drug product?
  - Is the drug a rescue medication, or is it a drug that is dosed daily that may be well suited for a prefilled syringe? Examples of prefilled syringe types of drugs are daily insulin doses or epinephrine in an auto-injector for anaphylaxis rescue.
  - Is the drug to be delivered as an IV bag or concentrate? Concentrates require attention to the risk of improper dilution and the potential for overdosing.
  - Will the drug be heavily used and, therefore, best developed as a multidose formulation? Local anesthetics are good examples of heavily used candidates for multidose formulation, but many medications are also candidates.
  - Is the drug intended for long-term delivery? If so, a depot formulation should be considered. Depot formulations are typically suspensions such as the aqueous polyethylene glycol system Depo-Provera or the nonaqueous vegetable oil system Depo-Testosterone.
    - If the product will be a depot formulation, is the depot product a suitable candidate for a nanoparticle or polymeric carrier approach?

Not all of these questions may have answers at the start of a program. Nonetheless, these and many more must be considered before beginning the development phase. Discounting or disregarding the ultimate use can result in wasted time and resources in the early phase of the project.

Unfortunately, some of the questions can be answered only by obtaining clinical data. In this situation, the development team must agree on a most likely scenario and, accordingly, on the most appropriate development strategy. In addition, the team must keep in mind that their agreed upon strategy may change over the clinical program as information is gathered through clinical use.

Once the requirements of the final formulation are established, the challenge of developing the commercial formulation can begin. Commercial formulation challenges include establishing, and in some cases overcoming, any solubility

requirements by preparing the appropriate dosage form. An appropriate dosage form might be an aqueous-based solution, a lipophilic-based system, a suspension formulation, a dry powder or lyophile, an implant, or some other alternative product.

1.3.1.1 Aqueous Solutions

Undoubtedly, the biggest hurdle in formulation development is solubility (Liu 2008). Solubility can be a double-edged sword. Apparent incompatibilities often arise from solubility issues. The following questions represent some of the issues that can be encountered.

- How do we formulate a compound for bolus or infusion delivery if the compound is poorly soluble?
- How do we deal with a drug intended for long duration dosing, e.g., depot, if it is too soluble?
- What if the salt form that is most soluble is also the most irritating, causing injection site issues?
- What if the API is only stable in its acid or base form and not as a soluble salt?

If an aqueous solution formulation is required and the API is poorly soluble, the salt form must be judiciously chosen. The process of salt form selection can raise additional complications and questions, as represented here:

- Can a salt be created in order to achieve the desired solubility without being on the border of saturation?
- If the choice of salt is driven by pK_a, is the resulting compound acidic or basic?

Typical salt counterions (hydrochlorides, phosphates, acetates, etc.) are not always an option due to stability issues or for physiological reasons. Some problems can be avoided by choosing a salt counterion that has a history of use. If the salt counterion being considered has not been previously used in a product, additional safety studies may be required, potentially draining resources and using valuable development time.

There are also many methods of improving solubility by using one of a myriad of excipients and solvent systems that are available. Surfactants are one way of improving solubility issues, as are cyclodextrins (Mishra et al. 2009; Shi et al. 2009).

Several marketed drugs use surfactants to solubilize and stabilize the administered product. Some of the more notable products include Taxol®, Taxotere®, Sandimmune®, and vitamin K. These systems use Cremophor® and polysorbate 80 to maintain the drug in solution. In particular, the Taxol and Taxotere use the surfactant micellar system to solubilize the drug at the point of administration—in the IV bag. Without the presence of the surfactants, many drugs precipitate almost immediately at the concentrations used for administration. However, in the presence of the surfactants, the drugs are kept in solution for several hours, allowing time for preparation and infusion.

Surfactants, however, are known to cause hypersensitivity reactions in some patients. Reactions can sometimes be fatal. Therefore, alternative modes of solubilization are still desirable. Cyclodextrins are also excellent in solubilizing insoluble

drugs, by encapsulating the drug within the central cavity of the cyclodextrin molecule. Thus, cyclodextrins can often provide much higher levels of solubility than first reported for the native molecule (Uekama et al. 1998). Chaudhari et al. have published an article combining cyclodextrins with a cosolvent (Chaudhari et al. 2007). Sreevallia et al. have published a review article on the subject, noting several examples in which cyclodextrin use is increasing, both as a solubilizing agent and on its own (Sreevallia et al. 2005).

The azoles, a series of antifungal compounds, are notorious for being insoluble. They are also well known to be very soluble in the presence of cyclodextrins. VFEND® (voriconazole) for injection is an example of a product using a cyclodextrin as a solubilizing agent. Voriconazole is considered only "very slightly soluble" by USP standards, but with the addition of the cyclodextrin, sufficient amounts are solubilized to allow delivery. Stella et al. (Stella and He 2008) note that compounds that are soluble in as little as 1 μg/mL quantities can be dissolved at levels as high as 1 mg in each mL when using cyclodextrins.

However, some cyclodextrins are known to be nephrotoxic. In these cases, cosolvent systems may also be an option (Jouyban et al. 2007; Stephens et al. 1999; Strickley 2004). Cosolvents are water-miscible organic solvents that can be used to increase the solubility of poorly soluble drugs in an otherwise aqueous solution. The use of glycerin, ethanol, or polyethylene glycol is not uncommon. Keep in mind, however, that the use of solvents such as ethanol has potential issues. First and foremost among the issues is patient sensitivity. Cosolvents can impact tonicity, tissue irritation, and toxicity. Another serious consideration is manufacturability. Ethanol is typically used in its absolute form. In that form, it is extremely flammable. Therefore, consideration to operator safety is imperative. Some groups have published articles outlining a statistical approach to formulation optimization using cosolvent systems (Stephens et al. 1999).

Additionally, cosolvents can enhance stability in situations where hydrolysis is an issue or where polar intermediates are an issue (Rubino 2006). However, attention must also be focused on administration. Similar to some surfactant systems, the formulator must assure that the drug will not hydrolyze or precipitate when diluted for administration.

1.3.1.2 Lipophilic Systems

If a cosolvent system does not provide adequate solubility or if the desired dose is not intended as an immediate release dose, the API can be dissolved in a suitable vegetable oil for long-acting release. Common excipients used in lipophilic formulations include sesame oil, soybean oil, and cottonseed oil. Delestrogen® (estradiol valerate) is an example of an oil-based formulation. Delestrogen contains benzyl benzoate and castor oil as the basis for the vehicle. The drug is injected intramuscularly. Formulating a product in this manner allows for monthly dosing and prolonged release. Other products, such as testosterone and fluphenazine, are similarly formulated for depot dosing.

In cases where a prolonged release is not desired, insoluble products can be formulated as emulsions or liposomes. An emulsion is a system containing two

immiscible liquids in which one is dispersed in the form of very small globules throughout the other. The emulsion may or may not be stabilized with a surfactant or, most commonly, a lecithin. Total parenteral nutrition products contain combinations of plant oils and lecithin to provide fats and nutrients to the patients.

Propofol, probably the most widely used drug product emulsion, consists of the drug contained in a soybean oil carrier in an egg lecithin and water emulsion.

In contrast to emulsion globules, liposomes are discreet vesicles made up of a phospholipid bilayer (Allen and Cullis 2013). They can be unilamellar or multilamellar and range in size from nanometers to microns. Liposomes should not be confused with micelles, which are unilayer. Liposomes can deliver small or large molecules (Swaminathan and Ehrhardt 2012). Drugs can be "captured" inside the lipophilic layer of the liposome and released over time by various mechanisms. This allows higher drug loading, better targeting, potentially reduced side effects, and the potential to deliver compounds that may not be amenable to parenteral delivery. The earliest approved liposomal systems included Abelcet® and Ambisome®, used for the delivery of amphotericin B. Another excellent example of the impact a liposome can have is doxorubicin. Traditional doxorubicin has severe side effects, limiting the dosing. As the liposomal formulation Doxil®, the side effects of doxorubicin are greatly reduced. Consequently, doses can be much higher.

Though there are great advantages to having liposomal formulations of toxic drugs, there are only a few products approved by the FDA to date. Challenges include the complexities of the manufacturing processes required to prepare reproducible supplies of the drug. The FDA has provided outlines on the development of liposomal formulations in order to assure the safety of the patients during testing (FDA Guidance for Industry 2002). Later chapters in this book will address the details and challenges of liposomal product development.

1.3.1.3 Suspensions

Another mechanism for delivering a delayed release dose is through the use of suspensions. An intentionally insoluble form of the API is selected and compounded as a finely dispersed powder in a vehicle. The vehicle may be an aqueous- or oil-based system. Such products may need a suspending agent in order to prevent agglomeration or particle size changes. In the phenomenon known as "Ostwald ripening," the drug compound comes to equilibrium between solid and dissolved material. The higher curvature of the smaller particles causes them to tend to dissolve, and the larger particles receive this dissolved material and grow in size. Since bioavailability is greater as the particle size decreases, growing particles is definitely not a desirable attribute in order to maintain the targeted bioavailability.

Examples of suspension injections include Celestone® Soluspan® (betamethasone) suspension injection and triamcinolone acetonide suspension injection USP.

Celestone Soluspan contains two forms of the active ingredient, sodium phosphate and acetate. In addition, it includes edetate disodium and benzalkonium chloride as a preservative. All are combined in a phosphate buffer adjusted to a pH of ~7.

Kenalog®, a formulation of triamcinolone, combines polysorbate 80 with sodium chloride for isotonicity, benzyl alcohol as a preservative, and carboxymethylcellulose sodium as a suspending aid.

Nanoparticles are being studied extensively as a way to deal with solubility and side effect issues. Commonly used in oral delivery to improve the overall bioavailability, nanoparticles are also being used in injection, ophthalmic, and other presentations (Diebold and Calonge 2010; Müller et al. 2011; Wong et al. 2008). For parenterals, often the drugs are conjugated to a polymeric nanomaterial. This conjugation allows for reduced toxicity as well as, in some cases, longer circulation times. Abraxane®, a nano-system of albumin-bound paclitaxel, is considered one of the first successful nanoparticle injections and was approved by the FDA in 2005. This formulation primarily contains just the drug bound to the albumin along with excess albumin adjusted to an appropriate pH.

1.3.1.4 Dry Formulations

When the compound of interest is too unstable in an aqueous vehicle and oil is not suitable for the dosing scheme, the formulator can explore lyophilization, freeze-drying, of the formulated liquid product (Nail et al. 2002). More information will be presented later in this book, but lyophilization is a field of study unto itself. However, many of the studies referred to in this chapter will still apply to the compound solution before lyophilization. In order to make use of lyophilization, tonicity, pH, short-term bulk stability, and product contact sensitivity must all be understood. Additionally, the use of cosolvents must be considered in order to improve cake quality and reconstitution times.

In situations where the product is not stable enough even for the short exposure to an aqueous system that lyophilization provides, dry powder filling can be an option (Hofmann 1986). Few products are filled as sterile powders because of the complexities and difficulties faced in the process. For example, the API must be received sterile and transferred in the sterile area. The powders must be managed in the filling process to keep the particulates within controlled levels during filling while preventing foreign contaminants. This is a tedious process and requires special equipment and engineering studies.

1.3.1.5 Implants

Implants are developed when the disease state requires a drug delivery time that is much longer than the drug's normal elimination from the body. Delivery time requirements can extend from days to months or even years. For example, Norplant® was an implantable silicon tube filled with levonorgestrel. Norplant was designed as a birth control device that delivered the drug over long periods of time. Later on, the implant (Norplant II, Jadelle®) was further developed with a polymer matrix device containing the same drug, and data show delivery of the active for up to 5 years.

There are very few implant devices approved for use, though there are several polymeric devices that are considered implants. Zoladex®, for example, is a polylactic acid/glycolic acid rod that delivers goserelin acetate for endometriosis for breast cancer in women and for prostate cancer in men. Nonetheless, a variety of studies are being performed in the area of implantable delivery systems. Studies range from polymer reservoirs to long-term insulin pump systems.

1.3.2 Formulation Development

As discussed above, formulation development typically starts with consideration of the solubility of the drug as it might relate to the mode of delivery. This analysis leads formulators to the type of dosage form required. Each dosage form requires a series of excipients or other ingredients that are not the active ingredient but are justified for the development of that dosage form (Rowe 2012). In any parenteral formulation, the use of nonessential ingredients should be avoided. This section presents general considerations for several types of excipients, including buffering agents, tonicity agents, antioxidants, preservatives, and bulking agents.

1.3.2.1 A Note on Excipients

When selecting excipients, the formulator should assure that any considered excipient is approved for use by the FDA. New excipients can be used, but they will require extensive toxicological evaluations in order to prove safety. The FDA provides an inert ingredient database (http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm) that lists excipients and the ranges of their use in approved products. Selecting from and conforming to this guide will help minimize development time and reduce the expense of additional toxicological studies.

Other chapters in this book will address the details for developing the various dosage forms. Remington's pharmaceutical handbook may also be a great help to the pharmaceutical formulator (Gerbino 2005). This chapter, as an overview, will discuss the general excipient decisions that are typically made during the development of a parenteral product.

1.3.2.2 Buffering Agents and pH

In aqueous systems, the effect of pH must be assessed. If at all possible, the pH of the system should be set to as close as physiological pH (~7.4) as possible. However, many systems require an alternative pH in order to assure the stability of the drug itself. Thus, questions such as the following must be addressed:

- Is there a pH range where the solubility is optimum?
- Is there an optimum pH for stability?

By preparing multiple solutions representing a pH range and studying stability when each solution is subjected to stress conditions like heat, pH can be narrowed to an optimum range.

In addition to the pH value, the strength of the buffer used to prepare the formulation should also be considered in relation to the location of the injection or dose. For example, if the dosage form is intended for intravenous use, the buffer capacity of the product should be considered relative to blood (Ellison et al. 1958). If the pH of the formulation is similar to blood, this concern is less of an issue. However, if the pH of the formulation is substantially outside the physiological range, the buffer strength of the formulation should be sufficiently low to ensure no significant change to the blood at the site of administration.

A pH analysis should be made for other delivery routes as well (Agarwal et al. 2002; Irani 2008; Moskowitz et al. 2007). Each delivery route (inhalation, intrathecal, intra-articular, ophthalmic, etc.) has specific issues that must be addressed with regard to pH and buffer capacity.

Typical buffers used in parenteral systems include phosphate, citrate, and acetate buffers (Gerbino 2005). Sodium or potassium salts are also commonly used. Buffer choice depends on compatibilities of the buffer system and the type of process intended for manufacture. For example, phosphate buffers are typically not used for lyophilized materials because the pH changes dramatically over the course of the very low temperatures experienced in the lyophilization process. Similarly, acetate systems are not always used in lyophilization because the acetate buffer may tend to flash off during the lyo process.

The analyses discussed above apply to both the biocompatibility of the formulation to the patient and to the stability of the drug substance. In addition, proteins and peptides are particularly sensitive to pH and to buffer choice (Carpenter and Manning 2002). Small shifts in pH and slight differences in buffer materials can result in undesirable unfolding and, consequently, instability or inactivity of the molecule. Often, protein and peptides are also sensitive to the overall ionic content of the formulation. Hence, care must be taken to optimize and balance the ion level in the formula while ensuring an appropriate buffer counterion and stabilizing pH.

1.3.2.3 Tonicity Agents and Osmolality

Similar to pH and buffer capacity, the tonicity of the formulation should be addressed in order to assure that the product is compatible with the tissues at the administration site (Agarwal et al. 2002; Gerbino 2005; Irani 2008; Moskowitz et al. 2007). The easiest way to assure that a drug substance is infused as an isotonic product is to administer the product in 0.9 % sodium chloride injection or in 5 % dextrose solution.

However, a more complex formulation is required at times, or a concentrated bolus dose of a solution formulation is necessary for rapid delivery. In such cases, sodium chloride is the most common tonicity-modifying agent added to the formula in order to assure biocompatibility at the injection site.

Even so, sometimes the molecule is not compatible with the chloride or the ions in general. In those cases, sugars are often used, in particular mannitol or trehalose. These sugars offer the advantage of having no troublesome ionic content that can affect molecules, in particular proteins and peptides. In addition, sugars offer an aesthetically pleasing cake for lyophilized products.

Regardless of the tonicity agent used, a target range from ~275 to ~320 mOsm/kg is typical for formulation development. This target range ensures that the tissues at the injection site will not be disrupted and that the drug product will not be painful during administration.

1.3.2.4 Antioxidants

Oxidation of the drug substance is a common challenge during the development of many drug products (Gerbino 2005; Tonnesen 2004). The simplest way to minimize oxidation is to replace the oxygen in the package with an inert gas such as nitrogen or argon. The headspace of the vial/container can be replaced with nitrogen during the filling process. If the drug substance is more sensitive, then the bulk product vehicle is sparged with nitrogen during the processing in order to displace the oxygen and minimize any degradation occurring during manufacturing.

In some instances, nitrogen sparging or headspace replacement is not sufficient to ensure the long-term shelf life of the product. In these cases, additional excipients must be added to scavenge the free radical oxygen atoms and prevent the degradation of the drug. Components such as butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) might be added. Typically, only very low levels of these excipients are used. The range tends to be between 0.0003 and 0.03 %. Extremely oxygen-sensitive products may benefit from an antioxidant such as bisulfites; however, the patient population must be considered in order to avoid reactions.

In addition, the product may be sensitive to trace metals that may cause oxidative reactions with the drug. Ethylenediaminetetraacetic acid (EDTA) is a good chelator at low concentrations. Citric acid may also be used to chelate metals, thereby improving the stability of the drug in the product.

1.3.2.5 Preservatives

If the product is intended for a multiple use presentation, an antimicrobial preservative should be considered (Meyer et al. 2007). Care should be taken here in considering the patient population. For example, neonates can react to benzyl alcohol, a commonly used antimicrobial preservative.

Cresols are also used for their antimicrobial properties. In particular, metacresol is common in biotechnology products. Benzalkonium chloride is also used in various parenteral applications such as ophthalmics and inhalation solutions. In addition, methyl and propyl parabens are also sometimes used in conjunction with each other for preservation of parenteral products.

Regardless of the preservative used, the formulator must still prove the antimicrobial nature of the excipient within the product itself. This means that the antimicrobial preservative effectiveness test should be performed on the drug product with a variety of levels of the antimicrobial present in the drug product. For example, testing should be performed at the target level as well as several levels below the target concentration. It is not necessary to show the concentration at which the preservative effectiveness fails. However, it is important to show that the preservative system is still effective at levels far below the target level. The specification for the preservative can then be set based on the results of the antimicrobial testing. In this way, the robustness of the formulation can be proven.

However, the antimicrobial preservative test is a labor-intensive, 28-day test (Moser and Meyer 2011). Therefore, once the lowest level of preservative effectiveness is shown, an alternative method for confirming an effective limit can be put into place. Typically, the alternative method is UV/HPLC. The lower limit specification for the preservative is then confirmed using this alternative test, but the alternative is predicated on the results of the antimicrobial work discussed above.

1.3.2.6 Bulking Agents and Cryoprotectants

Some products are so hydrolytically labile that they must be lyophilized to ensure long-term shelf life stability (Nail et al. 2002). Often, the level of drug substance in these products is in milligram quantities and is not sufficient to provide an elegant-looking cake. Sometimes, the level of the drug substance is even microgram quantities and cannot even be seen in the vial by the clinician. Therefore, an excipient is used to create the cake so that the vial appears to have product and give a visual indication that the product is in good condition. These excipients are called “bulking agents.”

Bulking agents range from various amino acids to sodium chloride to a host of sugars. Glycine is an example of an amino acid that is used for bulking. However, amino acids are expensive. Sodium chloride can be difficult to freeze-dry, depending on the circumstances. Therefore, sugars are the most commonly used bulking agents.

The most common sugar bulking agent in sterile product development is mannitol. It is simple to handle and relatively easy to freeze-dry because mannitol/water solutions have a eutectic point just below 0 °C. Therefore, freeze-drying can be done at a reasonably high temperature and completed in relatively short periods of time.

Sucrose is also used for various protein products; however, freeze-drying sucrose-based products can be more challenging because the glass transition temperature of sucrose/water solutions changes as the concentration of sucrose increases during the drying of the water. At times, though, the use of sucrose is warranted because the stability of the drug substance is improved using sucrose rather than mannitol.

Trehalose is also used, in particular for antibody formulations such as Avastin® and Herceptin®. Trehalose, like mannitol, is also fairly easy to dry. The use of trehalose is likely to become more common as the industry gains more field experience with its use.

Most sugar bulking agents are also known to protect proteins and other drug substances sensitive to freezing, hence the term cryoprotectant. The alcohol groups on the sugars interact with various functional groups on the proteins, maintaining the proteins' conformational structure during the extremes of drying. The interaction prevents aggregation or agglomeration of the protein that might be observed at the point of reconstitution. It also ensures the overall efficacy of the drug for long-term shelf life.

1.4 Stability and Compatibility

During the formulation development program, many formulations are studied in parallel in order to determine which offers optimum stability and compatibility. Based on the parallel studies, a final formulation is defined. Once this final formulation is chosen, more detailed studies demonstrate the range of stability during processing and for long-term shelf life. The results of these studies include establishing the required levels of headspace oxygen to confer oxidative stability over the shelf life, the impact of high temperature, the impact of low temperature, the effect of light on the formulation, and any interactions between the formulated product and the materials of construction used in the processing equipment and used in the packaging components. Of course, the finished product must also meet the general ICH stability requirements.

1.4.1 Oxidation

When headspace oxygen is to be minimized, studies are required to determine the allowable level of oxygen. Samples are prepared in an isolation chamber, such as a glove box or small lyophilizer, in which the oxygen level in the atmosphere can be adjusted and measured. The experimental design includes at least three treatment groups: 5, 10 %, and ambient (~21 %) oxygen content. All three groups are placed into an accelerated stability chamber, preferably in an upright position to avoid any stopper influence, and monitored for degradation. If differences in oxidation are observed, further studies are conducted to establish the acceptable limits in headspace oxygen. The limits will later be used as processing parameters.

Often, other parameters may also influence stability in conjunction with oxidation (Kasraian et al. 1999). The impurities in the excipients, the formulation pH, and the packaging may all influence the rate of oxidation in the drug product. A simple experimental matrix design that examines both oxygen headspace content and pH is presented in Table 1.1 below.

For the design above, the high pH and the low pH are set at the label limits. The assay includes potency, related substances, and appearance or other CQAs defined for that formulation and drug substance. Similar experiments would also be

Table 1.1 Experimental design: oxygen headspace study and pH matrix

Condition	2 weeks 40 °C	4 weeks 40 °C	6 weeks 40 °C	8 weeks 40 °C
Ambient O_2/low pH	Assay/pH	Assay/pH	Assay/pH	Assay/pH
Ambient O_2/high pH	Assay/pH	Assay/pH	Assay/pH	Assay/pH
10 % O_2/low pH	Assay/pH	Assay/pH	Assay/pH	Assay/pH
10 % O_2/high pH	Assay/pH	Assay/pH	Assay/pH	Assay/pH
5 % O_2/low pH	Assay/pH	Assay/pH	Assay/pH	Assay/pH
5 % O_2/high pH	Assay/pH	Assay/pH	Assay/pH	Assay/pH

conducted to examine the levels of the antioxidant, the contribution of impurities from excipients, and other factors to ensure the drug substance is appropriately protected throughout the course of the shelf life and the use of the product.

1.4.2 Thermal Stability

The final formulation must demonstrate that it can withstand an appropriate range of temperatures during manufacturing, shipping, and use. These temperature ranges can be quite extreme, ranging from sterilization temperatures of >120 °C to deep-freezing temperatures as low as −80 °C, depending on the product.

An obvious prerequisite for a parenteral product is sterility. The most desirable method of sterilization is terminal sterilization. In fact, the regulatory agencies require terminal sterilization or an assessment justifying why it is not appropriate for the product. Consequently, the ability of the product to withstand thermal stress must be assessed when considering autoclaving for terminal sterilization. The easiest approach is to prepare samples and process them at 121–122 °C, the temperature typically used for moist heat terminal sterilization. The duration of exposure is typically 15–20 min. If no degradation or other deleterious effect is noted (such as pH shift or discoloration), longer autoclaving cycles should be explored in order to determine an upper limit and establish a sufficient window of robustness.

If the product is able to withstand terminal sterilization, then stressing at lower temperatures typical of standard forced degradation studies adds little to understanding the product. However, if terminal sterilization is not an option, lower temperature forced degradation stress testing must be carried out (Maheswaran 2012). Typical stress conditions range from 40 to 60 °C for a period of weeks to months, depending on the sensitivity of the drug product. For example, a typical study could be 8 weeks long at an elevated temperature with a test done every 2 weeks. Upright and inverted positions should also be assessed. In extremely sensitive products, room temperature or even 15 °C can be the stress condition when 2–8 °C is considered the long-term label storage condition. Regardless of the temperature range, a study design might use the format shown in Table 1.2 below.

While it is clear that high temperature can degrade a product, the astute developer also recognizes that extremes of low temperature must also be considered.

Table 1.2 Accelerated temperature stress study

Sample times	2 weeks	4 weeks	6 weeks	8 weeks	12 weeks
Assay	X	X	X	X	X
Related substances	X	X	X	X	X
pH	X	X	X	X	X
Color/clarity	X	X	X	X	X
Particulate matter	–	–	X	–	X
Others[a]	X	–	X	–	X

[a]Other parameters (such as headspace oxygen, antioxidant, and preservative levels) may also be examined in the protocol, depending on the sensitivity of the product

Low-temperature studies are obviously required for lyophilized products where temperatures are cryogenic, ranging from −70 to −80 °C (Kasper and Friess 2011). However, low-temperature studies are also important for confirming the stability of the product during extremes in shipping conditions. For example, if drug product freezing inactivates the drug (e.g., causes precipitation), the user may not be aware, and the patient may not receive of a full dose. Worse, particles may be injected into the patient, creating potential for emboli formation. Low-temperature consideration should be given to both the drug within the product *and* to the container. If a glass container freezes, it may develop micro-fractures, and sterility could be compromised. However, if a container is unknowingly frozen and then thawed, as may happen in shipping, the user will not be aware of the potential for problems. Thus, the effect of low temperatures and freeze/thaw on the product should be evaluated.

In general, a series of samples are frozen for 24 h and then held at room temperature for 24 h. Some of those vials, whatever number of vials is required for testing, are removed from the test set and stored separately at an appropriate condition. The removed vials are marked “Cycle 1.” The remaining vials are then refrozen for 24 h and then thawed and held at room temperature for 24 h. Again, the required number of vials is removed from the test set. The removed vials are marked “Cycle 2” and stored appropriately. In the third cycle, the remaining vials are frozen for 72 h and then thawed and held at room temperature for 24 h. The vials are then collected, marked “Cycle 3,” and stored appropriately. At the end of each cycle, the vials and their contents should be inspected for physical changes, such as precipitation.

If time and resources are scarce, often only the Cycle 3 samples are tested for assay and related substances as well as appearance, subvisible particles, osmolality, and other appropriate CQAs. If the Cycle 3 samples pass, then Cycle 1 and Cycle 2 materials may not require testing. However, as noted above, after each room temperature period, the vials of that cycle should be inspected for physical changes.

The study design above accommodates short-term (1 day) and longer-term (3 days) freezing. It also accommodates a 5-day workweek, where Cycle 1 is started on a Monday, allowing for the freezing step of Cycle 3 to occur over a weekend.

It is interesting to note that prefilled, premixed IV bags are often frozen. Companies are offering a host of ready to use IV bags containing premixed drug products that typically have a very short period of usage at room temperature

(http://www.ecomm.baxter.com/ecatalog/browseCatalog.do?lid=10001&hid=10001&cid=10016&key=3480b31688beaeb98335eb9d7c46aa6). Freezing these products increases shelf life and saves time in the hospital pharmacy. In addition, some protein products are provided to the clinic frozen in vials due to their instability at any other temperature.

1.4.3 Photostability

Interestingly, many drugs can be very sensitive to light, be it in a solution or a solid state (Tonnesen 2004). Therefore, ICH has developed Guideline Q1B (FDA Guidance for Industry 1998), which defines the conditions for photostability testing. Two options are presented. Option 1 uses a chamber with concurrent white and UV light exposure. Option 2 allows for the use of a system that tests each condition independently. The Option 1 chamber's use of xenon or metal halide lamps may cause it to operate at a higher temperature than an Option 2 system. Data analysis can be easier using Option 2 because the impact of UV light is assessed independently. In all cases, proper controls must be run. One easy way is to simply wrap control samples in foil in order to protect them from the light then expose the wrapped samples along with the test samples. This way, degradation due to thermal effects alone can be determined, allowing the formulator to determine the effects of light alone in the non-control samples.

1.4.4 Material Contact Compatibility

The compatibility of the drug product with the materials with which it comes into contact must not only be assessed in relation to the primary packaging (e.g., a rubber stopper, a glass vial, or a plastic container), but it must also be assessed in relation to the materials to which the formulation will be exposed during manufacturing. For example, the formulation will likely be compounded in a steel tank, so the formulation's sensitivity to the appropriate grade of stainless steel must be evaluated. In addition, the filling system may have diaphragms or gaskets in the pumps, and the filtration system will include tubing and various filter materials and components. Is the operation being carried out in a disposal bag system? If so, the compatibility with the disposable bag system should be addressed. In all cases, appropriate studies must be conducted to assure product integrity.

1.4.4.1 Primary Packaging

The selection of an optimum closure system is critical. Ideally, a vial/stopper combination must be found that will provide both a sterile closure system and long-term

stability for the product. Some products require glass ampoules, which avoids any leachables or extractables from a vial's elastomeric closure system; however, a stoppered vial is the first choice. Stopper screening consists of exposing under stress conditions a series of potential elastomeric formulations to the lead formulation. For example, exposure of the lead drug product formulation to the elastomeric formulations at temperatures from 40 to 60 °C might be performed. The major stopper manufacturers can provide some guidance on elastomer selection and the requirements for meeting compendial standards, such as USP <381>. Nonetheless, it is still the obligation of the formulator to demonstrate that any manufacturer-recommended closure system is acceptable.

Once the elastomer has been selected, it must be qualified for use. Qualification includes confirming sterility assurance by demonstrating container closure integrity as well as by confirming physicochemical compatibility. Testing for container closure integrity can be done using either a dye ingress or a microbial ingress method (FDA Guidance for Industry 1999, 2008a).

Determining compatibility of a solution with an elastomer entails both assuring physical compatibility and chemical compatibility. Stopper elastomers can interact with the active ingredient, which causes degradation of the API, shifts in pH, introduction of undesirable materials, precipitation of material, and loss of material due to absorption or adsorption. In some cases, the product is so sensitive that a barrier is needed between the elastomer and the solution. Barrier options range from Teflon to polymeric coatings. In cases where a barrier is used, understanding the interaction of the base elastomer with the product is helpful because if the barrier is ever compromised, the resultant effect can be explained. USP <381> lists a series of tests, conditions, and specifications. This USP monograph also specifies who is responsible for the testing—the vendor, the user, or both.

USP Type I glass is the primary material for injection vials or ampoules; however, the use of plastic containers is increasing. A host of other packaging presentations are also common. For example, prefilled IV bags and prefilled syringes are more and more prevalent. Containers filled via blow/fill/seal technology are also on the rise. Pen injectors often have multiple components, including the glass cartridge, the needle assemblies that deliver the product, and the internal and external stoppers within the cartridge. Other products, such as implants, ophthalmics, and inhaled products, must also be shown to be compatible with the plastics, metals, and lining materials used for packaging.

Care must be taken with each of these materials, even with glass, to understand any potential interactions between the container and the product. Considerations such as oxygen migration, label adhesive migration, leachable material, extractable material, sterilization, and clarity are all issues to be examined.

1.4.4.2 Equipment Materials of Construction Compatibility

During the manufacturing process, the product will come in contact with a variety of equipment made from a variety of materials. Vessels, tubing, pumps, and filters

can be made out of steel, silicon, elastomers, and plastics. Federal regulations require the formulator to show that each of these materials does not interact deleteriously with the drug product (Code of Federal Regulations).

Stainless steel is the material of choice for compounding and storage of product, pump housings, fixed piping, and filter cartridge housings. To assess the compatibility of the product with stainless steel, coupons or strips of stainless steel that are of the same quality and finish as the manufacturing equipment are placed into contact with the formulation for extended periods of time, which depend on the estimated time of exposure during the overall process. Samples of the formulation are taken and tested in order to determine if any potency losses or increases in degradation are observed. Such studies should be performed at the temperature at which the process is expected to be run. Testing duration should exceed the expected process time in order to allow for the inevitable delays that will be experienced during manufacturing.

Platinum-cured tubing is often used for pumping and filling product. Tygon tubing may also be used; however, platinum-cured silicon tubing is typically more compatible with a wider variety of products.

Tubing does not usually cause degradation, but it can cause a loss of critical materials because both the active ingredients and the excipients can be removed from solution by adsorption onto the tubing surface. To assess tubing and formulation compatibility, lengths of tubing adequate to hold enough of the formulation for testing are sterilized or otherwise prepared as they would be in the production environment. One end of the tubing is sealed, either with a clamp or a glass stopper. The tubing is filled, and the other end is likewise sealed. The tubing is then held at the process temperature for a number of predetermined periods of time that are linked to the manufacturing conditions. At each time period, a length of tubing is emptied and tested for the potency of the active ingredient and/or excipients. If a loss of material is observed, loss data must be shared with the production personnel so that they can establish the period of time after which the equipment must be flushed if a line stoppage occurs. This flushing ensures that the low potency product is removed from the line and that the product will be fully potent during filling.

When testing the compatibility of the tubing, the question of potential leachable and extractable material may also be raised. In this case, the materials stored in the tubing are tested for the appropriate leachate. Often, the tubing vendor can assist with putting into place the appropriate test methods.

Filters are another source of potential interaction (PDA Technical Report No 26 2008). Solutions that contain surfactants, organic cosolvents, or all organic vehicles are of special concern because they can leach, or even dissolve, material from the membrane and/or housing, which sometimes ruins the product as well as the filter. To assess these effects, small-scale filters are filled with solution and held at the processing temperature for an extended period of time. These filters are then placed into a water bath, pressurized, and observed. Streams of bubbles may indicate leaks in the housings. In addition, the membrane is tested by performing a standard integrity test in order to confirm that the porosity of the membrane has not been affected by the prolonged presence of the formulation vehicle. An integrity test is typically

a bubble point test using either the filter wetted with the bulk solution product or the filter flushed with water to remove the bulk solution product.

Just as the vehicle can affect the filter, the filter has the potential to contaminate the formulation vehicle with leachable or extractable material. Again, working with the vendor can help address this, typically as part of the filter validation study.

Finally, similar to some of the studies previously described, the propensity of the drug and/or critical excipients to adsorb onto the filter must be addressed. Two types of studies can be performed—a static study and a dynamic study. In the static study, the bulk drug product solution is placed into the filter and held for extended periods of time. In the dynamic study, a specified volume of bulk solution is recirculated repeatedly through the filter. During each study, samples are taken at specified time intervals. For each sample, measurements of the concentration of the drug/excipient are made in order to determine drug and excipient losses. When losses are too great for the product to be filtered and filled in parallel, the filtration may be performed completely into a bulk receiving tank prior to filling so that losses are minimized. If a bulk receiving tank is not available, the bulk vehicle may be flushed through the filter system until the drug/excipient adsorption reaches saturation and the level of the drug/excipient in the bulk solution is at label strength. If neither of these approaches are an option, then alternative filter systems should be explored.

1.4.5 General Long-Term Stability Considerations

The studies discussed above represent one-time studies that are required for the registration of the formulation with the Health Authorities. Of course, specific long-term stability studies are required for full registration of the product, as outlined in the ICH Q1A(R2) guidance (FDA Guidance for Industry 2003). Nonetheless, long before the product is scaled up for registration stability, it is advisable to gather preliminary long-term stability data on lab scale batches, examining at the least the assay, the related substances, and the appearance. Other parameters critical to the quality of the product should also be identified and monitored. Although some formulators use preliminary studies at only the accelerated condition for several months, it is advisable to store samples at the label condition, under refrigeration, or in another controlled condition because the samples can serve as controls for investigation if unexpected results are obtained.

Per the ICH guidances, the accelerated condition for a room temperature product is typically 40 °C/75 %RH for 6 months. Alternatively, 30 °C/60 % humidity for 12 months can be performed if the 40 °C condition is too harsh. In some cases, a lower humidity condition is more appropriate. For example, sterile products stored in plastic bottles may be required to be held at a lower humidity in order to examine the effects of moisture migration out of the bottle. For refrigerated products, 15 or 25 °C may be considered the accelerated temperature. Similarly, for frozen products, an appropriately chosen higher temperature may be considered as an accelerated temperature.

In general the stability data for the registration of the product is likely to include at least 6 months of accelerated data and at least 12 months of real-time stability at the label use temperature. Based on these data, the agency will approve the product for a 24-month shelf life. However, it should be remembered that for biotechnology products the FDA will provide a shelf life only as long as the real-time stability data that has been submitted. For example, if 18 months of data are submitted for the real-time shelf life condition, then the approval will be only for 18 months.

1.5 Processability and Scalability

As the development team gains more experience with the product during the development stage, they should always keep in mind the scalability of the product for commercialization. They should keep in mind proper equipment requirements that will assure ease in processing and general stability of the drug product as the manufacturing passes from each unit operation to the next. In particular, aspects of compounding, filling, and sterilization should be considered. Unique unit operations like lyophilization or homogenization that might be required should also be reviewed to ensure that the product lends itself to a scalable process.

1.5.1 Compounding

During compounding, many factors can affect the quality of the product. Issues can arise with simple differences in the order of addition, in the temperatures used for processing, in mixing speeds required for dissolution or dispersion, and in the shear stresses associated with the mixing itself. Each factor must be assessed in order to confirm that the most optimum approach is being employed.

When developing a product, the order in which ingredients are added can have a significant impact on the quality of the active ingredient as well as the overall quality of the product. For example, an antioxidant would be added to nitrogen-sparged water before the active is added to the water in order to ensure the active is protected as much as possible. If the salt of the API is going to be generated in situ, the counterion may need to be added first in order to help with solubilization of the active ingredient. Excipients such as surfactants would likewise be added before the active in order to assist in solubilization. In addition, pH adjustment may have to be made early in the process and then verified and adjusted later.

As the materials are added, the time and temperature required for full incorporation should be recorded whether full incorporation means fully dissolved for solutions or fully dispersed for suspension systems. Sometimes, the processing parameters acceptable to laboratory personnel are not appropriate at all for a production environment. For example, the production preference for the compounding steps is often completion within one or two work shifts in order to keep the

bioburden of the drug product to a minimum. Therefore, a formulation in which the dissolution of the drug substance takes several days will not be desirable in production. Similarly, temperature should also be monitored to assure that the final processing temperature is optimum for commercialization. For example, if water must be heated to nearly boiling in order to allow for dissolution of the drug substance or other excipients, the production facility might find this difficult to accomplish for a pharmaceutical parenteral product.

During the course of the addition of the materials, the mixing speeds and impeller types required to incorporate the drug and excipients into the vehicle should also be considered and recorded. Often, the type of mixer used in the laboratory is not similar at all to the impeller systems available in production. The result may be the selection of mixing speeds and times that do not scale well into commercial production.

Mixing speeds and impeller types can be particularly important in the manufacturing of protein and other biotechnology products. The effect of shear on the denaturation of proteins is well known. Therefore, care must be taken to assure that the processing steps are robust enough to prevent the aggregation or agglomeration of the protein products. Even small differences in mixing can create subvisible particles in the protein product that can serve as seeds for aggregation over the long-term shelf life of the protein product.

1.5.2 Filling

Several product parameters can affect the efficiency of the filling process. For example, high viscosity materials can result in large variation in fill volumes due to pumping variability. If a product is water-like in viscosity, filling is often done easily and accurately. However, some products, such as those with high levels of polysorbate 80, polyethylene glycols, or other more viscous materials, are more difficult to fill accurately. In these cases, special filling pumps can assist in maintaining an accurate fill volume over the course of the manufacturing. In addition, slower filling line speeds can sometimes help in maintaining fill accuracy.

In addition to affecting the accuracy of filling, the viscosity of the product can also affect the observed shear within the product. Even when viscosity is not an issue, shear effects can be observed during the filling process because the filling needles used to target the vial or package are usually much smaller than the tubing leading into them. This decrease in diameter has a net effect of increasing the shear stresses in the bulk product solution. Thus, the effect of shear during filling should also be monitored for shear-sensitive products, such as proteins, peptides, and other biotechnology drugs.

Filling line speed is often set based on the type of product a manufacturing site has the most experience with. For most parenteral products, this means an aqueous-based vehicle of low viscosity and negligible degradation over the course of compounding and filling. However, some products are particularly labile and must be

filled quickly. In these cases, the batch size and temperature of compounding and filling must be optimized with consideration of the time required for completion of these operations. Therefore, it is incumbent upon the formulator to collect data on these factors and provide those data to the production personnel in order to assist in designing an appropriate process.

Another key aspect in the filling process is establishing the headspace requirements. The details of the experiments associated with formulation development were discussed previously in this chapter. The results of these experiments become key because the capability of the fill line to maintain the recommended headspace oxygen level is tested at this point. The variables that affect the efficiency of maintaining headspace oxygen include the vial size, the fill volume, the line speed, and the gas used for the overlay. It is helpful for the formulation expert to work in close conjunction with the production personnel in order to ensure that the formulation is sufficiently stable based on the headspace oxygen results the fill line can attain.

1.5.3 Sterilization

As previously discussed, the Health Authorities prefer that products are terminally sterilized. Therefore, the formulator must make an assessment of the robustness of the product when exposed to terminal sterilization. This process is typically performed via moist heat sterilization (autoclaving), but it can also be accomplished via gamma irradiation, electron beam, ethylene oxide, or other methods. Regardless of sterilization method, the formulator must confirm that the degradation profile is acceptable.

For cases in which terminal sterilization is not possible, aseptic filtration is performed. Formulations that are sterile filtered must have a low enough viscosity that the bulk solution can be passed through a sterile filter that has at least a 0.2 μm membrane. In some cases, a 0.1 μm membrane may be required. In addition, formulations must also be compatible with the materials making up the filter membrane and housing. The formulation must not affect the integrity of the membrane or the housing, and the filter must not affect the quality of the formulation. These concerns were also previously discussed in this chapter.

1.5.4 General Processing Requirements

In addition to the requirements associated with the unit operations discussed above, the formulation expert must also keep in mind other aspects of the production requirements. These include:

- The general overall time and temperatures required for processing relative to the stability and degradation of the product

- The light sensitivities in the production facility
- And any relative humidity requirements or sensitivities

In addition, the formulator must work with the production personnel to address any requirements associated with unit operations such as lyophilization or high-pressure homogenization, depending on the product.

Clearly, the temperature under which a unit operation is completed is coupled with the time required for that step to be completed. As discussed above, for example, a dissolution step must be performed to complete dissolution. However, for an active ingredient that also degrades readily, this step may need to be performed at lower temperatures, which may also slow the dissolution itself. Usually, jacketing the vessel with coolant is sufficient control; however, sometimes compounding a cold room may be required. Clearly, optimization of this mixing time and temperature must be established, and often the formulation chemist is at hand assisting the process engineer in these studies.

Light sensitivity can also require similar studies in order to answer questions like the following:

- What wavelength of light is the drug most sensitive to?
- How long of an exposure is acceptable and at what light intensity?
- What types of changes in the production environment are required to meet the specifications for the product?

Though the first two of the above questions are in the realm of the formulation expert, the results of the formulator's studies will assist in answering the third question. For example, for some drugs, simply minimizing white light exposure is sufficient. This can be done by covering the vessels, tubing, or other transparent apparatus with opaque material and keeping finished vials in opaque bins. In other cases, the use of yellow light is required in order to minimize or prevent the degradation of the compounds.

The relative humidity of the production environment can also present a challenge. Sometimes, the drug substance and/or drug product may be very hydroscopic or hydrolytically labile. When the drug substance is highly hygroscopic, strict control of the relative humidity must be enforced in order to, at the very least, ensure that the proper amount of drug substance is added to the batch. For example, drug substances that deliquesce often require tightly controlled low-humidity environments in order to ensure that the drug does not liquefy prior to addition. Other drugs that readily absorb moisture in the atmosphere might require a specific standard humidity for the weighing out of the compound in order to control the moisture content for reproducibility of the final assay. In each of these cases, the formulation chemist is responsible for establishing what the limits of the relative humidity should be to ensure quality.

In the case of a hydrolytically labile drug substance, the product is usually manufactured as a lyophilized product in order to ensure minimal hydrolytic degradation over the course of the shelf life of the product. Here, it is up to the formulator to establish the lyophilization parameters under which production occurs. The details

of this development are addressed in a separate chapter. Suffice it to say that the thermal properties that govern lyophilization are part of the requirements. Thermal properties include glass transition, eutectic melt, and robustness to thermal cycling, which includes adequate freezing, potential pH shifts, and overall stability of the drug substance throughout production.

Not all formulations, however, are solutions. Some are micellar, emulsion, or even liposomal systems. Some products are drug substance particulate or suspension systems. Each of these unique formulation types has unique unit operations associated with it. In each case, the formulation expert must define a formulation that is robust enough to be scaled to commercial quantities. Less typical unit operations such as homogenization may require formulations that can be pumped at extremely high pressures—perhaps tens of thousands of pounds per square inch for hours at a time. High-pressure homogenization used for emulsions and suspensions is an example. Sometimes, in particular in drug substance suspensions, the pressure and time can create different polymorphs of the drug itself, rendering the entire formulation unprocessable.

Clearly, the formulation development does not end once a preliminary formulation has been defined. Many additional parameters must also be considered as they relate to the final production process.

1.6 Other Considerations

As mentioned at the outset of this chapter, the formulation chemist must always keep the final use in mind. This means that patient use studies must also be considered during the formulation development. These studies include, but are not limited to, admixture studies, patient in-use studies, administration device development, and administration device compatibility.

Admixture studies are stability studies that focus on the chemical and physical stability of the drug at the point of administration. These studies include stability and compatibility of the drug with the syringes, needles, IV bags, and tubing used for the injection or infusion of the product to the patient. Typically, the regulatory authorities require a potency of at least 90 % of the initial dilution in order to establish the acceptability of the administration.

However, the Health Authorities also approve an expiry for the admixture for reasons other than just potency. For example, if an admixture stability is maintained for more than 24 h, then the anti-microbiological properties of the product may be required to be effective for twice the amount of time that the sponsor requests. As an example, a product that shows chemical stability for 48 h may also be required to exhibit antimicrobial properties for at least 96 h in order to gain a label of 48 h. Similarly, products that tend to precipitate in the admixture bag may also be required to show stability for at least twice the time period listed in the product label insert. For example, a product may be required to maintain physical stability for at least 16 h in order to obtain the label for 8 h use, including the infusion time.

For products that are delivered using particular devices, the effect of human factors must also be addressed (FDA Guidance for Industry 2011). This applies to the design of injectable pens and other devices. The FDA guidance regarding human factors analysis states:

> The intent is to improve the quality of the device user interface such that errors that occur during use of the device are either eliminated or reduced." In addition: "…manufacturers conduct a risk analysis that includes risks associated with device use.

These analyses are performed based on an understanding of the device users, the environment in which the devices are used, and the interface between the device and the users. Any new device in development is required to have these issues addressed.

Oftentimes, the nature of the formulation may also impact or be impacted by the human factors involved even if the product is not a part of the device. For example, what happens to the product if the shipper leaves the package out on the dock in the heat of summer or in the freezing temperatures of winter? What happens if the patient carries the pen containing the drug along with them as they perform their errands? Will the shaking of the product shear the drug enough to aggregate it or otherwise deactivate it? Planning ahead to address these and other end-use issues needs to be a part of the overall formulation development program.

1.7 Conclusions

Clearly, formulation development is not limited to the early stages of preformulation and API characterization. Formulation development is not limited to simple choices of excipients and simply mixing a cocktail of materials together to achieve a solution. All aspects of the formulation must be carefully considered so that possible complications can be addressed. Consideration must be given to details on issues like the stability of the API, excipient compatibility, materials compatibility, processability, and end-use requirements. In this way, a product can be designed with quality and the patient in mind.

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Chapter 2
Molecule and Manufacturability Assessment Leading to Robust Commercial Formulation for Therapeutic Proteins

Ranjini Ramachander and Nitin Rathore

Abstract The transfer of lead molecules from discovery into process development at a relatively fast pace requires a process of candidate selection that assesses if a candidate is not only active and safe but also "manufacturable." Formulation and process stability of potential candidates help narrow down lead candidates at an early stage, prior to large-scale manufacturing, by a process of rank-ordering properties generated from process and long-term stability studies. Such an assessment of the molecules' manufacturability is especially useful when binding affinity and bioactivity are comparable among the various candidates under question. This chapter reviews several case studies that explore the utility of early-stage molecule or manufacturability assessments in moving forward therapeutic candidate/s by finely balancing potency and pharmacokinetics with the manufacturing capability of the candidate/s under question.

2.1 Introduction

Molecule and manufacturability assessments are key components of commercial formulation development. Molecule and formulation selection based on manufacturability assessment is critical to designing robust drug product formulation. Molecule assessment involves work that includes assessing sequence attributes for product quality, robust cell line expression, desirable purification properties that include lesser propensity to aggregate under process conditions, and the evaluation of physical and covalent stability of biotherapeutics under formulation and storage

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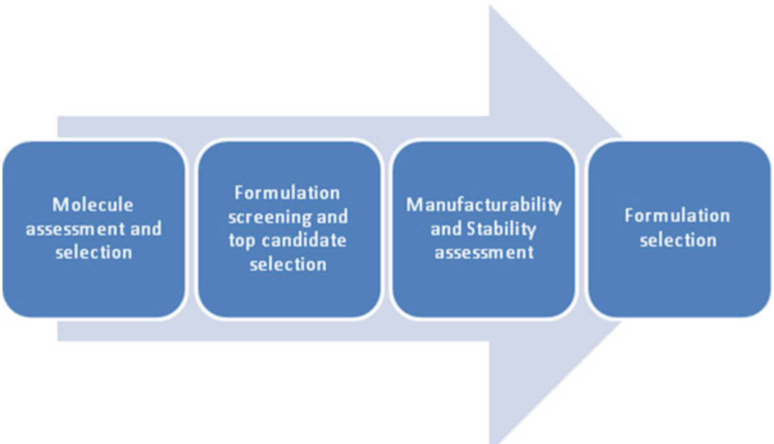


Fig. 2.1 Steps in molecule and manufacturability assessment process leading to selection of robust formulation

conditions (Narhi et al. 2010). These activities typically commence on a set of candidates that are initially chosen or narrowed down based on favorable target binding or bioactivity and pharmacokinetic considerations. Upon assessment and mitigation of potential unfavorable sequences that may result in product heterogeneity, optimal cell expression, and protein purification yield, the molecules are subject to several stability studies. These studies are not only designed to predict stability in the long term but oftentimes used to also define the formulation conditions to store the therapeutics in. The top formulation candidates can then be subjected to stress conditions representative of the manufacturing process to select the final formulation. Small-scale studies are often employed to conduct such manufacturability assessments. This kind of early evaluation is the holy grail of quality by design (QbD) where prior knowledge of the evaluated therapeutic is used to design more robust molecules with the desired properties and characteristics (Rathore and Winkle 2009).

Figure 2.1 provides an outline of the steps leading to robust formulation selection. This chapter will discuss the molecule assessment approach in detail. Case studies related to manufacturability assessments are also included.

2.2 Molecule Assessment

2.2.1 Protein Sequence Analysis and Assessment of Product Quality

In addition to maintaining product homogeneity for the therapeutic of interest, accurate determination of molecular weights and modifications is also highly desired. Uncommon framework sequences and non-germ line mutations that affect expression

are identified, and appropriate engineering is carried out on these residues. Sequences that show a greater propensity for chemical or covalent modifications are also mutated to less labile amino acids especially if they decrease stability or result in loss of activity. Further, residues that have a greater propensity to increase deamidation/oxidation are also identified and mutated if needed from a product quality standpoint or due to their possible detrimental effect on activity. Specific examples will be discussed under chemical stability in the case studies below.

2.2.2 Assessment of Physical Stability

Upon the completion of sequence analysis and engineering, candidates are moved forward based on desirable upstream expression and favorable downstream purification properties such as stability to common process conditions, including low pH for viral clearance for mammalian-derived proteins, exposure to denaturing buffers during chromatography steps, and resistance to formation of high molecular weight species (HMWS). The purified candidates are assessed for both physical and chemical stability. Depending on the molecular modality of the protein, physical and chemical stability assessment may also involve deducing the appropriate storage formulation buffer after purification in addition to understanding relative stabilities in different formulations under accelerated conditions.

2.2.2.1 Recommendation of Initial Formulation for a Single Candidate: Assessment of Stability at Different pH Conditions

Short-term and long-term formulation stability of the therapeutic or candidates is assessed by subjecting them to accelerated temperature stress in appropriate formulation conditions over time. Several assessments including melting temperature determination to predict long-term storage stability, presence of HMWS and clipped species, and other degradation products can be carried out. The candidates and formulations are then rank ordered based on predetermined stability criteria.

A formulation stability assessment was carried out on Fc-fusion protein Y in several potential formulations over a wide pH range due to lack of prior knowledge of stability at different pH conditions. Based on the stability results at 25 °C over a month, an appropriate storage formulation was recommended for further stability studies.

Figure 2.2 shows a comparison of the percent increase in HMWS generated under different buffer pH conditions at accelerated temperature for Fc-fusion protein Y.

It was seen that the greatest increase in HMWS was in the pH 4.8 buffer over a month long period, followed by pH 7.4 buffer. This Fc-fusion protein showed the greatest stability in the pH 8 buffer which was thus recommended as the ideal buffer to store the protein after purification.

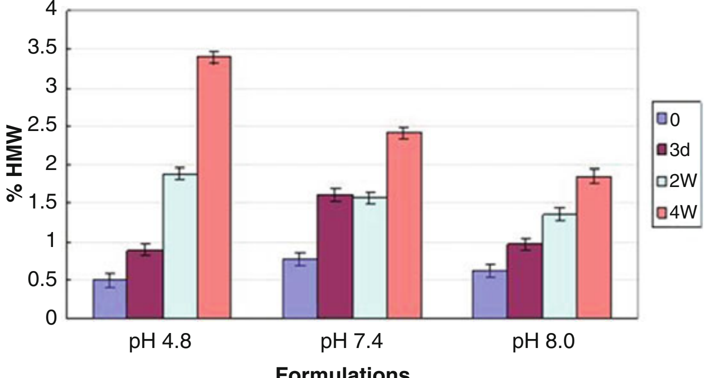


Fig. 2.2 Percent high molecular weight species (HMWS) formation over 4 weeks at different pH conditions at 25 °C for Fc-fusion protein Y. At each pH condition, the Fc-protein was assessed up to 4 weeks

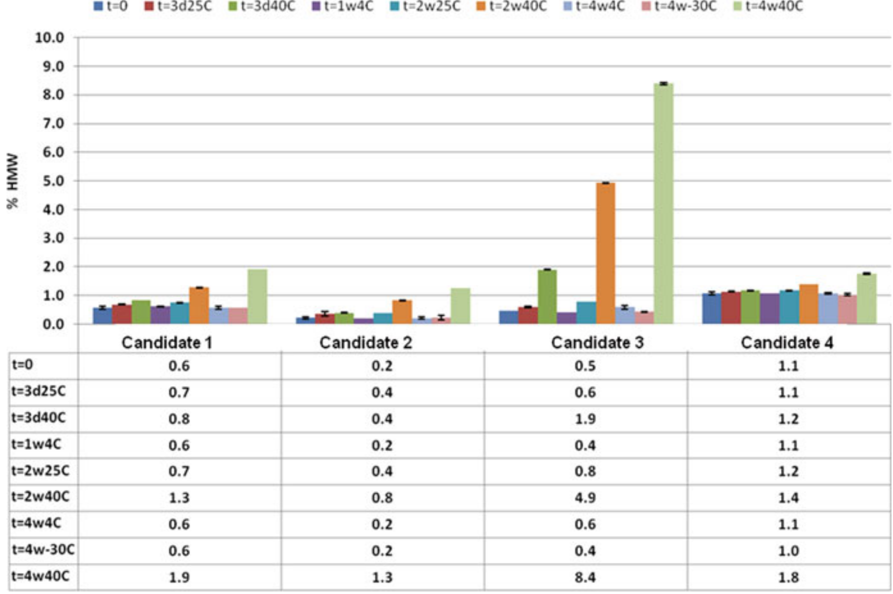
2.2.2.2 Stability of Several Candidate Molecules at a Single pH

Assessments are oftentimes also carried out across several candidates, especially antibodies or molecules with established stability in a standard storage buffer. These assessments rely on the fact that protein therapeutics generally have established storage stability in buffers and stability assessments for these buffers are made by accelerated temperature stress. Accelerated temperature stability experiments are carried out at 40 °C for more stable therapeutics to predict impact of long-term stability.

Figure 2.3 shows a comparison of the stability of different monoclonal antibody candidates at accelerated temperatures of 25 and 40 °C. Of all four mAb candidates evaluated, candidate 3 showed the highest HMW formation under the conditions tested, followed by candidate 4. In other experiments, candidate 1 also showed significant aggregation during low pH purification on a protein A column indicating process instability which was also reflected in a short-term stability study of the purified protein (data not shown). Candidate 2 was therefore selected as the lead for further development.

2.2.2.3 Stability to Physiological pH Conditions

The effect of the transition from formulation pH to physiological pH conditions is monitored by assessing the change in pH of the therapeutic going from actual formulation pH to that for phosphate buffer saline (PBS) (pH 7.4). The effect of this transition on precipitation or the formation of oligomers is monitored using a variety of tools including visual assessment, size-exclusion chromatography (SEC), and dynamic light scattering (DLS). The change is monitored in PBS at 37 °C over a



	Candidate 1	Candidate 2	Candidate 3	Candidate 4
t=0	0.6	0.2	0.5	1.1
t=3d25C	0.7	0.4	0.6	1.1
t=3d40C	0.8	0.4	1.9	1.2
t=1w4C	0.6	0.2	0.4	1.1
t=2w25C	0.7	0.4	0.8	1.2
t=2w40C	1.3	0.8	4.9	1.4
t=4w4C	0.6	0.2	0.6	1.1
t=4w-30C	0.6	0.2	0.4	1.0
t=4w40C	1.9	1.3	8.4	1.8

Fig. 2.3 Four monoclonal antibody (mAb) candidates were evaluated for their stability towards aggregation as a function of temperature and time. mAb candidate 3 shows a greater rate of HMWS formation of about 8 % compared to the other candidates over the same period of time at 40 °C. Candidate 2 showed the least amount of HMWS followed by 1 and 4

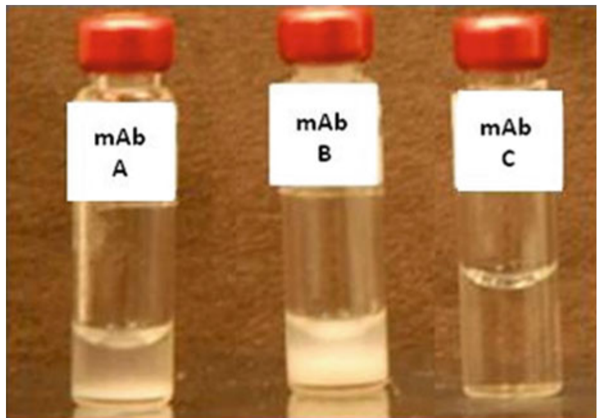


Fig. 2.4 Monoclonal antibodies A and B show visible precipitation upon transition from formulation pH of 5 to PBS at pH 7.4; mAb C remains clear even after 24 h

period of several hours (up to 24 h). Figure 2.4 shows the effect of the pH change on three antibody therapeutics, mAbs A, B, and C. It was seen that this pH change caused precipitation in two antibodies (A, B), while no visual change was observed for mAb C. Interestingly, SEC did not show significant change in smaller oligomers for any of these three antibodies (data not shown).

2.2.2.4 Stability to Low pH Conditions

Relative stability of therapeutic candidates is also assessed based on their ability to withstand purification process conditions. Low pH stability of proteins, particularly monoclonal antibodies, is determined by analysis of HMWS formation during elution from the protein A column and viral inactivation steps. This assessment is performed both during real-time purification and under accelerated conditions that simulate process excursions. Samples are checked for turbidity, and SEC is carried out to look for the presence of oligomers and other HMWS. For molecules that show propensity to aggregate under process conditions, structural integrity of these therapeutics is determined using biophysical techniques such as Fourier transform infrared (FT-IR) spectroscopy, circular dichroism (CD) spectroscopy, fluorescence spectroscopy, and differential scanning calorimetry (DSC). The reversibility of these changes is also determined. Criteria are set to eliminate candidates based on the amount of HMW species or aggregates obtained at the various process steps (data not shown). Only candidates that display robustness towards these conditions are moved forward through the pipeline (Ramachander 2008; Jiang et al. 2008).

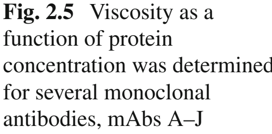
2.2.3 Solubility and Viscosity

Subcutaneous administration of high-concentration antibody therapeutics is desirable in order to minimize the volume injected in a single delivery. This often can result in significant challenges achieving the right viscosity for administration, targeted solubility at concentrations as high as 70–150 mg/mL, and overall stability under high-concentration formulation conditions. As part of screening therapeutic candidates during molecule assessment, solubility and viscosity are determined at a series of concentrations up to about 150 mg/mL. Molecules exhibiting very high viscosities are flagged and given a lower rank compared to other candidates with lower viscosities (Yadav et al. 2011). Shear-independent viscosities of candidates are initially screened using a plate-based DLS method followed by a shear rate-dependent viscosity method (typically a cone and plate method).

Figure 2.5 shows a plot of viscosities of a number of monoclonal antibodies as a function of concentration. mAbs A, B, and C show significantly high viscosity compared to the others and were flagged to indicate the possibility of greater challenges during manufacturing and delivery.

2.2.4 Stability to Agitation-Induced Particulation

Particulation or aggregation due to agitation can be encountered during fill-finish operations or during transportation (Kiese et al. 2008). Potential candidates are screened for their propensity to particulate under agitation by subjecting them to



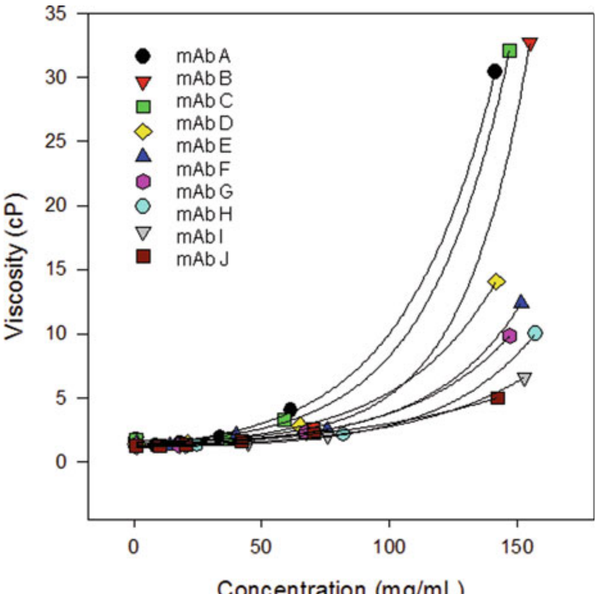


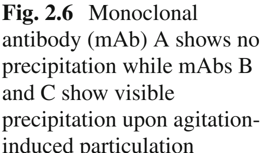
Fig. 2.5 Viscosity as a function of protein concentration was determined for several monoclonal antibodies, mAbs A–J

shaking stress on a tabletop orbital shaker at high speed over a significant period of time (up to 3 days). Samples are then observed for visual precipitation and tested by SEC for observing oligomers formation and DLS to look for a qualitative size distribution.

Figure 2.6 shows the precipitation of mAbs B and C under agitation stress, whereas mAb A remains clear even after 3 days. SEC of the precipitated mAbs also showed a greater increase in HMWS, and DLS showed a significant increase in light scattering corresponding to the larger species present in the sample (data not shown). Molecules with greater propensity to aggregate upon agitation are flagged for potential concerns during formulation development, and other candidates with desirable manufacturing properties are recommended.

2.2.5 *Chemical Stability*

Chemical degradations of monoclonal antibodies such as deamidation, isomerization, methionine and tryptophan oxidation, and succinimide formation in the complementarity determining regions (CDRs) regions may possibly lead to decreased potency of antibody therapeutics. Several of these "labile" sites may be modified or as part of the molecule assessment or the therapeutic candidates can be assessed for chemical modifications in the appropriate formulation buffer/s by subjecting them to accelerated conditions under which modifications such as deamidation, oxidation, or isomerization can be detected. While considering the various covalent



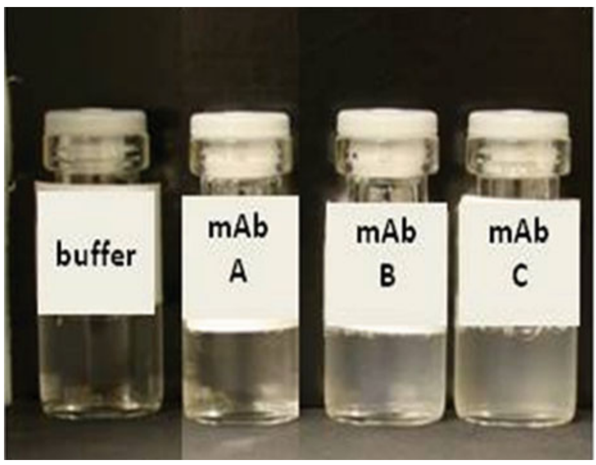


Fig. 2.6 Monoclonal antibody (mAb) A shows no precipitation while mAbs B and C show visible precipitation upon agitation-induced particulation

modification pathways, it appears that asparagine deamidation and aspartate isomerization in the CDRs are significant routes of degradation. One option that is commonly adopted is to substitute the asparagine of interest into another amino acid while still preserving antigen binding and not introducing any new routes of degradation.

An illustrative example is the study of panitumumab (antiepithelial growth factor receptor (EGFr) immunoglobulin 2 (IgG2) antibody) by Rehder et al. (2008), which contains an aspartate at position 92. Upon long-term storage, this aspartate was found to isomerize to iso-aspartate, which was captured as a separate peak in a reduced/alkylated reverse-phase assay that these authors developed, in conjunction with ion-trap mass spectrometry. The isomerization phenomenon increased with higher temperature and lower pH. These authors also developed an in vitro size-exclusion-based antigen-binding assay, which showed that a single molecule was able to bind to two EGF receptors, indicating that each antibody arm participated in binding one EGF receptor. Their data indicated that the isomerization event deactivated the binding reaction. Thus, the intact antibody was able to bind two EGF receptors, while panitumumab with one arm isomerized only bind one EGF receptor, and panitumumab with both arms containing iso-aspartate did not bind to EGF at all.

In another study, Nakano et al. (2010) detected an asparagine isomerization event in an anti-glypican 3 IgG1 antibody. This asparagine was found to deamidate significantly due to being present in an asparagine-glycine sequence. Since deamidation is highest in asparagine-glycine and asparagine-serine sequences, it was decided to mutate the glycine, and not the asparagine, to mitigate the problem. In this case, the authors mutated the glycine to an arginine which substantially reduced deamidation rates while still preserving the antigen-binding capability due to the continued presence of the asparagine residue in the mutated antibody as well.

It is thus recommended that antibodies with the instabilities mentioned above should be given lower priority as a clinical candidate. One option to mitigate such issues is by sequence engineering and applying structural analysis to generate

antibodies that do not contain residues which are prone to modification. In such cases, a backup antibody candidate could be designed that avoids or minimizes such tendencies. This area is still new and upcoming, and the effects of specific chemical modifications on potency, aggregation propensity, and immunogenicity are being intensely evaluated in the industry.

2.3 Manufacturability Assessment

Once the optimal molecule is selected, formulation screening experiments are conducted to identify the buffering conditions, ionic strength, and product concentration suitable for product stability in the desired primary container. Design of experiments (DOE) approach can be used for defining and characterization of formulation design space including identification of critical factors impacting product stability and the interactions among them. A series of forced degradation studies and DOE can be used to demonstrate the robustness of protein formulation under long-term storage (Grillo 2010).

Manufacturability assessment provides a mechanism to rank top formulation candidates for their robustness against stresses associated with manufacturing, storage, transportation, and end use. Bench-scale studies can be conducted to more closely simulate the stresses that are associated with the manufacturing process and are known to impact product stability. This requires identifying the key process parameters suspected to impact product quality and simulating them on a small scale. The following sections present some examples of such lab scale assessments.

2.3.1 Freeze–Thaw

Freezing of drug substance for long-term storage is a standard practice widely used in the industry for the obvious advantage it present in terms of product stability and manufacturing flexibility. However, freeze and thaw steps themselves could bring stresses that could potentially impact product quality. Chapter 7 discusses these stresses in details. Freezing of drug substance in large volume containers could result in cryoconcentration due to the exclusion of solutes from the ice-water interface (Singh et al. 2009; Lashmar et al. 2007). Such concentration gradients can be further amplified during the thaw step due to the migration of ice to the top of the container. For example, static freeze–thaw in a 10 L container showed protein concentration can increase by more than 50 % at the bottom of the container. At the same time protein and excipient concentration at the top of the container are reduced to half of the starting composition. Product destabilization may occur due to extended exposure of protein molecules to an environment significantly different from the recommended formulation conditions. The extent of gradient formation and the resulting impact on product quality attributes could be scale specific. Stability studies conducted at very small scale (e.g., vials) may not capture the full

Formulation Candidate	HIAC count (cumulative/mL)				
	2 µm	5 µm	10 µm	25 µm	50 µm
A	57	17	2	0	0
B	15588	3208	68	7	0
C	37	12	5	0	0
D	47	25	13	3	0

Fig. 2.7 Subvisible particle data and visual observations for post-freeze–thaw samples collected for four different formulation candidates. Formulation B exhibited higher turbidity as well as elevated particle count

commercial scale freeze–thaw 10 L carboys. It is therefore important to assess the stability in more realistic conditions. However, large amount of bulk is usually not readily available during the early stage of product development. An intermediate scale using a container made of representative material offers a reasonable mechanism to assess product sensitivity to freeze–thaw stresses and rank the top formulation candidates. Figure 2.7 shows the outcome of such an evaluation where freeze–thaw stability of four formulation candidates was evaluated at a scale of one liter. Unlike the freeze–thaw in vials where no impact was observed, results show that one of the formulation candidates experienced significant destabilization resulting in protein aggregation and formation of subvisible particulates. For controlled rate technologies such as Celsius Pak systems and CryoVessels, appropriate scaled down models are available that ensures heat transfer path length and freeze–thaw durations are consistent between small and large scale (Lashmar et al. 2007; Kolhe et al. 2009; Jameel et al. 2010). This provides the flexibility to conduct product quality assessments at lab scale using very limited material.

2.3.2 Filtration

After the bulk drug substance has been formulated, it goes through filtration step(s) that assures sterility of the filled drug product. Design of a robust fill-finish process requires understanding of the filterability of drug substance as well as the impact of

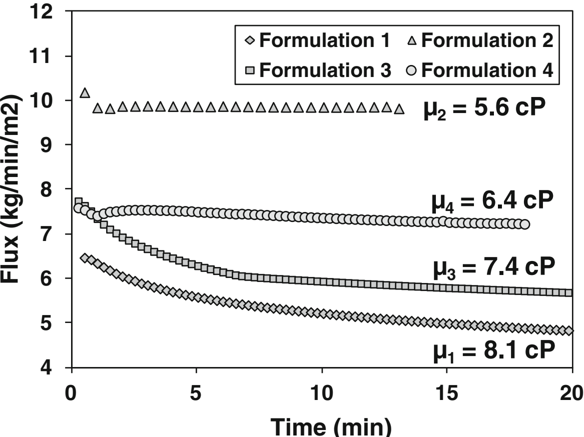


Fig. 2.8 Filtration flux as function of filtration time for four different formulations for a monoclonal antibody. The starting flux decreases with increase in viscosity; the decay in flux is specific to formulation candidate and directly impacts filtration time

filtration on drug product quality attributes. Such filterability evaluations can be conducted on small scale using filters of representative geometry and membrane but with smaller filtration area in order to lower the material demand. Various product attributes including viscosity, subvisible, and visible particle load impact the filtration process performance. Early during formulation development, bench-scale experiments can provide relative comparison for different formulation candidates. Filtration laws and mathematical models such as V_{max} methodology (Badmington et al. 1995) can also be used to estimate filter capacity and perform process scale-up for commercial scale.

Figure 2.8 shows filtration flux data for a monoclonal antibody in four different formulation buffers. The buffer excipients govern the viscosity of the final formulation as well as the particle load. The starting filtration flux is inversely related to solution viscosity, with less viscous solutions having higher starting flux. The particle load present in the protein solutions can also potentially plug the filter pores and result in flux decay during the course of filtration. Formulations 1 and 3 show significant flux decay in comparison to formulations 2 and 4, suggesting elevated levels of particle loads. Filtration models can be utilized to estimate the filter capacity for these candidates and rank them based on the expected process performance requirements (batch size, filtration time) for the large-scale process. In addition to filter capacity assessments, small-scale filtration experiments also help to assess impact of multiple filtration steps on product quality and to estimate any potential losses in protein or excipient concentrations due to membrane binding (Rathore and Rajan 2008).

Sections 3.1 and 3.2 provide two examples of commonly used small-scale manufacturability assessments and their application to ranking of formulation candidates. Similar studies can be designed to simulate other manufacturing stresses. Bench-scale filler assessments provide a mechanism to assess impact of fill operation on product quality. Recirculation of monoclonal antibody solution through a piston pump has been shown to contribute to higher turbidity as well as increase in subvisible particles (Cromwell et al. 2006; Nayak et al. 2011). Simulated transportation

studies that expose product to a range of temperature and pressure variations can be used to assess risk of product quality impact during actual shipments. Photosensitivity studies can be designed to mimic the exposure to light conditions that are representative or worse than the levels experienced in the manufacturing site and during end use.

2.4 Summary

Early-stage candidate screening during molecule assessment is one of the first steps in understanding the feasibility of manufacturing a therapeutic with the desired attributes. Establishing in vitro and in vivo potency and favorable pharmacodynamic attributes is key to every therapeutic program followed by high cell expression and a purification process that results in folded protein with minimal aggregates. Subsequently, molecules are evaluated for both short- and long-term stability that includes both storage and transportation stresses in addition to the effect of chemical modifications on stability, potency, and immunogenicity. Small-scale studies also provide mechanism to assess product stability against stresses associated with manufacturing, shipment, and storage. Candidates are rank ordered, eliminated, or moved forward based on these assessments for further scale-up and downstream processing. The predictability of these small-scale assessments during scale-up is currently being established. Most stability parameters at the early-stage assessment have been used to develop or streamline downstream formulation and scale-up processes. The desire to move a therapeutic with favorable potency and pharmacokinetics over the difficulties of processing the molecule with significant aggregation will have to be balanced by assessment of risks such as immunogenicity in administering such a therapeutic to a patient versus superior bioactivity.

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Chapter 3
Polymer- and Lipid-Based Systems for Parenteral Drug Delivery

David Chen and Sara Yazdi

Abstract The field of drug delivery technology research has continued to grow in recent decades, expanding from technologies traditionally focused on controlled release to now include nanotechnologies and functionalized molecular architectures for target-specific delivery. While these emerging novel technologies are beginning to mature, many of the clinical applications of controlled-release drug delivery technologies continue to focus on well-characterized biodegradable polymer particles, in situ forming gel depots, and lipid-based particulate formulations. This review principally focuses on the physicochemical and functional characteristics of in situ forming semisolid depot formulations and lipid-based drug delivery technologies and also discusses the broader considerations in bringing drug delivery-enabled products to market.

3.1 Introduction

Advances in molecular biology and genomics have revolutionized the treatment of human diseases, with an increasingly diverse array of molecular modalities appearing in the clinic to treat conditions that were once considered unmanageable. As the number of new molecular entities continues to increase, however, success in the clinic can often be limited by suboptimal stability, solubility, or pharmacokinetics, among other factors. Furthermore, as the biopharmaceutical market becomes

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increasingly competitive (Aggarwal 2011), pharmaceutical manufacturers are paying increasingly close attention on enabling strategies for product differentiation and life cycle management to maximize commercial value. Taken together, these challenges pose unique opportunities for novel drug delivery technologies to help bring new drug products to market.

Polymer- and lipid-based controlled-release drug delivery systems have long been considered as part of strategies to enable clinical and commercial success of a drug product. From a clinical perspective, these technologies are used to achieve constant plasma concentration levels of drug within the desired therapeutic window over an extended period of time, thereby reducing the possibility of side effects and reducing the frequency of administration. Increasingly, these technologies are considered in the early stages of clinical development to modulate the pharmacokinetic profile of a molecular candidate whose viability might otherwise be limited by short intrinsic half-life.

Drug delivery technology research has evolved over the years to encompass not only the well-known polylactide-co-glycolide (PLGA) family of polymers but also emerging novel functional biomaterials and an ever-dizzying repertoire of colloidal nanoscale systems for targeted drug delivery. Most current clinical applications of controlled-release drug delivery technologies focus on biodegradable polymer particles, in situ forming gel depots, and lipid-based particulate formulations. The encapsulation of biotherapeutic agents in PLGA-based materials has a storied history (Okada and Toguchi 1995) and will not be covered here. This review of the literature broadly discusses the functional characteristics of in situ forming semisolid depot formulations and lipid-based drug delivery technologies and also discusses the broader considerations to be taken into account in bringing such products to market.

3.2 Polymer-Based In Situ Forming Semisolid (Gel) Depots

In situ forming semisolid depots are injected as liquids and then undergo a phase transition to a semisolid gel at the site of injection to form the drug-releasing depot (He et al. 2008; Packhaeuser et al. 2004; Van Tomme et al. 2008). These in situ forming systems may offer advantages over microencapsulation by way of their relatively less complex manufacturing and more mild processing conditions. On the other hand, however, several of these systems tend to rely on precipitation from organic solvents, which may present challenges in maintaining the stability of the active ingredient, particularly macromolecules. Given that these systems are complex mixtures that must undergo phase transitions from liquid to semisolid or solid states, the phase behaviors of these systems are nonlinear and not fully understood thermodynamically; therefore, the formulation parameters that determine the release profile are highly empirical, making it critical to determine the appropriate fit between technology and the nature of the active molecule.

As the name implies, the hallmark of these formulations is the ability to be injected as a liquid and then undergo a phase transition at the site of injection. The

phase transition most occurs as a result upon solvent precipitation or in response to an environmental stimulus, usually temperature, pH, or solubility. Biomaterials exhibiting these behaviors are most frequently biodegradable or biocompatible copolymers, but in addition there is growing interest in amphiphilic lipids whose phase behavior may also lend themselves to depot formation with hydrophilic or hydrophobic molecules. The appeal of polymer systems is that the chemical space is sufficiently large, and the synthesis techniques are sufficiently advanced to allow precise control of molecular weight, polydispersity, and molar ratios such that specific polymers can be optimized to fit the desired release characteristics of a particular drug.

3.2.1 Solvent-Precipitating Depot Formulations

3.2.1.1 Atrigel Drug Delivery Technology

Initial reports of in situ forming gel depots as a result of solvent precipitation date back to the work of Dunn (1990). A nonaqueous-soluble polymer is dissolved in a biocompatible organic, but miscible, solvent and added to a drug to form a solution or suspension. The formulation is injected into the subcutaneous space and precipitates in situ as the organic solvent dissipates from the site of injection. Perhaps the most prominent example of an in situ precipitating gel depot is Eligard® (Sanofi-Aventis), a sustained-release leuprolide formulation based on the Atrigel delivery technology platform. The development history of this technology platform has been previously described and will not be repeated here (Sartor 2003; Dunn 2002). The Eligard® formulation is principally comprised of a PLGA copolymer dissolved in N-methyl-2-pyrrolidone (NMP) in a fixed ratio. By varying the PLGA copolymer molar ratios, the NMP to PLGA to drug ratio, several dosage strengths, each corresponding to a different dosing interval, are available. The various dosage strengths range from 7.5 to 45 mg, with recommended dosing from once monthly to once every 6 months, respectively.

As previously described, the thermodynamic phase behavior of PLGA solutions can be complex; as such, the formulation parameter space for these precipitating gels is highly nonlinear and therefore very sensitive to the formulation components, including polymer (lactide to glycolide ratio and molecular weight), choice of organic solvent, the solvent to polymer ratio, and drug load. A number of fundamental studies exploring the solution thermodynamics of PLGA-solvent mixtures have demonstrated that the release kinetics from these systems is significantly driven by solvent strength and water miscibility (Brodbeck et al. 1999; Graham et al. 1999).

The conspicuous drawbacks of these precipitating gel formulations is the rather cumbersome formulation preparation and mixing steps that require these injections to be administered in an outpatient or inpatient setting. Eligard® is presented in a single-use kit comprising two prefilled, interconnecting syringes: one containing

the Atrigel delivery system and the other containing the leuprolide powder. The contents of the two syringes are thoroughly mixed by pushing the mixture between the two syringes. Presumably due to viscosity, the formulation is injected using 18- or 20-gauge needle. The use of organic solvents in these formulations often leads to significant discomfort upon injection and local site reactions. For the prostate cancer patient population and the time interval between injections, these discomforts of administration are ostensibly acceptable; in looking at expanding the use of these systems to patients with chronic conditions, however, the viability of the dosage preparation and administration procedure may be limited.

3.2.1.2 Sucrose Acetate Isobutyrate-Based Formulations

Similar in principle to the Atrigel system, sucrose acetate isobutyrate (SABER, Durect Corp.) is a material that forms a semisolid depot as solvent diffuses from the site of injection (Reynolds 1998; Reynolds and Chappel 1998). With SABER being a non-polymeric material, however, there is less flexibility in tuning the specific molecular properties of the material itself; rather the drug release kinetics are driven primarily by the choice of solvent system and other added components of the general formulation. The formulation development of a SABER human growth hormone (hGH) depot highlights the interdependency between the protein formulation and drug delivery matrix formulation in achieving the desired release profile (Okumu et al. 2002). The general manufacturing process for the hGH SABER depot was also developed as a dual-container configuration, with the liquid delivery matrix and the spray-dried solid active drug being mixed together at the point of use. For the delivery matrix, the solvent system was a mixture of ethanol and benzyl alcohol, and the addition of the hydrophobic polymer polylactic acid (PLA) in the formulation allowed for improved control of the initial burst release, while the inclusion of sucrose in the protein formulation led to improved release of hGH primarily due to its well-known stabilizing effects. While sucrose acetate isobutyrate is commonly used as a food additive (Reynolds and Chappel 1998), the biocompatibility of this material as a drug delivery matrix for parenteral administration is still undergoing investigation.

3.2.2 Stimuli-Responsive Injectable Depots

Several other materials exhibit distinct phase transitions at around body temperature (Jeong et al. 2002), although these systems are in earlier stages of development than solvent-precipitating gels. The most common of these systems are two triblock copolymer systems: polypropylene oxide/polyethylene oxide systems and PLGA/polyethylene glycol systems. Both of these materials attract considerable interest because they are available in many different variations and are often used in preclinical proof of concept studies, though demonstrations of late stage development and commercialization are limited.

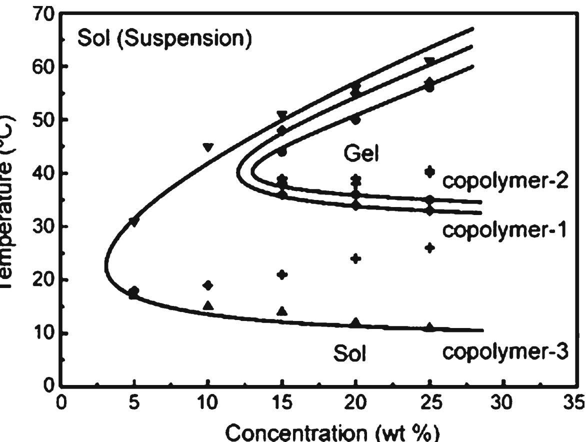

Fig. 3.1 Phase diagram of representative PLGA-PEG-PLGA solutions. The three triblock copolymers are distinguished by the number-averaged molecular weight, M_n, of each polymer block: copolymer-1, 1730-1500-1730; copolymer-2, 1740-1500-1740; and copolymer-3, 1400-1000-1400. Reprinted from reference (Yu et al. 2008), with permission from Elsevier

The triblock copolymers based on polypropylene oxide and polyethylene oxide, PEO-PPO-PEO, referred to as poloxamers (Poloxamers® or Pluronics®) are commonly investigated for drug delivery applications (Kabanov et al. 2002) because they are available in a variety of different compositions and the gelation behavior has been extensively studied. These materials exhibit physiologically relevant gelling behavior only at high concentrations, and therefore these systems may be at a disadvantage due to the osmolality of the formulations, viscosity, and frequently observed cytotoxicity (Sriadibhatla et al. 2006).

As an alternative to the poloxamers, the triblock copolymer made of PLGA-PEG-PLGA (ReGel®), polymers also undergo phase transition at physiologically relevant temperatures. Studies have shown that the block ratio of PEG:PLGA, molecular weight, block lengths, and polydispersity can all affect the properties of the gelation behavior (Fig. 3.1) (Chen et al. 2005; Yu et al. 2008). Although organic solvents are not required for these systems, a common problem is high drug burst. Peptide and protein drugs are often very hydrophilic due to exclusion of the aqueous phase during the course of the sol–gel transition. At this point in time, the application of the ReGel polymers is likely to be limited to hydrophobic drugs, such as paclitaxel (Elstad and Fowers 2009), and hydrophilic drugs with wide therapeutic windows.

3.3 Formulation Development of Lipid–Drug Delivery Systems

A close examination of an electron micrograph of an erythrocyte membrane, thinly sectioned and stained with osmium tetroxide, reveals a bilayer structure composed of phospholipids, in which polar head groups face outwardly to sequester the hydrophobic fatty acyl tails from the surrounding aqueous environment. It was a similar

Table 3.1 Examples of approved and licensed liposomal formulations in the US and EU, adapted from references (Janoff 1999; ElBayoumi and Torchilin 2009)

Product	Active pharmaceutical ingredient	Indication
Doxil®	Doxorubicin	Kaposi's sarcoma, ovarian cancer, and multiple myeloma
DaunoXome®	Daunorubicin	First-line treatment for advanced Kaposi's sarcoma
AmBisome®	Amphotericin B	Systemic fungal infections; visceral leishmaniasis
Amphotec™	Amphotericin B	Systemic fungal infections
Abelcet®	Amphotericin	Systemic fungal infections
Newcastle disease vaccine	Newcastle disease virus (dead)	Newcastle disease (chickens)
Avian reovirus vaccine	Avian reovirus (killed)	For vaccination of breeder chickens; for passive protection of baby chicks against reovirus infections
Epaxal(R) vaccine	Inactivated hepatitis A virions (HAV) (antigen: RG-SB strain)	Hepatitis A

microscopic observation of biomembranes that led to the seminal paper published by Bangham et al. (Deamer and Bangham 1976) in the 1970s on structures that went on to become modern day liposomes.

Liposomes in the broadest sense are self-assembled colloidal particles with phospholipids as their major molecular constituents. Phospholipids belong to a class of amphiphilic lipids (Fahy et al. 2005, 2007) (i.e., soaps, detergents), and their often polar and hydrophilic head group and adjoining nonpolar hydrophobic tail together have the ability to form a sheetlike structure that encloses on itself when presented to an aqueous environment under agitation, forming multilamellar vesicles. Liposomes' potential as drug delivery vehicles was once overshadowed by their inherent thermodynamic instability, though today, these challenges have been addressed and many of the "nonconventional" drug delivery systems approved or in development for parenteral administration for both human and veterinary applications fall into the liposomal formulation category (Tables 3.1 and 3.2) (Janoff 1999; ElBayoumi and Torchilin 2009). Most of the formulations approved for use in humans contain phosphatidylcholine (neutral charge), with fatty acyl chains of varying lengths and degrees of saturation (Langer 1990; Lian and Ho 2001). However, some of the important challenges associated with liposomal formulation remain, including their limited physical stability, burst release, low activity due to nonspecific tumor targeting, and nonspecific clearance by the reticuloendothelial system (RES) (Davis 2004; Wissing et al. 2004a).

3.3.1 Preparation and Characterization of Liposomes

The preparation of liposomes is broadly divided into two categories; one approach entails the physical modification of existing bilayers, while another approach

Table 3.2 Examples of liposomal formulations currently under clinical evaluation, adapted from references (Janoff 1999; ElBayoumi and Torchilin 2009)

Product	Active pharmaceutical ingredient	Indication	Approval status
Trivalent influenza vaccine	Hemagglutinin and neuraminidase from H_1N_1, H_3N_2, and B strain	Influenza	Phase III
Evacet™	Doxorubicin	First-line therapy for metastatic breast cancer	Phase III
Nyotran™	Nystatin	Candidemia, systemic fungal infections	Phase II and Phase III
Atragen™	All-trans retinoic acid	Leukemia and Kaposi's sarcoma	Phase II and Phase II
MiKasome®	Amikacin	Serious bacterial and mycobacterial infections	Phase II
SPI-77	Cisplatin	Advanced forms of cancer	Phase II
E. coli 0157:H7 vaccine	*E. coli* 0157:H7 (killed)	*E. coli* 0157 infection	Phase I
VincaXome™	Vincristine	Solid tumors	Preclinical development
SPI-119	CD4	HIV infection	Preclinical development

involves synthesis of new bilayers via removal of a lipid-solubilizing agent. For preparation of small unilamellar vesicles (SUVs) at a large scale, a thin film is either formed via lyophilization of lipid mixtures from tert-butanol or spray-drying followed by primary hydration of lipids. For final preparation of the liposomes, ultrasonication irradiation, and high-pressure homogenization or extrusion are often utilized (Lasic 1998).

3.3.1.1 Ultrasonication Irradiation

In this method, a probe sonicator is utilized to form SUVs rapidly and conveniently. The procedure is often carried out in presence of inert atmosphere and lowered temperatures to decrease the oxidation risk of unsaturated lipids. A cooling bath is used to dissipate the heat generated as the result of sonication. The final step includes centrifugation to remove the small titanium particles that may be shed during the course of sonication.

3.3.1.2 High-Pressure Homogenization or Extrusion

High-pressure homogenization or extrusion involves forcing multilamellar liposomes through defined size "straight-through" pores at high pressures. Repeated passage of

the liposomes through these small-sized pores at temperatures lower than the crystallization temperature (T_c) leads to deformation and reformation of lamellar layers in defined size and subsequently results in a more a monodisperse population.

3.3.1.3 Drug Loading

Neutral, hydrophilic drugs are introduced into the liposomal formulation in the hydration step, whereas hydrophobic drugs may be solubilized in the preliminary steps of lipid mixture solubilization and lyophilization. In the case of charged molecules, a pH gradient may be utilized to remotely load the liposomes. For example, an ammonium sulfate buffer gradient can be used to create an acidic interior, or sodium bicarbonate can be used to generate a basic interior.

3.3.1.4 Effect of Lipid Composition on Size and Surface Characteristics

The fate of liposomes in vivo is largely dependent upon liposome–liposome interactions as well as interactions between liposomes and their local environment; these interactions vary in nature from electrostatic to Van der Waals depending on the size and surface of liposomes. The optimal size for preparation of liposomes lies in the range of 10–100 nm. The lower bound is based on a threshold for first-pass elimination via kidneys and the upper bound is roughly based on the size of the leaky vasculature of mouse tumor models (Davis et al. 2008). As for the surface charge, studies have shown that particles with slight negative or positive charge have longer circulation times than those that are highly charged (positive or negative).

3.3.2 Representative FDA-Approved Liposomal Formulations

Two examples of FDA-approved and FDA-marketed liposomal formulations are Doxil® and DaunoXome® both of which are used to treat AIDS-related Kaposi's sarcoma (Table 3.1) (Janoff 1999; ElBayoumi and Torchilin 2009). The Doxil® (Johnson & Johnson) formulation is a liquid suspension containing 80–100 nm liposomal formulation composed of 2000 MW PEG-distearoylphosphatidylethanolamine-hydrogenated soybean phosphatidylcholine-cholesterol (20 mM) with doxorubicin hydrochloride. The active pharmaceutical ingredient (API) is loaded into the liposomes using an ammonium sulfate pH gradient method (Barenholz 2012). Doxil's long circulating formulation was granted accelerated FDA approval in 1995, with DaunoXome® following soon after.

DaunoXome® (Galen) is a solution of citrate salt of daunorubicin (DAU) encapsulated within the aqueous core of a SUV composed of distearoylphosphatidylcholine (DSPC) and cholesterol in a 2:1 molar ratio. The overall lipid to drug composition is 10:5:1 for DSPC:Chol:DAU, and similarly a pH gradient method is used to load DAU into the liposome (Forssen 1997). While the exact manufacturing details of

the aforementioned formulations are not available, they likely were prepared using high-shear homogenization or extrusion of the phospholipid derivatives followed by API loading using pH gradient method; the free drug is then further removed using dialysis under aseptic conditions, followed by aseptic filtration to ensure sterility of the final product.

3.3.3 Targeted In Vivo Delivery Using Liposomal Formulations: Stealth Liposomes and Immunoliposomes

3.3.3.1 Effect of Lipid Composition on In Vivo Fate: Stealth Liposomes

Early liposomal formulations were mainly composed of neutral and negatively charged phospholipids such as phosphatidylcholine or sphingomyelin, leading to their recognition and eventual endocytosis by cells of the mononuclear phagocytic system (MPS), mostly fixed Kupffer cells in the liver and spleen. This fate suits drug delivery to these organs, though it also prevents delivery applications elsewhere. Frank Davis and colleagues developed the first generation of polyethylene glycol (PEG)-conjugated proteins and peptides in the late 1970s (Davis 2002). Their findings showed that the PEGylated complex was 5–10 times larger than the free drug form due to the binding of water molecules to the ethylene glycol subunits, which decreases renal clearance rates and improves the pharmacodynamic and pharmacokinetic properties of PEGylated polypeptide drugs (Harris and Chess 2003). Additionally a closer look at the recognition and uptake mechanism by RES showed that adsorption of degraded blood proteins and opsonins onto the negatively charged liposomal surface expedited their clearance; employing a similar strategy to evade uptake by the RES and to prolong circulation time in the plasma culminated in development of PEG-coated, sterically stabilized liposomes (Harris and Chess 2003; Kozlowski et al. 2001; Skubitz and Haddad 2005; Spira et al. 2008). The density of the attached PEG groups showed a direct correlation to increased circulation time in vivo. The hydrophilic shield provided by the PEG groups increases the circulation time of liposomes in the system by reducing the rate of plasma protein adsorption on the hydrophilic surface. Further understanding of the extent of therapeutic potential of liposomal formulations were brought into light when a detailed understanding of lipid polymorphisms, physiological mechanisms of in vivo liposome deposition, and lipid–drug and lipid–protein interaction emerged and resulted in enhanced design of such systems with increased in vivo stability and improved biodistribution (Lian and Ho 2001).

3.3.3.2 Active Targeting: Immunoliposomes

Various candidate ligands have been examined to target liposomes to tumors with overexpressed receptors. Targeting ligands can range from macromolecules, such as antibodies and transferrin, to small-molecule ligands such as folate, lectins, and

others (Skubitz and Haddad 2005; Benesch and Urban 2008; Gabizon et al. 2003). The design criteria for a viable ligand with the potential to successfully target tumors include ease of ligand production in large scale, purification and stability, and the know-how of ligand-liposomes conjugation strategies without compromising the properties of either factor.

The use of monoclonal antibodies (mAb) is not straightforward; although the presence of two binding sites promotes mAb stability and high affinity, the Fc receptor-mediated response may lead to high spleen and liver uptake and subsequent increase in immunogenicity of the molecule. To counter this effect, modification and fragmentation of the whole antibody molecule has been undertaken and tremendous efforts are being made to improve the fate of antibody-coated liposomes. One such example is the so-called post-insertion method, in which ligands are conjugated to end-functionalized groups in PEGylated lipid micelles. The ligand–PEG–lipid conjugate is then transferred in an incubation step from micellar form to the outer monolayer of the already-formed liposomes (often Doxil®). This method has been used in studies of HER2-scFv conjugated liposomes for cancer therapy and anti-TfR scFv-lipoplexes for gene delivery. Another method relies on use of grafted maleimide-containing PEG lipids in liposomal preparation, followed by conjugation of cysteine-bearing antibodies at the c-terminus. While both of these methods can be rather cumbersome and difficult to control, the later may be more straightforward while the former combines the challenging insertion process with the task of separating the micellar ligand-PEG-lipids from the coated lipsomes (Puri et al. 2009).

The challenges that remain in the development of immunoliposomes vary from a continuous effort to improve their current design to determination of optimal ligand density on the liposomal surface and the choice of ligands for different tumor cell models. Evidence seems to indicate a balance between ligand density and ligand affinity, as well as the use of low affinity ligands may offer further penetration into the tumor environment (Puri et al. 2009).

3.3.4 Alternative/Composite Systems in Preclinical Development: Solid Lipid Particles

The need for development of an alternative lipid-based drug delivery system besides liposomes, which allows for higher control over drug release and better loading efficiency, has brought solid lipid carriers into light (Jaspart et al. 2007; Kohane 2007; Mehnert and Mader 2001; Muller et al. 2002, 2007; Reithmeier et al. 2001; Saraf et al. 2006). Solid lipid particles are made from solid lipids (i.e., lipids that remain solid at room and body temperature) and are stabilized by surfactants. By definition, the lipids can be purified triglyceride, complex glyceride mixtures (mono-, di-), and waxes (Muller et al. 2002; Wissing et al. 2004b). The main advantages of solid lipid particles are the excellent physical stability, protection of encapsulated labile drugs

from degradation, controlled release depending on the incorporation model, good tolerability, and the potential for targeted delivery (Wissing and Muller 2002).

Solid lipid nanoparticles (SLN) are usually made by means of high-pressure homogenization and are mainly characterized by their less than 200 nm size. Use of physiologically derived lipids and the absence of organic solvents in SLN's preparation minimize the risk for potentially acute and chronic toxic response and pose these delivery systems as strong contenders for parenteral delivery. Full characterization of SLN has shown that methods of preparation, lipid composition, and choice of emulsifier have a direct impact on the final size, charge, and stability of these particles. In addition, the degree of lipid crystallinity and degree of modification of lipids are strongly correlated with drug incorporation within SLN and their release rates (Gershkovich et al. 2008). The shortcomings of SLN delivery are similar to other nanotechnology-based platforms, in particular their rapid physiological clearance via the spleen and liver. Similar to liposomal formulations, this response is advantageous for those particular cases where spleen and liver are targeted delivery sites; however, it remains an undesirable outcome in case of delivery intended for other major organs.

3.4 Conclusion

Drug delivery technology research has blossomed in recent decades, expanding from technologies traditionally focused on extended release to now include nanotechnologies and other functionalized molecular architectures for target-specific delivery. Despite the extensive literature and high levels of research activity in developing novel delivery systems, however, the number of products on the market is few and far between due to the significant manufacturing, regulatory, and safety challenges that must be addressed.

From a formulation and manufacturing scale-up point of view, the addition of a controlled-release technology arguably adds an additional element of complexity to the formulation and process development of parenteral formulations, so the key consideration is to balance the cost and benefits in matching the appropriate molecule to the technology. Experience has shown that the large-scale reproducible production of drug delivery technologies is rarely straightforward from bench to industrial scale, and significant investments in the production process are very common. The challenges in scaling up production of these systems are large, and the publications on this topic are relatively rare. In the face of such challenges, and even more so as costs of development continue to soar, the overarching need within drug delivery is to develop technologies that can be applied widely across molecule classes, instead of the ostensibly bespoke formulations that are on the market thus far.

In spite of these challenges, academic and industry continue to invest in drug delivery technologies, a salient indicator of the needs for these enabling technologies in driving medical and marketplace differentiation for pharmaceutical

products. As these technologies mature and gain acceptance in the marketplace, drug delivery technologies will play an increasingly prominent role in meeting the current and future needs of medical providers and patients.

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Chapter 4
Formulation Approaches and Strategies for PEGylated Biotherapeutics

Roger H. Pak and Rory F. Finn

Abstract This chapter will begin with a brief review of the PEGylated biotherapeutic product landscape (commercial products and known molecules in development) and then discuss various product development approaches and issues. Several topics pertinent to PEGylated biotherapeutics formulation development include manufacturability, linker and conjugate stability, and viscosity and PEG reagent design. A discussion on each topic will be presented with a focus on strategies to overcome typical hurdles encountered.

4.1 Introduction

As the number of biotherapeutic molecules increases in the pipelines of pharmaceutical companies, it is becoming apparent that novel drug delivery technologies are needed to overcome some of the inherent challenges with some protein- and peptide-based therapies. Certain classes of biotherapeutics, such as small protein constructs, peptides, and oligonucleotides, have the potential for issues such as short plasma residence half-life, low solubility, aggregation, immunogenic effects, and enzymatic degradation. For such biomolecules, drug delivery systems may help overcome these challenges and lead to successful development and utilization as a therapeutic. The field of drug delivery technologies includes both non-covalent and covalent approaches. Non-covalent systems usually encapsulate the biotherapeutic molecule

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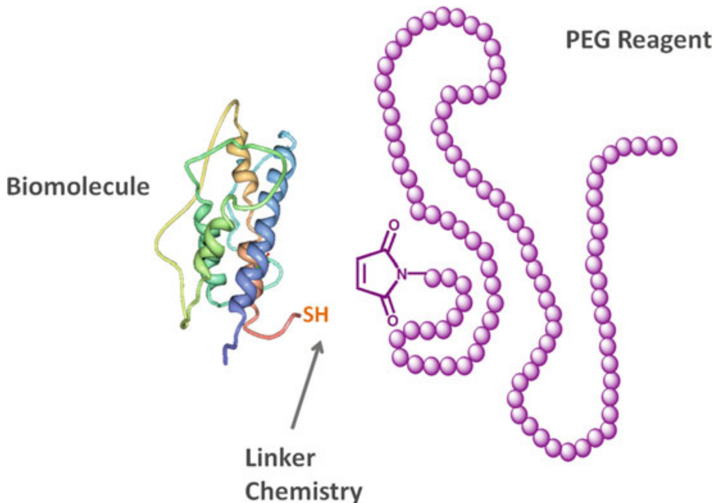

Fig. 4.1 Components of a PEG-biotherapeutic conjugate

in a lipid or polymer matrix and provide slow-release of the biomolecule over time. Typical systems include liposomes, emulsions, lipid or polymer gel depots, and encapsulating micro- or nano-spheres. Covalent systems include chemical conjugation to hydrophilic polymers (such as polyethylene glycol or PEG), fatty acids, or protein scaffolds, and protein engineered systems that express an appended amino acid sequence to the native biomolecule such as an Fc fusion protein or random polypeptide sequences. Covalent attachment of the polymer PEG has become a technology of choice due to its relatively straightforward approach and established correlation between hydrodynamic radius and in vivo half-life. This chapter will focus on the use of chemical conjugation of the hydrophilic polymer PEG to biotherapeutic molecules as an enabling drug delivery system.

4.1.1 Overview of PEGylation

One of the most commercially successful and widely used technologies for creating polymer bioconjugates is PEGylation of biotherapeutic drugs. PEGylation refers to the use of a linker-functionalized PEG polymer reagent for the chemical conjugation to certain reactive side chains of a peptide, protein, or oligonucleotide. The resulting bioconjugate is composed of the target biomolecule, a linker and the PEG polymer (Fig. 4.1).

This technology originated from the work of Frank F. Davis and Abraham Abuchowski at Rutgers University in the late 1970s (Abuchowski et al. 1977; Davis 2002). Davis postulated that the attachment of a hydrophilic polymer such as PEG might make a protein or enzyme less immunogenic and thus useful as a therapeutic. Further experiments on these PEG-conjugates in in vivo models indicated that not

R = CH_3- for mPEG
R = H- for diol PEG

Some Physical Properties of PEG
- Soluble in many aqueous and organic solvents (amphiphilic)
- Relatively nontoxic
- Relatively nonimmunogenic
- Large sphere of hydration
- Highly compatible with biologics
- Relatively chemically inert except for hydroxyl ends

Fig. 4.2 Chemical structure and some physical properties of polyethylene glycol (PEG). Methoxy-PEG (mPEG) is the predominant starting polymer for functionalized PEG reagents as the dihydroxy-PEG (diol PEG) would result in unwanted bifunctionalization during synthesis

Table 4.1 Clinical half-life extension of some PEG biotherapeutics

Commercial PEG-conjugate	Native protein $t_{1/2}$ (h)	PEGylated $t_{1/2}$ (h)
PEG-asparaginase	20	357
PEG-interferon α2a	3–8	65
PEG-uricase	4	154–331
PEG-erythropoietin	7–20	134–139

only were they less immunogenic but they also had increased circulation plasma residence times. Subsequent to those early experiments, it was found that the PEG polymer imparted a number of other desirable properties to PEG-conjugates. The physicochemical properties of PEG (Fig. 4.2) itself may give insight to the properties endowed to PEG-conjugates.

The PEG polymer has both hydrophobic and hydrophilic properties and is soluble in both aqueous and organic solvents (Harris 1992). This property makes it ideal for manipulation during reagent functionalization and preparation in organic solvents followed by conjugation to a biomolecule in aqueous solutions. The sp^3 carbon–carbon bonds and sp^3 carbon–oxygen bonds in PEG are all fully rotational and provide great flexibility to the polymer. This flexibility allows a PEG moiety to form a random coil around the biomolecule and provide a shielding effect and a volume effect (Lu et al. 2008) or, as more recent data indicates, allows the PEG to form a dumbbell configuration with the random coil PEG adjacent to the globular protein (Pai et al. 2011). Each ethylene oxide monomer retains approximately 2–3 bound waters. The resulting sphere of hydration of the PEG polymer presents a volume 5–10 times larger than the volume of a protein of comparable molecular weight (Harris 1992). The volume effects of PEG polymers (of typically greater than 20 kDa molecular weight) are used to prevent glomerular filtration and afford an increase in plasma circulation half-life. Some examples of increases in half-life for PEG-conjugates are shown in Table 4.1.

The large PEG moieties can also protect amino acid sequences that are prone to proteolytic degradation or immuno-recognition which consequently provides greater in vivo stability and lifetime. The solubility properties of PEG, meanwhile, can confer upon the PEG-conjugates a degree of increased solubility or prevention of protein–protein aggregation phenomena. For example, in Fig. 4.3, one poorly

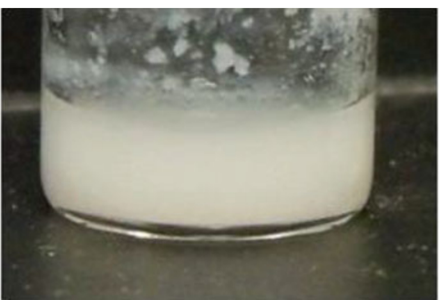

Fig. 4.3 Polyethylene glycol (PEG) solubilizing power for PEG-peptides. The *left* picture shows a solution of a poorly soluble peptide showing precipitation at 0.3 mg/mL in aqueous buffer, pH 7. The picture on the *right* shows the same peptide but now PEGylated showing solubility up to ~25 mg/mL (based on peptide mass only) in aqueous buffer, pH 7

soluble peptide (0.3 mg/mL aqueous solubility) displayed an increase in its solubility after conjugation to a 40 kDa branched PEG (~25 mg/mL aqueous solubility). All these properties (volume increase, half-life increase, protection from proteolytic degradation, and immuno-recognition and solubility) conferred upon a PEG-conjugate are desirable for a biopharmaceutical and may enable a biomolecule, lacking in one or more of these properties, to be developed into a viable biotherapeutic (Fishburn 2008).

4.1.2 Commercial Products

The first PEG-drug conjugate was approved by the FDA in 1990, PEG-Adagen (Levy, 1988). Since then there have been a total of ten FDA approved PEGylated biomolecules[1] (Jevševar et al. 2010; Schlesinger et al. 2011).

The diverse biotherapeutics utilizing PEGylation technology, as exemplified in Table 4.2, testifies to the broad acceptance of this drug delivery modality. The types of parent molecules range from enzymes to cytokines to growth factors and even include an aptamer (an RNA oligonucleotide). Indeed, several of these PEG biotherapeutics have been widely prescribed and have reached blockbuster drug status. Neulasta (pegfilgrastim), Pegasys (PEG-Interferon-α2a), and PEG-intron (PEG-Interferon-α2b) had combined sales in 2008 of approximately $5.7 billion (Hahn 2009). In 2010, Neulasta was the number 16 top selling product in the USA with approximately $3 billion in sales revenue (Bartholow 2011). Many of these innovative drugs that treat chronic debilitating diseases allow less frequent dosing which eases the burden on the patients, increases compliance (and thus therapeutic results) as well as potentially increases cost savings.

[1] After this manuscript had been submitted, Peginesatide had received FDA approval, making it the eleventh PEGylated biotherapeutic to gain approval.

Table 4.2 Commercial PEGylated biotherapeutics

Brand name (generic name)	Parent drug	Route	Indication	Year of US approval
Adagen (Pegadamase)	Adenosine deaminase	IM	SCID	1990
Oncaspar (Pegaspargase)	Asparaginase	IM, IV	Leukemia	1994
Peg-Intron (Peg-IFN α2b)	Interferon-α2b	SC	Hepatitis C	2000
Pegasys (Peg-IFN α2a)	Interferon-α2a	SC	Hepatitis C	2001
Neulasta (Pegfilgrastim)	GCSF	SC	Neutropenia	2002
Somavert (Pegvisomant)	hGH antagonist	SC	Acromegaly	2003
Macugen (Pegaptanib)	Anti-VEGF aptamer	Intravitreal	AMD	2004
Mircera (Peg-EPO)	Erythropoietin	IV, SC	Anemia	2007
CIMZIA (Certolizumab pegol)	Anti-TNFα Fab	SC	Crohn's disease, rheumatoid arthritis	2008
Krystexxa (Pegloticase)	Urate oxidase (Uricase)	IV	Chronic gout	2010

4.1.3 Molecules in Clinical Development

Following closely on the heels of the commercial products are the PEG-drug conjugates in clinical development (Kang et al. 2009). Table 4.3 shows a number of PEGylated biotherapeutic and small molecule candidates in clinical trials from Phase I to Phase III.

Some advanced stage candidates include Peginesatide (Footnote 1) (an erythropoiesis-stimulating agent), PEGylated Arginine Deiminase (carcinoma), and PEGylated Interferon β1a (multiple sclerosis). The parent drug in Peginesatide is a synthetic homodimeric peptide of approximately 5 kDa that requires polymer conjugation with a 40 kDa PEG for extension of half-life. Results from the Peginesatide clinical trials indicate that patients with chronic kidney disease (CKD) can increase and maintain hemoglobin levels when administered once every 4 weeks with this PEG biotherapeutic (MacDougall et al. 2011; Möller et al. 2011). Affymax and Takeda have filed an NDA for this drug in 2011 and, if approved, this drug could be the first once-monthly ESA marketed for the treatment of anemia associated with CKD in the USA (Affymax press release, May 2011).

Another advanced stage PEG-conjugate is PEGylated Arginine Deiminase or PEG-ADI 20 (Glazer et al. 2011). The use of an arginine-degrading enzyme, such as arginine deiminase (ADI), has exhibited tumoricidal activity by acting on tumor cells that show arginine auxotrophy, such as hepatocellular carcinomas. ADI is an enzyme that converts arginine to citrulline and would be able to deprive cancer cells of systemic arginine. However, ADI is not a native human enzyme and is very immunogenic and has a short circulation half-life. This mycoplasmic-derived enzyme, when conjugated to a 20 kDa PEG, shows decreased antigenicity, longer systemic lifetime, and was well tolerated in Phase II clinical trials. Further studies appear to be ongoing.

Table 4.3 Clinical stage PEGylated biotherapeutics

Conjugate name	Parent drug	Company	Indication	Clinical phase[a]	References
Peginesatide (Hematide)	Erythropoiesis stimulating agent (peptide)	Takeda/Affymax/Nektar	Anemia	Phase III (NDA submitted)[b]	(Affymax Press Release, May 31, 2011)
ADI-PEG 20	Arginine deiminase	Polaris Group Pharma	Hepatocellular carcinoma and melanoma; antiviral hepatitis C	Phase III	Glazer et al. (2011), Holtsberg et al. (2002)
PEG-IFN β1a (BIIB017)	Interferon β1a	Biogen Idec	Multiple sclerosis	Phase III	Baker et al. (2010)
PEG-hemoglobin (Hemospan, MP4OX)	Hemoglobin	Sangart	Perioperative hypotension; hemorraphic shock	Phase IIb	(Sangart Press Release Jun 14, 2011), Vandegriff et al. (2008)
PEG-SN38 (EZN-2208)	Small molecule metabolite of irinotecan	Enzon	Metastatic breast cancer; colorectal carcinoma	Phase II	www.clinicaltrials.gov and Pastorino et al. (2010)
PEG-hGH (ACP-001)	Human growth hormone	Ascendis Pharma	GH deficiency	Phase II (completed)	www.ascendispharma.com
PEG-hGH (ARX-201)	Human growth hormone	Ambrx/EMD Serono	GH deficiency	Phase II	www.ambrx.com and Cho et al. (2011)
PEG-Naloxol (NKTR-118)	Small molecule derivative of naloxone, an opioid antagonist	Nektar/Astra Zeneca	Opioid-induced constipation	Phase III	www.nektar.com
PEG-Irinotecan (NKTR-102)	Small molecule Irinotecan (topoisomerase I inhibitor)	Nektar	Metastatic breast cancer, ovarian cancer, GI and solid tumors	Phase II/III	www.nektar.com

PEG-Interferon lambda (PEG-rIL29) (BMS-914143)	Recombinant interferon lambda (interleukin 29, rIL29)	Bristol-Myers Squibb/ Zymogenetics (2010)	Chronic Hepatitis C	Phase II/III	www.bms.com and Muir et al. (2010)
PEG-PAL	Recombinant phenyl-alanine ammonia lyase (rPAL)	Biomarin Pharma	Phenylketonuria (PKU)	Phase II	Sarkissian et al. (2011))
PEG-rFIX (N9-GP)	Recombinant factor IX	Novo Nordisk	Hemophilia B	Phase I	Negrier et al. (2011)
PEG-rFVIII	Recombinant factor VIII	Baxter	Hemophilia B	Phase I (planned for 2011)	Jevševar et al. (2010)
PEG-BDD-rFVIII (BAY 94-9027)	BDD recombinant factor VIII	Bayer	Hemophilia A	Phase I	Mei et al. (2010)
PEG-glutaminase (GlutaDON)	Glutaminase enzyme in combo with 6-diazo-5-oxo-l-norleucine (DON)	New Medical Enzymes AG	Solid tumors	Phase I/II	Mueller et al. (2008)

[a] After this manuscript had been submitted, Peginesatide had received FDA approval, making it the eleventh PEGylated biotherapeutic to gain approval

[b] More information on specific clinical trials can be obtained at www.clinicaltrials.gov

A third late-stage PEGylated therapeutic is PEGylated Interferon β1a (PEG-IFN-β1a; BIIB017) being developed by Biogen Idec for multiple sclerosis (MS) (Baker et al. 2010). This chronic autoimmune disease affects the central nervous system and patients suffer progressive neurological disability. Although there are several products approved for treatment of MS, a significant number of patients choose not to initiate treatment due to perceived side effects, perceived lack of efficacy or avoidance of too frequent injections (1–3 times per week depending on therapy). A PEGylated version of IFN-β1a is being developed to meet the needs of this patient population. Preclinical studies showed improved PK and efficacy. In fact, in a mouse model, a single dose of PEG-IFN-β1a was more efficacious than nine daily doses of the unmodified IFN-β1a. Two Phase I studies supported the further development of PEG-IFN-β1a and this conjugate is currently in Phase III clinical trials for subcutaneous (sc) dosing every 2 or 4 weeks.

These advanced clinical candidates as well as those in early clinical trials show great potential and appear to provide an advantage over current medicines. The success of these conjugates shows the value that PEGylation has in enabling bioactive molecules to become medicines.

4.2 The Chemistry of PEGylation

In order to PEGylate proteins or other biotherapeutics, PEG polymers need to be activated. Typically, methoxy-PEG is preferred which contains a methoxy group at one terminus and a single hydroxyl group at the other end which is used for functionalization. This hydroxyl group can be converted to a number of active linker chemistries for reaction with amino acid side chains on the target biologic. The linker chemistry is chosen based on the target site of attachment of the biologic—such as lysines, histidines, cysteines, methionines, or N-terminal amino groups. The PEGylation reaction is typically performed using bioconjugation techniques that are generally mild and aqueous based. This is done to protect the protein binding site or enzymatic activity of the parent biotherapeutic. Functionalized PEG reagents are mixed with the parent protein drug and react with amino acid side chains in either a specific or nonspecific manner.

Commercial functionalized PEG reagents come in a variety of lengths, branching, shapes, and linker chemistries (NOF, Sunbio, BioVectra, and other vendor catalogs). On an investigational level, there is an even greater variety of linker chemistries being looked at across many academic and industrial labs. Several thorough reviews on PEGylation chemistry have been written (Bonora and Drioli 2009; Roberts et al. 2002; Veronese and Mero 2008). In this section, we will focus our discussion on the linker chemistries used in the commercial and clinical PEG-conjugates as well as those that may have clinical relevance. Table 4.4 describes the linker chemistries used for commercial and clinical conjugates.

Table 4.4 Linker chemistries used for commercial and clinical PEGylated biotherapeutics

Biotherapeutic	MW of PEG	Linker chemistry	Site of attachment
Commercial products			
Adagen (Pegadamase)	Linear 5 kDa	NHS ester	11–17 random sites: Lys, Ser, Tyr, His
Oncaspar (Pegaspargase)	Linear 5 kDa	NHS ester	Nonspecific Lys, Ser, Tyr, His
Peg-Intron (Peg-IFN α2b)	Linear 12 kDa	NHS carbonate ester	His 34 (major)
Pegasys (Peg-IFN α2a)	2-Branch 40 kDa	NHS ester	Lys 31, 121, 131 or 134 (Bailon et al. 2001)
Neulasta (Pegfilgrastim)	Linear 20 kDa	Aldehyde reductive amination	N-terminal Met
Somavert (Pegvisomant)	Linear 5 kDa	NHS ester	4–6 random sites: Lys 38, 41, 70, 115, 120, 140, 145, 158 or N-terminal Phe
Macugen (Pegaptanib)	2-Branch 40 kDa (Lys branching)	Active ester coupling to pentylamino linker	Pentylamino linker on 5′-phosphodiester terminus on a 28-oligonucleotide aptamer
Mircera (Peg-EPO)	Linear 30 kDa	NHS ester	Lys
CIMZIA (Certolizumab pegol)	2-Branch 40 kDa	Maleimide	C-terminal Cys
Krystexxa (Pegloticase)	Linear 10 kDa	*p*-Nitrophenyl-carbonate ester	10–11 random sites, Lys
Clinical products			
Peginesatide (Hematide)	Branched 40 kDa	Possibly *p*-nitrophenyl-carbonate ester	Dipeptide amino linker (MacDougall 2008)
ADI-PEG 20	Linear 20 kDa	Succinimidyl succinate	11 random sites, Lys (Holtsberg et al. 2002)
PEG-IFN beta 1a (BIIB017)	Linear 20 kDa	Aldehyde reductive amination	N-terminus (Baker et al. 2010)
PEG-Hemoglobin (Hemospan)	Linear 5.5 kDa	2-Iminothiolane and maleimide	~7–8 random sites: Cys 93 and thiolated (alpha chain) Lys 7, 16, 40 or (beta) Lys 8, 17, 59, 66, 95, 132 (Vandegriff et al. 2008)
PEG-SN38 (EZN-2208)	4-Branch 40 kDa	Active ester coupling to amine linker on SN38	4-Branch to four SN38 molecules (Zhao et al. 2008)
PEG-hGH (ACP-001)	Unknown	TransCon Releasable Linker	Unknown
PEG-hGH (ARX-201)	Linear 30 kDa	PEG-aminooxy and *p*-acetyl-phenylalanine	Y35pAcP mutation (Cho et al. 2011)
PEG-Naloxol (NKTR-118)	Unknown	Unknown	Unknown

(continued)

Table 4.4 (continued)

Biotherapeutic	MW of PEG	Linker chemistry	Site of attachment
PEG-Irinotecan (NKTR-102)	Unknown	Unknown	Unknown (Nektar website)
PEG-Interferon lambda (PEG-rIL-29)(BMS-914143)	Linear 20 kDa	Unknown	Unknown
PEG-PAL	Linear 5 kDa	NHS ester	Random sites: Lys (Sarkissian et al. 2008, 2011)
PEG-rFIX (N9-GP)	40 kDa	GlycoPEGylation	*N*-glycans
PEG-rFVIII	Unknown	Unknown	Unknown
PEG-BDD-rFVIII (BAY 94-9027)	Single large branched	Unknown	Specific amino acid (Ivens et al. 2010)
PEG-Glutaminase (GlutaDON)	Preferred 1–10 kDa	Unknown	Preferred 1–5 sites (Bausch et al. 2007)

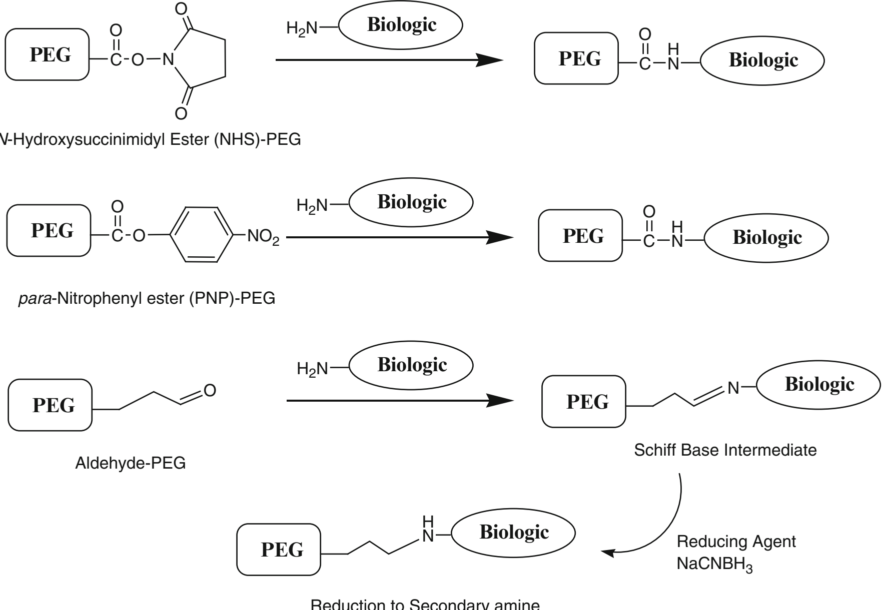

Fig. 4.4 *N*-Hydroxysuccinimidyl ester (NHS), *p*-nitrophenyl ester (PNP), and reductive amination (aldehyde-PEG) chemistries for PEGylation to an amine-containing biologic. In the case of reductive amination, the main target is the amino-terminus of a protein or peptide whereas the NHS and PNP reagents will typically react with available Lys

4.2.1 Conjugation via Amino Groups

The most common linker chemistry used by the commercial PEG products has been an amino-reactive *N*-hydroxysuccinimidyl (NHS) ester (Fig. 4.4) group that reacts with lysines and N-terminal amino groups but can also react to a lesser extent to other nucleophilic side chain groups (serines, tyrosines, histidines, etc.). When this active ester reacts with an amino group, the resulting linkage is a stable amide bond. Lysine tends to be the most abundant and accessible amino acid in proteins and in reactions with NHS-activated PEG reagents, a variety of lysines are possible sites of attachment. For example, in PEG-Interferon α2a (IFN-α2a, PEGASYS), four possible lysines (Lys 31, 121, 131, or 134) are known to be the site of PEG attachment using a branched 40 kDa NHS-activated PEG (Bailon et al. 2001). In Mircera, a 30 kDa NHS-activated PEG binds to either Lys 52 or Lys 46. When smaller size PEG is used, it is possible to attach multiple PEG polymers on the same parent protein. For example, Adagen is composed of multiple PEG polymers of 5 kDa molecular weight (Alconcel 2011, Booth 2009).

Another amino-reactive linker that has been used commercially in a PEG-conjugate is the *p*-nitrophenyl carbonate ester (Fig. 4.4). This functional group reacts with accessible lysines on the surface of the protein and forms urethane bonds.

In the case of Krystexxa (pegloticase aka peg-uricase), approximately 9 strands of 10 kDa PEG were attached to each uric acid tetrameric enzyme via the 12 accessible lysines (Sherman et al. 2008). Interestingly, in developmental studies on the uricase enzyme, a conjugate containing 6 strands of 10 kDa PEG per subunit were found to provide a significantly longer half-life in mice than conjugates with the same total mass of PEG (60 kDa per subunit) but fewer strands of PEG (i.e., 3 strands of 20 kDa or 2 strands of 30 kDa PEG).

Finally, the last amino-reactive PEG conjugation chemistry used commercially is the reductive amination on the N-terminal amino acid. One example of this chemistry is Neulasta (pegfilgrastim) where an aldehyde-functionalized 20 kDa PEG reacts under slightly acidic conditions (pH 5) to react more specifically at the α-amino group of the N-terminal methionine residue of GCSF (filgrastim) (Piedmonte and Treuheit 2008; Kinstler et al. 1996; Molineux 2004). The specific attachment at the N-terminus is afforded by the lower p*K*a of this α-amino group (p*K*a ~7.6–8.0) relative to ε-amino groups of lysines (p*K*a ~10.0–10.2) (Wong 1991). PEG-aldehyde reacts with the α-amine and readily forms an imine bond which is subsequently reduced with sodium cyanoborohydride (or other selective reducing agent) to form a very stable secondary amine (Fig. 4.4).

4.2.2 Conjugation via Thiol Groups

More recently, there have been efforts toward more specific attachment of PEG to provide less heterogeneity, decrease interference with enzyme active sites or protein binding sites and increase reproducibility of manufacturing. One route commonly used is to design into the protein a cysteine mutation in the amino acid sequence at a site distal from that involved in its biological activity. Cysteine is typically chosen for this because its thiol moiety can be much more reactive than amino groups depending on reaction conditions (pH, linker, etc.) affording some measure of specificity. This specificity is increased by the fact that cysteine residues are seldom found in protein sequences, and if they are present, many times they are oxidized and involved in a disulfide bridge and are less accessible and less reactive.

There are several thiol linker reagents that are reactive and somewhat more selective toward cysteine residues, such as maleimides, haloacetamides, haloalkyls, vinylsulfones, and disulfide reagents (Hermanson 2008). These reagents form stable thioether bonds after reaction with cysteine, although there are reports of retro-Michael addition reactions occurring for the maleimide reagents (Baldwin and Kuck 2011). The disulfide reagents undergo interchange reactions with the free thiol of the cysteine resulting in a mixed disulfide product bound to the biotherapeutic. This mixed disulfide bond is susceptible to further thiol interchange or to cleavage via reducing agents or reducing conditions in vivo. These disulfide reagents may have some role as a releasable linker for certain conjugates.

There are a number of clinical conjugates as examples for thiol PEG conjugation but the one commercial product is CIMZIA (Veronese and Mero 2008). This humanized anti-tumor necrosis factor (TNF)-α antibody fragment (Fab′) has an accessible

cysteine in the hinge region that is conjugated to a 40 kDa branched PEG maleimide reagent. Since the reacting thiol resides in the hinge region away from the antigen-binding pocket, there is essentially no loss in biological activity for the PEGylated antibody fragment compared to the native antibody.

4.2.3 Other Conjugation Types

The number of PEG reagents and attachment chemistries has increased over the past decade. Reagents are now readily available with PEG chains of molecular weights ranging up to 80 kDa and configured in various geometries from linear to multi-arm branched and pendants. Reagents range from PEGs with a single reactive group to PEGs containing homo- and hetero-multifunctionalities (Monfardini et al. 1995; Jevševar et al. 2010). Monodispersed PEGs with discrete MWs up to 4 kDa are also available (Quanta Biodesigns).

A number of alternate approaches to amine and thiol chemistries have been developed in order to improve attachment site selectivity. Directing PEG to a single site or location, regiospecificity, is likely to increase process yields, minimize the complexity of product purification and characterization, and potentially reduce PEG interference with bioactivity (Kinstler et al. 1996, 2002; Chapman 2002; Cox et al. 2007; Cazalis et al. 2004; Finn 2009; Buckley et al. 2008 and Greenwald et al. 2003b).

Groups have reported success in this area through the development of new PEG reagent functionalities. One approach (PolyTherics Ltd.) utilizes mono- and bis-sulfone-activated PEGs designed for conjugation to accessible disulfide bridges. Following reduction of disulfide thiols, these reagents undergo bis-alkylation with the two disulfide sulfur atoms to form a stable three carbon bridge in the location of the original disulfide to which the PEG is attached. An advantage of this technology is that it allows for the selectivity of other cysteine reagents when PEGylating biotherapeutics that do not possess free thiols and maintains the structural integrity of the original disulfide bridge (Balan et al. 2007; Brocchini et al. 2008).

4.2.3.1 Chemical Modifications

Many groups have examined chemical modifications, such as periodate oxidation of oligosaccharides and amino acid side chains, as a means to generate new reactive sites for PEGylation (Zalipsky 1995; Wolfe and Hage 1995; Wilchek and Bayer 1987; Dorwald 2007); others have reported site-selectivity through reversible blocking of possible side reaction sites (Tsunoda et al. 2001).

4.2.3.2 Enzymatic Approaches

Site-selective PEGylation of hGH was achieved through the enzymatic insertion of reactive handles via Carboxypeptidase Y (CPY) transpeptidation. Ketone or azide moieties were separately incorporated onto the hGH C-terminus for PEG coupling

using either oxime ligation or copper (I) catalyzed (2 + 3) cycloaddition reactions (Peschke et al. 2007). The term "GlycoPEGylation" refers to a novel PEGylation in which specific glycosyltransferases are used for conjugations of sialic acid-modified PEG reagents to serine and threonine residues and other potential O-glycosylation sites. This technology has been demonstrated in several clinically relevant biotherapeutics such as GCSF, GMCSF, interferon α2b, Factor VIIa, and Factor IX (DeFrees et al. 2006; DeFrees et al. 2007; Klausen et al. 2008). Another enzymatic approach utilizes transglutaminases (TG-ases) for PEGylation targeting glutamine residues. TG-ases catalyze reactions where the γ-carboxamido group on glutamine acts as an acyl donor which can react with amine PEG (PEG-NH_2) to form an amide linkage. Studies with several therapeutic proteins have demonstrated that these PEGylation reactions can be strikingly site-specific with PEGylation at only one or two specific glutamine residues in many cases (Sato 2002; Maullu et al. 2009). Analysis of the TG-ase reaction suggests that this selectivity results from enhanced enzymatic attack at flexible backbone regions (Fontana et al. 2008).

4.2.3.3 Incorporation of Non-native Amino Acids

In order to gain better control of site-selectivity, efforts have been made to introduce custom amino acids with orthogonally reactive functional side chains. This would allow for exquisite site-directed bioconjugation with PEG reagents that are nonreactive toward native amino acids but specific for the custom amino acid side chains.

One example is the addition of an azido-functionalized methionine analog to the media of an *E. coli* expression system in order to increase the incorporation of this non-native amino acid into the expressed protein (Cazalis et al. 2004). The azido-functional group was used for subsequent site-directed PEGylation using Staudinger ligation with a triarylphosphine-PEG reagent. Previous attempts to make the C-terminal cysteine mutant were unsuccessful while the use of this non-native amino acid method led to the successful generation of a bioactive site-specific PEG-conjugate.

In a second approach, Peter Schultz and coworkers have developed a novel technology for genetically incorporating non-native amino acids directly into exogenously expressed proteins (Deiters and Schultz 2005; Wang et al. 2001, 2003). The group is able to alter the cell's translational workings by incorporating a new *t*RNA/*t*RNA synthetase pair specific for the desired non-native amino acid. In this manner, non-native amino acids with functional groups for site-selective modification may be introduced into a protein sequence. Specifically, a keto-amino acid, *p*-acetyl-phenylalanine, can be incorporated into proteins as a chemical handle for the specific linkage of proprietary PEG reagents. Ambrx has used this technology to generate a number of PEGylated proteins. One molecule (ARX201), a PEGylated hGH, was shown to induce weight gains for a single weekly dose similar to that of daily doses of hGH in the hypophysectomized rat model (Cho et al. 2011). The molecule is currently in Phase II clinical trials.

4.2.3.4 Releasable PEGylation

Other groups have focused on generating PEG reagents with labile linkers for controlled release of the native biotherapeutic into the circulation or to targeted locations in the body. Releasable or reversible PEGylation has been investigated as a means to circumvent PEG-related protein inactivation through slow release of the PEG moiety to generate the active protein in a pro-drug manner. These reagents utilize labile sites in the linkers such as esters or other hydrolyzable groups. One example of this is a PEG benzyl elimination (BE) linker system consisting of a hydrolyzable ester trigger which initiates either a 1,4- or 1,6-benzyl elimination reaction for releasing the native molecule (Greenwald et al. 2003a; Zhao et al. 2006, 2008; Filpula and Zhao 2008). Ascendis Pharma has developed proprietary auto-hydrolyzing linkers (Transcon Technology) with rate of release controlled by pH and temperature (www.ascendispharma.com).

PEG reagents with linkers employing a labile 2-sulfo-9-fluorenyl-methoxycarbonyl (FMS) group have been demonstrated to slowly release several proteins and peptides including Interferon α2, hGH, Exendin-4, PYY, and others (Tsubery et al. 2004; Shechter et al. 2005). One such PEG reagent was demonstrated to slowly release Interferon α2 following subcutaneous administration in rats with active levels peaking at 50 h, with substantial levels still being detected 200 h after administration (Peleg-Shulman et al. 2004).

Another example of reversible PEGylation uses a hydrolyzable β-alanine linkage for slow release. A slow release PEG-hGH conjugate developed with this reagent demonstrated a similar growth response in rats with a single dose compared to daily dosing over a week (Pasut et al. 2008).

4.3 Manufacturing and Formulation Approaches for PEGylated Biotherapeutics

One area of PEGylation science that has not received much attention in the literature is the pharmaceutics of PEGylated biotherapeutics—the development of the formulation, manufacturing, and processes for a new PEGylated molecule into a pharmaceutically acceptable drug product form. A recent review of the product development issues for PEGylated proteins (Payne et al. 2011) covers many relevant areas for PEGylation. This review will cover additional areas of pharmaceutical development.

4.3.1 PEGylation Issues

Although PEGylation affords many desirable qualities for a biotherapeutic, there are challenges to bringing a PEG-conjugate to market: immunogenicity (anti-PEG antibodies), non-biodegradability, polydispersity, and reproducibility. These issues

have been discussed in the literature (Webster et al. 2007, 2009; Armstrong 2009; Jevševar et al. 2010).

During scale-up for clinical and commercial manufacturing, there are a number of formulation and process development considerations to overcome. Some of these include bioprocessing challenges (such as filtration and purification), analytical characterization, formulation issues (such as stability and viscosity), and the impact on the freeze-drying process. These will be discussed in the following sections.

4.3.2 Drug Substance Manufacturing Issues

4.3.2.1 Starting Materials

The quality of the starting material is critical to developing a reproducible, scalable GMP manufacturing process. Impurities in the PEG reagent and/or the biotherapeutic can result in heterogeneities in the subsequent PEGylated product which are extremely difficult to overcome (Seely et al. 2005). Although in recent years high quality low polydispersity PEG reagents have become available from an increasing number of manufacturers, ensuring adequate sourcing and availability of the required quantities of quality PEG reagents for manufacturing PEGylated biotherapeutics remains a key issue.

Concerns for PEG reagents include trace process impurities, PEG-related impurities, PEGylation reactivity, and stability of both the PEG chain and the linker between PEG and the reactive functional group (Gaberc-Porekar et al. 2008; Kumar and Kalonia 2006; McGary 1960; Seely et al. 2005). Susceptibility of PEG chains to oxidation and the reactive groups to hydrolysis and other degradative pathways necessitates detailed impurity profiling and “use” testing to monitor the PEGylation performance of reagent batches.

4.3.2.2 PEGylation Reaction Process Control

GMP manufacture of PEGylated biotherapeutics requires a well-defined PEGylation reaction process that is capable of consistently yielding products of comparable strength, composition and impurity profile, batch after batch. Ideally, the conjugation reaction would yield the desired PEGylated molecule with minimal residual un-reacted species and product-related impurities. A fully optimized reaction process can simplify downstream steps and ultimately lead to increased yields and decreased cost. Kinetic modeling and “design of experiment” DOE studies are recommended to determine optimized conditions for successful API manufacture (Buckley et al. 2008; Fee and Van Alstine 2006).

Conditions such as PEG/biomolecule reactant stoichiometries, concentrations, and reagent order-of-addition schemes; reaction temperatures and times; solution buffers and pH; reaction vessel sizes and geometries; and reagent, intermediate, and

product hold times need to be well defined and optimized (Payne et al. 2011). Understanding reaction kinetics is critical especially in cases where relatively non-selective PEG reagents are being utilized to reproducibly generate comparable batches of a PEG-biotherapeutic. This includes an understanding and control of the kinetics of the possible deactivation via hydrolysis or oxidation of the functional group on the PEG reagent during the reaction. Additionally, side reactions with buffer constituents or reagent impurities must be identified and controlled. And finally, in order to understand these issues, critical in-process control assays must be developed and in place (Buckley et al. 2008; Seely et al. 2005; Fee and Van Alstine 2006).

In cases where novel process steps are required to complete the PEGylation, optimization of those methodologies must also be thoroughly evaluated. For thiol PEGylations, limited reduction steps must be optimized in order to reduce disulfides or to maintain reactive sulfhydryl groups while limiting protein unfolding, disulfide shuffling, or dimer formation (Doherty et al. 2005). PEGylation of partially buried cysteine thiols may require transient denaturing conditions (Veronese et al. 2007) or use of a two-step protocol involving initial glycation of the buried cysteine followed by PEGylation of the oxidized glycosides (Salmaso et al. 2008). For reductive alkylation reactions, choice and concentration of reducing agents such as sodium cyanoborohydride or pyridine borane can be important and the safety implications should be well understood (Cabacungan et al. 1982). PEG reactions such as those catalyzed by enzymes (Peschke et al. 2007; DeFrees et al. 2006; Sato 2002) or those with chemical modifications such as periodate oxidation of polysaccharides prior to the actual PEGylation step (Wolfe and Hage 1995), may require additional in-process monitoring. Lastly, most PEGylation reactions will require a quenching step for stopping the reaction and inactivating residual reactive moieties.

Most PEGylation reactions are carried out in batch reactors where the extent of PEGylation is mainly controlled by fixed reaction conditions such as temperature, time, mixing speed, reagent stoichiometries, and concentrations. Another method to control the reaction is in the manner of addition. Adding the PEG reagent in a single portion, in separate multiple aliquots or a slow continuous feed can have an effect on the final reaction outcome (Fee and Van Alstine 2006). Several alternative approaches to batch PEG reactions have been reported. Size exclusion reaction chromatography (SERC) has been described in which PEGylation occurs in the mobile phase of a size exclusion column. This method utilizes size separation to control the degree of PEGylation and separate the reaction products in a single step (Fee 2003). Packed-bed or “on-column PEGylation” has also been reported where one reactant, either PEG reagent or biomolecule, is anchored to a surface through a covalent linkage with other reagents free in solution. Immobilization of biomolecules in this fashion may result in some control of regiospecificity depending on the orientation of bound biomolecules. Also, the resultant PEGylated biomolecule is attached to the surface which may facilitate its separation from the other components in solution (Monkarsh et al. 1997; Lee and Lee 2004; Baran et al. 2003).

4.3.2.3 Purification Considerations

Even a well-controlled PEGylation process will most likely yield a reaction mixture containing the desired product along with un-reacted PEG and biomolecule as well as reaction product impurities (e.g., PEGylated positional isomers, unwanted multi-PEGylated products, and aggregates). Techniques used for purification of unmodified proteins, peptides, and nucleic acids have been attempted for the purification of PEGylated biomolecules. Reaction mixtures are generally purified through subsequent chromatography and ultrafiltration/diafiltration (UF/DF). However, the performance of a particular PEGylated protein in chromatography and other downstream processes will be quite different from that of the unmodified protein (Fee and Van Alstine 2004; Buckley et al. 2008). Ion exchange chromatography (IEX) is often the first choice in commercial manufacture as conditions can generally be found where the protein-related species will bind, while residual PEG reagent does not. Subsequently, elution conditions can often be readily developed for separation of the protein species relative to the number of PEGs attached (Piquet et al. 2002; Seely et al. 2005; Kusterle et al. 2008; Chapman et al. 1999; Yun et al. 2005). PEG proteins have also been successfully purified by other chromatographic methods such as size exclusion chromatography (SEC) (Fee and Van Alstine 2004) and hydrophobic interaction chromatography (HIC) (Fee and Van Alstine 2006) or through combinations of columns (Clark et al. 1996). A comprehensive discussion on PEGylated protein purification considerations can be found in the review by Fee and Van Alstine 2006.

4.3.2.4 Polydispersity, Hydrodynamic Size, and Viscosity Considerations

The polydispersity, large hydrodynamic volume, viscosity, and other characteristics of PEG can make downstream scale-up considerations for PEG-biomolecules a challenge (Payne et al. 2011). The large hydrodynamic size of the PEGs typically used often interferes with the protein–resin interactions such that separation is dominated by the PEG physical properties and not those of the specific protein. Thus, PEGylated positional isomers become very difficult to purify. Column loading capacities can be greatly decreased through masking of charged residues, either indirectly, through steric interference of proximally located PEG, or directly, due to linkage at amines or carboxyl groups (Pabst et al. 2007). PEG polydispersity can broaden peaks and lower resolution and also complicate process analytics (Veronese and Pasut 2005). Commercial scale downstream processes (e.g., buffer exchange, column loading, and final formulation) often require steps at relatively high protein concentrations. The level of hydration on PEG can lead to PEGylated protein solutions that become very viscous upon concentration. This becomes important with PEGylated peptides where PEG:peptide weight ratios are 5–10:1. High viscosities in PEGylated protein solutions can have deleterious effects on chromatography such as decreased flow rates and increased pressures. These viscous PEG solutions may also slow or completely stop flux rates during UF/DF steps through membrane fouling (Fee and Van Alstine 2006).

4.3.2.5 Analytical Characterization

Process scale manufacture of a PEGylated protein requires well-defined analytical procedures for control of each step. Methods must be developed for PEGylation reaction product characterization and downstream fraction analysis, as well as final drug substance and drug product release.

Similar to purification approaches, traditional protein analytical techniques have been adapted for characterization of PEGylated proteins. For example, the extent and location of PEGylation and impurity profiles can be monitored by such techniques as sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Ramon et al. 2005), size exclusion (SEC)-HPLC (Fee and Van Alstine 2004; McGoff et al. 1988), IEX-HPLC (Pabst et al. 2007; Zhang et al. 2007; Ramon et al. 2005), reverse phase (RP)-HPLC (Park et al. 2009), HIC (Snider et al. 1992), mass spectrometry (MS), proteolytic digests (Kinstler et al. 2002; Schneiderheinze et al. 2009), and capillary electrophoresis. PEG hydrodynamic volume and polydispersity generally necessitates some modification to the technique and subsequent data analysis and often requires a combination of orthogonal approaches (Seely et al. 2005). Since PEG and proteins of similar molecular weights greatly differ in actual size, multi-angle laser light scattering (MALLS) is often used along with SEC to determine the molecular mass and hydrodynamic radius of a PEGylated protein (Kendrick et al. 2001; Koumenis et al. 2000; Fee 2007). Specific detection techniques for PEG such as iodine staining for SDS-PAGE (Kurfürst 1992), or in-line detectors on HPLC such as refractive index (RI) detection (Trathnigg and Ahmed 2011), corona charged aerosol (CAD) detection (Kou et al. 2009), or evaporative light scattering (ELS) detection (Trathnigg and Ahmed 2011) are commonly used in conjunction with standard protein detection methods. Due to the polydispersity of large molecular weight PEG, electrospray ionization mass spectrometry (ESI-MS) techniques of intact PEGylated proteins are usually not a viable option; alternatively, matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) has been used (Cindric et al. 2007). Identification of specific PEGylation sites generally requires proteolytic digests and mapping with HPLC; however, site conjugation is often confirmed through disappearance of PEGylated peaks as PEGylated fragments may be difficult to resolve and/or characterize (Kinstler et al. 2002; Schneiderheinze et al. 2009).

4.3.3 Formulation and Drug Product Manufacturing Issues

The formulation development of a PEGylated biotherapeutic is a complex task. Although the main goal is quite straightforward—to provide a stable commercializable drug product that is acceptable for administration to the patient—the actual work to develop that product is not so simple. Table 4.5 shows the variety of formulations used for some of the commercial PEGylated drug products. There are many things to consider but the two main principles are physical and chemical stability of the whole PEGylated biotherapeutic in solution phase or solid phase.

Table 4.5 Formulations used for commercial PEGylated biotherapeutics

Biotherapeutic	Excipients used	Highest strength dose concentration	Storage
Adagen (Pegadamase)	Monobasic sodium phosphate, dibasic sodium phosphate, sodium chloride, water for injection, pH 7.2–7.4	Liquid, single dose vial, 250 units/mL, 1.5 mL	Refrigerated. Do not freeze
Oncaspar (Pegaspargase)	Monobasic sodium phosphate, dibasic sodium phosphate, sodium chloride, water for injection, pH 7.3	Liquid, single dose vial, 3,750 IU/5 mL	Refrigerated. Do not freeze
Peg-Intron (Peg-IFN α2b)	Dibasic sodium phosphate, monobasic sodium phosphate, sucrose, polysorbate 80. Reconstituted with sterile water for injection	Redipen: Lyophilized, Dual Chamber PFS, 202.5 µg, 150 µg/0.5 mL	Refrigerated. After reconstitution, store refrigerated up to 24 h. Do not freeze
Pegasys (Peg-IFN α2a)	Sodium chloride, polysorbate 80, benzyl alcohol, sodium acetate, acetic acid, pH 6.0	Liquid, single use PFS, 180 µg/0.5 mL	Refrigerated. Do not freeze. Protect from light
Neulasta (Pegfilgrastim)	Acetate, sorbitol, polysorbate 20, sodium, water for injection, pH 4.0	Liquid, single use PFS, 6 mg/0.6 mL	Refrigerated. One freeze acceptable with thaw in refrigerator. Protect from light. Room temperature for 48 h is acceptable
Somavert (Pegvisomant)	Glycine, mannitol, sodium phosphate dibasic, sodium phosphate monobasic. Reconstitute with sterile water for injection	Lyophilized, single dose vial, 20 mg, reconstitute with 1 mL	Refrigerated. Do not freeze. After reconstitution, use within 6 h
Macugen (Pegaptanib)	Sodium chloride, monobasic sodium phosphate, dibasic sodium phosphate, hydrochloric acid and/or sodium hydroxide, water for injection, pH 6–7	Liquid, Single dose PFS, 0.3 mg, 3.47 mg/mL	Refrigerated. Do not freeze or shake vigorously
Mircera (Peg-EPO)	Sodium dihydrogen phosphate monohydrate, sodium sulfate, mannitol, methionine, poloxamer 188, water for injection	Liquid, PFS, 800 µg, 1,333 µg/mL	Refrigerated, PFS, 3-year shelf-life

CIMZIA (Certolizumab pegol)	Liquid: sodium acetate, sodium chloride, water for injection. Lyophilized: sucrose, lactic acid, polysorbate. Reconstitute with water for injection	Liquid, PFS, 200 mg, 200 mg/mL	Refrigerated. Do not freeze. Protect from light. After reconstitution, refrigerate up to 24 h
Krystexxa (Pegloticase)	Disodium hydrogen phosphate, sodium chloride, sodium dihydrogen phosphate, water for injection. Dilute 1 mL in 250 mL IV bag of 0.9 % sodium chloride or 0.45 % sodium chloride prior to dosing	Liquid single dose vial, 8 mg, 8 mg/mL	Refrigerated. Do not shake or freeze. Protect from light

References: Product labels for the respective commercial prescription drugs are available at www.fda.gov

The physical stability of a biotherapeutic protein or peptide refers to its conformational changes, aggregation and/or adsorption properties (Koppenol 2008). This will also be true for a PEGylated biotherapeutic. Some of the drivers for instability are temperature, pressure, pH, denaturing agents, surface interactions and mechanical disruption. Unfolding of the protein leading to aggregation (soluble aggregates or insoluble particulates) could result in decreased bioactivity and/or immunogenic responses. PEGylation is known to decrease aggregation for hydrophobic proteins such as interferon β1b (IFN-β1b) (Basu et al. 2006). When native IFN-β1b was formulated without any surfactant, the protein rapidly aggregated to quantitatively form insoluble particulates within 7 days at neutral pH. In contrast, a PEGylated IFN-β1b (40 kDa PEG) remained soluble during the same timeframe. In another study, it was shown that PEGylation slowed the aggregation rate and increased the solubility of aggregates for PEGylated GCSF (pegfilgrastim) compared to the non-PEGylated GCSF (Piedmonte and Treuheit 2008). It should be noted that although PEGylation may increase the physical stability of a protein, aggregation and other physical degradation pathways can still occur and formulation development is still necessary to minimize these phenomena. For example, the choice of buffering agent for PEG-GCSF at pH 4.0 proved to be important. Aggregation was affected by the choice of buffer (percent aggregates in parentheses): glutamate, acetate and formate (0.5–0.7 %) < citrate, succinate (1.3–1.5 %) < tartrate (3 %). Thus, formulation development is still needed to ensure the physical stability for PEGylated biotherapeutics.

The chemical stability of a biotherapeutic protein or peptide refers to its stability toward chemical degradation pathways such as deamidation of asparagine (Asn) or glutamine (Gln); oxidation of His, Met, Cys, or Trp; and/or hydrolysis of the peptide backbone (Bummer 2008). Side reactions with formulation excipients, leachables in packaging components and processing equipment should also be considered. For a PEGylated biotherapeutic, these all apply with the added burden of chemical stability of the linker (discussed in Section 4.3.3.1) and polymer backbone. Typically, formulation conditions such as pH, buffers, ionic strength (tonicifiers), solvents, additives (antioxidants, radical scavengers or metal chelators), and degassing as well as stabilization into the solid phase (via lyophilization) are used to temper the chemical potential of degradation pathways. For example, oxidation, deamidation and cleavage were observed for the PEG-GCSF conjugate (Piedmonte and Treuheit 2008). These degradation pathways were found to be pH dependent phenomena. Acidic conditions (<pH 3) favor the direct hydrolysis of the amide side chain while at mildly basic conditions (>pH 7) deamidation occurs through a cyclic intermediate (Bummer 2008). Consequently, pegfilgrastim was formulated at pH 4.0 to minimize these degradations. As this example shows, chemical stability still needs to be examined during formulation development of PEGylated biotherapeutics.

One example of how PEGylation can improve the chemical stability of a biotherapeutic against interaction with a formulation component is in the case of octreotide, an octapeptide analog of somatostatin, with the microsphere formulation excipient poly(D,L-lactide-*co*-glycolide) (PLGA) polymer (Na and DeLuca 2005).

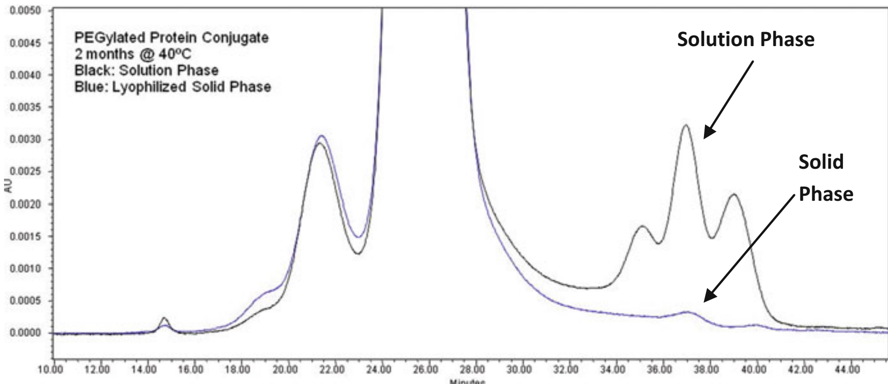

Fig. 4.5 SEC-HPLC overlaid chromatograms of PEGylated protein stability samples (2 months at 40 °C) in solution phase (*black line*) versus lyophilized solid phase (*blue line*). The lyophilized samples were protected from chemical degradation indicated by the reduction in low molecular weight species eluting at ~34–40 min

For this proposed slow release depot formulation, it was found that octreotide interacts with PLGA through an initial adsorption phase followed by acylation of amino groups on the octapeptide with ester groups within the interior of the PLGA polymer backbone. Amino-terminal PEGylation significantly improved the chemical stability by preventing adsorption and acylation by PLGA.

PEGylated biotherapeutics that do not have long-term stability in solution phase, in spite of formulation development efforts, may require storage in the solid phase. Three of the ten PEGylated products are manufactured by freeze-drying (Bhatnagar et al. 2011). Figure 4.5 illustrates an example of the difference in stability for a PEGylated protein in liquid form versus lyophilized (freeze-dried) form. In this example, the lyophilized PEG-conjugate sample had a reduction in the formation of low molecular weight degradation products compared to the solution phase sample. However, the physical degradation pathway forming high molecular weight aggregates appeared to be similar in both solid and solution phase. Typically, a well-formulated lyophilized protein product can be stable for months or years, even at ambient temperatures.

In spite of the increased long-term stability, the process of freeze-drying a protein can cause short-term stresses on the physical structure of the protein. Prevention or minimization of physical damage to the biotherapeutic is critical. The process steps of solution cooling, freezing, freeze-concentration, crystallization, thawing, annealing, vacuum drying, and reconstitution can all potentially lead to physical damage to the protein (Carpenter et al. 1997; Bhatnagar et al. 2007). Although PEGylation can lead to stable freeze-dried products, there is evidence that the PEGylation itself can create some issues one needs to be aware of. For instance, PEG crystallization may occur during freezing of PEGylated proteins. Bhatnagar et al. observed that for a PEGylated human growth hormone (PEG-hGH) and sucrose system, the PEGylation facilitated PEG crystallization during freeze-drying

compared to a control with unconjugated PEG (Bhatnagar et al. 2011). While PEG crystallization did not cause protein degradation during freeze-drying, PEG crystallization during storage appeared to be related to protein instability. The physical state of PEG (amorphous vs. crystalline), the amount of “free” water available for interaction, and the storage temperature appeared to influence protein stability. Water retention by PEG moieties apparently played a role in a separate study of PEGylated bovine serum albumin (BSA) (Tattini et al. 2005). Here it was found that at certain ratios of BSA:PEG, it was difficult to lyophilize. At a 1:1 ratio, the sample formed a “jelly-kind material (sic)” that caused cake collapse. Tightly bound water by the PEG was attributed as the likely cause. In contrast, Mosharraf et al. found that when PEG is covalently bound to protein (pegvisomant) the residual moisture measured in lyophilized sample cakes was lower than in samples of non-PEGylated protein (Mosharraf et al. 2007). They also found that dissolution of the PEGylated protein cake was faster than that for the free protein + free PEG sample. These examples highlight the importance of a thorough characterization of the phase behavior of PEGylated proteins during freeze-drying and storage.

4.3.3.1 Linker and Conjugate Stability

Although the polyethylene ether backbone of PEG polymers is relatively stable, PEG reagents must utilize a linker moiety to attach to a protein or peptide. This linker moiety may be susceptible to cleavage or other chemical stability issues. The linker chemistries and types of linkers were discussed in Sect. 4.2 and the linkers used in commercial and clinical PEGylated biotherapeutics are shown in Table 4.4. Here we will describe what is known on the stabilities of these linker types in regard to formulation development.

Active Ester Coupling Chemistry

The majority of commercial PEGylated biotherapeutics rely on the amino-reactive active ester couplings using PEG reagents functionalized with an NHS ester or *p*-nitrophenyl carbonate ester or in situ activating agents such as those used in solid phase peptide synthesis to activate a carboxylic acid on either the PEG or on the biotherapeutic and react that with an amino group on the other component. In all these chemistries, the resulting linkage is the same—an amide bond between the PEG and the biotherapeutic. Since an amide bond is quite stable, the active ester linker chemistry can be considered to result in a stable linkage. This bond is stabilized by the resonance of the lone pair of electrons on the nitrogen with the carbonyl group. However, the carbon atom may be considered somewhat electrophilic and the oxygen atom may be considered somewhat nucleophilic, thus potentially making the amide bond susceptible to intra- or inter-molecular cleavage or cyclization reactions. In formulations, the amide bond is susceptible to cleavage under strongly acidic or basic conditions. Metal ions are known to play a part in oxidative (or

hydrolytic) cleavage of amide bonds (Li et al. 1995, Rana 1991, Bantan-Polak and Grant 2002). Thus, formulation efforts for PEGylated bioconjugates with the active ester coupling chemistry will be in line with the chemical stabilization of the protein or peptide being formulated.

Reductive Amination Chemistry

Reductive amination results in a stable secondary amine linkage which is not readily hydrolyzed in aqueous environments. In chemical stability terms, this is a very stable bond. There are some considerations of reductive amination in physical stability terms, though, that need to be addressed. For example, in the case of PEG-GCSF, the reductive amination route using a PEG-aldehyde provided a fivefold decrease in aggregation versus an acylation route using a PEG-NHS ester despite the same site specificity at the N-terminus for both routes. Kinstler suggested that the charge neutralization caused by the acylation route may be the factor causing aggregation (Kinstler et al. 1996). During PEG reagent design and formulation development, one needs to remember that the basic amino moiety at the linkage site will be protonated in neutral buffer. For most cases, this should be beneficial as it keeps the charge density more similar to the original biologic. An additional advantage for reductive amination during drug product development is the N-terminal site specificity. Aldehyde reactivity is mainly targeted at the amino-terminus at lower pH which results in a more homogeneous mono-PEGylated product. This was apparent during the clinical development of PEG-IFN-β1a where the reductive amination route provided a PEG-conjugate with a single mono-PEGylated positional isomer simplifying drug product manufacturing and release testing (Baker et al. 2010).

Maleimide Linker Chemistry

Maleimide chemistry has been used for thiol-specific couplings and has gained popularity as a PEGylating linker chemistry with the approval of CIMZIA (C-terminal cys on anti-TNF Fab′ coupled to a branched 40 kDa maleimide-PEG). The maleimide linker provides an efficient and site-specific route to PEGylation. Although it appears the maleimide forms a stable thioether bond, the resulting succinimidyl ring can undergo hydrolysis in the formulation buffer and/or can undergo deconjugation or retro-Michael additions under reducing conditions in vivo (Baldwin and Kuck 2011; Lin et al. 2008). Interestingly, it was found that the microenvironment of the site of attachment of the PEG-maleimide may have an influence on the route of in vivo degradation: ring opening hydrolysis for positively charged sites or deconjugation/thiol exchange for plasma accessible sites (Shen et al. 2012). Figure 4.6 illustrates the degradation routes that the maleimide ring can undergo. Ring opening hydrolysis produces a succinamic acid moiety to the bioconjugate. During formulation stability studies on maleimide-linked PEGylated biotherapeutics, the appearance of new acidic species on analytical IEX-HPLC, isoelectric

Fig. 4.6 PEG-maleimide linker degradation routes. After the conjugation of PEG-maleimide to a thiol-containing biologic, the PEG-conjugate can degrade through two maleimide degradation routes, Retro-Michael addition or hydrolysis to form one of two possible isomers of the succinamic acid thioether

focusing (IEF), or imaging capillary electrophoresis (iCE) can be an indication of this ring opening phenomena (unpublished data). Further degradation of the ring-opened species could potentially lead to linker cleavage and loss of PEG polymer. However, ring opening could also lead to the formation of a related ("open-ring") form that is as active and stable as the original PEGylated biotherapeutic. During formulation development, the pharmaceutical scientist should be aware of ring-opening phenomena with a maleimide-linked PEGylated biotherapeutic.

4.3.3.2 Viscosity and Injectability of PEG-Conjugate Solutions

Among the several physical properties endowed upon a conjugate by PEGylation, the property of viscosity perhaps plays the largest impact on formulation development. Typically attributed to the self-association of PEG groups, the viscosity of PEGylated biotherapeutics increases with PEGylation number, size of PEG reagent, and concentration of PEG-conjugate required for dosing (Sahu et al. 2009). Increased branching of the PEG reagent appears to decrease the viscosity of the

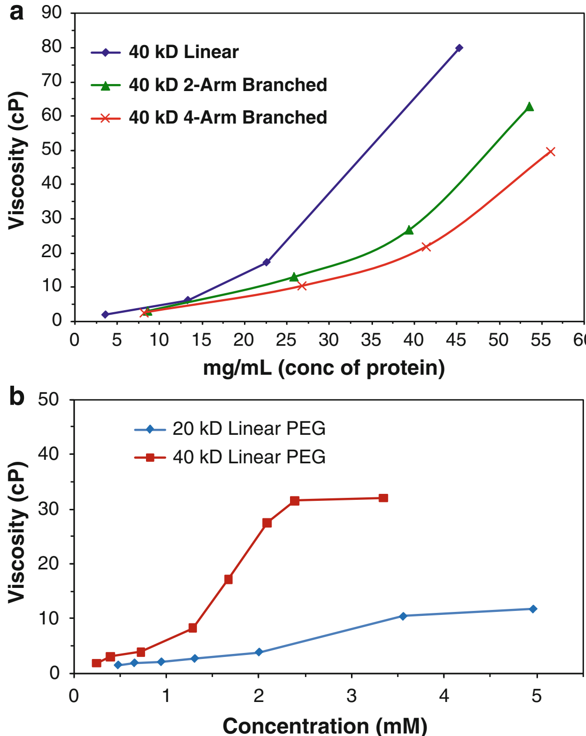

Fig. 4.7 The effect of PEG branching (*graph A*) and PEG molecular weight (*graph B*) for a series of comparable PEG-conjugates on their respective solution viscosities at different concentrations

PEG solution. Figure 4.7 displays the correlation of viscosity with PEG concentration, size of PEG and branching of PEG. For PEGylated biotherapeutics of low potency that require a large dose (and thus a high concentration) and/or for a PEG reagent of high MW, viscosity of the solution is becoming more of an issue (Payne et al. 2011; Bailon and Won 2009). Typically at low concentration, the viscosity of a PEGylated biotherapeutic is roughly proportional to its concentration. However, at higher concentration there is an inflection point and the relationship becomes more exponential. For PEGylated biotherapeutics with a viscosity over 50 cP (approximate limit for subcutaneous (sc) injections) (Du and Klibanov 2010; Liu et al. 2005), this can become problematic. Harris et al. point out the relationship that viscosity has with the syringeability of therapeutic antibody solutions, thus highlighting the difficulty patients and administrators may have with highly viscous solutions (Harris et al. 2004). Another factor to consider for the preferred sc injection is the limit in the volume that can be administered by this route (typically set at approximately 1.5 mL). In order to meet the required efficacious dose, this volume limitation requires that high concentrations may be needed. An example of a highly viscous PEGylated biotherapeutic drug product on the market is CIMZIA from UCB. The viscosity of this solution is estimated to be ~90–100 cP (unpublished data). This PEGylated Fab′ is dosed subcutaneously at 200 mg/mL and requires a

23 gauge needle (vial) or a 25 gauge needle (PFS) to handle the viscous solution for sc administration (CIMZIA product label, 2010). Therefore, for PEGylated biotherapeutics of high concentration, viscosity could be a major concern during formulation and can be a challenge to the successful development of a drug product targeted for subcutaneous delivery.

4.3.3.3 Excipient and Design Strategies for Stability and Viscosity

For the formulator faced with a PEGylated biotherapeutic with either stability or viscosity issues, there are excipient and design strategies available. When approaching stability issues, it is important to identify the type of degradation route involved. For our purposes, we will not discuss the major protein formulation strategies for addressing protein instabilities but rather focus on the added potential instabilities that might be present for a PEGylated biotherapeutic. These could include PEG linker instability, polymer cleavage, or polymer-catalyzed degradation. Additionally, excipient and design strategies for viscosity due to the PEG polymer will be discussed.

Among all the commercial and clinical PEG linker chemistries, the maleimide linker has the most well-known route of degradation: ring opening hydrolysis. For formulation development of a maleimide-linked PEG-conjugate, efforts should be made to reduce the hydrolysis of the thiol-adduct succinimidyl ring through pH and buffer considerations. Since ring opening is catalyzed by base hydrolysis (Kalia and Raines 2007), lowering the pH should help reduce the hydrolysis rate. Additionally, it was found that ring opening hydrolysis is catalyzed by molybdate (MoO_4^{2-}) and chromate (CrO_4^{2-}) metaloxo ions. Minimizing the levels of these metaloxo ions in container closure leachables, process contact materials and excipients should help to minimize this ring opening catalysis. Formulation development should keep these factors under consideration to stabilize PEG-conjugates using maleimide linker chemistry.

PEG polymer backbone cleavage, although less likely to occur compared to linker instability, may be attributed to peroxides or oxidative radicals in solution. Polyether-type polymers are susceptible to oxygen insertion or hydrogen abstraction which can lead to carbon–carbon or carbon–oxygen bond cleavage (Kerem et al. 1998). These oxidation degradation pathways can also be mediated by redox metal ions (Fe^{3+}, Cu^{2+}) and oxygen-derived species in solution. Note that if metal ion-catalyzed oxidation is the culprit, antioxidants may actually catalyze the redox cycle (Kamerzell et al. 2011). In one study, PEG polymer was found to degrade in the presence of ascorbic acid (0.2–4 mg/mL) and oxygen (Vijayalakshmi 2011). The degradation was likely due to ascorbate radical formation via molecular oxygen activation. However, these experiments were performed in saturated oxygen solutions and it is unclear how these data translate to biotherapeutic formulations. In a different study, de-PEGylation and PEG truncation were found to occur for a PEG (40 kDa)-conjugate where the Coomassie stained SDS-PAGE gels identified the protein portion of the conjugate resulting from de-PEGylation and truncation and an iodine-based PEG stain visualized the free PEG (40 kDa) and the truncated PEG (20 kDa) on the gel (Buckley et al. 2008). It was not determined whether the

truncation was from backbone oxidation/scission or by cleavage at a branching point (amide bonds). Typically, PEG oxidation is prevented by careful choice in the quality of PEG reagent procured from the vendor (low peroxide levels) and by carefully keeping the PEG reagent under inert atmosphere. In the rare case that PEG backbone cleavage is detected, nitrogen headspace in the vial, antioxidant additives (being prudent with the use of ascorbic acid) and/or chelators in the formulation could be useful preventative tools.

PEG polymer-catalyzed degradation is not a major occurrence but could have subtle effects on the stability of the protein, linker, or combinations of both. In spite of the fact that most PEG reagents are methoxy-capped and do not have a terminal hydroxy group, the type of PEG reagent used could have an effect on the degradation routes and rates for PEGylated biotherapeutics. For example, in one study of three different branching types of 40 kDa PEG (linear, 2-branched, and 4-branched), the apparent surface charge of the PEG-conjugate varied (Vugmeyster 2011) as well as the distribution of low levels of various low molecular weight degradant levels during accelerated stability studies (unpublished data). This seems to indicate that the structure and/or charge masking of the PEG protein had some subtle role in the degradation mechanism. Depending on the degradation route, formulation efforts may reduce the significance of any minor degradant. However, careful design of the PEG reagent (or polymer reagent), linker chemistry, and/or linker site could potentially modulate the formation of these cleavage degradation products. This would need to be determined on a case-by-case basis for each PEGylated biotherapeutic.

For the formulator faced with a PEGylated biotherapeutic solution of high viscosity, there are a few excipient strategies available. Many of the same viscosity challenges that are seen for high concentration protein formulations (Harris et al. 2004) are also found for PEGylated biotherapeutics; indeed, many of the solutions to those challenges may be the same. Understanding the factors that affect viscosity is important. Although this field is complex, a few of the main factors include net charge, nature of intermolecular interactions, and effective volume of the molecule (Yadav 2010). The intermolecular interaction or self-association of the PEG polymer chains is likely to be the cause of most of the increase in viscosity from the unPEGylated protein to the PEGylated version (Payne et al. 2011). However, little is known on the PEG–protein interactions and the effect of the PEG on the conformation of the protein (Kerwin et al. 2002), which could potentially play some role. Harris et al. point out the relationship that viscosity has with the syringeability of a therapeutic antibody solution and note that increases in NaCl concentration decreased the viscosity and increased syringeability (Harris et al. 2004). In contrast, Kerwin et al. found that for a PEGylated type I soluble tumor necrosis factor receptor (sTNF-RI), viscosity was affected by pH and size of PEG and that additives such as sorbitol and glycine were able to reduce viscosity greater than NaCl (Kerwin et al. 2004). In a different study, Nielsen and Ostergaard were able to show decreased viscosity for PEGylated GLA domain-containing proteins using divalent cations (Nielsen and Ostergaard 2009). Du and Klibanov studied the effect of hydrophobic salts and chaotropic anions on high concentrations of BSA and gamma-globulin and were able to show decreases in viscosity (Du and Klibanov 2010). The question remains whether

these excipients would decrease viscosity for PEGylated biotherapeutics. Indeed, there is little published in the literature on the effects of excipients with viscous solutions of PEGylated biotherapeutics.

Another way of reducing viscosity may be in the design of the PEG reagent itself. It is well known that the larger the molecular weight of the PEG polymer, the more viscous the solution (Holyst et al. 2009). Designing a PEG reagent that provides the shortest MW polymer to give a reduced viscosity but still afford the necessary half-life and efficacy required of the PEG biotherapeutic will be a balancing act for the project design team. As mentioned previously, in addition to length, the branching of the PEG reagent affects viscosity. Figure 4.7 illustrates the effect of branching on viscosity. From this graph, it is apparent that increased branching decreases viscosity. So, branching and reducing MW are design strategies for reducing viscosity in PEGylated biotherapeutics. Another design strategy is to investigate alternative polymers to PEG. This strategy is outside the scope of this review and has been discussed elsewhere (Vincent et al. 2009; Pasut and Veronese 2007; Pollaro and Heinis 2010; Knop et al. 2010).

4.4 Conclusions

For proteins and peptides that have short plasma half-life, low solubility, aggregation, immunogenic effects, and/or proteolytic degradation, PEGylation has proven to be an important enabling drug delivery tool. The number of commercial and clinical PEG biotherapeutics made it one of the most preferable tools to develop a successful product. However, the complexities of designing, manufacturing, and formulating PEGylated biotherapeutics have not decreased. Indeed, there are a number of improved PEG reagents and improved analytical, manufacturing, and formulation tools and knowledge; therefore, the pharmaceutical scientists who are taking on the challenge of PEGylating a biotherapeutic have many choices and need to be well-informed before embarking on their PEG project. Hopefully this review has provided some insight into formulation approaches and design strategies for development of a PEGylated biotherapeutic.

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Chapter 5
Considerations for the Development of Nasal Dosage Forms

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Abstract The anatomy and physiology of the nasal cavity provide unique advantages for accessing targets for local, systemic, and potentially central nervous system drug delivery. This chapter discusses these advantages and the challenges that must be overcome to reach these targets. The chapter then comprehensively reviews nasal dosage forms, analytical testing, and regulatory requirements in the context of existing nasal spray products. Since nasal sprays are moving towards being preservative-free, the chapter covers specialized methods of achieving a sterile product, namely, formulation strategies, manufacturing strategies, and the device landscape that support this upcoming platform. Finally, the chapter reviews various pathways for regulatory approval around the world, for brand and generic, with particular emphasis on the growing acceptance of in vitro data for locally acting nasal spray products.

5.1 Introduction

Preservative-free nasal spray drug products represent a small portion of the overall drug delivery market. However, the desire to remove preservatives from formulations driven by concerns over potential damage from long-term use coupled with innovations in device technology has allowed Pharma companies to consider preservative-free nasal sprays as a viable option. In this chapter, an overview of nasal

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cavity physiology will be presented along with a review of locally and systemically acting drug products. Current formulation and manufacturing strategies are discussed along with the device landscape that enables preservative-free formulations. Finally, the pathway for global regulatory approval will be outlined including considerations for in vitro analytical test requirements.

5.2 Nasal Physiology

A schematic of a human nasal cavity is shown in Fig. 5.1. Two nostrils, also referred to as the nasal vestibule, mark the entrance into the nasal cavities. At the end of the nasal vestibule, the diameter of each cavity decreases at a point called the nasal ostium (Newman 1993). The septum separates the two cavities, which extend, on average, 12–14 cm from the nostrils to the junction between the nose and pharynx (Vidgren and Kublik 1998; Marom et al. 1984). This junction is called the nasopharynx. The nasal-associated lymphoid tissue (NALT), an area that may be associated with inducing mucosal immunity, is located in the nasopharynx. Within the nose itself, the main nasal passage is further divided by three projections from the septum called turbinates (Pontiroli et al. 1989). The inferior, middle, and superior turbinates increase the total surface area of the nasal cavity to 150 cm^2 (Pontiroli et al. 1989). The total volume of each cavity is 7.5 mL.

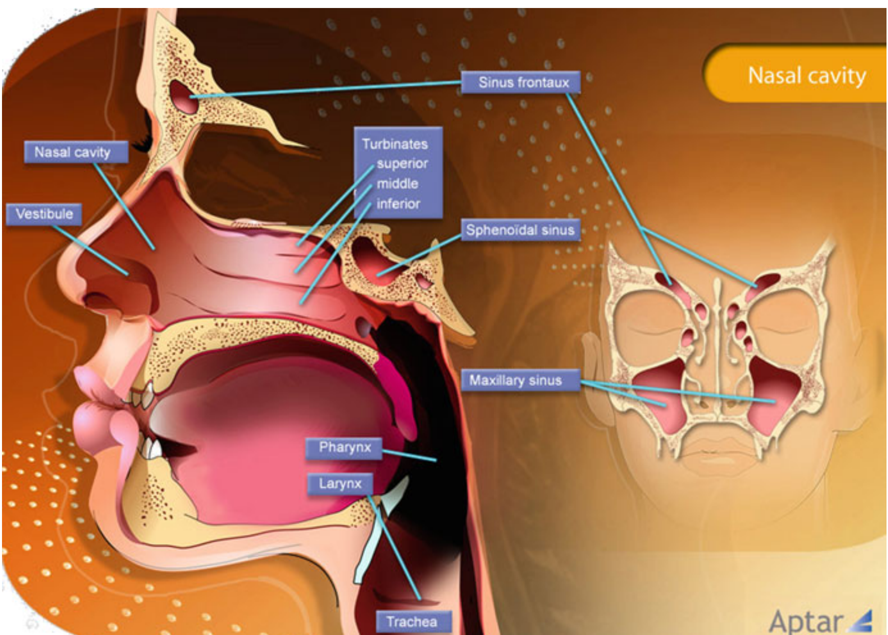

Fig. 5.1 Human nasal cavity anatomy (courtesy of Aptar Pharma)

The nasal mucosa is lined with stratified squamous, pseudostratified columnar, and transitional epithelia cells (Adams 1986). The stratified squamous and transitional types are mainly found in the anterior third of each cavity. Cells in this region are neither ciliated nor well vascularized. The columnar type, also known as respiratory epithelium, is located in the posterior two thirds. The respiratory region contains ciliated cells, mucous secreting goblet cells, and basal cells (Petruson et al. 1984). The respiratory epithelium is also highly vascularized, innervated, and drained by an extensive lymphatic network (Pontiroli et al. 1989; Schipper et al. 1991). The olfactory epithelium, which contains cells that provide a sense of smell, is located near the superior turbinate and adjacent to the nasal septum (Schipper et al. 1991). The main function of the nose is to warm and humidify inspired air and to filter inhaled, potentially toxic or infectious, particles from the airstream (Pontiroli et al. 1989). Thus, the nasal cavity primarily acts as a defense mechanism by protecting the lower respiratory tract (Andersen and Proctor 1983).

Inhaled particles or droplets are thought to deposit in the nose by three mechanisms: inertial impaction, gravitational sedimentation, and Brownian diffusion (Brain and Valberg 1979; Newman et al. 1982; Gonda and Gipps 1990). Of these, inertial impaction is the most predominant for two main reasons. First, the air passageway constricts sharply approximately 1.5 cm into the nose at the nasal ostium (Mygind 1985). This constriction accelerates the inhaled air and increases turbulence (Yu et al. 1998). Secondly, the air stream must change direction at this constriction to enter the turbinate region. Particles that are large or moving at high velocity cannot follow the air stream as it changes direction due to their high momentum. Such particles continue in their original direction of travel and impact the airway walls, particularly at the leading edge of the turbinates. Because the drug-laden droplets for most aqueous nasal sprays are so large (30–60 μm) (Chien et al. 1989), a high percentage of the spray impacts in the anterior third of the nasal cavity (Hardy et al. 1985). However, droplets that are smaller than 10 μm may bypass the nasal cavity and deposit in the lower respiratory tract, which may be deemed as a risk by regulatory agencies.

A particle that deposits on the nasal mucosa may exert a local effect and/or be absorbed into the blood stream. Absorption is facilitated by a highly vascularized, large surface area with relatively low enzymatic activity. Since blood leaving the nasal cavity bypasses the liver, first pass hepatic metabolism can be avoided, making the nose a suitable target for drugs with low oral bioavailability. However, cytochrome P-450-dependent monooxygenase has been reported to metabolize compounds in the nasal mucosa such as cocaine and progesterone (Dahl and Hadley 1983; Brittebo 1982).

Nasal absorption can be rapid. Concentration vs. time profiles similar to intravenous administration have been reported for nicotine and butorphanol (Henningfield and Keenan 1993; Bristol Myers Squibb Company 1999). Absorption is thought to take place primarily in the respiratory zone (posterior, ciliated two thirds) of the nasal cavity. However, the absorption rate at specific deposition sites has not been clearly defined (Vidgren and Kublik 1998). Animal studies have shown that drugs can be absorbed through transcellular and paracellular passive absorption, carrier-mediated transport, and by transcytosis (Bjork 1993; McMartin et al. 1987).

Caution should be exercised when extrapolating results from animal models to man, according to some published literature (Illum 2000). Rats, rabbits, sheep, pigs, dogs, and monkeys have all been used as models for nasal drug absorption. In man, the surface area/body weight ratio is 2.5 cm^2/kg (Illum 2000). The surface area/body weight ratios for the animals above range from 7.7 to 46 cm^2/kg except for sheep that have a ratio of 0.2 cm^2/kg (Illum 2000). In addition, animal's nasal cavities are structurally different than man because they lack a third turbinate. To deliver nasal sprays into the nose of many of these animals, the animal needs to be anesthetized or sedated, which also can affect drug absorption. In short, animal models produce absorption results that fail to accurately predict the results in man (Illum 2000).

The nose filters undesirable chemicals and bacterial and viral particles from the inhaled airstream. Particles depositing in the anterior regions are physically removed from the nose by wiping, blowing, or sneezing. Although these regions (nasal vestibule and leading edge of the turbinates) are non-ciliated, some of the surfaces are covered with mucus. Here mucus flow is slow, 1–2 mm/h, and occurs mainly due to its connection to the mucus layer in the posterior nose (Hilding 1963).

Unabsorbable particles that adhere to the mucus layer that lines the respiratory epithelium are swept towards the nasopharynx by ciliated cells through a process called mucociliary clearance. They are ultimately swallowed.

The mucus layer is predominately aqueous (90–95 %). However, glycoproteins in mucus give it a gel-like structure. The velocity of mucus transport in ciliated regions is about 6 mm/min (Andersen and Proctor 1983). Particles that partition into mucus or deposit on its surface are typically removed from the nasal cavity in 20 min (Andersen and Proctor 1983). Obviously, physical removal of particles either by wiping the nose or by mucociliary clearance is a major component of the nose's defense mechanism. For drug delivery, these processes can oppose local drug activity or absorption.

The rate of mucociliary clearance can be altered by pathophysiology such as a common cold or cystic fibrosis, environmental conditions that affect the mucus content, by drug-induced side effects, or potentially by excipients found in nasal spray formulations. A controversial example of such an excipient is benzalkonium chloride (BAC) which is used to prevent microbial growth. A review of BAC (Marple et al. 2004) studies suggest that BAC may cause changes to ciliary beat frequency, ciliary morphology, mucociliary clearance or may potentially damage the epithelial lining. However, after assessing all the literature, the reviewers concluded that BAC is safe to use in nasal spray formulations. A more thorough discussion of use of BAC in formulations is presented later in this chapter (Sect. 5.4).

When delivering drugs to the nose, one must consider the interplay between the formulation, device, and the patient. These three factors greatly affect where the drug-laden droplets or drug particles deposit within the nasal cavity. The site of deposition in the nose is recognized as one of the keys to success or failure of nasal drug therapy. Although this concept is widely recognized (Vidgren and Kublik 1998), only one study actually relates deposition pattern to biologic response (Harris et al. 1986).

Table 5.1 Initial site of deposition, clearance of radiolabel from the nose, and pharmacokinetics of intranasal desmopressin

Device	Initial deposition site	50 % clearance ($t_{1/2}$, min)	AUC (μg × h)	C_{max} (pg/mL)
Spray (2 × 0.05 mL)	Anterior	240	3,675	675
Spray (2 × 0.1 mL)	Anterior	120	3,556	587
Drops (Rhinyle catheter)	Posterior and nasopharynx	20	1,599	316
Drops (Pipette)	Posterior and nasopharynx	14	1,318	244

Results adapted from Harris (1986). Clearance of the radiolabel (^{99m}Tc-HSA) was determined by acquiring images with a gamma camera over an 8-h period

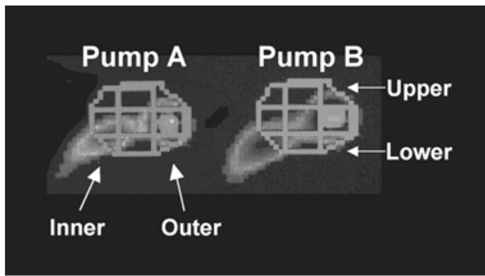

Fig. 5.2 This figure shows gamma scintigraphs following use of Pump A and Pump B in the same volunteer. The nasal cavity was divided into a nine region grid. Deposition in the upper, lower, inner, and outer regions of the grid was calculated as described previously (Suman 1999). The outer region represents the anterior portion of the nasal cavity including the nostrils

This detailed study related deposition pattern, clearance, absorption, and response for desmopressin admixed with radiolabeled HSA delivered by sprays and drops (Harris et al. 1986). The spray formulation deposited in the front of the nose (anteriorly) while the drops covered more surface area. Since the drops covered a larger surface area, it seems logical that the drops would have elicited a greater response. In fact, the opposite was true. The drops were cleared faster by mucociliary clearance since they deposited in posterior regions of the nasal cavity (where cilia move the mucus layer faster). The spray was retained longer, allowing more time for absorption of desmopressin to occur (Table 5.1). The levels of factor VIII in the blood in response to delivery of desmopressin were significantly greater after administration with the spray compared to the drops.

Today's generations of nasal devices typically deposit droplets in the anterior portions of the nasal cavity due to inertial impaction and the size and/or velocity of the droplets. For example, the deposition patterns from two commonly used nasal spray pumps (Suman et al. 2002) were compared in human volunteers. A radiolabeled nasal nicotine solution was administered in a crossover study. Deposition pattern was determined by gamma scintigraphy. The mean droplet sizes for each of the pumps were 47 and 53 μm for Pump A and Pump B, respectively. The results, Fig. 5.2, indicated that both pumps produced similar deposition patterns and that the

droplets were deposited primarily in the anterior regions of the nose and along the floor of the nasal cavity. In this case, the size of the droplets determined the primary site of deposition.

While nasal nebulizers have been shown to cover more surface area in the nasal cavity by decreasing droplet size (Suman et al. 1999), a simple reduction in droplet size alone does not guarantee an increase in the deposition pattern beyond the anterior nose. Nasal aerosols (Newman et al. 1987b) that utilize propellants to generate the spray have been shown to have smaller droplets compared to conventional nasal sprays. However, the deposition pattern is even more localized because of the exit velocity of the plume. The droplets cannot make the bend in the nasal airway and deposit in the front of the nose. This also leads to slower clearance from the nasal cavity for the pressurized formulation as the droplets deposit on non-ciliated regions of the nose.

Despite the challenges of delivery and maintaining contact with the nasal epithelium, the nose is a very attractive site for administration for both locally and systemically acting drugs.

5.3 Local vs. Systemic Action

The easy access to the middle meatus and turbinates gives nasal drug delivery a unique advantage for local pharmacological action, systemic delivery, and potential for nose to brain delivery. The turbinates are richly vascularized and have a large surface area, which makes them an ideal target for systemic drug delivery. In addition, both the olfactory nerve and trigeminal nerve innervate the nasal cavity, which makes them a potential target for nose to brain delivery (Dhuria et al. 2009). Drugs reaching these targets can be rapidly absorbed across the thin membranes and can achieve potentially faster onset of action at lower doses while avoiding the disadvantages of oral dosage forms, namely, first pass metabolism and side effects from drug interactions with other organs (Dhuria et al. 2009; Laube 2007).

By delivering directly to sites of action, nasal drug delivery offers greater convenience and safety. It is a noninvasive and a painless method of drug administration, encouraging greater compliance compared to other routes of administration. Another advantage of nasal drug delivery for patients taking multiple drugs is that a nasally delivered drug may act as an adjunct to another drug given orally or intravenously (Behl et al. 1998a; Costantino et al. 2007).

5.3.1 Local Targets for Allergies

For the treatment of allergies, nasal drug delivery can place therapeutic agents within close proximity of the middle meatus and turbinates, the sites of inflammation. Thus sufficiently high levels of potent corticosteroids, antihistamines, or decongestants (Newman et al. 2004) can reach receptor sites at the target tissue, while systemic blood levels of these drugs are minimized.

Reducing this systemic exposure minimizes well documented side effects (Trangsrud et al. 2002; van Drunen et al. 2005). For example, antihistamines are known to sedate and interfere with psychomotor abilities. Delivered intranasally, these symptoms are absent (Costantino et al. 2007) because the drug does not reach the blood. Locally acting drugs have minimal or low bioavailability, and any blood levels that are detected have no correlation to efficacy because the drugs act locally. Table 5.2 summarizes commercially available prescription treatments for locally acting drugs approved in the United States and EU.

5.3.2 *Systemic Delivery*

In addition to topical treatments, the vascular-rich turbinates lend themselves to systemic drug delivery. Absorption in the nose can be rapid, and allows some molecules to achieve a greater bioavailability compared to oral administration. The turbinates have a large surface area and thin membranes. When drug contacts these membranes, rapid absorption into the blood occurs (Laube 2007; Newman et al. 2004). Unlike oral dosing, this absorption into the blood happens without first undergoing enzymatic degradation in the gastrointestinal (GI) tract nor first pass metabolism in the liver (other than the small amount that may be swallowed). Bypassing these metabolic pathways for poorly absorbed drugs allows comparable or greater blood levels, faster onset, and at a lower dose. These advantages (e.g., improved bioavailability, faster onset of action, lower dose) are particularly beneficial for drugs with potential toxic effects on the liver. When delivered through the nasal cavity, only a fraction of dose that may be swallowed could potentially reach the liver, instead of the entire dose when orally administered. When given orally, all drugs that clear the gastrointestinal tract are then available for the liver. Systemically acting drugs could therefore be more effective and safer when delivered intranasally directly to the blood supply within the turbinates.

Several marketed products use the intranasal route of administration to systemically deliver drugs for conditions such as pain and osteoporosis. MedImmue's FluMist®, approved in 2003, delivers an annual influenza vaccine intranasally (see Product Profile) while Novartis' Miacalcin® and Unigne Laboratories' Fortical® are indicated for osteoporosis. Other systemically acting nasal products include pain medications for migraines: Imitrex® (sumtriptan nasal spray) marketed by GlaxoSmithKline, Migranal® (marketed by Valeant), and Zomig® (marketed AstraZeneca) and examples for pain management indications include Sprix® (marketed by Daiichi Sankyo) and Instanyl® (marketed by Takeda). Refer to Table 5.3 for a summary of the current commercial prescription landscape for systemically delivered nasal products in the United States and EU. Several areas of research and development are ongoing for nasal delivery routes of administration including the delivery of insulin for treatment of Type 1 diabetes (including Nasulin® under development by Cpex Pharmaceuticals) and the treatment of infectious diseases (including hepatitis C, HRV/SARS).

Table 5.2 Commercially available locally acting nasal prescription products in the United States and EU as of December 2011 (courtesy of Lauren Seabrooks, Merck and Co., Inc.)

Commercially available Rx locally acting nasal products				
Product	API	Delivery	Company	Indication
Veramyst Avamys	Fluticasone Furoate	Spray	GlaxoSmithKline	Allergic Rhinitis
Flonase Flixonase Flunase Fluxonal	Fluticasone Propionate	Spray	GlaxoSmithKline	Allergic Rhinitis Polyp, nasal
Patanase	Olopatadine	Spray	Alcon	Allergic Rhinitis
Otrivin	Xylometazoline	Spray	Novartis	Allergic Rhinitis
Syntaris Synaclyn Bronalide Lunis Bronalide Rhinalar	Flunisolide	Spray	Hoffmann-La Roche	Allergic Rhinitis
Nasacort HFA	Triamcinolone acetonide	Aerosol	Sanofi	Allergic Rhinitis
Astepro Azeptin Astelin Afluon Allergodil	Azelastine HCl	Spray	Meda	Allergic Rhinitis
Omnaris AQ	Ciclesonide	Spray	Sunovion Pharmaceuticals, Inc.	Allergic Rhinitis
Rhinaaxia	Spaglumic acid	Spray	Novartis	Allergic Rhinitis
Nasacort AQ TriNasal Allernaze	Triamcinolone acetonide	Spray	AstraZeneca	Allergic Rhinitis
Rhinocort Aqua Rhinicortol Topinasal Pulmicort Nasal Budecort Nasal Budecort Aqua	Budesonide	Spray	AstraZeneca	Allergic Rhinitis Polyp, nasal
Nasonex Nasonex AQ	Mometasone furoate	Spray	Merck	Allergic Rhinitis
Nasalcrom	Cromolyn sodium	Spray	Prestige Brands Inc.	Allergic Rhinitis
Atrovent	Ipratropium bromide	Spray	Boehringer Ingelheim	Rhinorrhea

Table 5.3 Commercially available systemically acting nasal prescription products in the United States and EU as of December 2011 (courtesy of Lauren Seabrooks, Merck and Co., Inc.)

Commercially available Rx systemic acting nasal products				
Product	API	Delivery	Company	Indication
Imigran Imitrex Suminant	Sumatriptan succinate	Spray	GSK	Migraine
Migranal	Dihydroergotamine	Spray	Novartis	Migraine
AscoTop Zomig	Zolmitriptan	Spray	AstraZeneca	Migraine
Sprix	Ketorolac	Spray	Daiichi Sankyo	Pain management
PecFent Lazanda	Fentanyl	Spray	Archimedes	Pain management
Instanyl	Fentanyl	Spray	Takeda	Pain management
FluMist	Cold-adapted trivalent influenza vaccine (CAIV-T)	Spray	AstraZeneca	Vaccine
Calsynar	Calcitonin	Spray	Sanofi	Osteoporosis
Miacalcin	Salmon Calcitonin	Spray	Novartis	Osteoporosis
Fosatur	Elcatonin	Spray	Therapicon	Osteoporosis
Salcatonin	Calcitonin	Spray	Therapicon	Osteoporosis
DDAVP Minirin Defirin Desmoressin Adiuretin	Desmopressin	Spray solution (Defirin)	Ferring	Diabetes insipidus

5.3.2.1 Product Profile: MedImmune's FluMist® (Influenza Vaccine Live, Intranasal)

FluMist® is an annual influenza vaccine that is delivered intranasally (see Fig. 5.3). It is a live attenuated influenza vaccination (LAIV, trivalent, types A and B) that is preservative-free and contains three live attenuated influenza virus reassortants recommended by the US Centers for Disease Control and Prevention (CDC) (identified for the Northern Hemisphere 2011–2012 flu season as an A/California/7/2009 (H1N1)-like virus; an A/Perth/16/2009 (H3N2)-like virus; and a B/Brisbane/60/2008-like virus) (Fiore et al. 2010; MedImmune, online 2003), the same three CDC-recommended influenza strains in the traditional flu shot (a needle injection which builds up the body's immunity to the flu through antibody production carried in the bloodstream—using inactivated (dead) virus (TIV)).

Once dosed intranasally (one 0.1 mL spray per nostril), the formulation stimulates an immune response by producing antibodies in the lining of the nose where the flu virus typically enters the body. FluMist is termed cold-adapted since the virus is engineered to replicate efficiently at temperatures below that of the body (25 °C) as is the case in the nasal passages (2003). Protective immunity is built up in the

Fig. 5.3 FluMist influenza vaccine live, intranasal (courtesy of MedImmune)

nasopharynx by the antigenic properties from the *ca*, *ts*, and *att* phenotypes derived from master donor virus (MDV) influenza strains (MedImmune, online 2003).

It was first approved by the FDA in June 2003 and is currently approved in five countries including Canada and EU (marketed by AstraZeneca as Fluenz® in select European counties). The original BLA for FluMist was submitted to the FDA for approval in 1998 and was subsequently rejected due to a lack of manufacturing validation and stability data (Food and Drug Administration 2003). MedImmune (formerly Aviron) was able to win US regulatory backing approximately 5 years later. Since FluMist contains a live virus, it is recommended for use by children, adolescents, and adults ages 2–49 years old.

In a placebo-controlled study in adults 18–49 years of age (study AV009), FluMist showed a decrease in any febrile illness of 10.9 % (95 % CI: −5.1, 24.4) and febrile upper respiratory illness of 23.7 % (95 % CI: 6.7, 37.5) (MedImmune, online). In comparative efficacy data between FluMist and an active control (study MI-CP111 using an injectable influenza vaccine made by Sanofi Pasteur, Inc.) FluMist demonstrated a 44.5 % (95 % CI: 22.4, 60.6) reduction in influenza rate in children <5 years of age as measured by culture-confirmed modified CDC-ILI (MedImmune, online). Given the comparative efficacy and safety of FluMist (Ambrose et al. 2011), US regulatory approval was received by MedImmune with four post-marketing clinical commitments (including a 60,000 patient safety trial, adverse event monitoring in patient subsets, an investigation of vaccine virus

shedding and immune response, along with providing additional revaccination data) and one nonclinical commitment (to complete additional reproductive toxicology studies) (U.S. Food and Drug Administration, online). FluMist 2011 revenue totaled $161 MM and $174 MM for full year 2010 (Astrazeneca, online).

5.3.3 Nose to Brain

Nasal delivery also offers the opportunity to bypass the blood–brain barrier and deliver drugs directly to the central nervous system. This barrier prevents systemically delivered drugs, whether delivered orally, intravenously, or by other routes, from reaching significant concentrations in the brain. Two cranial nerves, the olfactory nerve and the trigeminal nerve, pass through the nasal cavity. An intranasally delivered drug could use these pathways to reach tissue in the central nervous system and achieve levels necessary to be of therapeutic benefit. Additionally, there are other potential vascular, cerebrospinal, or lymphatic pathways as routes to the central nervous system (Dhuria et al. 2009).

Currently, no marketed drug products exist that act via nose to brain. One challenge is targeting deposition of sprayed droplets in the regions where olfactory neurons are located. However, there are research programs to treat Alzheimer's and Parkinson's diseases, some of which have shown some success (Dhuria et al. 2009). Given the overall difficulties with treating central nervous disease, nose to brain delivery could offer a promising way to achieve efficacy while minimizing side effects of drugs.

5.3.4 Challenges of Nasal Drug Delivery

Nasally delivering drugs to therapeutic areas of interest can make them more effective for local action, systemic action, and central nervous system action, at lower doses with minimum side effects. However, delivering drug to the specific regions of interest is challenging. As mentioned previously, these challenges arise because the winding and narrow geometry of the nasal airways filter most droplets into the anterior third of the cavity (Kimbell et al. 2007; Laube 2007; Hardy et al. 1985; Newman et al. 1987a; Suman et al. 1999; Vidgren and Kublik 1998). Most targets, though, are located in the posterior nasal cavity. Even less reach the access points for the nerves to the brain in the olfactory region. To overcome these challenges, new devices are in development to target drugs specifically to these regions (Djupesland et al. 2006). Also with these new devices come challenges to accurately assess how well they deposit within specific areas of the nasal cavity.

Another challenge with nasal drug delivery is mucociliary clearance. Most droplets landing within the therapeutically beneficial posterior nasal cavity are removed by mucociliary clearance within 20 min (Hochhaus et al. 2002). The drug,

therefore, must absorb and/or act quickly. Formulation changes, such as using absorption enhancers (Behl et al. 1998b; Costantino et al. 2007; Na et al. 2010) and using mucoadhesives to increase residence time (Ugwoke et al. 2005), are actively being researched in order to take advantage of benefits of nasal drug delivery.

5.4 Formulation Strategies

Until recently, nasal formulations were primarily prepared in the form of either solutions or suspensions and frequently required the use of preservatives (such as BAC) to prevent microbial contamination and microbial growth. Due to potential adverse events associated with the use of these preservatives, regulatory agencies from several countries, including Germany, requested that the manufacturers avoid the use of preservatives in the nasal formulations. These limitations necessitated the development of preservative-free formulations and thereby led to adoption and implementation of various strategies to circumvent the use of preservatives.

In order to develop preservative-free nasal formulations, novel approaches including the use of preservative-free devices and various sterilization techniques have gained widespread attention. Since the aforementioned approaches tend to rely heavily on the use of sterile techniques for manufacturing, compliance with the procedures related to the use of sterile techniques, as outlined in USP <797> Pharmaceutical Compounding-Sterile Preparation, is critical.

The following summarizes the current landscape of nasal formulation development, the limitations of using preservatives, and describes USP <797> regulations as they apply to manufacturing of nasal preparations under sterile conditions.

5.4.1 Current Landscape

The majority of commercially available nasal formulations are active pharmaceutical ingredient(s) (APIs) mixed with excipients such as preservatives, suspending agents, emulsifiers, or buffering agents. Microbial growth can occur in the nasal formulation preparations either during manufacture or while in use by the patient. During manufacture, the most commonly occurring sources of microbial contamination include the handling process and the use of contaminated excipients (Groves and Murty 1990).

These sources of contamination, either alone or in conjunction, can negatively impact the quality of the finished product and shelf life. Once the nasal product is used by the patient, factors such as unhygienic handling or the contact between the tip of the nasal delivery device and nasal cavity can further introduce contamination via migration into the nasal spray tip. Further, the conventional design of the nasal delivery device may allow microbial contamination to enter the formulation by the intake of unfiltered air.

5.4.1.1 Currently Adopted Approaches to Address Microbial Contamination

To avoid contamination and prevent microbial growth, manufacturers use some of the following approaches:

- Adding preservatives to the nasal formulations: This is the most commonly used approach and there are a variety of commercially available preservatives that are routinely employed.
- Preventing the entry of microorganisms through sterile manufacturing of the nasal formulation: This approach can be applied to unit-, bi-, as well as multi-dose products. The drug formulation is prepared under sterile conditions where no preservative is added or the product can be terminally sterilized. If the product is not sterile, then the finished product is generally subjected to radiation to ensure inactivation of microbial contamination (if any) after filling. A detailed description of sterile manufacture is discussed in Sect. 5.6.
- Selection of a preservative-free device: After manufacturing the formulation under sterile conditions, these devices (discussed in Sect. 5.5) require no preservatives. Several companies also manufacture preservative-free pumps for multi-dose formulations. The special tip seal and filter in these pumps reduce microbial growth upon repeated use. Another type of device platform, called "Bag-on-Valve" (BOV), also supports preservative-free formulations, as discussed in Sect. 5.5.1.3.

5.4.1.2 Use of Preservatives

Adding preservatives is a simple, robust, and cost-effective method of controlling microorganisms. The FDA guidance states that if preservatives are used in the nasal formulation, the minimum content limit should be demonstrated as microbiologically effective by performing a microbial challenge assay of the drug formulated with an amount of preservative equal to or less than the minimum amount specified. Although BAC is by far the most widely used preservative, other preservatives such as thiomersal, chlorhexidine, chlorobutanol and phenylethanol, potassium sorbate, and parabens are also routinely employed in the formulation of nasal drops and cosmetics. Table 5.4 includes the list of preservatives and the ranges of concentration used.

5.4.1.3 Limitations Associated with Preservatives

Although preservatives have been used for decades, and they are simple, they do have limitations. These include adverse effects on the nasal mucosa—particularly in children, and the potential of preservatives to cause discomfort, irritation, and other side effects after long-term use. In certain cases, preservatives affected the cilia in the nasal cavity by altering the elimination of the nasal mucus (in cases of nasal

Table 5.4 List of preservatives and the ranges of concentration (information from Boukarim et al. 2009; Marple et al. 2004)

Preservatives	Concentration range (% w/w)
Benzoic acid (sodium benzoate)	0.1–0.2
Benzalkonium chloride	Up to 0.1
Thiomersal	0.003–0.01
Chlorobutanol	0.5
Chlobutol	0.25
Potassium sorbate	0.1–0.2
Methyl paraben	0.1–0.25

infection) and slowing down or even stopping mucociliary clearance, an essential natural mechanism for protecting the upper airways. Several reviews have examined adverse events associated with the use of preservatives (Lebe et al. 2004; Mallants et al. 2007; Bernstein 2000; Merkus et al. 2001; van de Donk et al. 1980, 1982).

Preservatives also introduce formulation challenges due to drug stability/drug-device compatibility issues, and/or by modifying the smell and/or taste of the nasal drug products. For example, phenylethylalcohol can be perceived by some patients as causing an unpleasant odor, potentially reducing patient compliance.

Quite recently, several countries have expressed concern about the risk associated with the use of BAC. Therefore, manufacturers in Europe, Latin America, and more recently Japan have started to consider eliminating the use of preservatives and reformulating their nasal products. Although the FDA still allows the use of preservatives, the FDA has started to encourage manufacturers to actively adopt the use of preservative-free techniques.

5.4.2 Development of Preservative-Free Nasal Products

Since the goal is to avoid adding preservatives while ensuring that the formulation is sterile during the manufacturing and use period, it is important that the formulation is prepared and processed under aseptic conditions before and during transfer of the formulation into the nasal delivery device. Alternatively, terminal sterilization may be employed if suitable for the formulation and device.

Recently, several US manufacturers have ventured into the arena of aseptic nasal formulation processes for manufacturing nasal formulations. Since the processes related to sterile manufacturing techniques, as outlined in the United States Pharmacopoeia USP <797>, are considered the "gold standard," these processes have also been adopted by manufacturers of nasal products. USP <797> provides information on procedures and practices that may be adopted to prevent microbial contamination. The chapter discusses minimum quality standards based on state-of-the-art scientific information and the best sterile compounding practices.

It is important to note that the goal of USP <797> is to provide a global view of the various practices that can be adopted to prepare sterile formulations across the manufacturing spectrum, rather than describe the approaches that can be adopted for a particular formulation. Hence, although USP <797> does not specifically describe the application of various sterilization techniques in the context of manufacturing nasal formulations, the general principles outlined in USP <797> still apply to the manufacturing of nasal formulations.

Once the formulation is prepared under aseptic conditions using the principles outlined in USP <797>, the next step is to ensure that the nasal formulation delivered to the patient is free of microbial contamination. Therefore, it is critical that the device used for delivering the nasal formulation provides a sterile environment to the nasal preparation. For unit- and bi-dose formulations, a preservative-free pump is not needed because the formulations are designed for a single use. In some cases, the conventional pump used for multi-dose preservative-free formulations can be subjected to gamma radiation to ensure that the pump is free from microbial contamination. This could be performed before or after the filling process.

5.4.2.1 Ideal Design Characteristics of Preservative-Free Pump

Throughout the use of product life, the conventional nasal delivery devices can introduce microbial contamination by the following routes: the orifice, the venting air which replaces the dispensed liquid, or due to insufficient container/dispenser fit (Brouet and Grosjean 2003).

In order to ensure that nasal delivery devices are free of microbial contamination, it is vital that the device can be sterilized before or after the filling process. Therefore, specific polymeric materials such as high density polyethylene are a good choice for manufacturing of devices as they resist gamma irradiation and maintain their physical properties.

For unit-dose and bi-dose devices, creating a preservative-free environment for the device is not a major concern since the disposable devices are capable of delivering one or two shots only. However, for multi-dose devices, the following additional considerations apply to ensure that the formulation remains protected inside the container:

- Pump as a closed system: Metering spray pump should work as a closed system (full seal system). Unlike conventional metering nasal spray pumps, the closed system does not allow air to enter into the container and come into contact with the nasal drug product, thus preventing contamination from airborne germs.
- Using a filter: When metering spray pumps are equipped with a filter, the venting air is sucked through a filter assembled inside the pump, which eliminates the airborne germs and keeps them out of the container.
- BOV technology: Unpreserved product is stored in a pouch and dispensed through a valve. The content of the pouch is not in contact with the outside atmosphere.

- Using bacteriostatic agent: The agent such as silver ions could be added to device components so that liquid that comes in contact with them gets protected. Silver ions have a large antibacterial spectrum and low toxicity to humans.

5.4.2.2 Case Study on Preservative-Free Systems: Mechanical Spray Pumps

The orifice of any container is a contamination risk because it contacts the mucosa and/or skin, areas populated by microorganisms and body fluids. Some marketed systems use the oligodynamic activity of a silver wire in the tip of the actuator, a silver-coated spring, and ball (Groß 2000). These components control release of silver ions into the formulation over time. The system minimizes microorganisms between long dosing intervals, even when the tip is immersed into bacterial-contaminated fluid (Bagel and Wiedemann 2004). Silver ions are widely used for their antiseptic properties and are even used for wound dressings. They are safe and have no adverse effects. One must ensure, however, that the silver ions do not react with the formulation, e.g., chloride ions forming micro-precipitations. This effect may be overlooked because it is most relevant for spans of 6–12 h between individual actuations, intervals not usually evaluated during development.

Consequently, the most recent preservative-free systems follow a purely mechanical approach to minimize interactions between device parts and formulation. One way to prevent contamination via the orifice is “tip seal technology.” Both spray pumps and ophthalmic droppers use this technology. A spring-loaded valve is located directly below the opening of the tip orifice, not allowing any microbes to migrate from any surfaces or contacted liquids into the system, sealing the orifice under resting conditions. The tip seal keeps the system closed until a defined pressure (for sprays it is more than 3 bar) is reached by actuating the system. Once a defined pressure is reached, the system opens and formulation is forced through the orifice at a higher pressure than needed to open the valve. When the pressure drops at the end of the actuation, the tip seal immediately closes the orifice with an outward movement. Therefore, no backflow of potentially contaminated medication or other liquid is possible. Depending on the pump system, the fluid path may even be “metal-free,” which means the springs needed for the device operation do not come in contact with the formulation.

At any time when a liquid is dispensed out of a container, the pressure inside such container decreases gradually. To avoid contamination of the formulation via venting air, different technical solutions are used. The simplest way is sterile filtration of the venting air via separate filters or filter gaskets. For oxygen-sensitive formulations, the so-called collapsing bags or depressed systems are used. The formulation is filled in a special, microbial tight bag which is protected by a surrounding bottle. When dispensing the product, the bag collapses with the content not coming in contact with the ambient air. Some pumps are constructed in such a way that the entire system is air-tight and during use some vacuum (up to −300 mbar) is generated within the bottle. Those systems allow even a purging with inert gases to reduce oxygen content in the container headspace.

Fig 5.4 Examples of commercially available products from Brazil (*top left*), Australia (*bottom left*), France (*top right*), and Austria (*bottom right*)

While appearing complex, these approaches to avoid the use of preservatives for multi-dose devices are well established and matured technologies. Though not commercially available in the United States yet (as of publication), unpreserved multi-dose nasal sprays have gained substantial interest and market share in places like Europe and Latin America (Fig. 5.4).

As the development paradigm for nasal formulations shifts from preservative-based formulations to preservative-free formulations, in particular for Latin American countries, the information outlined in USP <797> will continue to provide the roadmap for manufacturing preservative-free nasal formulations prepared under a sterile environment which will ultimately benefit the entire healthcare community.

5.5 Device Landscape for Nasal Drug Delivery

5.5.1 Nasal Spray Devices: Liquid Formulations

Nasal spray devices for liquid formulations come in various dose and container volume sizes. The devices include unit-dose, bi-dose, and multi-dose delivery systems for both preserved and preservative-free. Fill volumes range from 125 μL

(for unit-dose) to 30 mL or larger (for multi-dose) and spray volumes range from 25 to 140 μL. The selection of the spray volume is driven by the therapeutic dose. The selection of the spray pump is driven by the volume of formulation that is required to support that dose. The selection of the fill volume is generally driven by the intended frequency of use of the drug product—for a chronic-use product (for example, for nasal allergies), a multi-dose device containing 1 month's supply might be selected; for an acute-use product (for example, for controlling seizures or pain management), a unit-dose or bi-dose device might be chosen.

5.5.1.1 Case Study for Characterization of Multi-dose Nasal Spray Devices

This case study reviews the steps typically taken when selecting a multi-dose nasal spray device. The selection procedure is based on the spray characteristics of the product formulation from the device in question.

A minimum of 12 devices from one lot were taken and filled with the product formulation for the study. The amount of dose delivered on $n=6$ actuations was determined by hand after priming. The number of doses delivered per bottle was then determined on $n=6$ devices. At this time a visual evaluation of the plume shape was made (a nicely formed plume should be evident rather than a liquid stream).

Once the formulation "sprayability" had been demonstrated, the device was loaded into a computer-controlled device actuator (supplied by Proveris Scientific) to determine stroke length, which is the distance moved when the spray pump is compressed. Using this value, and default velocity and acceleration parameters, the dose weight was determined. The droplet size distribution at 3 cm from the orifice at both the beginning and end of the container life was determined using the stroke length and default velocity and acceleration parameters.

Selection criteria:

- The number of doses delivered per bottle must meet the label claim.
- The dose weights obtained must meet the label claim, and the variation (% RSD) in the dose weight data (both hand-actuated and computer-actuated data) must be within the acceptable range.
- The droplet size distribution at the 10th, 50th, and 90th percentiles must be within the acceptable range at both the beginning and the end of the container life.

If droplets are too large, the formulation may deposit in the front of the nose and tend to drip out of the nose; on the other hand, droplets smaller than 10 μm may travel deeper into the nasal cavity and reach the lungs—which are not the intended delivery site. Ideally, the percentage of droplet smaller than 10 μm should be kept to a minimum. At the upper end of the size range (90th percentile), the majority of droplets should be less than 150–200 μm.

Fig. 5.5 Classic pump (multi-dose pump that is sterilized for a low preservative nasal spray application) (courtesy of Aptar Pharma)

5.5.1.2 Container/Closure Systems

Suppliers of nasal spray container/closure systems include: Becton Dickinson, Coster, MeadWestvaco, Rexam, and Aptar Pharma. Examples of the various systems currently available for use with liquid nasal sprays are shown in Figs. 5.5, 5.6, 5.7, and 5.8.

Classic spray pumps are widely used for local and systemic nasal drug delivery, and are used for preserved formulations. The extensive range of closures, actuators, and accessories available make this spray pump highly adaptable to fit customers' specific requirements. Classic pumps are incorporated into a number of drug products marketed in Asia, Europe, Latin America, and the United States.

The cartridge pump system (CPS) is a highly versatile spray pump. It is designed for the multi-dose delivery of preserved or non-preserved drug formulations. CPS can be used for a wide range of therapeutic applications including allergy, pain, and intranasal mass vaccination. CPS can be terminally sterilized by gamma irradiation.

Unit-dose liquid delivery systems are available for delivery of sterile or preserved single dose medicines. For unit-dose (UDS) and bi-dose (BDS) devices, a coated rubber stopper is placed in the device vial. This stopper contains "fins," which create a good seal to prevent evaporation of the formulation during storage, and prevent the ingress of microorganisms. During the insertion of the stoppers, the "fins" are compressed to allow air within the vial to escape and prevent a build-up of pressure within the sealed unit.

Fig. 5.6 Cartridge pump system, CPS (multi-dose nasal spray pump with microfilter air filtration system to protect non-preserved formulation) (courtesy of Aptar Pharma)

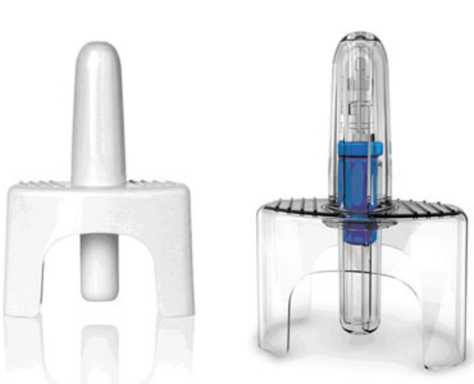

Fig. 5.7 Unit-dose, UDS liquid (single dose liquid nasal spray device) (courtesy of Aptar Pharma)

The pumps used with multi-dose devices contain a gasket which is compressed during application to give an air-tight seal. These pumps can be a screw-on, snap-on, or crimp-on design.

After manufacture, the sterility of drug products is maintained by the container/closure system. For single use or unit-dose devices, sterility is assured by the integrity of the container/closure system itself until the time of use. For multi-dose devices, however, the situation is not as straight forward, and sterility can be compromised when the device is sprayed for the first time, and on each subsequent use. One method used to maintain the sterility of the product in-use is by incorporating a 0.2 μm filter into the dispensing tip/actuator (see Fig. 5.6). With these devices, the return air that is introduced into the container after the dose is expelled is filtered

Fig. 5.8 Bi-dose, BDS liquid (two spray single dose liquid nasal device) (courtesy of Aptar Pharma)

through the 0.2 μm filter—thereby maintaining the sterility of the product throughout its in-use lifetime.

Alternatively, antimicrobial preservatives, as discussed in Sect. 5.4, may be included in the formulation to kill or to inhibit the growth of microorganisms inadvertently introduced during use. Single preservatives, and more often combinations of preservatives, are commonly used in pharmaceutical formulations (including some sterile formulations, for example, eye drops and multi-dose injections) to prevent the growth of bacteria.

5.5.1.3 Alternative Preservative-Free Nasal Product: The "Bag-on-Valve"

In addition to maintaining a sterile environment, engineering of the physical device also helps ensure that the preservative-free formulation remains free from microbial contamination. One example of how optimizing the design of the device can help prevent microbial contamination for saline nasal sprays is the BOV technology.

Briefly, BOV technology can potentially be used whenever it is important to separate the drug product from the propellant, thereby ensuring product purity. The major benefits of using the BOV technology include cost-effectiveness, better preservation of the drug product, and environmental safety. Figure 5.9 shows key components of the BOV system.

The product is sealed inside a pressurized container (generally an aluminum can) and is released by compressed air or nitrogen. The BOV technology offers several benefits to the consumer such as longer shelf use without the use of preservatives and ability to use at all angles because the spray is driven by compression of the bag by the propellant.

Fig 5.9 An overview of "bag-on-valve" (BOV) technology. The bag and valve are inserted into the canister during assembly. The canister is pressurized with the bag then filled with drug product (courtesy of Aptar Pharma)

5.5.1.4 Nasal Spray Characterization Testing and FDA Nasal Spray Guidance (Food and Drug Administration 2003)

In order to support a regulatory filing for a nasal spray product, the current FDA guidance documents make recommendations as to the characterization and test data. These test recommendations are summarized in Table 5.5. To date, the FDA has not issued guidances specific to unpreserved nasal spray formulations. Unpreserved formulations are generally manufactured sterile, and the testing in Table 5.5 applicable to that type of formulation is undertaken.

5.5.2 Nasal Spray Devices: Dry Powder Formulations

Following the success of liquid formulation nasal sprays, research and marketing interest has expanded to include dry powder nasal devices. Dry powder inhaler (DPI) systems were undertaken as an alternative to the pressurized metered dose inhalers (pMDI) that use ozone depleting propellants. Dry powder systems (both DPI and nasal) generally comprise a micronized active drug and suitable powdered excipients within an apparatus that is designed to aerosolize the formulation. Some of the advantages of dry powder drug delivery systems include formulation stability, a system that is propellant-free, and that less coordination between actuation and inhalation is required (Telko and Hickey 2005; Serra-Batlles et al. 2002). Also, because of the absence of moisture in the dry powder system, microbial growth is minimized or eliminated, and the use of preservatives is less critical than in the case of liquid formulations. Deposition efficiency, dose uniformity, complexity of manufacturing, and device to device performance remain as concerns for dry powder technology (Chan 2006; Islam and Gladki 2008).

Dry powder devices come in unit-dose, bi-dose, and multi-dose systems. Some of the container/closure systems currently available for use with dry powder nasal sprays are shown in Fig. 5.10.

Table 5.5 FDA test recommendations for nasal sprays

Test	Metrics	Unit-dose	Bi-dose	Multi-dose
Description	Appearance of container and contents	✓	✓	✓
Identification	Drug substance	✓	✓	✓
Drug substance assay	Concentration	✓	✓	✓
Impurities and degradation products	From drug substance	✓	✓	✓
Preservatives and stabilizing agents assay[a]	Concentration (preservatives, antioxidants, chelating agents, etc.)	✓	✓	✓
Microbial limits[a]	USP<61>, <62>	✓	✓	✓
Antimicrobial effectiveness[a]	USP<51>	✓	✓	✓
Sterility[a]	USP<71>	✓	✓	✓
Container/closure integrity	USP <671>	✓	✓	✓
Endotoxins[a]	USP<85>	✓	✓	✓
pH	USP<791>	✓	✓	✓
Osmolality	USP<785>	✓	✓	✓
Viscosity	USP<911>	✓	✓	✓
Pump delivery	Shot weight	✓	✓	✓
Spray content uniformity	Mass of drug per actuation	✓	✓	✓
Droplet size distribution	Dv_{10}, Dv_{50}, Dv_{90}, span, % less than 10 μm	✓	✓	✓
Spray pattern	D_{min}, D_{max}, ovality ratio	✓	✓	✓
Plume geometry[b]	Plume width, plume height, spray angle	✓	✓	✓
Net content/weight loss	USP<755>	✓	✓	✓
Particulate matter	USP<788>	✓	✓	✓
Particle size distribution[a]	Suspensions only	✓	✓	✓
Extractables/leachables	Elastomeric and polymeric components	✓	✓	✓
Priming/repriming[b]	Mass of drug per actuation, shot weight			✓
Tail-off profiling[b]	Mass of drug per actuation, shot weight			✓
Dose proportionality[a,b]	Multiple strength suspensions	✓	✓	✓
Effect of dosing orientation[b]	Mass of drug per actuation, shot weight			✓

[a]If applicable to formulation
[b]One time studies, not for routine testing

5.6 Manufacturing and Filling Nasal Delivery Systems

There are several different methods to achieving a sterile product that cover both aseptic manufacture and terminal sterilization. In aseptic manufacture, the drug product, container, and closure are first subjected to sterilization

Fig. 5.10 Unit-dose powder (UDP) and bi-dose powder (BDP) devices (courtesy of Aptar Pharma)

separately, and then brought together in an extremely high-quality environment. Terminal sterilization, on the other hand, involves manufacturing a low bioburden product in an environment designed to minimize microbial and particulate contamination, and then subjecting the final container to a sterilization process such as heat (e.g., autoclaving), chemical sterilant (e.g., ethylene oxide), or ionizing radiation (e.g., gamma or electron beam). Each of these methods of producing a sterile product has its own technical challenges.

5.6.1 Aseptic Manufacture

Various sterilization processes are employed for both the container/closure system (for example, glass containers are subjected to dry heat; suitable plastic containers such as high density polyethylene are subjected to ionizing radiation; rubber closures are subjected to moist heat) and the dosage form. Some options for producing a sterile dosage form are presented below.

5.6.1.1 Sterile Filtration

If the dosage form is a liquid solution, or a very low viscosity emulsion, sterilization can be affected by passing the solution through a filter with a pore size small enough to trap out any microbial contamination (0.2 μm). The filtered dosage form is then kept sterile until it is enclosed in the final container/closure system (see Fig. 5.11).

During process development, an assessment of the filter and filtration process must be carried out—including:

- Retention of the drug substance by the filter/loss of potency—by testing the dosage form pre- and post-filtration
- Testing the filter for potential extractables and leachables
- Testing the filter for microbial retention

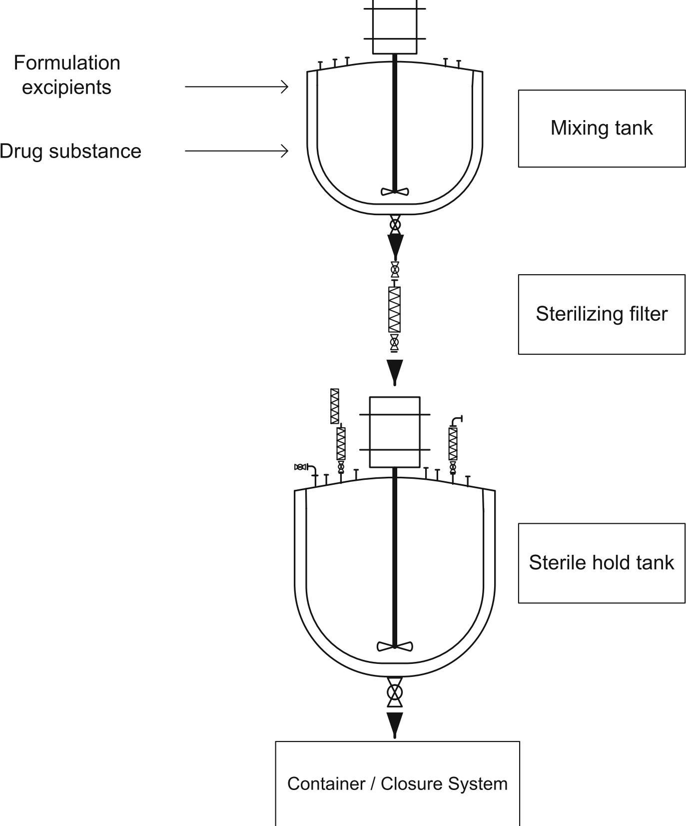

Fig. 5.11 Sterile filtered dosage form

The bioburden of the pre-filtered solution must be evaluated as part of the in-process testing to ensure that the filter does not become overloaded with contaminants. The integrity of the filter must also be checked following the filtration process.

Filtration cannot be used as the sole means of achieving a sterile bulk formulation in the case where the dosage form is viscous or contains suspended particles (for viscous or suspension formulations, see Sect. 5.6.1.2). The following case study summarizes work carried out to validate a sterilizing filter.

Case Study: Sterilizing Filter Validation

Purpose

The purpose of this study was to demonstrate that the sterilizing filter was acceptable and capable for the sterile filtration of the product base. This was achieved through a review of the supplied filter documentation, and by carrying out various verification activities.

Verification Requirements and Results

The critical operating parameters associated with the sterile filtration of the product base (temperature, flow rate, and pressure) that could potentially impact the performance and integrity of the filtration process must be within the design capability of the filter cartridge/membrane. Table 5.6 lists the verification requirements for the sterilizing filter, the acceptance criteria, and the results obtained.

5.6.1.2 Combination Processes

In cases where filtration cannot be used as the sole means of sterilization (for example, for viscous or suspension formulations), there are several combination processes available.

Dry Heat or Ionizing Radiation of Powders Followed by Aseptic Addition to Pre-filtered Base

Here, the powders are first packed into a suitable container, and then subjected to sterilization by dry heat (e.g., 170 °C for 1 h) or ionizing radiation (gamma or electron beam). The liquid formulation base is sterilized by filtration. The sterile powder is then added to the sterile formulation base by aseptic addition—for example, via a presterilized isolator attached to the mixing vessel (Fig. 5.12). Factors to consider when presterilizing powdered active ingredients include heat stability of the active ingredient; stability to ionizing radiation; packaging of the active ingredient; compatibility between the active ingredient and the packaging; and extractables and leachables from the packaging.

Aseptically Combining Phases Sterilized by Different Methods

Here, the bulk formulation is split into two distinct phases—for example, the oil and aqueous phases of an emulsion. The drug substance is dissolved in one of the phases. The oil phase is then sterilized by passing it from a phase tank into the final mixing tank, via a 0.2 μm filter; the aqueous phase is autoclaved in a second phase tank. These two phases are then combined in a final mixing tank, and held sterile until packaging (Fig. 5.13).

Once the bulk formulation has been produced sterile, the manufacturing environment for the downstream processes (filling and closing the nasal delivery system) needs to be kept and monitored at a very high quality (low bioburden and particulate levels). The whole manufacturing process needs to be validated at regular intervals (usually every 6 months) to demonstrate that the aseptic handling techniques and manufacturing operations do not compromise the sterility of the final product. This validation exercise involves carrying out media simulations, processing microbiological growth media through the entire process train and into the final container/

Table 5.6 Verification requirements for filter sterilization

Test	Description	Acceptance criteria	Results
Membrane attributes	Verification performed to document the pore size of the filter membrane in order to assess the retention capability by design	Filter membrane pore size, by process design, is to be a 0.22 μm size for microbial retention	Pore size was confirmed to be 0.22 μm
	Verify physical size—for information only	Document membrane characteristics for information only	Thirty (30) inches in length, with an effective filtration membrane surface area of 22 ft^2 (2.1 m^2)
Pyrogens	Verify that the filter is appropriate for use with sterile processes	USP Pyrogen test was performed to confirm that the filter cartridges are non-pyrogenic and suitable for parenterals	Meets current USP <151> requirements
Bacterial endotoxin	Verify that the filter cartridge meets USP requirement for parenteral use, <0.5 EU/mL	USP Bacterial Endotoxin test was run (using a solution of cartridge extract mixed with Limulus Amebocyte Lysate) to confirm that the filter cartridges are not contaminated with endotoxin	Extractables contain <0.066 EU/mL bacterial endotoxin (as determined using LAL test)
Material compatibility	Verify that the membrane meets USP requirements for the intended application (non-shedding/nonreactive)	Filter membrane must be constructed of non-shedding and nonreactive material	Polyvinylidene fluoride (PVDF) membrane and polypropylene components USP Class VI Plastic Meets criteria for non-fiber releasing filters as defined per Title 21 of the Code of Federal Regulations (CFR) parts 211.72 and 210.3 (b) (6)
Process capability	Verify that the filter membrane is designed to meet the requirements of the manufacturing process	Must meet the following: • Max. 25 psi pressure at 80 °C (product temp) • Sterilizing grade (capable of temperatures ranging from 121 to 123 °C)	Membrane design attributes: • ≥25 psi at 80 °C • Sterilizing grade (for temperatures up to 126 °C)

(continued)

Table 5.6 (continued)

Test	Description	Acceptance criteria	Results
Microbial retention	Verify that the filter can retain microbial organisms within actual production material providing sterilization through filtration	Sterilizing filter can retain challenge organism (*Brevundimonas diminuta*, ATCC 19146) when challenged at levels equal to or greater than 1×10^7 cfu per cm^2	The sterilizing filter effectively retained the challenge organism demonstrating sterilization through filtration
Bubble point challenge	Verify the product bubble point ratio for the PVDF filter membrane wetted with product base at a controlled temperature of 85 ± 4 °C and establish a recommended minimum bubble point for the product	The coefficient of variation for the bubble point ratio within each product lot and between the three product lots should be ≤5.0 %	The coefficient of variation for each product lot and among the three product lots was no more than 5.0 % with a recommended value of 22 psi
In-process performance testing	Verify product sterility and filter integrity following a minimum of three (3) production runs	Final filled product must meet sterility testing and the filter cartridge/membrane must pass the post-run filter integrity test	Review of in-process and final release testing for the product manufacturing process demonstrates that the final filled product is sterile and that the filter is integral (via post-run filter integrity testing)

closure system—and needs to include all anticipated process interventions, manual and mechanical manipulations, and machine downtime. The media is then incubated to determine if the process is contamination-free. If contamination is found, the contaminants need to be identified, and causes assigned to the failure of the aseptic operation.

In an aseptic operation, controlling the sterility of the drug product and container/closure system is relatively straight forward; it is the human interface that provides the biggest challenge and the most likely cause of contamination.

5.6.2 Terminal Sterilization: Heat

The use of heat (dry heat or autoclaving) to terminally sterilize the drug product can lead to challenges to the thermal stability of the formulation and formulation ingredients. Dry heat sterilization involves taking the product up to 170 °C for a set

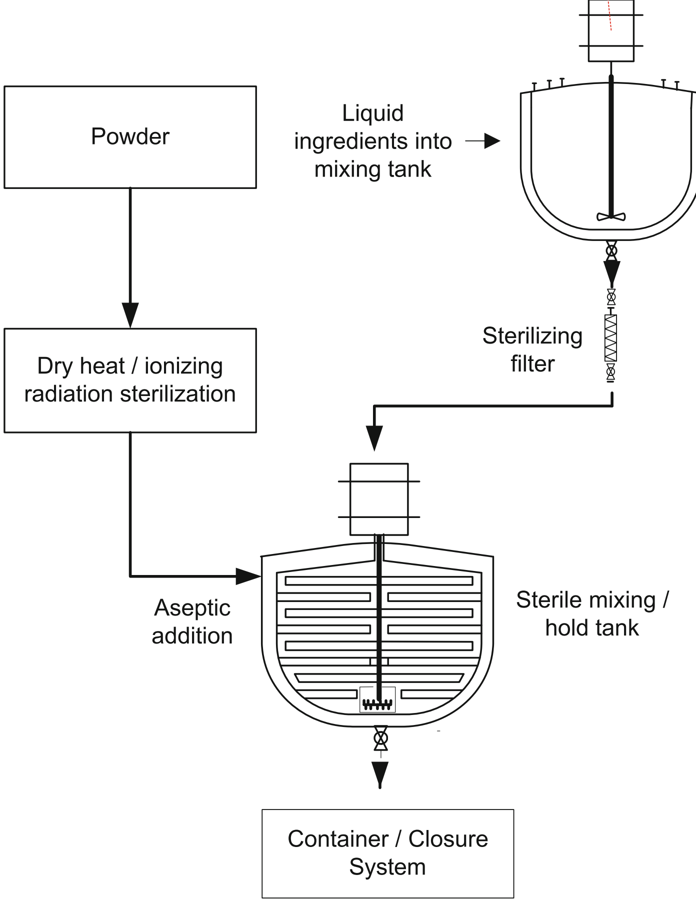

Fig. 5.12 Presterilized isolator attached to the mixing vessel

period of time; autoclaving (or wet heat sterilization) involves heating to 121 °C. Whereas dry heat uses the heat itself to bring about sterilization, autoclaving uses the water contained within the formulation to achieve this. As the temperature of the product increases during autoclaving, the vapor pressure within the container/closure also increases. To prevent package rupturing, the pressure within the autoclave chamber must be controlled to match that within the container/closure.

Many drug substances will degrade or denature at high temperature, leading to loss of potency and the generation of degradation products. Also, many formulation bases will fail when exposed to such high temperatures—for example, ointment bases will lose viscosity and lead to sedimentation of any suspended solids; emulsions will exhibit phase separation. Aqueous gels are typically the most tolerant formulation type to the effects of heat sterilization—usually being sterilized by autoclaving.

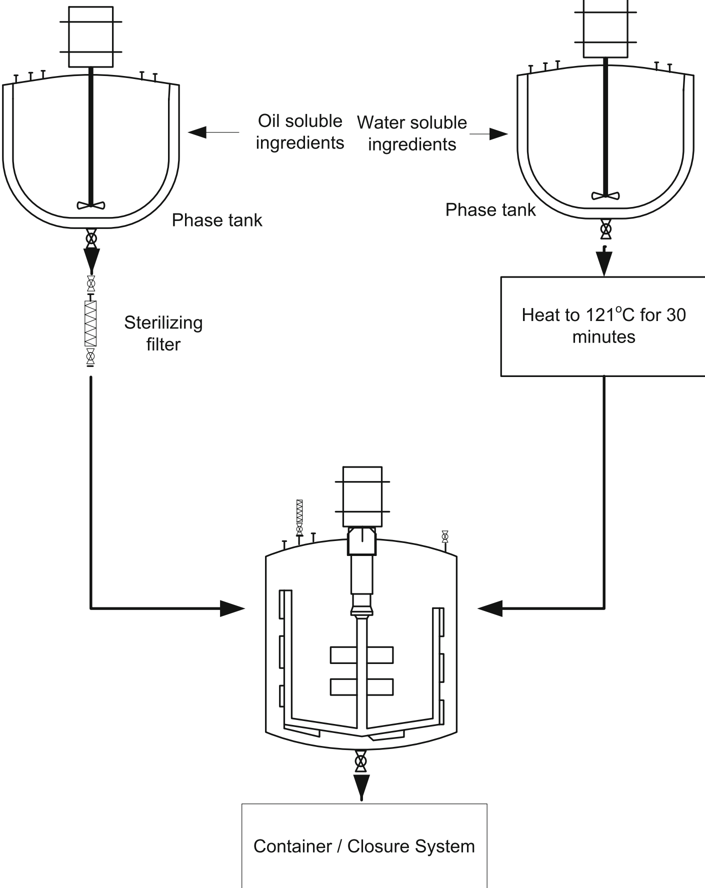

Fig. 5.13 Aseptic combination of sterilized phases

5.6.3 Terminal Sterilization: Chemical Sterilant

Chemical sterilants are highly reactive and affect sterilization by oxidation. Approved chemical sterilants are ethylene oxide, hydrogen peroxide, and ozone. The technical challenges with the use of chemical sterilants are (a) getting the chemical sterilant into the pack so that it can interact with any microbial contaminants, (b) ensuring that the sterilant doesn't affect the potency of the drug substance, and (c) getting the sterilant (and any degradation products) back out of the pack after the sterilization process is complete.

Semipermeable packaging (to enable gas transfer) is used to allow penetration and removal of the sterilant. As a result, this method of sterilization is unsuitable for liquid and semisolid formulations.

Desorption studies are carried out on the sterilized product to ensure that the chemical sterilant and any degradation products are reduced to acceptable levels before the product can be distributed and used. In the case of ethylene oxide, the degradation products are ethylene glycol and ethylene chlorhydrin. These degradation products have toxic effects and their acceptable level is controlled.

Because of the challenges with the use of chemical sterilants, they are mainly used for the sterilization of device components rather than the finished nasal spray product.

5.6.4 Terminal Sterilization: Ionizing Radiation

The use of radiation to bring about terminal sterilization is very effective, but poses technical challenges regarding the stability of the drug substance and drug product to its ionizing effects. Aqueous-based formulations are unsuitable to sterilization by this route due to the formation of hydroxyl radicals, which then react with other chemicals within the formulation. Although the majority of nasal spray formulations are currently aqueous-based, a few nonaqueous-based liquid formulations exist in the development phase.

Radiation can also affect polymers causing either cross-linking or chain scission. This can manifest itself in many ways; for example, some plastics can become brittle; some plastics can discolor; gels can lose viscosity; adhesives can become hard and less sticky; or, conversely, adhesives can become stringy and more sticky. The effect of ionizing radiation needs to be monitored on a product by product basis, and over an extended period of time—as these effects are not always apparent immediately after processing.

The following two case studies summarize work carried out to validate a gamma irradiation cycle for nasal spray device components, and to determine the effects of gamma radiation on the extractable and leachable profile of a gamma-irradiated delivery device.

5.6.4.1 Case Study: Radiation Sterilization of Nasal Spray Device Components

Purpose

The purpose of this study was to qualify radiation sterilization as an acceptable means of sterilization for nasal spray device components. The ANSI/AAMI/ISO 11137-2: 2006 ($VDmax_{25}$) guideline was followed to achieve a sterility assurance level of 10^{-6}.

Definitions

- *Bioburden*: Population of viable microorganisms on a material (e.g., product, package, or component).
- *Dosimeter*: Device or system having a reproducible and measureable response to radiation, which can be used to measure dose exposure.
- *Sterility assurance level* (*SAL*): Probability of a viable microorganism being present after sterilization (normally expressed as 10^{-n}).
- *Sterility testing*: Test performed to determine if viable microorganisms are present.
- *Verification dose*: A radiation dose estimated to produce a sublethal SAL for a material. Verification doses are used in dose setting to establish or confirm the sterilization dose.

Dose Setting

First, the mean bioburden of the components was determined by evaluating ten unirradiated samples randomly selected from each of three separate production lots. Aerobic and fungal bioburden counts were performed on each sample. A verification dose (SAL 10^{-1}) was selected based upon the average bioburden results, adjusted for recovery efficiency, and referencing Table 9 in ANSI/AAMI/ISO 11137-2: 2006. The closest number greater than or equal to the average adjusted bioburden was selected for dose determination.

Next, a sublethal dose verification experiment was carried out. Samples were exposed to the verification target dose, ±10 %. Calibrated dosimeters were used to verify the dose. After exposure, each sample was visually checked for damage and/or compromised packaging prior to sterility testing. Sterility testing was performed by adding the test sample to Soybean Casein Digest Broth and Fluid Thioglycollate Medium, and incubating for 14 days at 20–25 and 30–35 °C, respectively. Bacteriostasis and fungistasis testing was also carried out.

Acceptance/Rejection Criteria

If, after completion of the verification dosing, the results of the sterility test showed that one or fewer positives were observed, the sterilization dose of 25 kGy minimum would be considered valid. If, however, the results of the sterility test showed more than two positives, and if after repeat verification dosing, the results of sterility testing still showed positives, the adequacy of the 25 kGy sterilization dose might not be acceptable.

Establishing Sterilization Specifications and Revalidation

Factors to consider when establishing sterilization specifications include a description of the material to be sterilized and its packaging, carrier loading configuration,

dose mapping, minimum dose (to give acceptable sterilization), maximum dose (for materials compatibility), and placement of dosimeters.

Once established, routine dose auditing exercises are carried out (e.g., every 3 months) to assess the ongoing material bioburden and continued effectiveness of the sterilization cycle. Any changes in the material or manufacturing location must be evaluated for their possible influence on the sterility validation.

5.6.4.2 Case Study: Extractable and Leachable Study on a Gamma-Irradiated Delivery Device

Purpose

The purpose of this study was to identify any extractable and leachable materials present in a delivery device that was to be used in contact with a sterile product. The device in question was to be pre-irradiated using gamma irradiation, and then aseptically filled with the product.

Method Development: Volatile and Semi-volatile Materials

Initially, a headspace GC/MS method was developed to analyze the device for the presence of any volatile and semi-volatile components prior to irradiation.

Sections of material were placed into a 20 mL headspace vial and analyzed using GC/MS at a range of oven temperatures between 80 and 230 °C. The results demonstrated that the temperature which yielded the maximum number of extractable peaks was 230 °C.

Next, a headspace vial equilibration time study was conducted to determine the optimum time at which peak areas were maximized. The peak areas of four randomly chosen peaks were monitored at six different vial equilibration times ranging from 10 to 120 min. For the four peaks studied, a common trend of the peak area plateauing after 30 min was exhibited. This 30 min vial equilibration time was then used in an attempt to characterize all unknown peaks.

The identities of the peaks were confirmed by injecting pure standards of the components proposed by the GC/MS NIST library, and matching the R_t values and mass spectral fragments. In total, nine peaks were identified and confirmed by MS.

Method Development: Nonvolatile Materials

In order to characterize the nonirradiated device material, a solvent extraction procedure was developed. GC/MS and LC/MS methods were also developed for the analysis of any possible nonvolatile species.

Initially, an 8 % ethanol in water solution was used in contact with the device housed in a stoppered graduated cylinder. This was placed into a water bath and

incubated for 2 h at 65 °C. The cylinder was then cooled, and 15 mL of the extract was pipetted into a conical glass vial and evaporated to dryness using N_2 gas. After complete evaporation, the remaining extract was reconstituted with 0.5 mL of solvent, and then analyzed using the GC/MS conditions previously developed. As no extractable peaks were observed, the extract procedure was repeated using a solution of 3 % acetic acid in water, and incubated for 2 h at 100 °C. Again, no peaks were observed.

Since the last two approaches failed to yield any extractable peaks, a more aggressive solvent (*n*-heptane) was chosen. Initially, a blank of *n*-heptane was analyzed by GC/MS. Many peaks were observed in the *n*-heptane solvent that could possibly interfere with any extractable peaks, so *n*-hexane was chosen as an alternative. The change in solvent from *n*-heptane to *n*-hexane resulted in fewer solvent peaks and a cleaner baseline. Therefore, *n*-hexane was implemented as the extraction solvent.

Initially, *n*-hexane was used with an incubation time of 2 h at 50 °C. After evaporating to dryness with N_2 and reconstituting the remaining extract with 0.5 mL of *n*-hexane, analysis by GC/MS showed no additional peaks other than those present in the solvent. As a result, longer incubation periods of 6 and 24 h were implemented. Even with the increased incubation period at 50 °C, no components were extracted. Additionally, no components were extracted when solvent studies were carried out at room temperature and incubation periods up to 168 h.

Overall, the GC/MS solvent extractable studies conducted on the nonirradiated device material showed that only solvent peaks were present and no extractables were observed.

An LC/MS method was then developed and used to analyze the *n*-hexane solvent extract which had been incubated for 2 h at 50 °C. In this case, 0.5 mL of acetonitrile was used to reconstitute the extract. An electrospray positive (ES(+)) mode of ionization was initially employed. Comparing the extract chromatogram to that of blank acetonitrile, a single peak at $R_t = 25$ min was observed. Upon further investigation, the mass spectral pattern of the unknown peak was also observed in a control sample where the *n*-hexane solvent had been evaporated to dryness using N_2 gas, and then reconstituted with acetonitrile. This suggested that the peak was related to the solvent, and not an extractable peak. Even when the incubation period was increased up to 24 h at 50 °C, or 72 h at room temperature, no other peaks were observed other than those present in the solvent control or the blank. Furthermore, no additional peaks were observed on changing the mode of ionization from ES(+) to Atmospheric Pressure Chemical Ionization positive (APCI(+)), ES(−), or APCI (−).

Evaluation of Irradiated Devices

Devices that had been exposed to gamma radiation at both a nominal dose (26.0–26.5 kGy) and a higher dosage (51.7–53.3 kGy) were analyzed using the same conditions listed above. The results from this analysis showed that the irradiation performed on the devices removed the volatile components previously observed in

the headspace analysis of the nonirradiated material. Likewise, no peaks were observed using the solvent extraction procedure.

Finally, a leachable study was performed on an aged product sample to determine if any extractables previously detected were present. Product that had been stored in an irradiated device at 40 °C for 6 months was analyzed and compared against the formulation base stored in a glass jar, not exposed to the device material, to observe if any leachable peaks were present. GC/MS and LC/MS analysis showed no peaks were present in the stability sample other than those present in the placebo.

5.7 Analytical Techniques and Drug Product Characterization Studies for Nasal Spray and Nasal Aerosols

Analytical tests that are used to characterize the performance include methods that measure the size of emitted droplet, the shape of the spray, as well as critical formulation components such as viscosity and content uniformity. A list of these tests is shown in Table 5.5 and is described in more detail as this section progresses. These tests can be used to characterize the reproducibility of performance and make decisions regarding device selection and formulation optimization. Of the in vitro tests that will be discussed, droplet size is likely to be the most important parameter to predict where droplets may deposit in the nasal cavity. It should be noted that these tests can be used to facilitate development and can also be used as quality control tests. One should be careful to denote the differences between the two applications. To date, a significant correlation between in vitro analytical tests such as spray pattern and in vivo outcomes has not been established.

Because of the importance of deposition, many researchers (Shah et al. 2011; Shah et al. 2013; Suman et al. 2006; Newman et al. 2004; Laube 2007; Aggarwal et al. 2004; Schroeter, et al. 2006; Djupesland et al. 2006; Djupesland and Skretting 2012; Cheng et al. 2001; Foo et al. 2007; Guo et al. 2005; Hughes et al. 2008; Kundoor and Dalby 2010) have turned to nasal casts and computational fluid dynamic (CFD) models to assess the deposition patterns of new nasal devices and/or formulations. Often used in early development, nasal cast studies have become easier to perform with increasing ease in the creation of nasal casts from MRI and CT scans (Fig. 5.14). With rapid prototyping techniques, nasal casts can be machined for use in a lab setting. These casts are typically coated with a material to simulate the mucus layer and to prevent particle bounce. These casts can provide both a qualitative and quantitative picture of the sites of drug deposition, and can be combined with impaction-based techniques to quantify the mass of drug exiting the nasal cavity.

CFD modeling can also be used to simulate changes in airflow, angle of insertion, disease state, or patient geometry as a mechanism to predict nasal deposition. Several studies (Chen et al. 2010; Segal et al. 2008) have been cited in the literature that have simulated nasal hypertrophy and to assess potential patient to patient variability. With the availability of software from Mimic and Fluent, the end user can perform analysis of many different simulations.

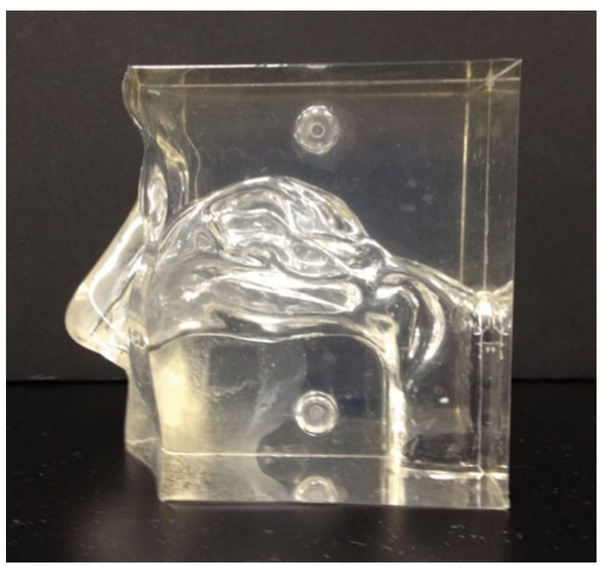

Fig. 5.14 Silicon model of a nasal cast

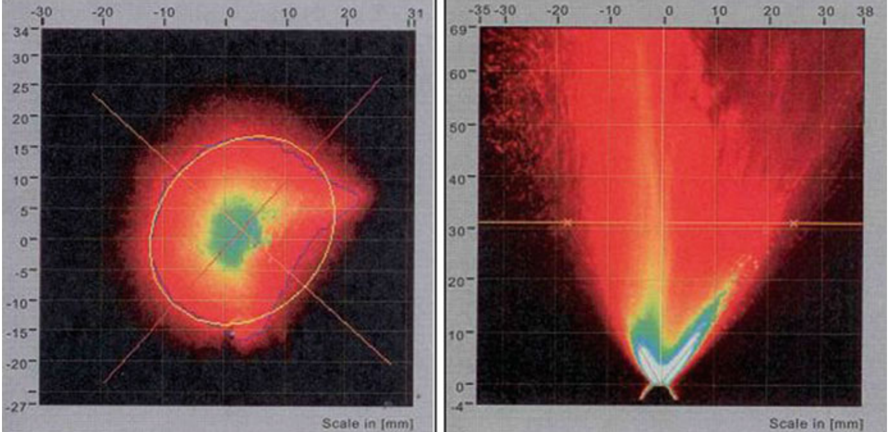

Fig. 5.15 Spray pattern (*left*) and plume geometry (*right*) images (courtesy of Proveris Scientific)

Reverting back to the traditional tools to characterize performance, spray pattern (Fig. 5.15) and plume geometry (Fig. 5.15) are in vitro tests used to define the shape of the emitted spray and to confirm that the molding process of the pump components was successful. These tests are performed from the analysis of a two-dimensional image of the emitted plume. Traditionally spray pattern and plume geometry have been performed with impaction systems such as TLC plates and fast speed cameras. Nowadays, spray pattern and plume geometry analyses are mostly

performed using non-impaction laser sheet-based instruments. Spray pattern is characterized by the D_{max}, D_{min}, ovality ratio, and area. D_{max} is the longest diameter measured on the resulting spray pattern image. D_{min} is the shortest diameter measured on the resulting spray pattern image. Ovality ratio is the ratio of D_{max} to D_{min}. This ratio provides a quantitative value for the overall shape of the spray. The spray pattern area is automatically detected by the software. Percent area is the ratio of the spray pattern area to the entire image area. Plume geometry is characterized by the plume height, spray angle, and plume width. Spray angle is the angle of the emitted plume measured from the vertex of the spray cone and spray nozzle. Plume width is the width of the plume at a given distance (e.g., 3 cm) from the spray nozzle. Plume height is the height of the emitted plume measured from the tip of the device. While specifications may be set for all spray pattern parameters, FDA recommends using area and ovality ratio for statistical comparison (Food and Drug Administration 2003) to establish bioequivalence between test and reference nasal drug products. In case of plume geometry, FDA recommends using spray angle and plume width for statistical comparison (Food and Drug Administration 2003).

Droplet size distribution is an important in vitro test based on laser diffraction principle to characterize droplet size distributions from nasal sprays. The droplet size distribution is characterized by the volume distribution (Dv_{10}, Dv_{50}, and Dv_{90}), span, and percentage (%) less than 10 μm. Dv_{50} is the volume median diameter. It indicates that 50 % of the distribution is contained in droplets that are smaller than this value while the other half is contained in droplets that are larger than this value. Similarly the Dv_{10} and Dv_{90} values indicate that 10 % and 90 %, respectively, of the distribution are contained in droplets that are smaller than these values. Span is calculated by the following equation: ($Dv_{90} - Dv_{10}/Dv_{50}$) and quantifies the spread of the droplet size distribution. Percentage (%) less than 10 μm is the cumulative volume of the particles with size less than 10 μm. This cumulative fraction provides a risk estimate of particles from nasal spray that may be inhaled into lung. For bioequivalence assessment, FDA recommends using Dv_{50} and span for statistical comparison (Food and Drug Administration 2003) to establish bioequivalence between test and reference nasal drug products. Droplet size is also a quality control test.

Single actuation content (used for in vitro bioequivalence) or spray content uniformity (SCU) through container life and pump delivery (PD) through container life testing are used to characterize the delivery of drug discharged from the actuator of an aerosol or nasal spray against the label claim through container life. This test ensures that the product delivers the label claim over the labeled number of actuations. This test is also used to confirm the number of priming and repriming shots under different storage conditions and orientations. Typically the spray from the nasal unit is collected in a collection tube or glass bottle and the mass of drug is quantified by HPLC. Pump delivery is calculated from the weight difference of the collection tube or the glass bottle before and after shot collection. Single actuation content/SCU and pump delivery are performed at the beginning and end of the unit life for multi-dose drug products. Drug mass per single actuation is recommended by FDA (2003) for bioequivalence assessment.

For suspension products, drug particle size distribution by microscopy can estimate the rate of dissolution. Drug particle size distribution and extent of agglomerates are characterized in the spray or aerosol formulation prior to actuation, and in the spray following actuation. A sample from a nasal spray unit is sprayed onto a substrate (e.g., a microscope slide or a gridded filter paper). A polarized light microscope is used to analyze the size of the primary drug particle present in the sample. A count-based particle size histogram and a cumulative particle size graph are reported. Optical microscopy coupled with Raman spectroscopy (Kippax et al. 2011) imaging techniques (Klueva et al. 2008) can provide an improved method to establish equivalent particle size distribution between Test and Reference products that can be in accordance with FDA's critical path initiative (Food and Drug Administration 2003). While current optical microscopy relies on the morphology of the drug particle, Raman spectroscopy or imaging techniques can provide chemical information and hence can improve the specificity and accuracy of the method through ingredient-specific particle size analysis.

Aerodynamic particle size distribution by cascade impaction is intended to determine the amount of drug in small particles/droplets. Small droplets defined as droplets smaller in size than the nominal effective cutoff diameter of the top stage of the cascade impactor may potentially be delivered to regions of the airways beyond the nose which may be a safety issue. The amount of drug in small particles is typically measured by an Andersen Cascade Impactor (ACI) operated by drawing the sample laden air through the ACI at 28.3 L/min. ACI is made up of classification stages consisting of a series of jets and impaction surfaces. At each stage, an aerosol stream passes through the jets and impacts upon the surface. Particles in the aerosol stream with significant inertia will settle upon the impaction plate. Smaller particles pass as aerosols on to the next jet stage. By designing the following consecutive stages with higher aerosol jet velocities, smaller diameter particles are collected at each subsequent stage giving the cascade affect of separation. The ACI is assembled to a 2 L glass nasal induction port and a pre-separator. Aerosol collected in the induction port, pre-separator, and the impactor is analyzed using HPLC to quantify the mass of drug. The amount and % of drug less than 9 μm and the mass balance are reported. Deposition profile (i.e., distribution of mass deposited on various components of the ACI and associated accessories) is recommended by FDA (Food and Drug Administration 2003) for bioequivalence assessment.

5.8 Global Regulatory Perspective

The regulatory landscape for nasal spray drug products is well established in the Western world. However, as utilization of nasal sprays, particularly generics, gains momentum in the BRIC countries (Brazil, Russia, India, China), regulatory bodies such as ANVISA in Brazil and CFDA in China are looking to adopt regulatory strategies similar to FDA. The following sections will discuss analytical regulatory expectations from both a new drug and generic drug approval perspective.

Table 5.7 Variations in regulatory requirements from FDA, Health Canada (HC), and EMA

Metric/study	FDA	HC/EMA
Spray pattern	X	
Plume geometry	X	
Droplet size distribution	X	X
Physical characterization[a]		X
In vitro dose proportionality	X	

[a]Development phase. One may consider evaluating highly functional excipients throughout development and stability

5.8.1 New Drug Approvals

From a Chemistry, Manufacturing, and Controls (CMC) perspective (Food and Drug Administration 1999), nasal spray product performance depends on the interaction between the formulation and delivery device. Hence, analytical requirements for the approval of the drug product consist of techniques that assess the chemical and physical stability of the formulation and the functionality of the device. While the relationship between certain spray characteristics and the efficacy of a product is still under investigation, FDA currently requires 12 different techniques for characterizing the spray and device for nasal spray product New Drug Applications (NDAs) and Abbreviated New Drug Applications (ANDAs) (refer Table 5.5 in Sect. 5.5). These methods are used to support stability, batch release, and drug product characterization for NDAs. The extensive nature of analytical requirements puts nasal drug products in a category of most highly tested dosage forms when compared to, for example, oral solid dosage forms.

Developers are cautioned to perform these tests even if they are not planning to market in the United States. Neither Health Canada nor EMA (Health Canada 2006; European Medicines Agencies 2006) require spray pattern and plume geometry analyses (Table 5.7), and specifications on pump delivery, SCU, and droplet size distribution vary between regulatory bodies. However, if later there is interest in launching the product in the United States, and these tests had not been performed, extensive reformulation or device design may be required for FDA approval, requiring new clinical studies. It should also be noted that both ANVISA in Brazil and the CFDA in China are beginning to incorporate analytical requirements into their expectations for these drug products. In India, the World Health Organization (WHO) requires analytical testing, such as droplet size, for devices used for nasally administered vaccines.

Both preserved and preservative-free nasal spray drug products will be required to complete the series of testing outlined above. If the drug product is manufactured in a sterile environment, then sterility testing will be required. An antimicrobial active may be self-preserving and, therefore, may not need routine preservative effectiveness testing. As previously discussed, BAC, phenylethyl alcohol, EDTA, and potassium sorbate have a history of use as preservatives in nasal spray formulations. If an alternate or novel preservative is used that does not have a history of use in the

nasal cavity or respiratory tract, regulatory bodies may require additional toxicological studies on the excipient.

Nasal powders and pressurized nasal aerosols can be considered alternates to preservative-free systems. For both of these, particle size characterization by cascade impaction to quantify the mass of drug less than 9 μm will be required. This will be used to address the potential for lung deposition via the nasal cavity, which is a FDA and EMA safety concern. Cascade impaction may also be required on a routine basis by FDA for nasal powders and aerosols. HFA-based nasal aerosols may also need to follow some of the analytical tests outlined in the Inhalation CMC Guidance (Food and Drug Administration 1999; European Medicines Agencies 2006). For passive nasal powders, where the patient's inspiratory effort aerosolizes the powder, spray pattern and plume geometry would not be required.

Recently, FDA has requested that sponsors submit additional CMC data with the Investigational Drug Application (IND), and spray performance measurements can provide some of that data. Spray characterization data appropriate for this stage might include any or all of the following: pump delivery (PD), SCU, droplet size distribution, spray pattern, and plume geometry.

In the case of a solution formulation, pump delivery (PD) may serve as a surrogate for SCU to conserve resources at this phase of development, since PD takes only minutes to complete compared to hours for SCU. However, confirming first that the correlation between the PD and SCU exists is prudent. Since the distribution of API in suspensions might result in differing amounts of API in each actuation, PD might not equate to SCU, therefore suspension formulations always require SCU.

FDA requires evaluation of potential leachables on stability. Leachables, which may be seen as a potential contaminate and harmful to public health, are also on the radar of the CFDA. To address, an extractable study is required to determine if potential components from the device may leach into the drug product. If the extractable profile reveals entities above the analytical evaluation threshold (AET) that require monitoring, a leachable study is necessary. For practical and financial reasons this study should take place concurrently with your registration stability batches because units can be stored for both studies at the same time under the same conditions. In order to have sufficient planning time for the leachable study, you will need to complete the extractable profile at least 6 months prior to the scheduled start of registration stability studies.

FDA requires testing of three registration batches prior to submission of an NDA. In addition to analysis of physical characteristics and microbiological testing over the course of the stability study, most sponsors also choose to include spray pattern, although spray pattern and plume geometry are not required. These registration stability study designs (Table 5.8) typically involve the analysis in excess of 10,000 units over a 2- to 3-year period. As a result, poor planning, such as failing to place a sufficient number of units in the stability chambers, can result in a disastrous loss of time and money.

Drug product characterization studies on samples from three registration batches should also take place along with clinical batch release testing. One-time drug product characterization studies performed at this stage include (where appropriate)

Table 5.8 Example stability design for a multi-dose nasal spray suspension drug product with preservatives

	Time (months)						
Storage condition	1	3	6	9	12	18	24
25 °C/60%RH	NT	A	A, B, C	A	A, B, C	A	A, B, C, D
40 °C/75%RH	A	A, D	A, B, C, D	NT	NT	NT	NT
30 °C/65%RH	E	E	E	E	E	NT	NT

NT not tested
A appearance, assay, pH, viscosity, weight loss, degradation and impurities, preservative content, spray content uniformity (SCU), pump delivery (PD), droplet size, particulate matter, particle size (API), spray pattern
B microbiological testing
C Preservative effectiveness
D Leachables
E Reserve samples tested in the event of a failure during 40 °C/75 % RH

photostability, temperature cycling device robustness, profiling, effect of dosing orientation, prime/reprime, and cascade impaction for nasal sprays to determine the percentage of droplets less than 10 μm.

5.8.2 Generic Drug Approvals

The global interpretation of qualitative and quantitative (Q and Q) sameness, as required for generic drug products, may actually vary from country to country. In the United States, FDA's interpretation of Q and Q is well defined in that the active must be the same and the inactive excipients must be with 5 % of the reference label drug (RLD) for nasal sprays. Health Canada and EMA have a similar approach. However, the similarities may end there. In the United States, the FDA expects that the patient has the same experience when using the device. In other words, there is a need to have the same type of device, e.g., CPS pump to CPS pump, used for the RLD and generic. In Brazil, the regulatory bodies allow omission or alternate excipients for generic drug products. For example, Budecort (budesonide, RLD) is available on the market as a preserved multi-dose nasal spray and a generic budesonide formulation that is non-preserved is also on the market. This Brazilian example would not meet FDA's expectations of a generic drug product because it is not Q and Q from a formulation and device standpoint.

Other regulatory differences are the bioequivalence guidances that request in vitro analytical testing (FDA and ANVISA) or deposition studies (EMA). There is a draft FDA Bioequivalence Guidance (Food and Drug Administration 2003; European Medicines Agencies 2006) that outlines a series of analytical tests that can be used to determine equivalence. These tests include droplet size by laser diffraction, drug in small particles/droplets as determined by cascade impaction, spray pattern, plume geometry, single actuation content uniformity, microscopy for suspensions, and priming and repriming. A combination of statistical approaches is

Table 5.9 Statistical approaches to determine equivalence

In vitro test	Statistical process
Single actuation content uniformity	Population bioequivalence (PBE)
• Drug mass per actuation	
Droplet size	PBE
• Dv_{50}	
• Span	
Spray pattern	PBE
• Ovality ratio	
• Area	
Plume geometry	Point estimate
• Width	
• Angle	
Particle size by microscopy	N/A
Drug in small particles by cascade impaction (sprays)	Comparison of means by PBE
Priming/repriming	Point estimate

used to determine equivalence (Table 5.9). For a locally acting nasal spray solution, equivalence of these six tests (no particle size by microscopy) may allow the generic sponsor to avoid performing pharmacokinetic, pharmacodynamic, or clinical endpoint studies.

In Europe, the EMA has defined a stepwise approach for approval of generic drug products (European Medicines Agencies 2006). While this model is meant for inhaled drug products, in theory, this could also be considered relevant for nasal sprays. The stepwise approach relies on similarity of in vitro tests as the starting point. The question for nasal sprays is that the exact in vitro tests are not defined. However, if in vitro tests are not equivalent, the next step may be a deposition study with a technique like gamma scintigraphy and demonstrating similar systemic exposure. Unlike FDA, deposition studies could be used as a tool for bioequivalence in the EU.

In October 2010, ANVISA issued a guidance (Brazil National Health Surveillance Agency 2008) similar in design to the FDA draft bioequivalence guidance. ANISA has also reissued a more detailed guidance in March 2012 (Brazil National Health Surveillance Agency 2013). The tests required for generic approval in Brazil are spray pattern, droplet/particle size by laser diffraction, uniformity of delivered dose, number of actuations, priming and repriming, and general assays for the drug product found in pharmacopoeias (Brazil, USP, EP, etc.). The statistical approach is not defined in this guidance; however, it is believed that an approach defined by FDA will be utilized to determine equivalence.

5.9 Conclusion

In the future, there is likely to be an increase in preservative-free formulations, especially in certain countries, both in the prescription and over-the-counter markets. Preservative-free nasal sprays are made possible by the device platforms that allow

for sterilization before or after manufacture of the drug product. In addition, preservative-free devices add another barrier by preventing microbial ingress during use by the patient. Sterile manufacturing technology is adaptable to preservative-free nasal sprays and the regulatory pathway is similar to that of traditional nasal spray drug products.

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Chapter 6
Formulation Approaches and Strategies for Vaccines and Adjuvants

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Abstract In order to create safe and efficacious vaccines, formulations that confer stability must generally be developed. In this chapter, formulation considerations consisting of solution conditions, particles, delivery route, endotoxin level, and preservatives will be covered along with the addition of adjuvants currently approved for use in vaccines and adjuvants currently being researched. Methods to increase vaccine stability and analytical techniques used to monitor vaccines will be discussed.

6.1 Introduction

Currently, there are 30 diseases that are preventable by vaccination (WHO et al. 2009), and numerous new vaccines currently are under development. Since vaccines prevent disease at a low cost, they have become the most cost-effective healthcare intervention (WHO et al. 2009) and offer the hope for combating a number of challenging diseases, including malaria, tuberculosis, human immunodeficiency virus, and cancer. For the full promise of vaccines to be realized, formulations must be developed that allow optimal immune responses while at the same time providing for retention of activity during storage, transportation, and delivery to patients. This chapter will discuss topics in vaccine formulation such as types of vaccines,

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current and future adjuvants, particulate formulations, route of delivery, endotoxin levels, preservatives, stability, and challenges associated with analytical techniques needed for vaccines.

6.2 Vaccine Versus Protein Formulations

There is now a significant literature dealing with strategies for developing formulations that are appropriate for therapeutic proteins (Volkin et al. 2002; Wang et al. 2007; Carpenter et al. 1997; Frokjaer and Otzen 2005; Stolnik and Shakesheff 2009; Kamerzell et al. 2011; Chang and Hershenson 2002; Akers et al. 2002). Vaccine formulations have much in common with these formulations, but differ in a critical aspect: the desirability of an immune response. A strong immune response to a vaccine is a requirement, whereas an immune response to a therapeutic protein formulation could be very detrimental to the patient and disease treatment (Nechansky and Kircheis 2010).

To help stimulate a suitable immune response to an administered antigen, adjuvants are frequently added to vaccine formulations. These adjuvants are typically used as suspensions of nano- or microparticles. Although the addition of such particles lowers the required amount of antigen needed to create an appropriate immune response, formulation design is also complicated because the physical and chemical stability of adjuvants as well as antigens must be considered.

Vaccines are able to create strong immune responses with relatively low concentrations of protein (10–100 μg/mL) (FDA 2012a) due to the high native immunogenicity of the antigen being used or the presence of an adjuvant in the formulation. Therapeutic protein formulations require much higher protein concentrations to be an effective treatment of a disease such as antibody formulations which often require as much as 100 mg/mL of protein (Shire et al. 2004).

Although the mechanism of action for protein therapeutics and vaccines is very different, both types of formulations need to be stabilized. Excipients used to stabilize protein therapeutics are often used to also stabilize vaccines. Methods to monitor stability and increase formulation stability will be discussed in later sections.

6.3 Types of Vaccines

Depending on the characteristics of the pathogen of interest and target population to be vaccinated, different types of vaccines can be formulated. There are three main types of vaccines: live attenuated, killed/inactivated, and subunit vaccines. Live attenuated vaccines consist of a weakened version of the pathogen. Since live attenuated vaccines are normally immunogenic enough on their own, they rarely require

an adjuvant (Pulendran and Ahmed 2011). Live attenuated vaccines can be problematic if they revert back to a stronger form of the pathogen, which could potentially cause harm in non-vaccinated or immunocompromised people (Singh et al. 2006). To avoid a pathogen from being able to revert to a stronger form, killed, also known as inactivated vaccines are created using whole pathogens that have been either heat or chemical treated. The safest type of vaccine is the subunit vaccine where only a portion of the pathogen is used (Pulendran and Ahmed 2011). Although the subunit vaccines have less risk in terms of the potential for the pathogen causing the disease, they are also less immunogenic because they are highly purified. The low immunogenicity often requires the vaccine to contain an adjuvant or be given in multiple doses (Pulendran and Ahmed 2011).

Since subunit vaccines only contain a portion of the actual pathogen, they can come in many forms depending on which portion of the pathogen they include. Examples of specific types of subunit vaccines are toxoid vaccines, conjugate vaccines, and DNA vaccines (Pulendran and Ahmed 2011). Toxoid vaccines are used when an invading pathogen secretes a toxic material to the body. Toxoid vaccines contain an inactivated version of the toxic material, so that in the event of exposure to the actual toxic material the body would be protected. A conjugate vaccine takes advantage of the immune system being able to recognize bacteria coated in polysaccharides by linking the antigen of interest to polysaccharides. A DNA vaccine carries genetic material, DNA, which the body can then use to produce the desire antigen and create an immune response.

The main focus of this chapter will be on subunit vaccines. The main components of subunit vaccines typically are the antigen, adjuvant, stabilizer, buffer, and preservative when necessary.

6.4 General Formulation Considerations

Intuitively, one might expect based on physiological conditions that buffer pH values near 7 might be optimal for a vaccine formulation. However, a broader range of pH (e.g., 5–8) may be explored for vaccine formulations. Practical limitations on formulation conditions include the relatively rapid rate of deamidation reactions observed at alkaline pH, and acid-catalyzed degradation reactions that can be accelerated at acidic pH values. Stability for many proteins is optimal in solutions formulated at pH 5–6. Pain on injection may be dependent on formulation pH, tonicity, osmolarity, solution temperature, drug concentration, and injection volume (Brazeau et al. 1998), but can sometimes be mitigated by using formulations with reduced buffer capacity. The buffer solution should also be adjusted so that the overall vaccine formulation is isotonic. Isotonicity of the vaccine will reduce tissue damage and pain of injection. Preservatives can be added to vaccines in cases where potential contamination is a concern, such as in multidose vaccine formulations.

6.5 Adjuvants

Adjuvants are materials that are used along with the antigen in a formulation with the primary goal of eliciting a stronger and more efficacious immune response compared to the antigen alone. In addition, an ideal adjuvant should possess the following properties:

1. By eliciting a strong immune response, an adjuvant should be capable of lowering the required antigen dose (Vogel 2000; O'Hagan and De Gregorio 2009), hence reducing or eliminating any antigen-induced toxicity effects, and reducing the per-dose cost for expensive antigens.
2. The adjuvant should induce both cellular and humoral immune response to the antigen (O'Hagan and De Gregorio 2009).
3. Adjuvanted formulations should be capable of producing a rapid onset and prolonged immune response (O'Hagan and De Gregorio 2009).
4. Adjuvants should aid in creating an immune response in populations not able to originally create an immune response such as elderly, young children, and immunocompromised people (Vogel 2000; O'Hagan and De Gregorio 2009).
5. Any interactions between the adjuvant and the antigen should not result in a loss of structural or chemical integrity of the antigen (O'Hagan and De Gregorio 2009).
6. The adjuvant should be safe and easy to formulate (O'Hagan and De Gregorio 2009).

Currently in the United States, the Food and Drug Administration (FDA) has approved two aluminum-based adjuvants. The first approved adjuvant is alum which is most commonly present as the mineral salts aluminum phosphate or aluminum hydroxide. The second approved adjuvant is AS04. AS04 is an adjuvant system containing monophosphoryl lipid A (MPL) adsorbed to aluminum. In addition to alum and AS04, the European Union (EU) has approved three other adjuvants for use in vaccines. The oil-in-water emulsions MF59 and AS03 have been approved along with virosomes (Rappuoli et al. 2011).

6.5.1 Aluminum Salt Adjuvants

Aluminum salt adjuvants have been used safely in vaccines for over 70 years. The two main aluminum salt adjuvants used are aluminum hydroxide and aluminum phosphate. Aluminum hydroxide is also known as Alhydrogel®, and aluminum phosphate is also known as AdjuPhos®. The type of aluminum salt chosen for the vaccine formulations is based on the mechanism of antigen adsorption to the adjuvant. The antigen can adsorb to the adjuvant surface through van der Waals forces, hydrogen bonding, electrostatic forces, and ligand exchange. Since van der Waals forces and hydrogen bonding provide much weaker binding of antigen to adjuvant,

we will focus on only the other two stronger mechanisms of adsorption. The World Health Organization (WHO) recommends that over 80 % of antigen be adsorbed to adjuvant based on a tetanus vaccine (WHO 1977). However, studies with aluminum salt-adjuvanted vaccines based on recombinant protective antigen (Berthold et al. 2005), lysozyme (Clausi et al. 2008a; Chang et al. 2001; Romero Mendez et al. 2007), dephosphorylated α-casein (Romero Mendez et al. 2007), and ovalbumin (Romero Mendez et al. 2007) have shown that antigen need not be fully adsorbed to adjuvant to be effective (Clapp et al. 2011).

To maximize attractive electrostatic interactions and encourage adsorption of antigen to adjuvant, the antigen and adjuvant should have opposite charges (Seeber et al. 1991). Critical parameters for design of adjuvanted formulations thus include the isoelectric point (p*I*) of the antigen and the point of zero charge (PZC) of the adjuvant. At these two pHs, the protein and adjuvant, respectively, will exhibit net charges of zero. For aluminum hydroxide to PZC is approximately 11, and for aluminum phosphate the PZC is between 4 and 5.5 (Peek et al. 2007). Based on the pH of the vaccine formulation, the charge of the antigen and adjuvant will change; stronger binding is generally seen at solution pH values where the antigen and the adjuvant are oppositely charged (Seeber et al. 1991). To optimize the PZC for aluminum salt adjuvants, aluminum hydroxide can be treated with phosphate ions. In the presence of phosphate, aluminum hydroxide surfaces are converted to the more thermodynamically favored aluminum phosphate, thus lowering the PZC (Hem et al. 2010). Due to the relatively high ionic strength found under physiological conditions, antigens that are adsorbed via electrostatic interactions can often desorb from aluminum salt adjuvants once injected into the body (Hem et al. 2010).

Ligand exchange is another means by which antigen can be attached to adjuvant surfaces. Phosphate groups on antigens may exchange with adjuvant hydroxyl groups (Hem et al. 2010). To reduce the amount of ligand exchange between the antigen and aluminum salt adjuvant, the aluminum hydroxide adjuvant can be treated with phosphate ions, thus reducing the number of site for potential ligand exchange (Hem et al. 2010). Adsorption strength was varied by pretreating aluminum hydroxide adjuvant with phosphate ions, and it was found that the strength of adsorption was inversely proportional to the immune response for HIV gp140 antigen (Hansen et al. 2011), In-labeled alpha casein (Noe et al. 2010), and hepatitis B surface antigen (Egan et al. 2009). Since ligand exchange is a stronger mechanism of adsorption than electrostatics, antigen will not readily elute from the antigen-adjuvant complex once it is injected into the body and comes into contact with fluid (Hem et al. 2010).

The US Code of Federal Regulations recommends that vaccine formulations contain less than 0.85 mg Al^{3+} per dose when assayed and less than 1.14 mg Al^{3+} when calculated, whereas the WHO and European standards recommend less than 1.25 mg Al^{3+} per dose (Vecchi et al. 2012). The toxic levels of aluminum were evaluated to be around 36.42 mg of Al^{3+}, in an acute toxicity study in rats (Titkov and Oganesian 1995), which is 43 times greater than the FDA recommended dose. The regulatory agencies have presumably recommended a low dose of aluminum to avoid possibilities of chronic toxicity.

Many vaccines can protect people against a disease through a humoral response wherein antibodies are produced once a pathogen invades the body. The antibodies can help the immune system clear the invading pathogen from the body. Some pathogens, however, require the body to initiate a cellular response in order for the pathogen to be cleared. A cellular immune response is important in vaccines protecting against intracellular pathogens (Mbow et al. 2010). In particular, malaria and tuberculosis vaccines require a cellular immune response to be effective (Wilson-Welder et al. 2009). Since aluminum salt adjuvants create a humoral immune response which is not ideal for vaccines protecting against all pathogens, other vaccine adjuvants need to be investigated (Garcon et al. 2007).

6.5.2 MF59

MF59 was the second approved vaccine adjuvant after alum (Rappuoli et al. 2011). MF59 is an oil-in-water emulsion. In the emulsion, squalene oil nanodroplets approximately 160 nm in diameter are surrounded by the nonionic detergents polysorbate 80 (Tween 80) and sorbitan trioleate (Span 85) (Schultze et al. 2008). When stored at temperatures between 2 and 8 °C, MF59 is able to retain a constant particle size for up to 3 years (Schultze et al. 2008). MF59 is commonly used as an adjuvant in influenza vaccines (O'Hagan et al. 2011). In one study, the antigens diphtheria toxoid, tetanus toxoid, group C meningococcal conjugate, hepatitis B surface antigen, and recombinant MB1 were formulated with both aluminum adjuvant and MF59 adjuvant. For all antigens except diphtheria toxoid, formulations containing MF59 adjuvant were able to create a stronger immune response than corresponding formulations containing aluminum, as shown by geometric mean IgG titers after two doses of the vaccine (Singh et al. 2006).

6.5.3 AS04

AS04 is an adjuvant system created by GlaxoSmithKline Biologicals that contains 3-*O*-desacyl-4′-monophosphoryl lipid A (MPL) adsorbed to an aluminum salt. Lipopolysaccharide (LPS) is known to stimulate Toll-like receptor (TLR) 4, helping create a cellular immune response. MPL comes from the portion of LPS found in the cell walls of gram-negative bacteria (Casella and Mitchell 2008). Since LPS is too toxic to be used directly as an adjuvant, MPL is derived from LPS to have a similar effect on TLR 4 without the unwanted toxicity (Baldridge et al. 2004). The AS04 adjuvant can help create both humoral and cellular immune responses (Garcon et al. 2007).

AS04 is currently included in the FDA-approved human papillomavirus (HPV) vaccine Cervarix (Descamps et al. 2009). The AS04 adjuvant present in a hepatitis B vaccine was tested in comparison to a hepatitis vaccine without AS04.

It was found that after one dose of vaccine containing AS04 adjuvant, the patient seropositivity rate was 77 %, whereas patients receiving vaccine without AS04 had only a 37 % seropositivity rate. After injections at 0 and 6 months, the AS04 group had 98 % seroprotected, and after injections at 0, 1, and 6 months, the group without AS04 had 96 % seroprotected, showing that the vaccine formulated with AS04 was equally effective as the vaccine without AS04 but required fewer doses (Boland et al. 2004).

6.5.4 AS03

AS03 is an oil-in-water emulsion adjuvant system created by GlaxoSmithKline Biologicals. This adjuvant contains squalene and α-tocopherol, a form of vitamin E. Hepatitis B surface antigen (HBsAg) formulated with AS03 had a 10 times higher geometric mean titer than antigen alone formulated with alum after two intramuscular doses (Morel et al. 2011). A significantly higher antibody titer was also seen when an H5N1 influenza vaccine was formulated with AS03 in comparison to vaccine without an adjuvant (Morel et al. 2011). In addition to producing higher antibody titers with HBsAg, the AS03-adjuvanted influenza formulations were able to produced both Th1 and Th2 cytokines in greater amounts than alum (Morel et al. 2011). To be most effective AS03 should be injected in the same location and at the same time as the antigen (Morel et al. 2011).

6.5.5 Virosomes

Virosomes are viruslike particles containing portions of virus envelope without genetic material of the virus. When virosomes are used as an adjuvant, they can create both a humoral and cellular immune response (Reed et al. 2009). Virosomes are approximately 100–200 nm in diameter (Bachmann and Jennings 2010). Viruslike particles can be found in hepatitis A and B, human papillomavirus, and influenza vaccines licensed in Europe (Moser et al. 2011).

6.6 Future Adjuvants

Adjuvants are an integral part of an effective subunit and inactivated microorganism vaccine formulations, and scientists have consistently directed their efforts to discover new adjuvant molecules that are safer and more effective than alum. However, new adjuvant research involves thorough in-depth understanding of the mechanism of action, stability pattern, toxicity profile across various doses and populations, as well as compatibility with the vaccine candidate in the desired formulation.

6.6.1 OM-174

In a review by Corradin and Giudice, several adjuvant candidates were discussed (Corradin and Giudice Giuseppe 2005). They classified the adjuvants based on solubility (aqueous or oil soluble) (Corradin and Giudice Giuseppe 2005). The water-soluble adjuvants included OM-174, which is a derivative of MPL. The authors reported "excellent safety" of this adjuvant when used with a malaria synthetic subunit vaccine. Also, it was mentioned that this adjuvant can be either administered alone in a formulation or as a co-adjuvant with alum.

6.6.2 QS-21

QS-21 is another water-soluble adjuvant. Chemically, it is an acylated 3.28-o-bisdesmodic triterpenoid saponin derived from the bark of the *Quillaja saponaria* tree (Kensil et al. 1991). This adjuvant has been tested in several clinical trials for vaccines against infectious diseases such as HIV-1 (Evans et al. 2001), influenza (Mbawuike et al. 2007), and malaria (Stoute et al. 1997), as well as in cancerous patients with melanoma (Helling et al. 1995), breast cancer, or prostate cancer (Kensil and Kammer 1998). QS-21 has been extensively used with MPL in the malaria vaccine with satisfactory results. However, being a natural product, QS-21 exhibits variability in composition depending on the source, and also can be expensive to extract and purify (Kamstrup et al. 2000). Also, dose-dependent immune responses for QS-21 pose a challenge in cancer patients, who develop local erythema and flu-like symptoms at doses greater than 150 μg (Adams et al. 2010). Additionally, QS-21 degrades during long-term storage in aqueous solutions (Cleland et al. 1996). Synthetic saponins have been investigated to overcome these problems (Adams et al. 2010).

6.6.3 Immunostimulating Complexes

Another adjuvant that contains a saponin is immunostimulating complexes (ISCOM). ISCOM contains cholesterol, phospholipids, saponin, and protein. ISCOMATRIX is similar to ISCOM except it does not contain protein (Pearse and Drane 2005). When the ISCOMATRIX components combine, they form approximately 40 nm, cage-like structures (Pearse and Drane 2005). The ISCOMATRIX has been shown to be stable when refrigerated for 2 year, stored at 40 °C for a few months, after freeze-thaw cycles and during freeze-drying (Pearse and Drane 2005). Both humoral and cellular immune responses can be generated with this adjuvant (Sun et al. 2009). An increased amount of local reactions to the ISCOMATRIX in a clinical trial for human papillomavirus (HPV) vaccine was seen in comparison to

the group containing no adjuvant (McKenzie et al. 2010). In vaccine trials for HPV, hepatitis C virus, and influenza, ISCOMATRIX was found to be safe (McKenzie et al. 2010).

6.6.4 Montanide ISA

Montanide ISA 720 is a squalene-based adjuvant designed for human use that consists of mannide monooleate emulsifier and forms stable water-in-oil droplets with the idea of promoting sustained release of antigen at the injection site (Aucouturier et al. 2002). In clinical studies involving a malaria vaccine (*P. falciparum* CSP C-terminal fragment 282–283) formulated with ISA 720 and alum, high antibody titers were obtained along with good lymphocyte proliferation and production of IFN-γ that is critical for the elimination of malaria parasite (Roestenberg et al. 2008; Lopez et al. 2001). Another compound in this category is Montanide ISA 51, which is based on mineral oil that can be metabolized has also been shown to be safe for human use (Aucouturier et al. 2006).

6.6.5 Microorganism Compounds

Components derived from microorganisms such as bacteria hold promise as "immunopotentiating" adjuvants. For example, specific mutants (produced by site-directed mutagenesis) of heat-labile enterotoxin derived from *Vibrio cholerae* or *Escherichia coli* have been investigated as candidates for mucosal adjuvants that provoked increased serum IgG levels in mice and pigs when administered nasally in a microsphere delivery system (Vajdy et al. 2004). However, toxicity of such molecules has limited their use in humans (Vajdy et al. 2004). Another example in this category is a fusion gene (CTA1 gene from cholera toxin fused with a synthetic analogue of *S. aureus* protein A encoding gene) that exhibited less toxicity compared to wild-type cholera toxin (Lycke 2004).

6.6.6 Cytokines

Cytokines can also be potential adjuvant candidates. However, a variety of interleukins (IL-1, IL-2, IL-12) evaluated for this purpose exhibited in vivo stability and toxicity issues (Vajdy et al. 2004). Another example is IRX-2 which contains a natural mixture of Th1 cytokines (IL-1, IL-2, and IFN-γ) that enhances the antigen-processing capacity of lymph nodes by stimulating the Th1 pathway (Naylor et al. 2010).

6.6.7 *Toll-Like Receptors*

TLRs are pattern-recognition receptors produced by cells of the innate immune system. The TLRs bind to a variety of infectious agents and stimulate pathways that finally protect the host cells from the pathogen. Therefore, synthetic or purified TLRs have been the interest for adjuvant purposes (Steinhagen et al. 2011). One such example of TLR agonist is a repeating sequence of CpG dinucleotides, which has been found to be immunostimulatory and has been tested as an adjuvant in hepatitis B vaccine (Cooper et al. 2005). Imiquimod and resiquimod are small molecule TLR-7/TLR-8 agonist molecules, which are being studied as a topical adjuvant for skin disease (Gnjatic et al. 2010).

6.6.8 *Polymer Particles*

Micro- and nanoparticle formulations can also be employed for vaccine delivery resulting in sustained-release vaccine formulations. Such formulations involve the use of biodegradable polymers such as polylactic acid (PLA), poly-lactic-*co*-glycolic acid (PLGA), polyethylene glycol (PEG), and polyphosphazene to formulate the micro- or nanoparticles (Oyewumi et al. 2010). Researchers have tried to correlate immune response and particle size. In general, smaller particles can cross biological barriers via tight junctions or via endocytosis and get to systemic circulation, which might be expected to result in better efficacy. However, smaller particle sizes do not always correlate with enhanced immune response. For example, HBsAg entrapped in PLA particles of diameter 2,000–8,000 nm produced greater anti-HBsAg antibody response than HBsAg entrapped in 200–600 nm PLA particles (Kanchan and Panda 2007). Various formulation parameters such as formulation materials, dose, antigen loading method, uniformity of particle size, and various routes of administration can be held responsible for such contrasting observation (Oyewumi et al. 2010).

6.7 Vaccine Particles

The size, shape, and surface molecular organization of antigens have been found to affect the immune response (Bachmann and Jennings 2010). By using adjuvants of controlled sizes, vaccine particles can be made to be of sizes similar to those of the target pathogen (Bachmann and Jennings 2010; O’Hagan et al. 1997). Viruslike particles and immunostimulating complexes can be on the same order of magnitude of viruses. Emulsions, liposomes, and virosomes can be on the same order of magnitude of size as larger viruses, bacteria, fungi, and protozoa. Microparticles and mineral salts can be on the same order of magnitude size as bacteria, fungi, and protozoans (Bachmann and Jennings 2010). In addition to adjuvant particles being

a similar size to potential pathogens, it is also important for adjuvants to be taken up by antigen presenting cells.

Freeze-drying parameters can be varied to create vaccines containing a range of aluminum particle sizes (Clausi et al. 2008b). In a study conducted with a model freeze-dried lysozyme vaccine, formulations containing aluminum particles ranging in average size from 2 to 14 μm all produced similar anti-lysozyme IgG1 titers after two doses (Clausi et al. 2008a).

6.8 Route of Delivery

An ideal vaccine should be effective, safe, and administered in a minimally invasive manner. The route of vaccination is a very important consideration as some infectious disease pathogens invade the host cells on mucosal surface; in such cases, the ideal vaccine needs to induce systemic immunity as well as mucosal immunity (Devriendt et al. 2012). Oral administration of vaccine is one of the routes of administration that yield the highest patient compliance and does not require syringes or trained personnel. However, a vaccine delivered via the oral route must be robust enough to survive the acidic pH in the stomach and proteolytic enzymes and should be suitably transported across the gastrointestinal tract in order to reach the systemic circulation. Approaches to modulate delivery across the gastrointestinal tract includes altering physicochemical properties of the vaccine for enhanced uptake or formulating the vaccine in micro- or nanoparticles that protect the antigen from acid degradation in stomach. However, particle-based formulations face a major hurdle in crossing the intestinal barrier and therefore generally offer very poor protection at the mucosal site. Several ligand-based delivery systems have been recently explored to identify gastrointestinal surface receptors as vehicles of delivery of antigen via endocytosis to elicit a strong immune response. Such ligands include lectin-based targeting, bacterial adhesins, bacterial toxins, and antibody-mediated targeting (Russell-Jones 2000). Live attenuated vaccines are administered orally as the antigen needs to have an inherent ability to attach to mucosal cells. Presently, the vaccines that have been approved for oral administration include cholera, influenza, polio virus, rotavirus, and *Salmonella typhimurium* (Holmgren and Czerkinsky 2005).

The nasal route of administration can also produce mucosal and systemic immune responses. It is an attractive alternative to oral vaccines as the antigen is not subjected to acid degradation. Also, this route of administration is easily accessible, highly vascularized, and ideal for mass immunization. However, the vaccine still needs to overcome the nasal mucosal barriers to produce systemic effects. Solution, dry powder, or suspension formulations can be delivered via this route. Nasal vaccination possibly demonstrates a more rapid onset compared to oral vaccines (Davis 2001). Flumist® is an example of nasal delivery system consisting of temperature-sensitive attenuated influenza virus.

The most common route of vaccine administration is via intramuscular or subcutaneous injection. Intramuscular injection optimizes the immunogenicity of the

vaccine and greatly reduces any adverse reaction at the site of administration. Transcutaneous vaccination has also become a topic of interest for vaccine delivery. The skin is the largest organ in the human body and is the first natural barrier against harmful pathogens. However, the transport of antigens across the stratum corneum represents a significant barrier to this route of vaccine delivery. It is expected that adjuvants such as alum, MPL, and bacterial endotoxins will have limited penetration across the skin due to their large size. However, preclinical transcutaneous studies indicate that cholera toxin (CT) and heat-labile *E. coli* toxin (LT) can be used as adjuvants as they stimulate immune response against other antigens. The most successful delivery via transcutaneous route consisted of physically disrupting the skin barrier with the help of microneedles followed by delivery of the formulation (Bal et al. 2010).

6.9 Endotoxin Levels

Endotoxin comes from LPS found in the cell membranes of gram-negative bacteria (Magalhaes et al. 2007). LPS commonly contains distinct regions of an O-antigen region, core oligosaccharide, and hydrophobic lipid (lipid A), with the lipid A region being responsible for toxicity (Magalhaes et al. 2007). Endotoxin can be introduced into formulations when components of the vaccines are produced in gram-negative bacteria, such as recombinant proteins produced in *E. coli* (Magalhaes et al. 2007). When the body is exposed to large dose of endotoxin or small doses of endotoxin systematically, an inflammatory reaction occurs which can cause shock, tissue damage, or death (Magalhaes et al. 2007). To avoid damage caused by endotoxin, endotoxin levels should be kept low in formulations. The threshold pyrogenic dose of endotoxin in humans is 5 EU/kg (Malyala and Singh 2008), making it desirable to keep endotoxin levels below this amount. Although specific endotoxin limits have not been set by United States Pharmacopeia (USP), it is recommended to keep endotoxin levels low. Brito and Singh suggested upper endotoxin limits for different types of vaccines based on DTwP and cholera vaccines as follows: genetic vectors 10 EU/mL, recombinant subunit 20 EU/mL, polysaccharide 20 EU/mL, live attenuated 200 EU/mL, inactivated 500 EU/mL, and toxoid 200,000 EU/mL (Brito and Singh 2011).

Endotoxin present in formulation is most commonly measured by the gel clotting in the Limulus Amebocyte Lysate (LAL) test. If levels of endotoxin are too high, endotoxin can be removed throughout steps in the vaccine manufacturing process. Since endotoxin is stable at high temperature, heat sterilization will not inactivate endotoxin unless temperatures exceeding 250 °C for 30 min and 180 °C for 3 h are used (Magalhaes et al. 2007; Gorbet and Sefton 2005). Concentrations of acids and alkalis above 0.1 M are capable of inactivating endotoxin. Endotoxin present in protein solutions can be removed by LPS affinity resins, two-phase extractions, hydrophobic interaction chromatography, ion exchange chromatography, gel filtration chromatography, sucrose

gradient centrifugation, and membrane adsorbers. If protein is not present in the desired solution for endotoxin removal ultrafiltration can be used (Magalhaes et al. 2007).

6.10 Preservatives

Although preservatives are not normally used in single-use vials, preservatives are normally added to multidose vials to prevent growth of microorganisms as recommended by the United States Code of Federal Regulations for vaccines not containing live attenuated viruses. Preservative that have been used in US FDA-approved vaccines include thimerosal, phenol, benzethonium chloride, and 2-phenoxyethanol (Geier et al. 2010). At an acidic pH thimerosal is able to kill bacteria and at an alkaline or neutral pH thimerosal prevents bacteria and fungus from replicating (Rowe et al. 2009). Thimerosal is not compatible with aluminum and should therefore not be used with an aluminum salt adjuvant (Rowe et al. 2009). Vaccines recommended for children under 6 years old, except for influenza vaccines, have had the thimerosal reduced to trace levels or lower (FDA 2012b). Thimerosal is currently used in tetanus toxoid vaccine, influenza vaccines, and multidose Menomune-A/C/Y/W-135. Phenol is able to be used against both gram-negative and gram-positive bacteria, mycobacteria, some fungi, and viruses (Rowe et al. 2009). Phenol is currently included in Pneumovax 23. Benzethonium chloride has an optimal antimicrobial activity from pH 4–10 and is not compatible with anionic surfactants (Rowe et al. 2009). Benzethonium is currently included in BioThrax. 2-Phenoxyethanol is able to protect against gram-negative organisms but has reduced activity when non-ionic surfactants are present (Rowe et al. 2009). 2-Phenoxyethanol is currently included in inactivated poliovirus vaccine (IPOL).

6.11 Stability

In order for vaccines to be economically feasible and able to be delivered to patients, they generally should have a shelf life of 2 years or longer. To determine the stability of a given formulation, both real-time stability studies and accelerated stability studies can be conducted. In accelerated stability testing, a stress such as elevated temperature, elevated humidity, light exposure, agitation, freeze-thawing, extremes of pH, or redox conditions (Chang and Hershenson 2002; Brandau et al. 2003) is applied to the formulation, and the rates at which the formulation degrades is monitored. Extrapolation of degradation rate data as a function of stress level allows an estimate of shelf life in the absence of stress to be obtained.

Many parameters such as pH, ionic strength, osmolarity, and the type and concentration of excipients present may play a role in vaccine stability. pH affects vaccine stability by changing the rate at which hydrolysis and deamidation reactions

occur. pH also changes the charge of molecules in solution which can then cause changes in protein structure or changes in adsorption of protein to adjuvant or other surfaces (Brandau et al. 2003). Lower ionic strength can increase the solubility of biomolecules, and the solution ionic strength can change how molecules assemble (Brandau et al. 2003). Excipients can also be added to formulations for stability (Brandau et al. 2003).

Excipients are commonly added to formulations to increase the formulation stability, maintain pH, modify tonicity, or help increase antigen solubility. Excipients commonly added to increase stability consist of surfactants, sugars, salts, and antioxidants (Chang and Hershenson 2002). Surfactants are commonly used to prevent unwanted protein adsorption to surfaces. Proteins often denature when adsorbed to surface. Sugars in solution are able to protect proteins from denaturing by preferential hydration and excluding sugar molecules from the protein surface. Sugars protect lyophilized formulations by slowing molecular motions in the dried solid state, and by providing hydrogen bonds with protein in the place of water. Salts can be added to formulations to increase the formulation ionic strength and can be added to help maintain a particular pH. Antioxidants are used to protect against oxidation.

To predict the formulation conditions and excipients that maximize the vaccine formulation stability from complex data sets, empirical phase diagrams can be used to better interpret the data (Maddux et al. 2011). Empirical phase diagrams take mathematical data collected from a variety of spectroscopic techniques and convert it into colors. Similar colors represent similar stabilities. Techniques commonly used in collecting the spectroscopic data for phase diagrams consist of circular dichroism, near-UV absorbance, extrinsic fluorescence, dynamic light scattering, OD 350, and intrinsic UV fluorescence (Maddux et al. 2011). To determine regions of stability, controlled formulation parameters (e.g., temperature, pH, excipient concentration, protein history, or other relevant conditions) need to be varied.

Since vaccines have potential to experience both hot and cold temperatures before being delivered to patients, the vaccine stability should be tested with several cycles of freezing and thawing. Loss of or decreased potency has been observed for vaccines containing an adjuvant (e.g., Alhydrogel) due to freeze-thawing (Braun et al. 2009). Several studies in the literature have implicated freezing-induced agglomeration of Alhydrogel for loss of potency (Diminsky et al. 1999). A study by Jones et al. subjected hepatitis B and DTaP vaccine formulations to controlled freeze-thaw cycles; they also evaluated the freezing-induced protection effects provided by additives such as glycol, PEG 300, and glycerin (Braun et al. 2009).

To increase stability and allow for higher storage temperatures, vaccines can be dried. In the dried solid state, degradation reactions occur at a much slower rate and much less water is present allowing for less degradation. Methods of drying that have been used consist of lyophilization (Carpenter et al. 1997; Clausi et al. 2008a, 2009; Amorij et al. 2008), Xerovac (a dehydration process not involving freezing) (Worrall et al. 2001), spray drying (Amorij et al. 2008; Bowey and Neufeld 2010; Sou et al. 2011), spray freeze-drying (Amorij et al. 2008), and carbon dioxide-assisted nebulization with a Bubble Dryer® (CAN-BD) (Amorij et al. 2008; Burger et al. 2008) (a drying process used to produce an inhalable powder).

Although vaccines can be created with antigen and adjuvant produced separately and then mixed together before administration in the clinic, it is recommended to have antigen and adjuvant formulated together. If the antigen and adjuvant will be stored separately, both components of the vaccine will need to undergo stability studies separately and then throughout the stability study antigen and adjuvant will need to be combine to test the whole vaccine. Variations in the vaccine such as adsorption of antigen to adjuvant caused by amount of time combine and mixing conditions will be created when the antigen and adjuvant are combined before use by different people. Slightly variations in the mixing procedure used could cause potential changes in the vaccine. These variations in the vaccine could potentially cause a loss in efficacy or safety.

6.12 Challenges of Analytical Techniques

When developing antigens to include in vaccine formulations, high-resolution techniques such as X-ray crystallography, nuclear magnetic resonance (NMR), and cryo-electron microscopy (cryo-EM) should be used (Maddux et al. 2011). When vaccine formulations are monitored over time, lower-resolution, faster techniques are more appropriate (Maddux et al. 2011) and will be focused on for the rest of this section. Changes in the vaccine formulations could be an indication of instability leading to a loss of safety and efficacy. Since vaccines frequently contain adjuvants which can scatter light as well as low protein concentrations, analytical techniques can often become difficult.

Primary structure can be looked at by breaking apart the antigen of interest through proteolysis and then analyzing the fragments with mass spectroscopy for areas of degradation. The amino acids, glutamine, and asparagine are more prone to deamidation and should be monitored through a loss of carboxylic acid group. The glutamine and asparagine residues should be especially monitored for deamidation when surrounded by a glycine residue allowing for greater flexibility for the deamidation reaction (Manning et al. 1989). Oxidation is more common in the aromatic residues tyrosine and tryptophan and along with cysteine and methionine.

Secondary structure has been examined by infrared spectroscopy. A study conducted with the six model proteins, cytochrome c, ovalbumin, α-chymotrypsinogen A, recombinant human IL-1ra, IgG1, and sTNF-R1 compared the standard solution infrared spectrum at protein concentrations of 15 mg/mL to lower protein concentrations of 1.0 and 0.5 mg/mL with protein adsorbed to Alhydrogel adjuvant and found that the spectra were very similar (Dong et al. 2006). The technique developed of looking at the secondary structure through infrared spectra of adjuvant-protein pellet would be applicable to vaccines formulated with aluminum adjuvants containing low concentrations of antigen. The secondary structure of proteins adsorbed to aluminum hydroxide, glass, and cellulose was able to be examined by a similar method (Bee et al. 2009; Fradkin et al. 2011).

Tertiary structure has been examined by tryptophan fluorescence quenching for protein adsorbed to glass, cellulose, silica, and alum (Bee et al. 2009; Fradkin et al. 2011). Since proteins contain the amino acid tryptophan which gives off a fluorescent emission depending on how buried the tryptophan residues are in the protein, the amount of unfolding can be monitored by measuring how easily the fluorescence from these residues can be quenched. The Stern-Volmer constant can be used to help determine the amount of quenching taking place. The Stern-Volmer equation uses the ratio of fluorescence intensity without quencher present, F_o, to fluorescence intensity with quencher present, F, equaling one plus the Stern-Volmer constant, K_{SV}, multiplied by the quencher concentration $[Q]$. The Stern-Volmer equation is as follows (Bee et al. 2009):

$$\frac{F_o}{F} = 1 + K_{SV}[Q]$$

Aggregation of vaccine antigen and particles present in vaccine formulations can be examined by many different techniques based on the size of particles present in the formulation and the desired information (particle count, particle size distribution, particle images). Imaging particle size techniques using instruments such as micro-flow imaging (MFI) or FlowCAM can count, size, and image particle if particles are greater than 2 μm. Nanosight instruments are capable of sizing particles in the nanometer range. If only the particle size distribution is required, laser diffraction can be used for formulations when particles are much smaller in the range of 0.04–2,000 μm. For small particles on the order of nanometers, dynamic and static light scattering can be used.

To monitor the thermal stability of vaccines, differential scanning calorimetry (DSC) can be used to find to melting temperature (T_m). A higher melting temperature would be more desirable for a formulation. Studies have been conducted to compare melting temperatures of formulations with different excipients with and without adjuvant to determine the formulation with the best thermal stability (Peek et al. 2007). In addition, enthalpy of unfolding can also be determined for proteins in which the heat-induced conformational change is reversible (i.e., no aggregation) (Vessely et al. 2009). Peek et al. employed DSC as a method of looking at thermal transitions of proteins adsorbed to Alhydrogel in the absence and presence of stabilizers. The overall increasing T_m of protein-Alhydrogel samples in presence of stabilizers (e.g., sorbitol, caprylate) indicate that proteins adsorbed to adjuvant are stabilized (Peek et al. 2007). In another example, measles vaccine powder was analyzed using DSC where the various energy-related (endotherms and exotherms) transformations were seen such as glass transition (T_g), T_m, and recrystallization (LiCalsi et al. 2001). However, a powder form may be quite complex consisting of various additives and excipients, and in such cases it becomes challenging to assign peaks to particular components or events.

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Part II
Process, Container Closure and Delivery Considerations

Chapter 7
Challenges in Freeze–Thaw Processing of Bulk Protein Solutions

Hari R. Desu and Sunil T. Narishetty

Abstract Freeze-thawing is a common unit operation during the production of protein-based therapeutics. Bulk protein solutions are often stored in frozen state for extended periods, and thawed to room temperature prior to downstream process operations including lyophilization, add-in formulation ingredients step, and fill and finish processes. However, freezing can induce protein denaturation stresses, such as cold denaturation, ice–liquid interfacial denaturation, and cryoconcentration. Many of these stresses are manifested as unfolding, reversible aggregation, and insoluble particle formation, while some can cause loss of structure and therapeutic function. Numerous studies have been attempted to understand and mitigate protein denaturation during freeze-thawing, and thereby provide guidelines for optimization of formulation and process variables. This chapter presents an overview of freeze-thawing-induced stresses, cryopreservation aspects, and containers, associated with manufacturing of bulk protein solutions.

Abbreviations

°C	Degree Celsius
ΔG	Gibb's free energy
ANS	1-Anilino-8-napthalene sulfonate
BSA	Bovine serum albumin

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CMC	Critical micelle concentration
C_p	Heat capacity
DTPA	Diethylenetriamine penta-acetic acid
EDTA	Ethylenediamine tetra-acetic acid
IgG	Immunoglobulin G
IR	Infrared
kJ	Kilojoules
LDH	Lactate dehydrogenase
LPT	Last point to thaw
LPTF	Last point to freeze
LYS	Lysozyme
mAb	Monoclonal antibody
mL	Milliliters
PE	Polyethylene
PEG	Poly(ethylene) glycol
PETG	Poly(ethylene) terephthalate glycol
PFK	Phosphofructokinase
PP	Polypropylene
PTFE	Polytetrafluoroethylene
PVP	Poly(vinyl) pyrrolidone
rhFXIII	Recombinant hemophilic factor XIII
rhIFN-γ	Recombinant human interferon-γ
T_c	Crystallization temperature
T_e	Eutectic temperature
T_f	Equilibrium freezing point
T_g	Glass transition temperature of solids
T_g'	Glass transition temperature of frozen solution
T_{het}	Heterogeneous nucleation temperature
T_{hom}	Homogeneous nucleation temperature
T_m	Melting temperature
TRE	Trehalose
Trp	Tryptophan
T_x	Devitrification temperature

7.1 Introduction

Majority of protein-based therapeutics are stabilized and preserved by freeze-thawing or freeze-drying operations (Carpenter and Change 1996; Pikal 1994). Bulk protein solutions of finished products are often frozen during production as frozen material offers (Rathore and Rajan 2008) (a) increased stability and improved shelf-life, (b) flexibility to the manufacturer in process planning, (c) stockpiling of intermediate step pools for later processing on "as need" basis, (d) decreased microbial contamination, (e) buffering volume between manufacturing facilities with

differing capacities, thus eliminating potential production bottlenecks, and (f) elimination of foaming during transportation. Owing to their flexible and extremely sensitive 3D-macromolecule structure, proteins may undergo conformational changes due to freezing-induced stress phenomena, such as cold denaturation, crystallization, phase separation, and ice-formation, resulting in the loss of biological activity of proteins. Therefore, commercialization of safe and efficacious therapeutic proteins poses significant challenges for the development and manufacturing groups.

A thorough understanding of the impact of freeze-thawing process parameters on the formulation composition of proteins facilitates to design robust and scalable formulations, which can withstand manufacturing and storage conditions. Numerous studies have been published on the cryopreservation of proteins, ranging from seminal contributions on mechanisms behind protein stabilization to process and scale-up optimization studies during freezing. This chapter presents an overview of freeze-thawing-induced stresses that bring microenvironmental changes in protein formulations, which impact the structure and stability of proteins. Further, sections of the chapter focus on process, formulation, scale-up, and primary packaging considerations during freezing-thawing of bulk protein solutions.

7.2 General Aspects

Proteins themselves do not represent an administrable dosage form. To constitute proteins in a stable dosage form, various excipients are added to impart protection to proteins and bulkiness to the dosage form. In many instances, aqueous phase is the primary medium for forming a homogeneous mixture of proteins and excipients. It also determines the dynamic and kinetic behavior of proteins and solutes in solution, frozen, and dry states. As a first step in becoming familiar with cryopreservation of proteins, it is advisable to consider the properties of proteins and physical behavior of water as a function of temperature.

7.2.1 Protein Structure and Stability

Proteins, made of sequence of amino acids, are characterized by a unique three-dimensional structure. The native structure of proteins is the result of a balance of interactions including covalent and non-covalent interactions (electrostatic interactions, hydrogen bonding, hydrophobicity, and van der Waals forces). Hydrogen bonding is important for formation of secondary structures, while electrostatic and hydrophobic interactions are needed for stabilizing tertiary structure of proteins. Both intramolecular and external environment interactions determine the stability of 3D-structure of proteins. For large multi-domain proteins, gentle conditions are sufficient to initiate protein unfolding, whose free energy is reported to be quite small (21–63 kJ/mole) (Mozhaev and Martinek 1990). Since the folded state of

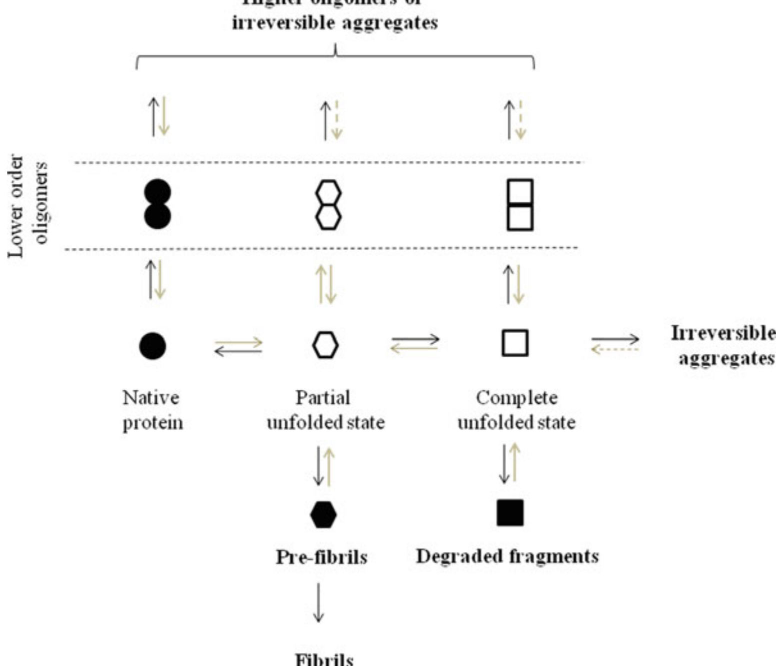

Fig. 7.1 The figure illustrates many conformational changes that proteins undergo in the presence of environmental conditions including excipients and temperature. During reversible equilibrium, *dark arrow* represents a thermodynamically favored equilibrium towards a particular state. *Dotted brown arrow* represents the least favored thermodynamic equilibrium. Modified and adapted from Dobson (2003)

protein is marginally stable than the unfolded state, changes in external conditions (e.g., temperature, pH, additives) trigger conformational changes (e.g., unfolding, aggregation, oligomerization, and fibrillation) (Fig. 7.1). Moreover, partially unfolded states are more susceptible to aggregation than the native or unfolded state, due to exposure of contiguous hydrophobic regions that are buried deep in the native state or inactivated in the denatured state.

Protein aggregation can be physical or chemical or both, resulting in the formation of high order oligomers (e.g., trimers, tetramers, or hexamers). Physical aggregation involves non-covalent interactions arising from the hydrophobic regions of the protein unfolded state, while chemical aggregation arises from covalent interactions, such as disulfide bond formation. Depending on the spacial- and time-scale of exposure to extreme conditions, aggregation phenomenon can be either reversible or irreversible. The high energy of activation of irreversible aggregation makes the process slow, but can have direct impact on manufacturing processes (e.g., sterile infiltration), drug potency, and immunogenicity. These aggregates (physical or chemical) can be either soluble or insoluble, and can form at the same

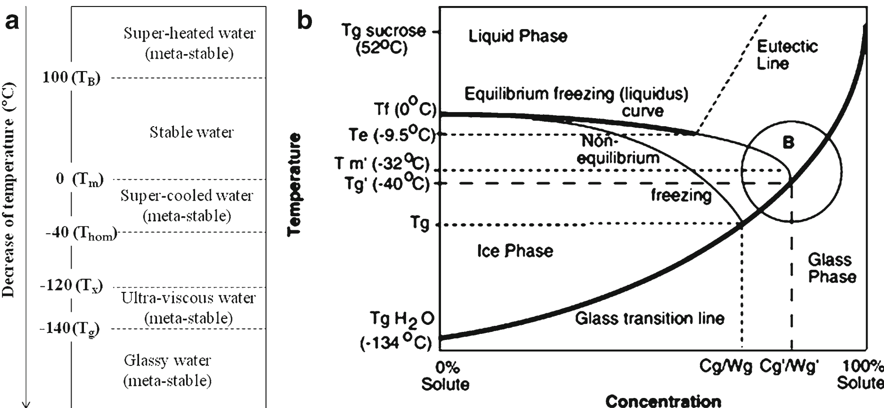

Fig. 7.2 (**a**) Hypothetical phase diagram of liquid water including super-heated and cryo-quenched states. Phase and state transition temperatures at atmospheric pressure are indicated: T_B, boiling point; T_m, melting point; T_{hom}, homogeneous nucleation; T_x, devitrification temperature; T_g, glass transition temperature. Modified and adapted from Mishima and Stanley (1998). (**b**) A schematic temperature–concentration state diagram for an aqueous carbohydrate solution, showing the glass transition curve, which extends from T_g (glass transition temperature) of pure water (−134 °C) to T_g of pure solute (52 °C), the equilibrium freezing (liquidus curve), which extends from T_m (melting temperature) of pure water (0 °C) to eutectic temperature (T_e) of the solute. The liquidus curve extends below T_e in a nonequilibrium state to intersect the glass transition line at T_g', which represents the glass transition temperature of the maximally freeze-concentrated solution. W_g' represents the amount of unfrozen water (100 % solute, C_g) entrapped in the glass. Point T_m' represents collapse temperature of the glass during warming. T_g and W_g represent a temperature–concentration relationship in a glass formed as a result of less than maximal ice-formation. Adapted from Goff et al. (2003)

time during processing and storage. Other degradation reactions that inactivate proteins include protein fragmentation, aspartate isomerization, oxidation, deamidation, and hydrolysis.

7.2.2 *Water: States of Matter*

Frozen storage and freeze-drying are used as preservation technologies for food and pharmaceuticals. The product shelf-lives can be closely correlated to water and solution transitions that occur during freezing and freeze-drying processes (Franks 2003). Figure 7.2a shows different phases and states of water as a function of temperature. The phase and state behavior of water becomes even more complex in the presence of solutes. This has led to the construction of state diagrams, combining conventional solid–liquid and solid–solid phase coexistence data with temperature-compositions relating glass transition profiles. Figure 7.2b illustrates a popular state diagram for the sucrose–water system.

Under equilibrium conditions, cooling of an unsaturated solution leads to eutectic point (T_e), where liquidus and solidus curves meet and anhydrous sucrose precipitates. This temperature is termed as eutectic temperature. At ordinary cooling rates, probability of sucrose nucleation at T_e or below is low. Further, continued cooling produces supersaturated solution undergoing vitrification (solidification of super-cooled solution without ice or solute crystallization) at glass transition temperature (T_g'; −32 °C). At this point, solution consists of 80 % w/w sucrose and represents amorphous, super-cooled, and high viscous system, referred to as maximum freeze-concentrated phase. In general, biologics are stored frozen below T_g' to avoid crystallization of solutes and associated pH changes which can cause protein denaturation.

However, the challenge of cryopreservation by vitrification is avoidance of ice-formation. Ice-formation consists of nucleation followed by growth. For nucleation to occur, molecules in the liquid water are subjected to small transient energy and density fluctuations during Brownian diffusion. Occasionally, these fluctuations lead to formation of clusters similar in dimensions to ice-crystal. The temperature at which crystallization is favored is approximately −40 °C in pure water, termed as homogeneous nucleation temperature (T_{hom}). The value of T_{hom} decreases by approximately 2 °C for every 1 °C decrease in T_m as solute concentration is increased (Mehl 1996). Below T_{hom}, solution is unstable against ice-formation because homogeneous nucleation occurs quickly. Between T_m and T_{hom}, solution is metastable against ice-formation. Ice can form, if nucleation is assisted by particles or surfaces that lower free energy barriers to nucleation. This is termed as heterogeneous nucleation, which can occur at higher temperatures (T_{het}) than homogeneous nucleation temperature. Heterogeneous nucleation is the predominant mechanism of ice-formation in biologics.

The solute concentration at which T_{hom} crosses T_g' is the lowest concentration at which it is possible to avoid homogeneous nucleation. The presence of solutes (e.g., cryoprotectants) in water depresses T_{hom}, and thereby inhibits homogeneous nucleation. The lower the solute concentration, the faster cooling must proceed to avoid ice-formation (Fahy and Rall 2007). The minimum cooling rate necessary to avoid significant ice-formation during cooling is the “critical cooling rate” of a cryoprotectant solution. The minimum warming rate to avoid significant ice-formation during warming from a super-cooled/vitrified state is the “critical warming rate.” Critical warming rates are typically 2–3 orders of magnitude greater than critical cooling rates. Ice-formation during warming is termed as “devitrification,” which happens faster than cooling because ice nucleation occurs at lower temperatures than ice growth. Nucleation at very low temperatures primes the solution for extensive ice growth at relatively warmer subzero temperatures. Nucleation rate increases by a factor of nearly 50 for each 1 °C lowering of temperature.

Nucleation and growth are kinetic processes, and therefore are determined by rate of cooling. Cooling rate determines the degree of cooling, but could not directly control. In the absence of nucleation, rapid cooling rates produce greater degree of cooling. Nucleation in a low temperature super-cooled solution will be rapid and leads to

the formation of more nuclei of small size. Post-nucleation, a number of factors influence the number, size, and shape of ice-crystals. Not all the crystal dimensions pose challenges to the stability of proteins. For example, dendritic ice-crystals produce uniform distribution of solutes and prevent cryoconcentration of solutes.

7.3 Freezing-Induced Stresses

During freeze-thawing, proteins undergo conformational stresses as a result of significant physical changes in their formulation composition. Low temperatures during freezing may induce partial unfolding of proteins, termed as cold denaturation (Privalov 1990). Rapid cooling rates may result in the formation of small ice-crystals, and thus relatively a large surface area of ice–liquid interface, which increases the propensity to denature proteins. With temperature decreases below the equilibrium freezing point of water, freezing-induced partitioning of solution into different thermodynamic phases (freeze-concentrated liquid phase and ice phase) occurs. As ice-crystallization proceeds to completion, further increase of solute concentration in the unfrozen liquid phase leads to changes in pH, ionic strength, osmolarity, and viscosity (Randolph 1997; Izutsu and Kojima 2000). This phenomenon is termed as cryoconcentration. Simultaneously, phase separation of crystalline and noncrystalline (amorphous) solutes may occur, which deprive proteins of the necessary protein–solute molecular interactions required for protein stabilization. Overall, these freezing-accompanied changes trigger protein denaturation (Soliman and Van den Berg 1971; Carpenter and Crowe 1988).

7.3.1 Cold Denaturation

Cold denaturation involves unfolding (i.e., loss of 3D-structure) of proteins at temperatures below 0 °C. It is a property of globular proteins well predicted by Gibbs–Helmholtz equation, and is believed to be driven by the hydration of polar and nonpolar groups as well as decrease of hydrophobic interactions. In fact, cold denaturation has been attributed to an increase in the solubility of hydrophobic residues in aqueous solutions at low temperature (Privalov and Gill 1988). The Gibb's free energy change during unfolding is a direct measure of the thermodynamic stability of the protein, which exhibits a parabolic profile of Gibb's free energy as a function of temperature. It has been shown that proteins denature at low (~ −20 °C) and high (~60 °C) temperatures. Unlike heat denaturation, cold denaturation appears to be fully reversible even at high protein concentrations (Franks and Hatley 1991; Hatley et al. 1987). If cold denaturation is reversible, proteins will refold when thawed, although the material would remain denatured during frozen storage. In contrast, if cold denaturation is irreversible, it would result in the formation of aggregates.

Although, the exact mechanism of cold denaturation is still not clear, hydration of hydrophobic nonpolar residues of proteins is largely believed to be a causative phenomenon (Privalov 1990; Dias et al. 2010; Lopez et al. 2008). As discussed earlier, the folded state or self-aggregated state (native state) of proteins can be imparted to hydrophobic interactions among nonpolar amino acids. The hydrophobic effects can be measured by the heat capacity of transfer from bulk liquid to surrounding water via *Kirchhoff equation*, $\Delta C_p = C_p^{water} - C_p^{bulk}$. For globular proteins, ΔC_p is positive, which is due to the hydration of nonpolar amino acids. Frank and Evans computations revealed that hydration of nonpolar solutes results in an increase of order of water molecules (shell-water) around the nonpolar solute (e.g. protein), i.e., increase in the number of hydrogen bonds between ordered water molecules (Franks and Evans 1945). In most of the orientations around protein, shell-water has at least one free (unsaturated) hydrogen bond towards the protein, which facilitates free-water molecules to infiltrate folded protein and passivate unsaturated hydrogen bonds of shell-water. At the same time, hydrophobic association between nonpolar solutes destabilizes 3D-structure of proteins (Dias et al. 2010).

Cold denaturation temperature may be dependent on solution pH, protein concentration, and presence of additives (Tang and Pikal 2005; Lazar et al. 2010). Protein formulations may suffer pH changes during freezing due to crystallization of buffer components. As a result, cold denaturation temperature might increase, causing protein cold denaturation at higher temperatures. On the other hand, Tang and Pikal reported a lower cold denaturation temperature in the presence of additives (e.g., sucrose and trehalose) and high protein (β-lactoglobulin) concentration in protein formulations. Therefore, cold denaturation temperatures of protein formulations must be evaluated before considering the storage temperature of biologics. It is believed that storage temperatures in the range −70 to −80 °C would provide a low risk towards cold denaturation, and subsequently cause a slow aggregation. While, storage temperature in the vicinity of −20 °C could present a risk of aggregation, since glass transition (T_g') and crystallization phenomena for many solutes occur at −20 °C, which could increase the probability of protein aggregation.

7.3.2 Ice–Liquid Interfacial Denaturation

A liquid is said to be super-cooled, if the temperature drops below the equilibrium freezing point (T_f) of water, and remains unfrozen. Unless seeded with crystalline ice, pure aqueous solutions can be super-cooled to −40 °C (Franks 1993a). Below −20 °C, the specific heat of aqueous solutions decreases rapidly leading to ice-crystallization with decreasing temperature. Factors, such as cooling rate, temperature, nucleation density, and heterogeneity of the substrate/solute, determine the dynamics of ice-formation, such as the number, size, and shape of ice-crystals. In general, faster cooling rates result in a high degree of super-cooling and high nucleation rates. Freezing produced from fast cooling results in the formation of a large

number of small ice-crystals, leading to large planar ice–liquid interfacial areas, which are considered to be detrimental to the stability of proteins.

Protein unfolding at ice–liquid interface has been demonstrated for a number of proteins including lactate dehydrogenase (LDH), IgG, and azurin. Schwegman et al. compared the infrared (IR) spectra of IgG and LDH in initial solution (prior to freezing), interstitial space (unfrozen liquid), and ice-crystal (Schwegman et al. 2009). IgG infrared spectral data from initial solution and interstitial space overlapped each other, indicating that the native state of IgG has been retained in interstitial space solution. In contrast, there are qualitative and quantitative differences between the spectra recorded through the ice-crystals relative to the spectra of solutions prior to freezing or in the interstitial space. An increase in the intensity of bands (1,668, 1,690, and 1,625 cm^{-1}), characteristic of intermolecular β-sheet structures (main component of aggregates) was observed. For LDH, IR spectra collected from ice-crystal showed a decrease in the intensity of bands characteristic of α-helix (1,654 cm^{-1}) and intramolecular β-sheet (1,638 cm^{-1}) structures, and an increase in the intensity of band corresponding to intermolecular β-sheet structure. The intermolecular β-sheet structural changes associated with ice-crystals of LDH and IgG indicate protein aggregation at the ice–liquid interface, while the changes in α-helix and intramolecular β-sheet structure reflect the loss of native protein structure. Addition of polysorbate 80 (protein stabilizer against surface-induced denaturation) to LDH prior to freezing resulted in the decrease of nonnative intermolecular β-sheet signal in the spectra of LDH protein adsorbed to ice interface, indicating that ice–liquid interface contributes to denaturation of proteins.

As discussed earlier, nucleation, cooling rate, and temperature influence the nature of ice-formation. In a controlled ice-formation study via seeding, catalase enzyme was frozen after prior equilibration at −2 or −10 °C by seeding with ice (Fishbein and Winkert 1977). Lower ice nucleation temperature (−10 °C) resulted in greater denaturation (60 % loss) than that observed at higher nucleation temperature (−1 °C/25 % loss). For samples frozen by seeding at −2 °C and cooled to −25 °C at different cooling rates, the extent of damage was greater for samples subjected to faster cooling post-freezing. Irrespective of the approach used to produce freezing (seeded or non-seeded) in protein solutions, a greater degree of super-cooling results in irreversible protein degradation (Cao et al. 2003). Jiang and Nail investigated the effect of different freezing methods on the activity of LDH using liquid nitrogen-induced freezing, precooling, and ramp cooling of shelves (Jiang and Nail 1998). Higher degree of super-cooling and low stability was observed with liquid nitrogen-induced freezing; whereas low super-cooling and high stability were observed with precooled shelf method; and ramp cooling method resulted in intermediate stability values. The authors hypothesized that even though ramp method is the slowest freezing method, it resulted in a greater degree of super-cooling and a better thermal equilibration than precooled shelf method. When ice nucleation occurred, the freezing rate in ramped samples was faster than the rate in samples placed on precooled shelf. It is therefore important to separate effects of cooling rate from those of freezing rate in the study of freeze–thaw stability of proteins.

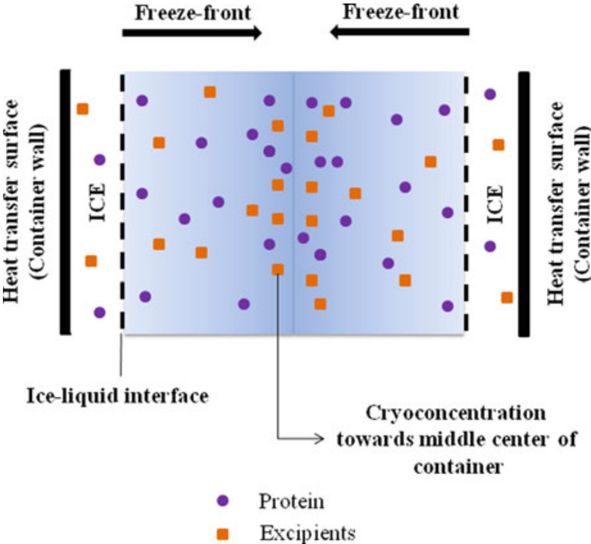

Fig. 7.3 Representation of cryoconcentration of solutes (proteins and excipients). Freezing-front moves from the storage container wall surface (heat transfer surface) towards the center of the container. Slow freezing rates cause solute exclusion from ice-liquid interface, and result in solute cryoconcentration towards the middle and center of cryovessels (Kolhe and Badkar 2011)

7.3.3 Cryo (Freeze)-Concentration

Cryoconcentration involves partitioning of solution into ice phase and freeze-concentrated liquid phase (unfrozen liquid containing proteins and excipients) of solutes. As freezing continues, growing ice-front preferentially takes up water molecules from the ice–liquid interface and excludes other solutes due to crystallographic dissimilarities. As shown in Fig. 7.3, exclusion of solutes by the moving ice-front leads to an increase in concentration of solutes in the residual unfrozen water (Franks 1993a; Schneider et al. 1973). The excluded solute, which is concentrated near the ice–liquid interface, is moved away from ice phase by diffusion and convection (Butler 2002a). Due to segregation of ice phase, an increase in the concentration of solutes, such as salts and buffers in the unfrozen liquid phase leads to changes in pH, ionic strength, osmolarity, and viscosity. Together, these physicochemical changes could cause protein denaturation.

Inside the freeze-concentrated liquid phase, solution viscosity increases with increasing solute concentration. When ice-crystallization is complete, the freeze-concentrated liquid phase reaches a maximum in its concentration. Now, the viscosity of freeze-concentrate increases several orders of magnitude than that of the initial solution over a narrow temperature interval, during which the physical state of freeze-concentrate changes from unfrozen liquid state through visco-elastic rubbery state to “glass-like” solid state. The temperature at which the freeze-concentrate liquid phase undergoes this liquid/solid transition is termed as glass transition temperature (T_g') (Carpenter and Crowe 1988; Franks 1993b).

Formation of "glassy" homogeneous protein–solute matrix is dependent on the formulation composition and cooling rate. At slow cooling rates, crystalline solute with least solubility in unfrozen solution may first crystallize out at a temperature, referred as crystalline temperature (T_c). A further decrease of solution temperature may crystallize together the least soluble solute and ice as a mixture. This temperature is referred to as eutectic crystallization temperature. Crystalline and eutectic crystallization temperatures occur in between melting (T_m) and T_g' of frozen matrices. As a result of crystallization, the freeze-concentrated liquid solute phase may further evolve into eutectic and/or amorphous phases, reflecting the separation of crystalline and noncrystalline solute phases, respectively. As described above, noncrystalline solutes transform into a glassy state with decrease of temperature. Both crystallization and phase separation of excipients from the homogeneous mixture deprive the proteins of their stabilizing effects.

7.3.3.1 Crystallization of Solutes

Crystallization phenomenon excludes protective excipients from the vicinity of proteins and imposes stress on the stability of proteins. Particularly, crystallization of buffer salts causes large shifts (3–4 pH units) in the pH of frozen phosphate and carboxylate buffer systems (Pikal-Cleland and Rodriguez-Hornedo 2000; Van den Berg and Rose 1959; Lam and Constantino 1996). The pH changes during freezing are a function of buffer salt, concentration, and freezing protocol (ice seeding vs. non-seeding). In sodium phosphate buffer, the pH changes could be due to solubility differences of the mono- and disodium salts, whose eutectic temperatures are −9.5 °C and −0.5 °C, respectively. Therefore, disodium salt is less soluble than the monobasic salt, leading to its precipitation during freezing. Exclusion (crystallization) of disodium salt from the solution alters the ratio of basic to acidic salt species in the buffer solution causing a pH decrease. At high buffer concentration, the pH changes of the frozen solutions are even more dramatic due to crystallization of large amount of salts, which hampers buffering capacity. Also, the presence of salts, such as sodium chloride in sodium phosphate buffer increases the ionic product of the dibasic salt of sodium phosphate buffer, and thus exacerbates pH shifts.

Polyols are used as stabilizers in protein formulations. Sorbitol, a polyol, is an effective protein stabilizer in formulations, Neulasta® (pegfilgrastim) and Neupogen (filgrastim) in liquid state. In frozen state, sorbitol can exist as an amorphous solute (T_g' is −44 °C) (Levine and Slade 1988). Piedmonte et al. reported that aggregation of Fc-fusion protein occurred in sorbitol containing formulations stored at −30 °C. Aggregation of Fc-fusion protein was attributed to the crystallization of sorbitol in frozen solutions stored above its T_g'. Due to depletion of sorbitol from the vicinity of protein, stabilizing interactions between the amorphous sorbitol and protein were removed, leading to protein aggregation. Unlike mannitol, sorbitol crystallizes at a slower rate, which should be considered during freezing of formulations and process intermediates (Piedmonte et al. 2006). In addition, these protein stabilizers (e.g., mannitol, sorbitol, and trehalose) undergo polymorphic transformations, which may exhibit different degrees of stabilization effects during freezing and varied solubilities upon thawing.

7.3.3.2 Phase Separation

Solutes in a freeze-concentrated unfrozen liquid phase are either miscible or immiscible (Izutsu et al. 1996). During freezing, certain combination of solutes with steric hindrance or repulsive interactions separates into eutectic and/or amorphous phases. The phase separating polymer excipients may stabilize or destabilize proteins. Phase separating excipients may protect the multimeric proteins (e.g., LDH) by stabilizing subunit interactions. The repulsive interactions between protein and polymer co-solutes shift the equilibrium between the subunit association (e.g., monomers, dimers, and tetramers) towards a stable multimeric protein. In contrast, phase separating excipients can deprive proteins of their stabilizing interactions. It is not uncommon that a solution composed of two polymers with different miscibilities separates into more than one amorphous phase. And preferential partition of protein into one of the phases may deprive protein of the protective effects of the other stabilizers.

Relative contribution of each individual stress, i.e., cold temperature, cryoconcentration, and ice-formation, on the overall freezing-induced denaturation of proteins is still unknown. From the literature evidence on freeze-thawing-induced stresses, it can be inferred that the contribution of cold denaturation to the overall protein denaturation can be considered negligible, which implies that ice-formation- and cryoconcentration-associated changes are the potential contributing factors to the destabilization of proteins.

7.4 Cryopreservation

Proteins undergo physical and chemical degradation (e.g., unfolding, aggregation, and insoluble particulate formation) on exposure to a multitude of stresses during manufacturing operations, which can negatively impact both efficacy and safety of the therapeutic products (Rosenberg 2006). A combination of formulation (e.g., pH, ionic strength, excipients, and protein concentration) and process parameters (e.g., freezing and thawing rates, temperatures) determines the robustness of bulk frozen protein formulations and thus the shelf-life. Kueltzo et al. illustrated interrelation of various parameters on protein stability during freeze-thawing (Kueltzo et al. 2008). Studies showed that aggregation was prevalent at pH 3 and 4, involving the formation of aggregation-prone conformational states. Aggregation at pH 3 has been attributed to acid-induced aggregation, while aggregation at pH 4 could be due to adsorption to ice–liquid interface and container surface. Process conditions, such as cooling and thawing rates influenced the recovery of monomer levels. In addition, containers made of different material and geometry caused a variation in monomer and aggregate levels of proteins.

Only if the frozen bulk protein formulation has acceptable stability, formulation can further be subjected to thawing, sterile filtration, and fill and finish operations before administration to patients. It is therefore essential to define product

characteristics that assure a stable protein formulation. Freeze-thaw stability characteristics of proteins may drive formulation and process development operations to achieve target product profile. Often, the process of identifying critical product characteristics coincides with development studies that could be performed as part of the freeze–thaw process studies. These studies not only address freeze–thaw unit-operations, but also provide suitable guidance to the design of a stable protein formulation.

7.4.1 Formulation Composition

Often, excipients are used to suppress protein degradation, if not enhance the stability of proteins (Akers et al. 2002). In relevance to the cryopreservation of proteins during freeze-thawing, excipients used will be either the same or a subset of those of the final drug products; however, their concentrations may differ. As shown in Table 7.1, various categories of excipients used to stabilize proteins include small molecular weight ions (e.g., salts, buffers), intermediate-sized solutes (e.g., amino acids, sugars), and larger molecular weight compounds (e.g., polymers, proteins). Excipients that require water for protein stabilization may operate under the same mechanism (preferential interaction hypotheses), as water is still present during freezing. However, freezing-induced stresses, such as freeze-concentration, ice-formation, excipient crystallization, and pH changes may alter the ability of protein stabilizing excipients. Both weak and strong interactions contribute to the overall interaction between excipients and proteins. These interactions can be classified into two groups based on the presence of excipients or water in the vicinity of protein surface. As illustrated in Fig. 7.4, the presence of high concentration excipients in the vicinity of proteins results in "preferential interactions," while the presence of water around protein surface results in "preferential hydration or exclusion" indicating the absence of excipients in the vicinity of protein. Many sugars, polyols, polymers, and certain salts, which stabilize proteins, decrease their solubility and are preferentially excluded from the vicinity of protein (Carpenter and Crowe 1988; Arakawa and Timasheff 1982a, b).

Preferential exclusion of solutes from protein surface results in an unfavorable thermodynamic condition, which increases the free energy of native state of the protein. Even a greater exclusion of solutes is expected for the unfolded structure due to larger surface area than the folded/native state. In the presence of solutes, unfavorable interaction, and thus free energy, would increase even more so for the unfolded state, which leads to a greater energy difference between native and unfolded structures, i.e., more energy is required to unfold proteins in the presence of preferentially excluded solutes. It can be understood that high solute concentration stabilizes the native state of proteins through preferential solute exclusion phenomenon.

Solutes (e.g., saccharides) have been postulated to protect proteins through other mechanisms, but two have been able to describe many observations, water replacement hypothesis and vitrification. For the former mechanism, direct interaction is a

Table 7.1 Pharmaceutical excipients for use in frozen bulk protein formulations

Category	Representative examples	Stabilization mechanisms	Factors affecting stabilization	References
Buffering agents	Phosphate, histidine citrate, acetate, Tris	Maintains solution pH; Buffer ion-protein-specific interaction	pH may change with temperature; Crystallization during freezing; Decomposition during frozen storage	Akers et al. (2002), Chang and Randall (1992), Stoll and Blanchard (1990), Gomez et al. (2001), Pikal-Cleland et al. (2002), Kolhe et al. (2010), and Sundaramurthi and Suryanarayanan (2011)
Sugars, polyols	Sucrose, trehalose, lactose, mannitol, sorbitol	Preferential exclusion; Hydrogen bond formation Glassy-state formation; Tonicity agents	Requires high concentration; Crystallization during freezing	Carpenter and Crowe (1988, 1989), Reategui and Aksan (2009), Clegg et al. (1982), Crowe (1971), Crowe et al. (1993), Dong et al. (2009), Nema and Avis (1993), Carpenter et al. (1993), and Levine and Slade (1992)
Amino acids	Alanine, glycine, lysine, proline, 4-hydroxyproline	Preferential exclusion; Buffering capacity; Inhibition of crystallization	Concentration-dependent crystallization	Fishbein and Winkert (1977), Pikal-Cleland et al. (2002), and Arakawa et al. (2007)
Salts	NaCl, KCl, $MgSO_4$, Na_2SO_4, $CaCl_2$	Tonicity agents; Stabilizing or destabilizing effects on proteins, especially anions	Nonspecific interactions with proteins; Metal impurities cause oxidation; Lowers T_g' which may affect bulk solution frozen storage temperature; Corrosive to metal surfaces	Carpenter and Crowe (1988), Arakawa and Timasheff (1982a, 1984), Kauzmann (1959), Strambini and Gallieneri (1996), and Chen and Cui (2006)
Surfactants	Polysorbate 20 and 80	Competitive inhibitors of protein adsorption; Concentration-dependent inhibition	Trace metal and peroxide impurities; Peroxide impurities trigger oxidation; May degrade during storage	Nema and Avis (1993), Philo and Arakawa (2009), Bam et al. (1995), Chang et al. (1996), Randolph and Jones (2002), and Hillgren et al. (2002)
Polymers	PVP, PEG, HSA	Amorphous phase formation; Competitive inhibitors of protein adsorption	Phase separation during freezing; dependent on cooling/freezing rates	Crowe et al. (1993), Minton (2005), Arakawa and Timasheff (1985a, b), and Pikal et al. (1991)
Antioxidants and chelators	EDTA, DTPA, methionine, and histidine	Bind metal ions; Free-radical scavengers	Light exposure accelerates oxidation; Nonspecific interactions with proteins	Hovorka and Schoneich (2001), Abernethy et al. (2010), Wasylaschuk et al. (2007), Valliere-Douglass et al. (2010), Qi et al. (2009), and Wang (1999)

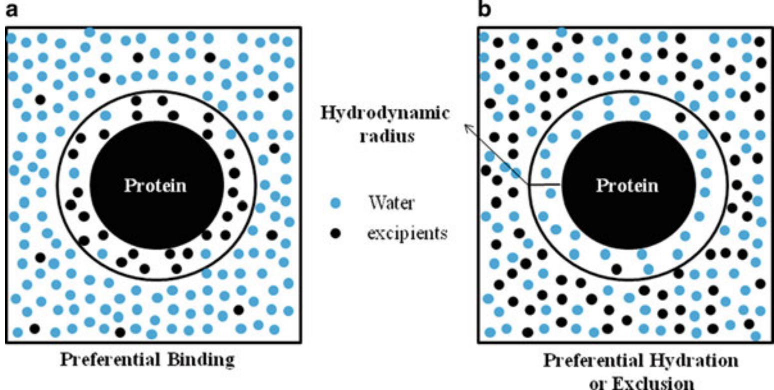

Fig. 7.4 Schematic representation of (**a**) preferential binding and (**b**) preferential hydration (preferential exclusion) in a solution. The protein is represented by the *dark circle*. Hydrodynamic radius represents the area excluding the solutes. Modified and adapted from Ohtake et al. (2011)

prerequisite (Carpenter and Crowe 1988, 1989), while formation of an amorphous glass is required to impart protection through retarding molecular motion and providing physical separation between proteins (inhibition of aggregation) for vitrification (Franks 1993a). In solution and frozen states, hydrogen bonding and hydrophobic interaction between nonpolar residues of the proteins are major forces responsible for maintenance of native state of proteins (Kauzmann 1959). Carpenter and Crowe concluded that intermolecular carbohydrate hydrogen bonding is decreased in the presence of sugar and that dried proteins form hydrogen bonds with carbohydrate (Carpenter and Crowe 1988). Also, the spectra (vibrational spectroscopy) of hydrated protein were strikingly similar to dried protein in the presence of carbohydrate, indicating the presence of hydrogen bonding. The degree of structural protection conferred by saccharides, such as sucrose and trehalose has been correlated with the extent of hydrogen bonding between sugar and protein (Allison et al. 1999). Hydrogen bonding, though necessary, is insufficient to confer stability during freezing and freeze-drying (Allison et al. 1999; Chang and Randall 1992). Along with direct interaction, existence of proteins and excipients in the same amorphous phase and stable pH are other major factors responsible for protein stabilization in solution and solid states.

7.4.1.1 Buffers

The physical stability and chemical stability of proteins are highly dependent on solution pH, and therefore buffers are used to control pH and impart stability to proteins. Common buffers used in the pH range 3–10 include acetate, citrate, histidine, phosphate, Tris, and glycine (Akers et al. 2002; Stoll and Blanchard 1990). At a given pH, different buffer ions can have specific ion effects on the chemical and

conformational stability of proteins, which drive the buffer selection. Certainly, freeze-concentration-induced pH changes can impact protein degradation via crystallization of buffer salts. Chang and Randall have classified salts into three types based on their amorphous glass-forming tendency at a given cooling rate (Chang and Randall 1992): (1) crystallizing salts, (2) partially crystallizing salts, and (3) glass-forming salts. Buffer salts that exhibit a lower crystallization potential and a high collapse temperature (e.g., citrate, malate) are preferred over buffers with a high crystallization tendency (e.g., succinate) and a low collapse temperature. However, if a crystallizable buffer has to be included in the formulation, one can suppress buffer crystallization by decreasing the amount of buffer and including amorphous bulking agents or crystallization inhibitors.

Numerous studies examined the crystallization of carboxylate and phosphate buffers in frozen state (Gomez et al. 2001; Pikal-Cleland et al. 2002; Kolhe et al. 2010; Sundaramurthi and Suryanarayanan 2011). Sundaramurthi et al. studied the freezing-induced crystallization and associated pH changes in carboxylate buffers (Sundaramurthi and Suryanarayanan 2011). Based on their crystallization potential in frozen solutions (−25 °C), carboxylate buffers were rank-ordered as succinate > tartarate > citrate > malate. Malate buffer showed no evidence of crystallization and hence negligible pH shifts. As illustrated in Fig. 7.5a–c, succinate buffer exhibited pH changes as 4.0–6.1 and then to 8.0, due to sequential crystallization of succinic acid and monosodium succinate, respectively. Pikal-Cleland illustrated the crystallization potential of phosphate buffer in the presence of glycine, a crystallization inhibitor (Pikal-Cleland et al. 2002). Glycine suppressed pH changes via inhibition of buffer crystallization, and stabilized multimeric proteins against freezing-induced denaturation.

7.4.1.2 Sugars/Polyols

Saccharides are commonly used as protein stabilizers during freeze-thawing and freeze-drying (Carpenter and Crowe 1988). Sugars/polyols typically prevent protein unfolding via preferential exclusion (Clegg 1982), reduced mobility (Reategui and Aksan 2009), and water replacement mechanism (Clegg et al. 1982; Crowe 1971; Crowe et al. 1993). Phase separation and crystallization of sugars have been implicated in the loss of specific interactions (e.g., H-bonding) between proteins and sugars, leading to the loss of biological activity of proteins. As illustrated in Fig. 7.6a, b, Dong et al. used lysozyme (LYS) solutions with varying amounts of trehalose (TRE) to quantify and characterize freezing-induced micro-heterogeneity and phase separation in the ice phase (Dong et al. 2009). At low TRE/LYS ratio, LYS and trehalose are relatively homogenous in the freeze-concentrated liquid phase. A high hydration level of trehalose combined with low LYS/TRE ratio in the freeze-concentrated liquid phase could be reasoned to three possibilities: (a) preferential interaction of TRE with the remaining unfrozen water molecules (which conforms to preferential exclusion hypothesis), (b) increase in TRE–TRE intermolecular interactions, and/or (c) preferential binding of TRE and LYS (which is in accord

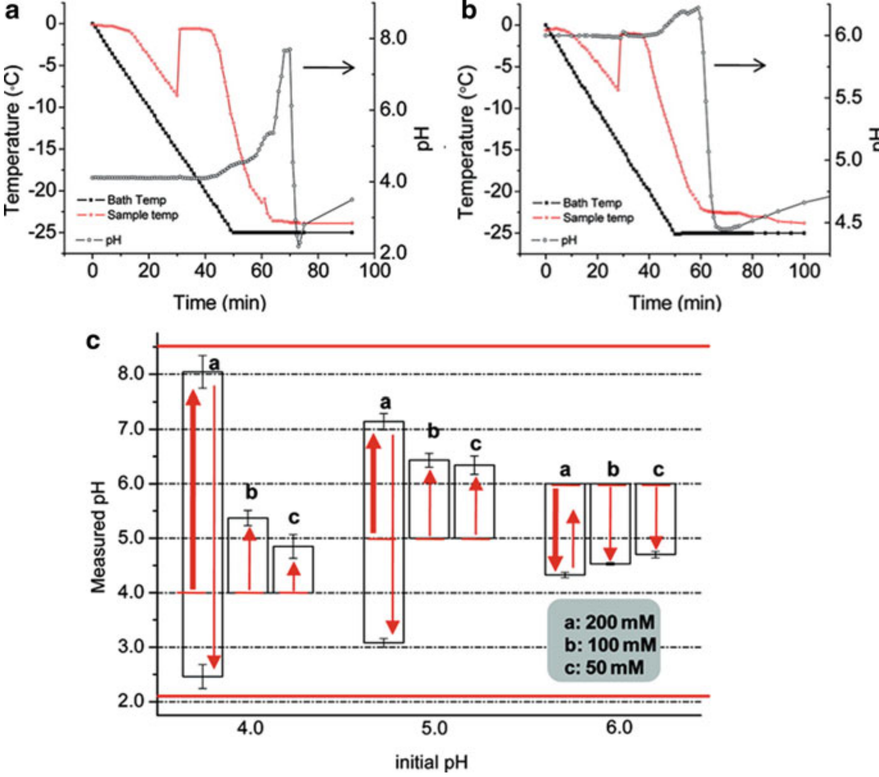

Fig. 7.5 (**a**, **b**) Graphs represent low temperature measurement of succinate buffer solution during cooling followed by isothermal hold at −25 °C. The solutions had been buffered to pH values of 4.0 (**a**) and 6.0 (**b**) at room temperature. Low temperature caused a dramatic pH shift in both the buffered solutions, more predominantly in the solution buffered to pH 4.0. (**c**) Graph represents the magnitude and direction of pH shift observed in the frozen buffer solutions. All the three concentrations (200, 100, and 50 mM) were initially buffered to pH 4, 5, and 6 at room temperature. The *solid horizontal lines* at the *bottom* and *top* represent, respectively, the pH of 200 mM succinic acid and disodium succinate solution at room temperature. At higher buffer concentration, succinate buffer solution showed a large pH swing, due to crystallization of disodium succinate component of the succinate buffer

with the water replacement hypothesis). Trehalose has been shown to interact with trehalose in the freeze-concentrated liquid preserving a high degree of native structure. In contrast, LYS/TRE ratio was 2–4 times higher in ice phase indicating a preferential rejection of TRE than LYS during freezing. Also, an increase in LYS intermolecular β-sheet structures was confirmed during freezing, which has been attributed to the loss of native structure in the ice phase.

Protein stabilization effects of sugars have been shown to be dependent on their concentration (Nema and Avis 1993). Low concentrations of sugars or polyols may

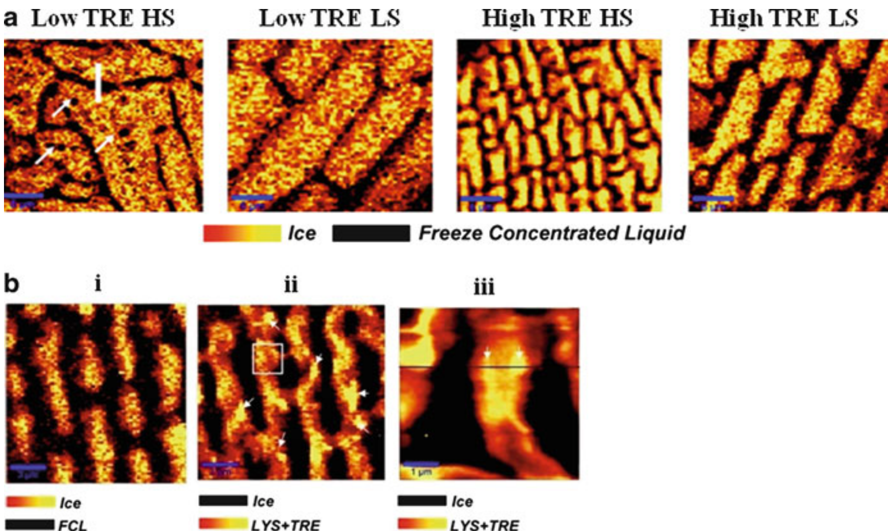

Fig. 7.6 (**a**) Confocal Raman microscopy (CRM) images and line scan analyses of frozen TRE+LYS in 1× PBS solution. Column low TRE HS, 20 mg/mL LYS+100 mg/mL solution; column low TRE LS, 20 mg/mL LYS+100 mg/mL solution; column high TRE HS, 20 mg/mL LYS+300 mg/mL solution; column high TRE LS, 20 mg/mL LYS+300 mg/mL solution. *Note*: *HS* high degree of super-cooling, *LS* low degree of super-cooling. Figures adapted from Dong et al. (2009). (**b**) (i and ii) CRM images of frozen 20 mg/mL LYS+300 mg/mL TRE solution (HS). *Arrows* in (**b**) (ii and iii) show LYS aggregation and possibly TRE crystallization in the freeze-concentrated liquid (FCL) phase. Figures adapted from Dong et al. (2009)

not afford protection to proteins during freeze-thawing or freeze-drying (Carpenter et al. 1993). A minimum sugar concentration (equivalent to form a monolayer on protein surface) is required for direct interaction with exposed polar residues of protein to exert maximum protein stabilization effects during freeze-drying. Tanaka et al. determined that maximum stabilization of catalase occurs at maltose/catalase weight ratio of 0.4 (Tanaka et al. 1991). A further increase of sugar/protein weight ratio to a certain level may reach limits of stabilization or even destabilize protein during freeze-drying. For example, an increase of trehalose concentration from 50 to 150 mg/mL resulted in the increase of freeze-dried recovery of phosphofructokinase (PFK) activity. A further increase of trehalose concentration to 400 mg/mL resulted in the loss of protein activity (Carpenter and Crowe 1989). The loss of PFK activity at high trehalose concentration has been implied to trehalose crystallization, which deprives the required hydrogen bonding to the dried protein.

The level of protection afforded by different sugars can be similar or different, depending on the formulation composition, compatibility with proteins, stabilizer concentration, and freezing rate. Ward et al. showed several sugars including trehalose, sucrose, mannitol, lactose, and maltose exhibited similar level of protection

towards tetrameric L-asparaginase during freeze-drying (Ward et al. 1999). Among polyols, disaccharides (e.g., sucrose and trehalose) have been shown to be effective stabilizers (Arakawa et al. 1993). Trehalose seems to be a preferred protein stabilizer over sucrose due to its higher glass transition temperature (Crowe et al. 1992) and the absence of internal hydrogen bonds, which allows formation of hydrogen bonds with proteins. However, Levine and Slade contended that sucrose and trehalose could be equally effective in protecting proteins during freeze-drying and are rather protein-specific (Levine and Slade 1992).

7.4.1.3 Amino Acids

Amino acids, such as histidine, glycine, and arginine can stabilize proteins by a variety of mechanisms including preferential hydration and direct binding as well as buffering capacity or antioxidant properties (Arakawa et al. 2007). A few amino acids could act as cryoprotectants, for example, freezing PFK in liquid nitrogen caused denaturation, but the inclusion of amino acids, such as glycine, proline, or 4-hydroxyproline recovered protein activity (Carpenter et al. 1986). LDH, a native tetramer, and recombinant human interferon-γ (rhIFN-γ), a multimer, are freeze-labile proteins, and in part undergo denaturation due to pH changes. Freezing-induced acidification of sodium phosphate buffer dissociated LDH and rhIFN-γ to monomers, which are prone to aggregation (Pikal-Cleland et al. 2002). At low concentrations (≤50 mM), glycine suppressed the pH decrease observed during the freezing of sodium phosphate buffer and sodium succinate buffer in LDH and rhIFN-γ formulations, respectively. Inhibition of pH changes has been ascribed to the decrease of buffer salt crystallization. At low concentrations, preferential exclusion of glycine in its amorphous state and inhibition of buffer crystallization might have stabilized the native protein structure against freezing-induced denaturation. On the other hand, high concentrations (>100 mM) of glycine showed a greater degree of crystallization during freezing, leading to protein denaturation.

7.4.1.4 Surfactants

Protein aggregation occurs through several different mechanisms (Philo and Arakawa 2009). Surface-induced aggregation is a common form of aggregation, in which native proteins first adsorb to an interface, after which they undergo conformational changes or partial unfolding (Fig. 7.7a). The resulting nonnative or denatured states then serve as a starting point for aggregation at the interface or in solution or on the surface. Protein binding at the ice–liquid and air–liquid interface has been attributed to hydrophobic interactions, while solid–liquid interface (e.g., vial surface–liquid) often contributes to electrostatic interactions. Preferential location of surfactants at solid–liquid interface (e.g., ice–liquid or vial surface–liquid) and air–liquid interface during freezing and thawing processes may inhibit the adsorption of proteins and prevent their subsequent denaturation.

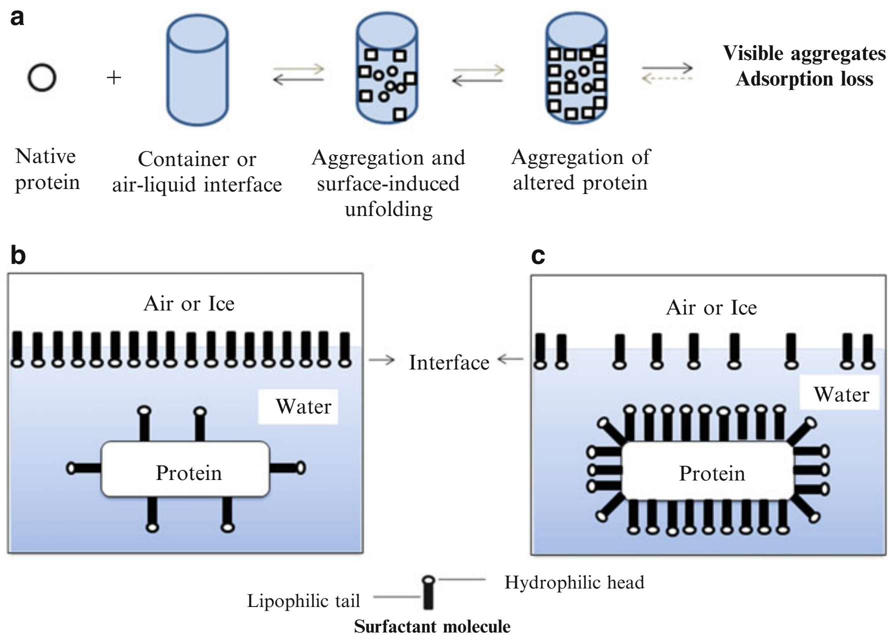

Fig. 7.7 (**a**) Schematic representation of surface-induced aggregation, with specific reference to containers. Figures redrawn from Philo and Arakawa (2009). (**b**) Mechanisms of protein stabilization by surfactants, which may (**a**) adsorb to the interface and thereby exclude proteins from the interface, and/or (**c**) form complex with proteins and thus prevent aggregation. Interface may be air or ice, which represents potential surface site for protein aggregation. Figures redrawn from Lee et al. (2011)

Surfactants are amphipathic molecules, which tend to adsorb or associate with surfaces and interfaces. As illustrated in Fig. 7.7b, c, surfactants stabilize proteins by two major mechanisms during freeze-thawing: (a) by preferentially locating at an interface and precluding protein adsorption, and/or (b) by associating with proteins in solution. Some surfactants may follow one of these mechanisms, while others may adopt both the mechanisms. Protein stabilization by surfactant adsorption at the interfaces has three possible outcomes: complete hindrance, reduced amounts, or increased amounts of protein adsorption. Complete hindrance is attributed to the faster diffusion of surfactant molecules to the interface, as compared to the much larger protein molecules. The adsorbed layer coats the interface and sterically prevents protein adsorption or aggregation. In this scenario, any adsorbed protein may be displaced by surfactant on account of a stronger surfactant–surfactant association. Reduced or increased amounts of adsorption are attributed to the formation of surfactant–protein complexes with reduced or increased surface affinity, respectively. Protein stabilization by association of surfactants requires strong surfactant–protein interaction, and would be effective in reducing protein adsorption, independent of the strength of surfactant–surface binding.

Among various classes of surfactants, nonionic surfactants exhibit low toxicity and low sensitivity to electrolyte species. Polysorbates are the commonly used

nonionic surfactants in protein formulations (Daugherty and Mrsny 2006). Polysorbate 20 (polyoxyethylene sorbitan monolaurate) and polysorbate 80 (polyoxyethylene sorbitan monooleate) are the available commercial grades. Low concentration of nonionic surfactants is often sufficient to prevent protein denaturation (Bam et al. 1995). At 0.01 % w/v concentration, polysorbate 80 protected LDH from denaturation during freeze-thawing (Chang et al. 1996). Other surfactants reported to protect proteins from surface-induced denaturation include Brij 30, Brij 35, and pluronic F127 (Nema and Avis 1993; Chang et al. 1996).

As the surfactant concentration increases further, interface becomes saturated and excess surfactant concentration nearing the critical micelle concentration (CMC) form micelles. However, CMC does not completely describe the surfactant effect on proteins. If the surfactant has high affinity for a surface, then surfactant concentration near the CMC tend to stabilize protein against surface-induced denaturation. In contrast, if the surfactant stabilizes proteins by directly binding to them, the effective surfactant concentration is related to the ratio of surfactant to protein rather than CMC (Randolph and Jones 2002). It has been demonstrated that polysorbate 80 concentrations less than CMC are large enough to cover ice surface to afford complete LDH protection from denaturation (Hillgren et al. 2002).

7.4.1.5 Protein Concentration

At high concentrations, proteins are often more resistant against freezing-induced protein denaturation. Allison et al. correlated the activity of freeze-labile proteins post-freeze-thawing with initial protein concentration (Allison et al. 1996). For example, increasing initial concentration of rhFXIII (recombinant hemophilic factor) from 1 to 10 mg/mL increased the recovery of native rhFXIII during repeated freeze-thawing (Kreilgaard et al. 1998). About 90 % LDH activity was recovered when the concentration was increased to 500 mg/mL (Anchordoquy and Carpenter 1996). An increase in the concentration of bovine and human IgG species markedly decreased lyophilization-induced protein aggregation (Sarciaux et al. 1998). However, some proteins do not show concentration-dependent protection. For example, in the absence of a stabilizer, the percentage of lyophilization-induced denaturation of catalase (65 %) was reported to be independent of protein concentration in the range, 1–5,000 mg/mL (Tanaka et al. 1991).

The mechanism of proteins' self-stabilization during freezing/freeze-drying is still not clear. Two hypotheses have been reiterated to explain the concentration-dependent protein stabilization upon freezing (Allison et al. 1996). First, unfolding of proteins at high concentrations during freezing may be temporarily inhibited by steric repulsion (preferential exclusion) of neighboring protein molecules. Second, the surface area of ice–liquid interface formed upon freezing is finite, which limits the amount of protein to be accumulated and denatured at the interface. In addition, favorable protein–protein interactions (formation of dimers or multimers) may contribute to the increased protein stability at high concentrations (Mozhaev and Martinek 1984).

7.4.1.6 Salts

Salts are included in protein formulations to impart tonicity as well as protect proteins during freeze-thawing, freeze-drying, and storage. Hydrophobic interactions are major forces responsible for the folded state/native state of globular proteins (Kauzmann 1959). Salts strengthen these hydrophobic associations through preferential interaction with proteins, which is a balance between salt-binding and salt-exclusion phenomena. Arakawa and Timasheff hypothesized that salts stabilize proteins in aqueous solutions by preferential exclusion from the surface of proteins (Arakawa and Timasheff 1982a). Carpenter and Crowe reported that preferential hydration of proteins may stabilize proteins not only in aqueous solution but also during freeze-thawing (Carpenter and Crowe 1988; Arakawa and Timasheff 1984). In accordance with preferential exclusion hypothesis, salting-out salts would stabilize proteins by preferential exclusion from protein surface during freezing and thawing (Carpenter and Crowe 1988).

The stabilizing effects of salts depend on the nature of ions and follow Hofmeister series, which relate the effectiveness of ions to their solubility in solutions. The anions in this series have been shown to have a more dramatic effect on protein stability ranging from kosmotropes (salting-out salts), such as sulfate ions (prevent protein unfolding and reduce protein solubility) to chaotropes (salting-in salts), such as thiocyanate ions (induce protein unfolding and increase protein solubility), which show preferential binding to proteins. Strambini and Gallieneri illustrated the stabilizing effect of anions on azurin in frozen state (Strambini and Gallieneri 1996). In the presence of anions, the frozen state stability of azurin follows the Hofmeister series in the order: sulfate > citrate > acetate > chloride > thiocyanate, with sulfate, citrate, and acetate stabilizing the protein relative to chloride. The authors further hypothesize that azurin partially unfolds at the ice interface and that binding of citrate and acetate to the ice interface may inhibit protein adsorption and denaturation. Chen and Cui demonstrated that $MgSO_4$, NaCl, and Na_2SO_4 can stabilize LDH in freeze-thawing studies (Chen and Cui 2006). Chloride anions, the most commonly used ion in protein formulations in the form of NaCl, are ranked in the middle of the Hofmeister series, i.e., in the borderline between salting-out and salting-in salts (Strambini and Gallieneri 1996; Chen and Cui 2006). From the Hofmeister series, the protective effects of cations are in the order, $Mg^{2+} > Na^{+} \sim K^{+}$ ions. Hofmeister series can be used as a general guide to determine the protective effects of ions against freezing-induced damage to protein.

7.4.1.7 Polymers

Polymers have been evaluated as bulking agents and stabilizers in protein formulations. Some of the polymer examples include poly(ethylene) glycols (PEGs), polysaccharides, and proteins. Polymers stabilize proteins by a multitude of mechanisms, which are dependent on protein, polymer, concentration, and states of matter (Minton 2005; Arakawa and Timasheff 1985a). During freezing, bovine serum albumin (BSA) (Dawson 1992) and poly(vinyl) pyrrolidone (PVP) (Gombotz et al. 1994) polymers

prevented LDH subunit dissociation via Timasheff's preferential exclusion mechanism (Arakawa and Timasheff 1985b). Not only the inhibition of LDH subunit disassembly, but also the polymers' subsequent immobilization of protein in its high viscous glassy matrix accounts for the preserved activity of the enzyme (Crowe et al. 1993; Pikal et al. 1991). High concentrations of polymers suppress freezing-induced pH shifts via inhibiting buffer salt crystallization, particularly in the case of sodium phosphate buffer, thus retaining protein structure during freezing or drying.

Proteins tend to adsorb to ice–liquid interface through hydrophobic interactions and undergo protein unfolding. Amphiphilic polymers, such as PEG and poloxamers can compete with protein and prevent its adsorption-mediated aggregation. In solution state, the stabilizing effect of nonionic polymers, such as PEGs is purported to be a balance of two opposing effects, i.e., stabilizing effect due to steric exclusion (preferential exclusion) and destabilizing effect due to hydrophobic interaction (Arakawa and Timasheff 1985a). However, in frozen state, the hydrophobic interactions of PEGs may flip-flop from being destabilizing to stabilizing the proteins. During freezing, hydrophobic surfaces of PEGs compete with proteins for the ice surface, and thus protect proteins from being denatured at the ice–liquid interface. As polymers are competitive inhibitors for surface adsorption, their use at low concentrations may be sufficient to cover ice surfaces. On the other hand, hydrophobic surfaces of PEGs can bind to proteins in unfolded state to a greater extent and may stabilize the unfolded state leading to aggregation.

7.4.1.8 Antioxidants and Metal-Chelating Agents

Oxidation of certain amino acid residues in proteins (e.g., Met, Cys, His, Trp) is a common degradation pathway (Hovorka and Schoneich 2001). Trace metal impurities (e.g., buffer salts) and hydrogen peroxides (e.g., polysorbates) in many pharmaceutical excipients initiate these oxidative reactions in proteins (Abernethy et al. 2010; Wasylaschuk et al. 2007). In addition, some buffer ions can decompose during storage and their degradants can interact and destabilize proteins; for example, citrate buffer upon exposure to Fe-ions and light caused covalent acetonation of recombinant monoclonal antibodies (mAbs; Valliere-Douglass et al. 2010). Metal-chelating agents, such as disodium edetate (ethylenediamine tetra-acetic acid [EDTA]) and diethylenetriamine penta-acetic acid (DTPA) have been used to inhibit oxidation of proteins. Other strategies to protect proteins against metal-catalyzed oxidation include addition of amino acids, such as methionine and histidine as well as optimizing solution pH and protection from light (Qi et al. 2009; Wang 1999).

7.4.2 Freeze-Thawing Procedures

Freeze-freeze, freeze-thawing, and isothermal incubation in the frozen state studies are useful to (1) test the stability of frozen bulk drug substance or drug products, (2) assess the robustness of a product intended for refrigerated storage (2–8 °C) against

Table 7.2 List of various possible steps during freeze-thawing of enzymes

Process variable	Thermal parameter
Step I: Cooling (−20, −30, −50, or −80 °C)[a]	Slow (<1 °C/min)
	Intermediate (1–10 °C/min)
	Rapid (10–900 °C/min)
Step II: Freezing	With seeding
	Without seeding
Step III: Isothermal hold	
Step IV: Thawing (2–8 °C, 20 °C, 35 °C)[a] (with stirring or shaking)[b]	Slow (1–5 °C/min)
	Intermediate (5–10 °C/min)
	Rapid (>10 °C/min)

Modified and adapted from Bhatnagar, B.S., M.J. Pikal, and R.H. Bogner, *Study of the individual contributions of ice formation and freeze-concentration on isothermal stability of lactate dehydrogenase during freezing*. Journal of Pharmaceutical Sciences, 2008. **97**(2): p. 798–814

[a]Targeted freezing or thawing temperature

[b]Stirring or shaking condition adopted during thawing; stirring expressed in rpm and shaking expressed in shakes per minute

accidental freezing, and (3) identify excipients that impart protection to proteins during freezing or frozen storage. During freezing-thawing, proteins are exposed to stress factors including interfacial stresses, cryoconcentration, solute crystallization, phase separation, and pH shifts. Although, freeze–thaw is the most direct method available to determine the extent of freezing-induced irreversible protein denaturation, difficulties in controlling heat transfer during these processes have made results from the studies difficult for interpretation. Therefore, there has been continued interest in delineating the effects of freezing and thawing on protein stability. A variety of approaches used to freeze–thaw proteins are summarized in Table 7.2. The processes that occur during various stages of freeze–thaw are discussed in detail below:

7.4.2.1 Cooling

Cooling is the first step of freeze–thaw cycle and can be achieved at slow (<1 °C/min), intermediate (1–10 °C/min), and rapid (10–900 °C/min) rates (Bhatnagar et al. 2007). Rapid cooling rates ranging from 80 to 900 °C/min are obtained by plunging sample tubes in a subzero ethanol bath or liquid nitrogen. Rapid cooling in liquid nitrogen or by placing small volume vials in a freezer at −70 °C leads to the formation of many small ice-crystals, and consequently large interfacial area (Hawe et al. 2012). Some proteins may be sensitive towards surface-induced denaturation. To avoid formation of large ice–liquid interfacial areas, it is necessary to promote moderate/fast cooling of a solution during freezing. Cooling be performed in a controlled manner to promote formation of dendritic ice-crystals, which may attenuate freeze-concentration of solutes during freezing.

7.4.2.2 Freezing

Often, freezing of a solution occurs following cooling. Cooling and freezing terms are used interchangeably which has led to misinterpretations in describing effects of freezing. It is important to draw a distinction between cooling rate and freezing rate. Cooling rate is the rate at which solution is cooled, while freezing rate refers to post-nucleation ice-crystal growth. Ice-crystallization is exothermic, and therefore an abrupt temperature increase is observed upon freezing followed by a decrease in temperature due to subsequent cooling of samples (Franks 1993a). It is important to notice that applied cooling rate is not necessarily predictive of the resulting freezing rate.

Two important parameters that define freezing process are degree of super-cooling and rate of ice-crystallization. Freezing can be controlled (i.e., seeding with ice) or uncontrolled where freezing occurs spontaneously without any external intervention. In the methods involving seeding with ice, degree of super-cooling and ice nucleation temperature can be controlled. It enables separation and determination of the effects of super-cooling and freezing rate on protein stability. In the absence of seeding, super-cooling and ice nucleation phenomena cannot be controlled, which leads to spontaneous freezing of protein solution. Also, the latter phenomenon has deleterious effects on the reproducibility of freezing conditions.

7.4.2.3 Isothermal Hold

Isothermal incubation of frozen protein formulation can be performed at temperatures between −80 °C and the thawing temperature of products to elucidate thermal effects on solute cryoconcentration and excipient crystallization. The effects of excipient crystallization and cryoconcentration are rather temperature-specific, and stability extrapolations to other temperatures may result in erroneous results (Singh et al. 2011). Also, freeze-freeze studies can simulate the influence of temperature fluctuations on the frozen state of products during storage and transportation. For example, when a bulk product is stored at –20 °C and shipped at lower temperature.

7.4.2.4 Thawing

The process of warming of frozen samples is referred to as thawing. Similar to cooling, thawing can be produced at slow, intermediate, or rapid warming rates (Hawe et al. 2012). Further, there can be various levels of control over the heating rate similar to cooling step. Controlled thawing can be obtained by warming frozen samples using a programmable water bath (Bhatnagar et al. 2005). In general, moderate cooling combined with rapid warming results in highest retention of biological activity following a freeze–thaw compared to other cooling/thawing combination (Cao et al. 2003; Pikal-Cleland and Rodriguez-Hornedo 2000; Anchordoquy and

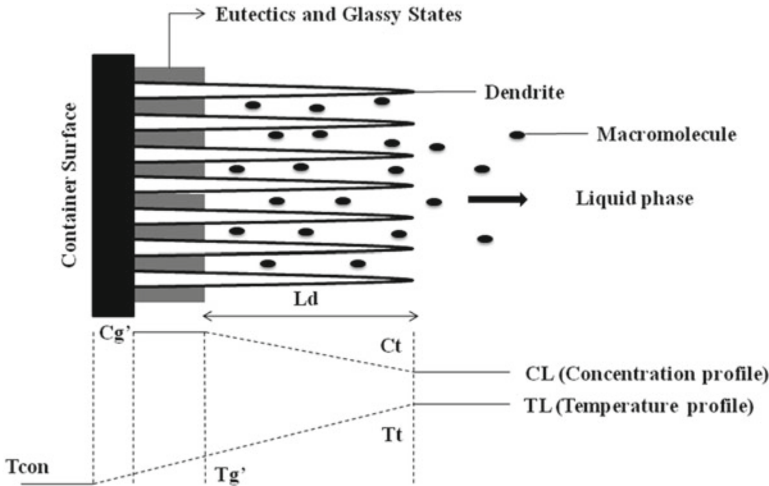

Fig. 7.8 Graphic representation of dendritic ice-formation in a solution. Fast freezing rates raise ice-front velocity resulting in dendritic ice growth. Ice between the dendrites traps solutes before being removed from the ice-front by diffusion and convection, resulting in the uniform distribution of solutes. CL and TL represent concentration and temperature of the unfrozen liquid phase at time *t*; C_g' and T_g' represent the concentration at the glass transition temperature of the solution; T_{con} is the temperature of the container wall or heat transfer surface at time *t*; and L_d represents path length of dendritic ice-crystal

Carpenter 1996; Anchordoquy et al. 2001). Precise monitoring of thermal profiles during cooling, freezing, or thawing may enable to segregate and evaluate the contribution of these processes to protein denaturation.

7.4.3 Dendritic Ice-Formation

Ice-dendrites are protruberances formed at the ice-front due to controlled freezing of super-cooled liquid layer (Langer et al. 1978; Weiss et al. 2008). These dendrites grow from ice mass into unfrozen solution. As illustrated in Fig. 7.8, solutes are retained (by inhibition of solute diffusion and convection) in the inter-dendritic space allowing uniform distribution of solutes within the ice mass, which is in contrast to slow or fast freezing regimens (uncontrolled), where ice-crystals form a flat front, leading to heterogeneous distribution of solutes (Wilkins et al. 2001; Butler 2002b).

Super-cooling in various directions can lead to growth of dendritic ice-fronts once nucleation starts (Ayel et al. 2006). During super-cooling, a negative temperature gradient is formed as ice undergoes dendritic growth into cold liquid. The dendritic ice-front grows rapidly at the tip, whereas the liquid trapped in the inter-dendritic spaces freezes much more slowly. Both thermal (freezing rate) and constitutional (high solute concentration) factors may drive the growth of dendrites (Butler 2002b;

Schoof et al. 2000). Constitutional super-cooling occurs when the planar ice interface becomes unstable, i.e., when the temperature gradient in the liquid (imposed by the freezing rate) is smaller than the gradient of the local freezing point depression (due to build-up of concentration gradient) along the moving ice-front. Due to a high concentration at the interface, a region of liquid will exist ahead of the interface that has a lower temperature (concentration-induced freezing point depression) than the equilibrium freezing point. In the constitutional super-cooled region, even a small protrusion of solidified material experiences a driving force for dendritic growth than the planar ice interface (Schoof et al. 2000). Fast-freezing rates form dendrites close to throughout the entire cross-section. The dendrites inhibit natural convection currents as well as diffusion, leading to more efficient trapping of the protein between the dendrites, resulting in a more uniform solute concentration profile. In general, dendritic ice-formation is considered highly desirable for cryopreservation of proteins (Wilkins et al. 2001).

7.4.4 Scale-Down Studies

Full-scale procedures in the early developmental stages can be very expensive, as they require large amounts of protein that could be lost at the end of experimental campaign. Rational studies to evaluate the dominating effects in scale-down models are critical in the design of freeze–thaw procedures. These studies can become "design space" studies for the identification of critical process parameters. A scale-down model can be used to mimic the large-scale system when it demonstrates equivalency in (a) product temperature and time profile, and (b) freeze–thaw events (e.g., cryoconcentration, ice–liquid interfacial area). Both temperature–time profile and thermal events depend on freezing/cooling rate and ice-front velocities, which in turn depend on freezing path length. Design of a scale-down container with constant freezing path length as that of large-scale unit mimics similar rate of freezing, which is predominantly determined by one-dimensional heat conduction. It is believed that one-dimensional heat conduction can be easily obtained with rectangular geometry vessels than cylindrical designs. Volume adjustments of the scaled-down models can be achieved by adjusting other dimensions of the container (e.g., diameter and height). Now, freezing temperature is the only variable that determines the rate of freezing, and a consistent freezing temperature between small- and large-scale systems results in scale-independent freezing. Although, super-imposable product temperature–time profiles between scale-down model (e.g., CryoWedge™) and large-scale unit (e.g., Cryovessel™) can be obtained, still scale-down model cannot be used to develop a large-scale freeze–thaw process. It can only be used to study the effect of process parameters on the stability of proteins. Full-scale freeze-thawing processes can only be developed in large-scale units.

Excipient crystallization often is considered to be a statistical event more likely to happen in larger volumes (Singh et al. 2011). Therefore, it is suggested to assess nucleation of excipients in the frozen state, with small-scale models that use seeding

of freezing nucleation to induce crystallization (Sundaramurthi et al. 2010). In some instances, extrapolation from small-scale freezing experiments to large-scale may not be possible as freezing at small-scale is highly different from large-scale (Singh et al. 2009a, b). For example, small volumes (e.g., 1–5 mL) can produce homogeneous frozen matrix in contrast to a significant solute cryoconcentration that occurs towards the middle and bottom of large-scale containers (Kolhe and Badkar 2011; Maity et al. 2009).

7.5 Containers

Primary packaging containers for freezing protein solutions include bottles, bags, and vessels. Container material may influence the stability of proteins, which could be due to adsorption of proteins to container surface. Scale of the container is another key parameter used to evaluate freezing-induced stresses, such as cryoconcentration and excipient crystallization, which normally are observed in large-scale containers. Therefore, it is suggested to perform freeze–thaw studies in the final packaging material. However, this is not feasible in the early stages of development when only limited amount of protein is available or when the protein concentration, container volume, and final formulation have not been defined. In such situations, bridging experiments, comparing the stability of a protein in different packaging materials and different scales, can give insight into compatible packaging materials and scalable volumes. Based on heating–cooling mode of operation, containers can be classified as passive and active systems.

7.5.1 Passive Systems

Passive systems are containers, which are devoid of active heating–cooling operations. Passive systems are modeled for small-scale to intermediate-scale hold volumes ranging from a few millimeters to a maximum of 20 L, but represent poor systems for full-scale transformation. These containers can be bottles, bags, carboys, and vessels, which can be used to process materials down to −70 to −80 °C. Containers (bottles and vessels) made of 316 L grade stainless steel can be used in reusable formats, while plastic (polyethylene (PE), polypropylene (PP), poly(ethylene) terephthalate glycol (PETG), Teflon) containers (bottles, bags, and carboys) are available in disposable and reusable formats. Plastic systems can become fragile, if the storage temperatures are below the T_g of the plastic material, and therefore can limit its use. It is therefore critical to know the glass transition temperatures of product solutions and plastic material. For example, polycarbonate and Teflon containers, whose T_g is −135 °C and −270 °C, respectively, can be useful for storing protein solutions at low temperatures.

Passive systems are filled with protein solutions to a specified volume to allow freezing expansion, and then transferred to freezers. Freezing and thawing

parameters, such as freezing and thawing times, rates, and temperatures are determined using a placebo formulation. Freezing fronts move from all sides of the container and reach the middle center of the container as the last point to freeze (LPTF), while last point to thaw (LPT) would be the top center. The freezing process time is the time to reach the target freezing temperature at LPTF from +10 or +20 °C, and thawing process time is determined as the time to raise the temperature at LPT to target thawing temperature. The temperatures are monitored by placing temperature probes at LPTF and LPT in bulk solution. Thawing is performed by placing containers in a refrigerator or at room temperature. Thawing process times are longer than freezing process times due to low thermal conductivity of ice. In the absence of mixing, thaw times can be quite long depending on container size. During this period, significant concentration and temperature gradients develop in each container. Adequate mixing can be achieved through gentle rocking, shaking, or stirring which prevents concentration and temperature gradients across the container. However, care should be exercised to avoid formation of bubbles at air–liquid interface, which is a potential site for protein denaturation.

The conventional stainless steel/plastic containers are simple to use, and, if a protein formulation is robust and stable under a wide range of freeze–thaw conditions, this is a preferred mode of operation. However, freeze–thaw operation in passive systems remains largely uncontrolled, which leads to cryoconcentration effects. Loading a number of small containers filled at room temperature into a freezer can overwhelm its cooling capacity, leading to long and variable freezing times among these containers. Therefore, freeze–thaw parameters must be well defined, qualified, and validated for fill volumes, loading patterns, spacial placement, and maximum and minimum loads.

7.5.2 Active Systems

A line of stainless steel vessels (e.g., CryoFin™) have been designed for controlled bulk freeze–thaw processing, transportation, and storage of proteins. The unique design of active systems allows performing freeze–thaw operations of proteins in a reproducible manner. Active systems can be programmed to freeze-thaw solutions using pre-determined temperature and time profiles. These systems can be validated for freeze-thaw parameters easily. However, active systems do not control the degree of super-cooling and nucleation rate. The active container systems can be supplied with appropriate hardware for clean-in-place and steam-in-place operations. The active heat transfer containers are available in various sizes to meet scalable requirements.

7.5.2.1 Large-Scale Containers

Large-scale Cryovessels™ made of 316 L stainless steel can hold volumes in the range 20–300 L with working temperatures as low as −60 °C (Fig. 7.9a, b). A

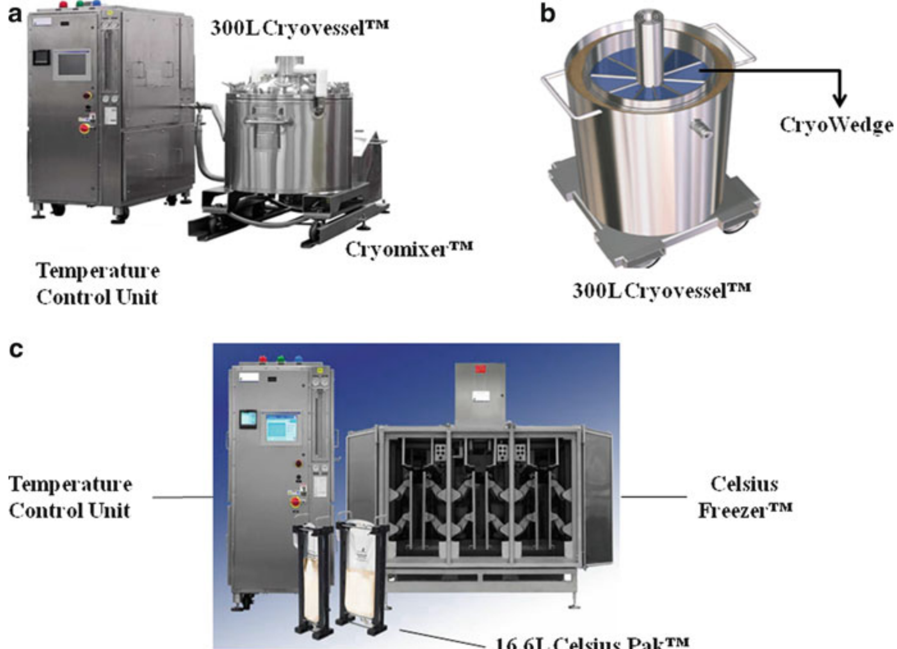

Fig. 7.9 (**a**) Graphic representation of 300 L capacity large-scale Cryovessel™; active mixing is provided by Cryomixer™ on a roller track; freeze–thaw parameters of Cryovessel™ are controlled by temperature control unit. (**b**) Cryowedge™, a scale-down unit of Cryovessel™. (**c**) Graphic representation of 16.6 L capacity intermediate-scale Celsius Pak™, which is compressed between heat transfer-enabled plates. Embedded Celsius Pak™ is transferred into freezer, whose thermal parameters are controlled by temperature control unit

combination of efficient heat transfer, tight control over freezing conditions, and short freezing path lengths avoids cryoconcentration. The wedge-shaped units of Cryovessel™ (e.g., CryoFin™) facilitate even and rapid freezing through internal active and passive heat transfer. The CryoWedge™ design promotes constant growth of dendritic ice throughout the freezing process to avoid solute cryoconcentration. The vessels are kept stationary through freezing to prevent solute motion and avoid incongruous growth of dendritic ice. Cryomixer inside the vessel allows aseptic and low turbulence mixing for rapid thawing of the frozen material. Some of the variations of vessel designs allow agitation of the whole vessel to remove concentration hotspots and maintain uniform solution temperature.

7.5.2.2 Intermediate-Scale Containers

Intermediate scale (Sartorius-Stedium™) freezing bags are available for freeze–thaw processing of protein solutions down to −70 to −80 °C (Fig. 7.9c). The bags (made of ethylene vinyl acetate) filled with protein solutions are held under slight

compression between plates which serve as controlled heat-exchange surfaces. These plates also serve to minimize agitation-induced damage to frozen material during transportation. Normal bag sizes are 8.3 and 16.6 L with fill volumes ranging 2.1–8.3 L and 4.2–16 L, respectively. Six bags can be simultaneously processed in one unit for a combined total volume of 100 L. The bags are kept stationary during freezing, but the whole unit is agitated during thawing to promote homogeneity of the solution.

7.5.2.3 Small-Scale/Scale-Down Containers

Scale-down cryo-vessels are available to model and monitor freeze–thaw operations at small-scale (e.g., CryoWedge™, CryoCassette™, and CryoPilot™) in a controlled manner (Wilkins et al. 2001). Its wedge shape is designed to mimic one compartment of the symmetric compartments of the large-scale Cryovessel™ with identical configuration of heat-exchange surface angles, freezing path length, and construction material. A stepwise freeze–thaw program can be developed using CryoTool™ software associated with CryoWedge™, to mimic freeze-thawing conditions of Cryovessel™. Each of these containers may hold volumes from a few millimeters to a maximum of 4 L. Shamlou et al. designed rectangular bulk freezing containers to maintain a constant heat transfer path between small-scale (30 mL) and large-scale vessels for easy transfer of freeze–thaw parameters to large-scale units (Shamlou et al. 2007).

7.5.3 Leachables and Extractables

Freeze–thaw processes may trigger container (e.g., stainless steel or plastic) surfaces to generate reactive molecular or ion species, which cause destabilization of proteins. Stainless steel used in various processing equipments has been reported to be a cause of protein aggregation or fragmentation (Bee et al. 2011). Distinct physical or chemical instabilities with proteins may be caused by (1) steel surface itself, (2) steel particles shed from the surface, and (3) Fe-ions leached from equipments. For example, exposure to steel surface combined with additional shear stress resulted in aggregation of a mAb; surface-induced soluble aggregation of a mAb; Fe-ions caused hinge-fragmentation of a mAb; Fe-ions leached from steel caused oxidation and aggregation; and Fe-ions directly bound to a protein resulted in aggregation. The impact of the formulation on container surface may play a large role in potential adverse interactions. For instance, exposure of steel to chloride ions at low pH caused corrosion and release of Fe-ions that subsequently catalyzed the oxidation of methionine residues.

Stainless steel surfaces are typically passivated with chromium-oxide to create a corrosion-resistant surface. Frequent passivation of stainless steel surfaces and avoiding exposure of steel surfaces to extreme low pH in the presence of chloride

ions are some of the strategies to eliminate deleterious effects of Fe-ions on proteins. Surface-induced aggregation of proteins can be completely suppressed using surfactants (polysorbates 20 and 80) in formulation compositions (Kueltzo et al. 2008). Addition of antioxidants and metal chelators may eliminate metal catalyzed oxidation, fragmentation, or conformational destabilization reactions of proteins (Lam et al. 1997; Zhou et al. 2010).

Disposable plastic containers are also common containers used in freeze–thaw operations. Leachables and extractables from plastic containers have been reported to cause destabilization of proteins. For example, polytetrafluoroethylene (PTFE) and polyethylene containers caused protein aggregation during freeze–thaw processing (Kueltzo et al. 2008). Teflon vessels or Teflon-coated vessels offer relatively inert surfaces to conduct freeze-thawing operations. Formulation compatibility studies with container surfaces during freeze-thaw processing can be useful in the selection of containers.

7.6 Conclusions

A number of factors can destabilize proteins during freeze-thawing process including cold denaturation, cryoconcentration, pH shifts, phase separation, and recrystallization of components from solution. These unwarranted processes either alone or together contribute to protein denaturation. An understanding of mechanisms of freeze-thawing-induced stresses will assist in the identification and optimization of formulation, process, and storage-related critical variables that affect the stability of proteins. Post-freeze-thawing, a stable bulk protein formulation enables completion of filtration and fill–finish manufacturing operations. During clinical trials, bulk protein solution stable to multiple freeze–thaw cycles facilitates administration of the same lot to participating subjects, and minimizes variation in therapeutic responses and aggregation-related immunogenicity issues.

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Chapter 8
Best Practices for Technology Transfer of Sterile Products: Case Studies

Leonore C. Witchey-Lakshmanan

Abstract Technology transfer of products, processes, and testing methods is a way of life in the pharmaceutical industry. Transfer is the movement of knowledge from an originating team and site to enable a receiving team and site to perform the manufacturing, testing, and releasing of the final pharmaceutical product. This includes the manufacture of the active pharmaceutical ingredient, the manufacture of the drug product, and the execution of all the tests associated with the quality assurance of these materials. The case studies discussed in this chapter are based on actual incidences that have occurred in various technical transfer activities. From receipt of excipients and characterization of drug substance to documentation preparation, clarification of compounding and lyophilization steps, to release testing of finished drug product, these case studies provide insight into the types of areas and weaknesses that can arise during the transfer processes, providing clear solutions in upfront communication to avoid such issues during a technology transfer.

8.1 Introduction

Technology transfer of products, processes, and testing methods is a way of life in the pharmaceutical industry. Transfer is the movement of knowledge from an originating team and site to enable a receiving team and site to perform the manufacturing, testing, and releasing of the final pharmaceutical product. This includes the manufacture of the active pharmaceutical ingredient (API), the manufacture of the drug product, and the execution of all the tests associated with the quality assurance of these materials.

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The compliance of transfer activities is governed by the current Good Manufacturing Practices governing each site. In addition, guidance documents are available to assist in the process. Such documents include the ISPE Good Practice Guide: Technology Transfer (2003) and the Annex 7, WHO Guidelines on Transfer of Technology in Pharmaceutical Manufacturing (2011).

According to the ISPE Guide:

> Technology transfer can be considered successful if a Receiving Unit can routinely reproduce the transferred product, process or method against a predefined set of specifications as agreed with a Sending Unit and/or a Development Unit.

According to the WHO Guidelines:

> Transfer of technology is defined as 'a logical procedure that controls the transfer of any process together with its documentation and professional expertise between development and manufacture or between manufacture sites.'

Companies engage in constant process analysis to make sure they have organized their personnel in a manner that moves concepts through development and into production in the most cost efficient way possible. Consequently, the industry has seen the advantage of creating specialized teams of technology transfer technicians to reduce the number of problems that may be introduced during the transfer process. Even so, millions of dollars per year are lost to problems that can be avoided if the industry embraces a constant communication of experience that lets each group in a company learn from the experiences of others. Even better, companies can learn from one another if they are willing to present their experiences to one another.

The development and transfer of sterile products from lab to lab or from site to site is no different. The following case studies represent experiences that include both the problems discovered and the solutions that can help keep them from being repeated. The sequence of the review below begins with the receipt of materials and flows through production and finished product release; however, many of the lessons learned in the cases below can be applied in any phase of the overall technology transfer process.

8.2 Materials Release Testing

The first portion of any manufacturing process flow is the release of the materials and components. This activity includes the release testing and quality control of the API, the excipients, and any other components related to the product. This section will focus on case studies specific to the release tests for API and excipients.

8.2.1 API Release Testing

In the case of technology transfer as it relates to the API and excipient release testing, company management often believes that these methods are "routine." Some of these methods may in fact include routine compendial tests, such as pH, moisture,

microbiological limits, and the like. These are methods that each laboratory would typically have in place and run routinely for most of their raw materials. Be they compendial or in-house standard operating procedure-based (SOP), the methods would not require full validation or more detailed technical transfer. Thus, the API assay methods are usually the main focus of method transfers for release of the raw materials, since these are typically the "new" methods for the new site.

8.2.1.1 Case: Confounding Methods for API Release

Sometimes, the details of even routine tests can become problematic. Take, for example, the assay test for an API with a high moisture content, greater than 10 % w/w.

In one instance, the receiving lab had performed the assay on three lots of drug by using the procedures provided for preparations toward registration stability batch manufacture. The three lots had previously been tested by the API supplier and had passed all specifications. However, the finished product site test results showed that two of the lots passed, but one lot failed with a value below the specification for potency. Immediately, the team mobilized to analyze the assay in order to determine what had gone wrong in the HPLC method used for the analysis.

However, the root cause did not lie in the HPLC analysis. Instead, the culprit was the method of moisture content analysis.

When the transfer first began, the team had agreed on a protocol. The receiving lab would use the moisture for the API in their labs as the correction for the purity. They would also take the moisture content for the reference standard from the CoA supplied by the vendor. What the receiving lab did not realize was that the moisture content of the API was dynamic. The API and reference standards were kept frozen. At the time of use, the samples were removed from the freezer and brought to room temperature for testing. However, no care was taken to adjust or control the relative humidity of the headspace in the containers. So, when the containers were frozen and then thawed again, the activity of the water (in effect, the relative humidity) in the headspace changed, changing the moisture content of the API and, consequently, *the reference standard*. Once the labs realized what was happening, they developed a more uniform handling and sampling program to assure that the drug was always exposed to the same relative humidity so that both the API and the reference standard could be reproducibly and reliably used in testing.

8.2.1.2 Case: Double Transfer of API and Drug Product

Another interesting event that can occur during technology transfer to the production site is the management requirement for a "double transfer." In a typical NCE program, drug substance experience is far ahead of drug product experience because the API must be produced to support the initial proof of concept studies as well as the early toxicological programs. These studies do not use the final dosage form proposed; rather, they use some simplified dosage form that allows for adequate delivery

to the subject in order to confirm the drug's mode of action and general activity. Thus, many of the challenges of the synthetic chemistry (for a small molecule drug substance), or of the fermentation and purification (for a biotech drug substance), are fleshed out long before the commercial drug product formulation is finalized.

However, at times the API scale up/technology transfer is occurring in the same relative timeframe as the drug product scale up/technology transfer. This may occur for any number of reasons. Perhaps a key starting material is discontinued and a new pathway must be found. Perhaps, in order to make the drug substance more economical, the company may decide to switch technologies for the API process. This simultaneous scale up and transfer of both API and drug product has particular challenges and caveats, especially when the product is a biotech material.

Such was the case when one company decided to move from a batch fermentation process to a continuous fermentation process for its monoclonal antibody. The drug product formulation had been established based on the pre-formulation data obtained on the batch-manufactured antibody. However, as production moved to continuous fermentation, drug product analysts began to notice changes in the overall sensitivity, stability, and impurity profile in the antibody. Moreover, these changes were not apparently consistent between lots manufactured using the same continuous process. The differences were eventually traced back to slight differences in the continuous processing conditions from lot to lot. In this particular case, the team reverted to the batch process to minimize the differences and mitigate the risks.

A shift from batch to continuous processing during technology transfer may appear to be an obvious pitfall if performed at a late stage in product development. However, in many varied situations, technology transfer teams find that they must adjust previous plans in order to provide added value mandated by management perceptions of the needs of the patient and the company. When this occurs, the technology transfer team members must be able to articulate the risks clearly to corporate management, and management must work with the technology transfer team to support them in appropriate mitigatation of the risks for the business and, more importantly, for the patient.

8.2.2 Excipient Release Testing

The technology transfer of the release methods for excipients may seem trivial at first glance because excipients are usually common from manufacturing site to manufacturing site, so the release methods are often already in place at a receiving site. In addition, the release methods for each excipient are typically taken from the respective compendia and, therefore, do not require full validation.

Nonetheless, it is not unusual for a formulator moving a product to a manufacturing site to be told that the excipient supplier must be changed. However, the general perception is that most excipients are compendial, so the quality should be identical, right?

Wrong.

8.2.2.1 Case: Differences in Compendial Excipients

Even for compendial grade excipients, impurity profiles vary. These small differences in impurity profiles can alter the stability or quality of the drug product.

One example of this type of transfer dilemma is the use of parenteral excipients like polyethylene glycols (PEGs). PEGs are used in many non-parenteral formulations and can be found in the USP/NF and other compendia. Because compendium monographs list similar specifications from one compendium to another, there can be a perception that one vendor's compendial PEG is the same as any other vendor's compendial PEG. So, at transfer, a product formulator may be asked to switch vendors of the PEG used in the product in order to use a vendor specific to and already qualified at the receiving site. The site's source allows conservation of resources and avoids the expense of auditing a new vendor, confirming the quality of the materials, maintaining additional inventory for testing, etc.

However, PEG synthetic pathways can be very different from vendor to vendor. These alternate pathways result in trace impurities in their excipients. These trace impurities may interact in unanticipated ways with the API or with other excipients.

In one case, the same formulation was prepared with three different sources of compendial PEG and placed on stability. Within a few weeks, one vendor's PEG maintained a clear, colorless product containing minimal related substances, but the other two were either golden yellow or dark brown and contained much higher levels of related substances and, in some cases, even different related substances. Though several months were lost in the timeline, in the end, the development team and the commercial site agreed to a PEG vendor that resulted in a quality drug product.

8.2.2.2 Case: Confirming Excipient Grade

At the outset, transfer specialists may be readily aware of the need to monitor the more unusual excipients. However, they must also ensure during the transfer process that other more typical excipients for parenteral products are also of the quality desired.

One such excipient is mannitol. For most parenteral products, the mannitol grade used is a low bioburden, low endotoxin grade specifically prepared for parenteral applications. However, when moving a product to a fairly new parenteral facility, the transfer specialist does well to confirm the parenteral grade of the mannitol and, in particular, its low endotoxin grade.

Imagine the unfortunate consequences for companies who have discovered in post-registration batch production that the mannitol used in the lots turned out to be oral tableting grade because transfer personnel did not ask sufficiently detailed questions regarding the grades and specifications of the excipients used.

Another issue that can arise, particularly with mannitol, occurs when the transfer is being done from the West to the East. Many eastern countries, such as India and China, have their own sources of excipient staples, such as mannitol. These sources may even go by the names of favored western vendors. However, the experienced

transfer specialist knows that even though the excipient source may have a favored name on the label, the actual manufacturing facility may not produce the same quality as a same-name excipient from Europe or the USA. Therefore, it behooves the transfer specialist to obtain the complete manufacturing pedigree of excipients prior to the transfer. Additionally, it is a good idea to confirm the compatibility of excipients with the API if the pedigree is different from the excipients used in development. (This same argument also applies to APIs, of course. It particularly applies when the products under development are generic products and the APIs are available from a variety of vendors.)

8.3 Production

To the uninitiated, technology transfer may first conjure up images of the manufacturing process itself—for example, equipment preparation, document preparation, compounding, lyophilization, and sterilization. And, of course, production technical transfer can be fraught with issues. Many potential problems can be mitigated by attention to detail during the development process and by transfer specialists taking special care to communicate production nuances to one another.

8.3.1 Preparations

The first step in the transfer of a production process is to confirm that the batch record documentation and the appropriate equipment are in place. These would seem to be fairly simple. For example, the transfer specialists take the batch records for which they have been manufacturing to date and provide the records to the receiving site to allow them to prepare the new records. Or, the transfer specialists exchange the information regarding the equipment, and the receiving site procures the new equipment or modifies existing equipment and/or operating procedures for the new process. Nonetheless, issues can still arise, even in the simplest cases.

8.3.1.1 Case: Preparing the Batch Record

When the process description is transferred from one site to another, a flow diagram and an example batch record are typically provided to the receiving site. However, this example batch record must then be entered into the documentation system of the receiving site. Often, the receiving site's documentation system is more detailed, and therefore more complicated, since the receiving site is likely a commercial site and has an added layer of compliance complexity to address. After the new master batch record is developed, the "flow of information" will look very different to the original formulator or process engineer. This means that the original staff must be

even more diligent in ensuring that all the appropriate details of information are transferred.

In one case, the original batch record was prepared on a volume basis. However, production typically prefers to prepare the product on a weight basis for ease of handling large amounts of materials. However, the originating staff had not provided a density for the conversion from volume to weight, so the production personnel assumed a density of 1 and prepared the formula sheet on a weight basis. The originating staff didn't catch the adjustment to a weight basis because, as they performed their review, they were looking for the same sequence of numbers, assuming that the units were on a volume basis. However, once the product was completed, the assay amounts were nearly out of specification because of the difference in the basis of the formulation.

Once the difference was caught, the adjustment was made in the batch record and future lots met the specification with ease.

In another case, personnel were working on the technical transfer of a fairly high pH product to a CMO for lyophilization. The transfer team had agreed that the CMO would prepare the basic vehicle for the pilot scale lot the day before the arrival of the client representatives. Then, with the client present, the key step of the addition of the API would be performed, and the rest of the process would proceed.

All seemed to go well with the production to the team; however, once the drug product was removed from the lyophilizer, the previously elegant cake appeared lacey and thin. Since the product still passed all release specifications, the team attributed the appearance during scale up to the larger pilot lyophilizer. The team agreed to proceed to the commercial scale demonstration lot.

As with the pilot lot, the CMO prepared the basic vehicle the day before and proceeded with production of the drug product in the presence of the client. Once the product was filled and the lyophilizer door was closed, one of the floor technicians commented to the client on the unusually large amounts of base that were required in the product. After further inquiry, the client came to realize that the CMO was adding over 10 times more base than the client had intended. The excess base caused the lacey, thin cakes after lyophilization.

This error occurred because of a simple miscommunication. The client had requested a pH adjustment to a particular pH range, and the CMO had interpreted that pH range as a specific pH. During the review of the master batch record prior to production, the client had one idea in mind and the CMO another, though both were looking at the same language in the batch record. The different perception of the pH adjustment step had not been caught even after the pilot lot was prepared because each set of personnel had assumed a particular process was being performed and had read the executed batch record in different ways.

Fortunately, the issue was caught during the lyophilization of the demonstration batch, and the correction was made for the registration lots. The lyo cakes returned to their original level of elegance, even at commercial production scale. Nonetheless, the lessons learned included making sure that the master batch record is unambiguous, encouraging the floor technicians to ask questions, and ensuring that the supervising staff listens closely to the questions and experiences of the floor personnel.

8.3.1.2 Case: Preparing the Equipment

In an instance using isolator technology, the methods of decontaminating the isolators ended up affecting the product quality. In this case, new isolators were built to house the new commercial process for the product. As the transfer specialists began the manufacture of the product, they noticed new degradation peaks increasing within the final product-related substance test results from lot to lot. They could not track down an assignable cause to any of the excipients or to differences in processing. Eventually, they came to understand that the cause was due to the mode of airflow into and out of the isolator during decontamination of the isolators prior to processing.

Their product, it turned out, was unusually susceptible to the residual vaporized hydrogen peroxide (VHP) used during the decontamination of the units. If the units were decontaminated a few days in advance, the final product was fine because the traces of VHP remaining in the isolators had been flushed away prior to production. However, if the units were decontaminated just prior to production, the levels of residual VHP, though immeasurable by the means used in the facility, were still sufficient to affect the product. In this case, both changes in the VHP procedure as well as adjustments in how the VHP was measured improved the reproducibility of final product quality.

8.3.2 Compounding

Simple aspects of compounding, such as order of addition of the ingredients, including pH adjustments, mixing systems, etc., can cause grief during technology transfer if personnel do not attend to details.

8.3.2.1 Case: Order of Addition

Surfactant addition sequence is an example. Is a surfactant added prior to or after the active ingredient?

In some cases, it may be necessary to add a surfactant prior to the API in order to assist the drug in dissolution. If the addition is not done prior to the API, the drug may be mixed for very long times but still not attain sufficient solubility. In other cases, surfactant addition may need to occur after the API in order to ensure an even coating on the drug suspension particles. If the addition is not done after the API, the drug particles may not exhibit sufficient stability over the shelf life of the product.

In the case of pH adjustors and additives, care must be taken to ensure that no local concentrations of extreme pH are created during the addition of later excipients. For example, pH may be increased in order to ensure the dissolution of a basic drug; however, certain drug substances and even excipients, such as mannitol, may not have sufficient stability if concentrated sodium hydroxide is added to the

solution in order to create a desired increase in pH. Therefore, during the transfer of the steps of the process, care must be taken that the order and type of addition as well as the appropriate amount of mixing are tested and in place.

8.3.2.2 Case: Mixing Systems

Not only should the order and type of addition be established, the appropriate amount and type of mixing should also be confirmed. Often in the laboratory, simple marine-type impeller mixing blades are used. However, at scale up and transfer, marine-type impeller blades are rarely the most efficient for commercial scale production. Thus, as the technology is being transferred, the effect of the new, and potentially more efficient, impeller systems needs to be evaluated. This is particularly important in the case of biotech materials that are sensitive to shear.

Of course, shear-induced aggregation of proteins is a classic example of failure during technology transfers. Not only can a change in impeller systems cause the formation of agglomerates, but also changes in pumps and filters can create issues. In one case, the switch from a peristaltic pump to a piston pump resulted in a marked increase in the level of agglomerates observed in a protein-based drug product. Therefore, each proposed system change should be evaluated prior to moving the product to the new site.

Evaluation of proposed changes also extends to items such as tank configurations. When mixing at one site is being performed with a specific impeller/tank geometry, assuming that an impeller/tank geometry at the new site will be identical and result in identical, adequate mixing can create problems.

In one case, a novice transfer specialist had prepared a simple solution in the original site using a 500 L tank that had been cylindrical, approximately twice as high as it was wide. The original impeller system had been located along the axis of the tank and included a shaft that extended the full length of the tank with two appropriately sized impellers positioned properly along the shaft. Dissolution of the drug in the original 500 L batch was rapid and complete.

At the start of the transfer to the receiving site, the specialist was told that the receiving production facility also had a 500 L tank/impeller system that would be suitable for making up the simple solution required for the product. Because of inexperience, the specialist did not consider asking specific questions regarding the geometry and configuration of the mixing system. The team believed that the impeller/tank geometry was suitable because the drug concentration was substantially below the solubility limit. The only requirements for the tank/impeller system were that the tank could hold 500 L and that the impeller shaft was sufficient for mixing. It was assumed that dissolution would not be an issue.

When the transfer team arrived at the receiving site to begin production, they found that the new tank was approximately twice as *wide as it was high*. In addition, the impeller shaft was mounted nearly radially, entering at one side of the tank and extending to the bottom center of the tank. Moreover, only one, very small, impeller was attached to the mid-point of the shaft.

Needless to say, when production commenced, it became quickly obvious that mixing was not efficient at all within this system. Instead of easily mixing and dissolving, the drug powder collected in a dead space on the opposite side of the tank from the small impeller. This configuration was replaced with a more efficient system for long-term, commercial production.

In another instance in which the overall tank configuration affected final processing, a transfer specialist was moving the production of a "simple" solution to a commercial facility in which the final quantity of product in the tanks was determined by volume using sight glasses attached to the outside. This was something of a surprise to the transfer specialist because most sites use floor scales or tanks on load cells to measure the batch quantities by weight rather than by volume. Nonetheless, the transfer continued using the sight glass method because neither floor scales nor load cells were available for processing.

As is common, the compounding procedure first required that ~80 % of the total amount of water for injection (WFI) be added to the tank. The remaining materials were added and dissolved into the water. Once all the excipients and drug were added to the tank and well mixed, the batch was topped off to volume using the sight glass, and the batch was mixed one last time.

Unfortunately, during the technology transfer, the assay for the drug was mysteriously out of specification, though all aspects of the operation had run very smoothly. Of course, an investigation ensued. During the investigation, the overall manufacturing process was repeated in the lab using lab equipment and the resultant product met all specifications. However, when the process was repeated in production using production equipment, the assay continued to be mysteriously out of specification.

Eventually, the technology transfer team realized that the issue lay with the use of the sight glass. Specifically, when the WFI was added to the tank, it filled the sight glass with WFI. As the other ingredients were added, they did not penetrate the already filled sight glass. Therefore, the density of the product in the sight glass was not representative of the density of the material in the tank. As the product was brought to final volume, the differences in density between the material in the tank and the material in the sight glass caused a sufficient difference in the assay to prevent the final product from meeting specifications. Once this issue was recognized, adjustments were made in the manufacturing procedure. Eventually use of the sight glass was eliminated and the plant added a floor scale, which resulted in quality product with each manufacturing lot.

8.3.3 Lyophilization

The most common challenges in moving a lyophilization process from one facility to another are typically associated with the changes in scale that occur. However, not all the issues are actually specific to scale. Lyophilization problems can also come from lyophilizer program variations, overlay gas purity, radiative heat flux effects, convective heat flux effects, and glass transition temperatures.

8.3.3.1 Case: Lyophilizer Program Variations

For example, as a cycle is developed, the focus is to create a lyo cycle program that results in a specific temperature profile that results in the desired final quality attributes for the cake. Documentation of the desired cycle and temperature profile are required elements of the transfer to the receiving site so that receiving site personnel can determine how best to program the cycle into their lyophilizer. When the cycle is moved from lab to pilot to commercial scale, the nature of the lyophilizer program can change based on the control points of the lyo cycle control system. For example, some lyophilizers use “soak” times to establish time durations. Some lyophilizers use temperature ramp rates with specific temperatures as end points. Operators must be cognizant of the peculiarities of each lyophilizer programming strategy as the cycle is being transferred from one site to another.

Once the cycle programming strategy is established and the first cycle is running product, thermocouples are often placed into the product in the vials in order to assure that the product is in fact seeing the same temperature documented during development. The thinking is that if the product sees the same temperature, pressure, etc., then each vial will dry virtually the same way as the vial dried in the laboratory-scale equipment.

A key indicator that floor technicians check is the “temperature break,” the point at which the product temperature begins to increase toward the shelf temperature during primary drying. At the start of primary drying, the vial temperature is typically below that of the shelf temperature due to the evaporative cooling effect. However, once the bulk water has completely sublimed, this evaporative cooling effect is no longer present. The temperature of the product increases, approaching the set point of the shelf temperature. This temperature change is often referred to as a “break” in the temperature, or thermocouple reading, and can be used as an indication that primary drying is nearing completion.

However, during actual transfers, the “break” is not always observed in the temperature reading of every thermocouple. Inevitably, at least one thermocouple does not exhibit this behavior. When no break in temperature is observed within the timeframe expected, often the lack of observed break is blamed on the thermocouple itself—that perhaps it wasn’t connected properly or placed in the appropriate location.

In other words, observers decide that the lack of break is due to the probe and not the product.

However, the probe may not be at fault. The absence of a break may also be indicative of differences in the performances of the freeze dryers from one site to another. For example, the specific location of a dryer can result in the absence of a break. In such cases, often the break is eventually observed if the cycle is allowed to run long enough.

8.3.3.2 Case: Overlay Gas Purity

Another difference that is often overlooked between sites is the purity of nitrogen or inert gas used in the overlay of the vials at the end of the cycle. The moisture,

oxygen, and/or carbon dioxide content of the nitrogen can affect the final product, depending on the drug's sensitivities. Nitrogen, for example, can come from small cylinders that feed only the lyo. It can also come from huge, typically outside, liquid nitrogen tanks that feed entire buildings. Each source may have slightly differing impurity contents and may need multiple treatments prior to use in the lyo (e.g., drying tubes).

8.3.3.3 Case: Radiative and Convective Flux Effects

Of course, differences between radiative and convective flux in lyophilizers may also change the quality of product at various locations. Specifically, radiative heat flux occurs in a lyophilizer because the room temperature at the face of the lyophilizer is higher than the interior. Therefore, the heat travels from the room into the lyo, resulting in a slight temperature gradient in the vials from the front to the back of the lyo. Convective heat flux occurs within the lyo simply because heat rises. Consequently, the product on the upper shelves of the lyo may experience an environmental temperature slightly higher than the product on the lower shelves. Consequently, differences in drying may be particularly noticeable at the front of the top shelf. Often, melt back is observed in the vials that are located on the top shelf in the front corners, where the radiative and convective heat flux meet.

This effect can be particularly problematic for sucrose-based products in which the transition temperature changes during the course of the drying process. However, the problem can also occur in mannitol-based products in which high levels of additional key excipients can depress the overall transition temperature.

8.3.3.4 Case: Glass Transition Temperature

In some cases, it is not the transition temperature during primary drying that is problematic. Problems can also arise that are dependent on the glass transition temperature of the final cake.

In one case, the laboratory that developed the product established that the glass transition temperature of the final cake was between the long-term stability temperature used for the product and the accelerated temperature. Analysts consistently observed that the cake consolidated during accelerated stability, shrinking from a marvelously elegant cake to a shrunken plug. However, all of the data regarding the chemical stability and the reconstitution time over the course of stability remained virtually unchanged. Additionally, the originating site personnel made no mention of the physical appearance to the receiving site personnel. Therefore, based on the data provided to them at the time of transfer, the receiving site personnel had no indication of this consolidation during stability.

Consequently, one can imagine the consternation that arose during the registration stability program. Within the first month of testing, the lyo cake samples began

to appear shrunken when held at accelerated conditions. A quick discussion with the developing laboratory confirmed that this was "normal" for this product and did not affect the overall product quality.

8.3.4 Sterilization

The sterilization process is applied not just to the final drug product to render the liquid sterile; it is also applied to the intended container and closure prior to filling the product. In addition, most of the equipment is at least sanitized, if not sterilized, prior to use in order to ensure the bioburden is eliminated as best as possible.

The transfer teams do well to recognize the nuances of both the terminal sterilization operation intended for the final product, such as aseptic filtration and gamma irradiation, and the nuances in preparing components, such as the autoclaving of lyophilization stoppers, to ensure the long-term quality of the product.

8.3.4.1 Case: Autoclaving of Lyophilization Stoppers

Products are lyophilized because they are not stable in the presence of water. The moisture content of the final cake is an important parameter in the long-term stability of the product. However, the novice transfer specialist may not be appreciative that stoppers actually absorb water during the autoclave process designed to sterilize the stoppers for use. This moisture can then migrate into the cake over the shelf life of the product and compromise the drug over the course of time.

Thus, development of the stopper autoclaving process should also include development of a cycle to dry the stoppers to the desired moisture content. The cycle parameters that will establish the moisture level in the stoppers might include the vacuum levels at the end of the cycle, the time period held under vacuum, and perhaps even a number of pulses from vacuum to pressure to assist in eliminating moisture from the stoppers.

Because differences exist between load configurations for each site and between cycle programming available for each autoclave unit, attention must be paid to these details during transfer in order to assure that stoppers are dried to sufficient levels. Most contract manufacturers who also do lyophilization have a specific set of cycles that they have already established as effective with their stock supply of stoppers. However, if such data are not available at the receiving site, stopper vendors can assist in determining the water content of the stoppers after various cycles are run.

8.3.4.2 Case: Aseptic Filtration

Justification must be given for the mode of sterilization for each product, and justification must particularly be made if terminal sterilization cannot be performed.

The most common mode of terminal sterilization is autoclaving, simply because the process is well established and lends itself well to aqueous-based solutions. Of course, during autoclaving, the drug product is pressurized and heated to at least 121 °C and held there for several minutes to eliminate the microbial load.

However, cycles validated in one unit are not always transferrable to another. This occurs because the load configuration might be different. Consequently, the same cycle may not offer the same level of bioburden reduction (F_0) or might end up providing a heat load that harms the product.

Most notably, proteins and other biological products are denatured by extreme processing conditions such as terminal autoclaving. In these cases, solutions are processed aseptically by sterile filtration through a 0.2-μm membrane to remove the microbial load.

To support the filtration process for commercial production, a series of tests are performed to validate that the given filter is suitable for the sterilization of the product. Testing includes ensuring that the membrane is compatible with the product and thereby able to retain any bioburden present in the bulk product solution. However, often plants forget to confirm that the filter cartridge itself is compatible with the product.

In one instance, a novel sterile formulation had successfully been manufactured at lab scale using a specific filter type. This information was transferred to the pilot clinical facility. That facility, in turn, purchased the next size up of the same filter membrane, not paying full attention to the details regarding the construction materials of the filter housing.

When the novel formulation was processed, the liquid leaked out of the filter cartridge, compromising the quality of the lot. Fortunately, the transfer specialists quickly realized that the materials from which the new pilot scale cartridge was made were different from those of the lab scale cartridge, and they were successfully able to replace the filter with a different model more closely mimicking that used in the lab, saving the remaining portions of the campaign.

8.3.4.3 Case: Gamma Irradiation

Gamma irradiation is another mode of terminal sterilization that is used, particularly for sterile solids. The industry standard is that 25 kGy is considered sufficient to sterilize. However, the effects of sterilization at those levels must be confirmed. If the effects are deleterious to the product, other levels must be determined to balance the level of bioburden reduction required vs. the degradation of the materials.

In one case, the desired final product was a sterile suspension for injection. The original laboratory process included aseptic compounding using a low bioburden drug substance followed by terminal sterilization via autoclaving. When the product was to be transferred to the clinical manufacturing facility, the company decided to sterilize the drug substance prior to aseptic compounding via gamma irradiation. This was done in order to further assure that the drug suspension was fully sterile after autoclaving. Unfortunately, when the final product was manufactured,

additional degradation products that previously had not been observed were formed due to the gamma irradiation.

The presence of these degradation products presented the transfer team with two issues. First, even though they were present in very small quantities, the levels of degradation products were high enough to fail the finished product lot. Second, the analytical methods weren't validated for these degradants. So, both the processing and the methods had to be revisited to achieve the desired goal.

8.4 Product Release Testing

Most aspects of drug product release testing are shared with other dosage forms. However, those that are specific to sterile products include visual inspections, particulate matter testing, and sterility testing.

8.4.1 Visual Inspections

Appearance is one of the simplest methods of inspection for a product. However, because it is the simplest method, it can also be the most subjective. Therefore, in a technical transfer procedure, it is imperative to be very specific and descriptive during the training of personnel on the appearance of the product. The aspects of appearance can range from general appearance, defined by color and clarity of the solution as per USP <1>, to defining the nature of the lyophilized cake, to the expectation of the presence of visible particulates.

8.4.1.1 Case: "Clear" vs. "Colorless"

Ensuring that the staff understands that "clear" and "colorless" are not synonyms is critical. Specifically, "clear" means "no particles" and "colorless" means without color. The concepts must be explicitly addressed at transfer time. In one case, the receiving site took clear to mean colorless. So, when the registration stability data were transferred back to the research team for compiling into the NDA, there was some concern that the commercial site had not mentioned the color of the product. Eventually, the confusion was rectified, but the documentation had to be corrected for the entire stability program, starting with the initial data set.

8.4.1.2 Case: Defining Color

In addition, problems arising from such simple term confusion can be avoided by defining shades of color and offering a specific tool to the new site in order to

allow them to distinguish acceptable shades. Indeed, most Health Authorities are requiring a tool that quantifies the level of color. The tool chosen depends on the nature of the product. For example, solution colors may be established using a UV/VIS spectrophotometer with which the absorbance at a particular wavelength or range may be measured.

Even more simply, solutions are often visually compared with the EP color standards established in EP Method 2.2.2, "Degree of Coloration of Liquids." In the EP color standard system, standards are prepared in combinations of yellow, brown, green, and red. These are also diluted to varying degrees of intensity, and then the specification is set both as a specific color range and as a specific intensity of color.

For powders or lyophilized cakes, a tri-stimulus analytical measurements system may be used for a specific range of numbers, providing a color indication. Alternatively, color charts may be used, such as Munsell charts for white to off-white colored cakes or Pantone charts for brightly colored cakes. Regardless, current industry standards require that color be defined as quantitatively as possible and that the tool is appropriately justified.

8.4.1.3 Case: Lyo Cake Libraries

A compilation of descriptions of the types of lyophilization cakes a product may exhibit is often called a "lyo cake library." This library is most easily developed using photographs of cakes that were created during the lab phase or the early scale up phases. Photos assist the technical staff with understanding the difference between cake descriptions like "melt back" and "shrunken." It is possible that the former would be unacceptable while the latter would be acceptable.

The acceptability of the appearance of a lyophilized cake can be established by performing the battery of release tests on cakes that appear to be compromised and comparing the results to the release specifications. In addition, it is a good idea to subject some of the samples with differing appearances to long-term stability testing. If those samples that have a different appearance still meet specifications, then the data prove the quality of the product.

Often, however, the challenge to the transfer team is to remember to collect the library samples. In one instance, the team remembered to collect samples during the scale up of the demonstration batch that was produced on the equipment and at the scale of the planned registration lots. These samples were then used for qualification of the semi-automated visual inspection of the product. However, team members neglected to save the samples or take photos for training of future inspectors. Once it was noted that the samples were destroyed, new samples had to be prepared, which cost time and resources and delayed other key activities. Clearly, the lesson learned in this case is to compile the samples *and photos* of the various types of lyophilization cakes that are observed starting early in the development program, and maintain the library in such a way that the information is not lost or destroyed.

8.4.1.4 Case: Visible Particulate Matter

For protein solutions or reconstituted solutions, the size and nature of acceptable visible particulates should also be established.

Some biotech materials are by nature particulate. Consider the case of an adenoviral vector gene therapy agent. The adenovirus had an average size of 150 nm and, consequently, presented as an opalescent suspension. The analysts were trained to distinguish the difference between particulates and opalescence. Opalescent standards were prepared to assist the analyst in determining the quality of the product. Similar approaches to standardizing visual confirmation have also been taken for nanoemulsions.

In these cases, and in the case of injectable small molecule drug suspensions, the concept of "foreign" particulates becomes important. Since suspensions, in general and by nature, have "particulates," inspectors need to be trained as to what types of particles are foreign in nature. This means that the drug suspension needs to be properly characterized prior to the transfer in order to allow for adequate training. Low magnification microscopic methods sometimes come in handy for these analyses, allowing the new analyst to become more familiar with the vagaries of the product and to distinguish more clearly the nature of the particulate matter in the product.

8.4.2 Particulate Matter Testing

Subvisible foreign particulate matter testing, also known as USP <788>, is a requirement for all sterile products. This testing can be done using a light obscuration test in which product is passed through a light path. The size of the particles is measured, and the particles are counted, automatically. Testing can also be performed using a microscopic method in which the product is filtered onto a membrane and then examined under a microscope. In this case, the particles are sized and counted manually. The microscopic method is typically used if light obscuration is not feasible for the product.

One of the main difficulties during technology transfer is communicating the details of the test procedures between the laboratories. When a product is a simple liquid solution, communicating testing details is typically not a problem because the method is often a light obscuration test taken straight from the USP. However, often products do not lend themselves to a simple, unmodified USP <788> light obscuration test method.

The product aspects that make it difficult to use the standard USP <788> light obscuration test method may include the following:

- Protein products that contain protein agglomerates, which are not considered foreign matter
- Non-aqueous products that dissolve the gaskets and parts of the light obscuration equipment

- Viscous products in which bubbles persist
- Suspension products

Some products, such as proteins and certain oncolytic products, are filtered prior to administration to a patient. In those cases, the product may therefore be filtered prior to USP <788> light obscuration testing in order to mimic the product at the time of use. In these cases, it is important to ensure that the filters used both in the originating lab and the receiving lab are the same make and grade in order to ensure the same performance.

For products that may dissolve the parts of the light obscuration equipment, the originating lab should have already determined if the product can be diluted and still tested using light obscuration. If a dilution is used, then the originating lab needs to be sure to specify the dilution medium and ratio as well as any other handling details that are necessary to run the method.

Although this step may seem obvious, there seems to be a tendency for testing laboratories to simply refer to the USP chapter and expect the receiving lab to do the same. The laboratories must appreciate that USP chapters often give several different options regarding how to approach the test, depending on the type and nature of the drug product.

When aspects of the testing are developed to address the nature of the specific product being transferred, the receiving site needs to have those aspects documented so that they can qualify the method at their site—as per the GMP adage: "If it is not documented, it didn't happen." In the same vein, if the receiving lab is instructed to follow the USP <788> procedure, the receiving lab will assume (and rightfully so) that no other special handling is required.

8.4.2.1 Case: Light Obscuration for Viscous and Suspension Products

Viscous and suspension products can offer particular challenges, especially during a technology transfer. The originating lab should document the details of the method in order to ensure that the lab personnel repeat the method in the same fashion each time. However, documenting all the necessary details for a given test can be challenging due to variances in training in the art associated with the testing.

In one case, the lab developing the test for a viscous product noted the option in USP <788> to sonicate the product in order to disperse air bubbles prior to light obscuration testing. Therefore, sonication was recommended for this purpose. However, the duration of time and allowed amount of ultrasonic energy were not documented. Consequently, when the method was transferred to a new analyst, the new analyst believed that "more was better." While the originating analyst had sonicated the sample for only a few seconds, the receiving analyst placed the sample in the bath, turned it on, and walked away, thinking his procedure was acceptable so long as the sonication was within the limits of the USP. Neither analyst realized that additional sonication actually created cavitation within the product, resulting in the creation of more bubbles rather than fewer. The problem was not discovered until

failing data appeared and forced investigation. Only through the lost time and resources of the investigation did the analysts realize the issue and adjust the method accordingly.

8.4.2.2 Case: Microscopic Examination

The microscopic method for particulate matter can offer similar challenges when moving the test from one lab to another. For example, the collection of a sample in the filter prior to examination under the microscope can be a technique-driven activity. As discussed above for the light obscuration test, documenting the method in sufficient detail ensures that a receiving site understands and executes the method the first time and in the most efficient manner.

In one case, the method documentation simply stated that the sample should be filtered. Then, the filtered sample should be recovered, dried, and examined for particle count. However, the originating analyst was meticulous and carefully dripped the sample onto the filter drop-by-drop in order to ensure that all of the suspension was in a readable portion of the filter. The receiving analyst didn't know this technique was used. Consequently, the receiving analyst poured the bulk sample onto the filter. This approach resulted in an unreadable test filter because all the suspension was agglomerated at the edges of the filter.

In cases such as this, it may be useful, and even imperative, that the originating analyst visit the receiving site and review the step-by-step procedure in the laboratory with the receiving analyst. In this way, the originating analyst will be able to observe and communicate directly the details of handling the sample and/or filter in order to ensure an appropriately readable test result.

8.4.3 Sterility Testing

Another requirement for all sterile products is the sterility test method (i.e., USP <71> or equivalent). This test can be performed either through a membrane filtration test or by using direct inoculation. To summarize, the membrane filtration test filters the sample through a commercially supplied, sterile membrane capsule so that organisms present in the sample are collected onto the membrane. Growth media is then poured into the capsule, and the capsule is incubated at the prescribed temperatures for the defined periods of time for the organisms being tested for. If turbidity is observed, the sample is considered failing. If it remains clear, it is considered passing.

The direct inoculation test is typically used when the sample does not lend itself to filtering, as is the case with a suspension product. During a direct inoculation test, the product is directly inoculated into the growth media. The media are then incubated for the prescribed temperatures for the defined periods of time, as with the membrane filtration test.

At this point, the two tests differ. Because direct inoculation is done only when a sample cannot be filtered, the media is usually already turbid as soon as the drug product sample is added to the media. Therefore, a simple visual test is not sufficient to know if the sample passes or not. Consequently, the media/product mixture is sampled and, after the prescribed incubation period, plated and incubated again. If colonies are observed on the plate, then the sample is considered as failing. If the plate is without microbiological growth, then the sample is considered as passing.

Given this background and the fact that sterility testing is ubiquitous in every parenteral facility, transfer specialists sometimes believe that such a test doesn't need a "transfer" at all. However, mistakes do happen, usually when the receiving lab assumes something about the product.

8.4.3.1 Case: Inexperience

In one case, an oncology product moved to a lab in a new facility that was just beginning to gain experience with oncology products. When the time came to perform the test, the sterile hoods were being used and timelines were tight. A new microbiologist in the lab decided to execute the method and associated procedures in the open lab rather than in a controlled hood, believing that the oncology product was already self-preserving. Unfortunately, the test failed. Worse, the mistake was not caught until after the test failed again. Only then did investigation bring to light the inappropriate method of execution. Though the failed method had been an attempt to succeed under tight timelines, the delay it created was worse. The test had to be repeated yet again with the appropriate handling in order to demonstrate sterility of the product.

8.4.3.2 Case: Membrane vs. Direct Inoculation

Membrane tests can be considered fairly simple compared to the complexities inherent in direct inoculation. Membrane tests are typically performed with commercially available pre-sterilized units so that the product can be poured and filtered directly from the vial into the unit and the unit itself can be incubated. However, for direct inoculation, the incubation vessel is typically pre-sterilized by the lab in preparation for the test. Additional plating manipulations take place at the end of the test.

In one test transfer case, the receiving laboratory sterilized the direct inoculation vial in their laboratory autoclave, as noted in the method. However, they prepared the vial by stoppering and sealing the empty vial in their laminar flow hood. They then placed the vial into the steam heat autoclave and ran their typical 121 °C cycle for 20 min. They fully believed that this would be sufficient to sterilize the receiving vial. After all, this was their validated cycle for all their equipment.

What they failed to appreciate was that in order for the interior of the vial to be sterilized during the steam heat autoclave cycle, water had to be present. So, when the sterility test failed, showing the presence of *B. subtilis*, they believed that the

contamination was present in the final product. The production personnel found this difficult to comprehend because the final product was terminally autoclaved to an F_0 of approximately 40.

A series of experiments in which vials were prepared in the hood, with and without inoculation of *B. subtilis*, proved to the laboratory that their preparation procedures were in error. An appropriate procedure for preparing the direct inoculation vials was developed and implemented in the sterility test method.

8.5 Conclusion

"Murphy" rules in the areas of technology transfer of sterile products from one location to another. Therefore, as the program is moved, both teams of scientists, the originating personnel and the receiving personnel, need to expand their thinking in order to try to anticipate where issues might develop. Potential issues are not limited to the technical challenges of equipment and process. They also develop from the challenges of achieving clarity in language and technique.

As described in the text above, often the main issues that develop center around assumptions and lack of clear concise communication. It may not always be expedient to include every detail in the protocol or in the associated method. In such cases, encouraging the scientists to visit each other's laboratories or facilities can go a long way toward ensuring an efficient transfer on the first try.

In this way, the team can work toward the efficiency of doing it right the first time rather than doing it over again and again.

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Chapter 9
Transfer Across Barrier Systems: A New Source of Simplification in Aseptic Fill and Finish Operations

Benoît Verjans

Abstract With the emergence of barrier systems to isolate the operator from the aseptic fill and finish operations, new technical issues appeared regarding the introduction of material inside the barrier without compromising its integrity. The first transfer systems were complex and were not absent of contamination risks. New technologies have appeared with two major aims: simplify the operations and increase the reliability of the transfer. In this chapter, various transfer systems are identified, their advantages and weaknesses are assessed and finally various case studies are presented to illustrate the selection process which could lead to most appropriate solutions.

Abbreviations

CIP	Clean-in-place
PBT	Polybutylene terephthalate
RABS	Restricted access barrier system
SIP	Steam-in-place
VHP	Vapor hydrogen peroxide
WFI	Water for injection

9.1 Introduction

In the last century, aseptic fill and finish activity has made huge improvement in terms of quality for the patient. Various improvements targeted operator mistakes with the aim to prevent their occurrence significantly and to reduce their impact.

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Among the most famous ones are the introduction of performing automated equipment and the setup of efficient gowning procedures.

Probably the most impactful solution was to separate the operator from the equipment by use of a barrier system. The operator is the major source of contamination because he/she carries a multitude of living organism and he/she can make frequent mistakes such as passing above open vials. Therefore, to avoid these contaminants to penetrate the processing area and to reduce the occurrence and/or the impact of a mistake, a physical separation turned to be extremely powerful to reach such goals. Various barrier systems have been implemented along the time. The first ones were flexible barriers allowing direct intervention of the operator in case of equipment problem. This design allows effective separation of the operator from the filling area when his/her close presence is not required but does not prevent close presence in case of intervention. In the late 1980s, more advanced barrier systems have emerged such as the restricted access barrier systems (RABS) and the isolators (Lysfjord and Porter 2010). In case of isolator, the operator intrusion inside the filling area is completely prevented by security systems such as interlocked doors, … The only way to act inside the barrier is through glove ports.

Isolating the inside of the barrier led to a new issue as the original procedures to enter material were not applicable without compromising the integrity of the isolators. This challenge obliged the pharmaceutical industry to be innovative and to create various solutions of transfer system with not only different concepts but also different aims.

This chapter focuses on the most common solutions for material transfer through barrier. Fill and finish equipment for liquid surrounded by isolator has been taken as an illustrative example. Transfers are frequent for other aseptic applications such as formulation of liquid products, filling of powder products, performance of sterility tests, … The content of this chapter is usually applicable to all these applications with very limited differences.

9.2 Materials to Be Transferred

Various materials must be transferred to ensure optimal fill and finish operations. The most classical ones are:

- Packaging components such as containers, stoppers, caps, and plungers
- Sterile liquids and suspensions to be filled
- Tubing and dispensing needles
- Environmental monitoring equipment and consumables
- Tools for various operations

For each of these categories, some solutions are available to the pharmaceutical industry. Before moving towards the solution, it is necessary to have a clear vision on various material characteristics to select the most appropriate solution. Therefore, the above listed materials must be classified according to the material state, its need

for additional sterilization, and its transfer frequency. The best is to answer to the following three questions:

What is the physical state of the materials to be transferred inside the barrier?

- Liquid such as a solution to be filled
- Suspension such as a solution containing particles to be filled
- Solid such as needle and environmental monitoring tool

What is the suitability of the material to the range of sterilization techniques available?

- Not suitable to any sterilization process: as an example, many vaccines consists in aluminum particle suspension and can therefore neither be autoclaved (leading to antigen destruction or modification) nor be filtrated.
- Suitable for liquid filtration: this filtration, made through a 0.22 μm filter, has been designed to eliminate bacteria from liquid solution. It cannot be claimed as a sterile process but well as an aseptic process as it is always possible that a bacteria can pass through. Nevertheless, such risk is almost eliminated, thanks to the high quality of filters provided and the high sensitivity of the methods to test the filter integrity.
- Suitable for autoclave: many materials can be sterilized efficiently by autoclave such as stoppers and also silicone tubing and stainless tools.
- Suitable for gamma-irradiation: many disposables are made of polymer which can resist to sterilizing dose of gamma-irradiation. This technology is used more and more for multiple materials as it presents the advantage to penetrate in all hidden parts of the materials. Among these materials are container components (e.g., sterile caps), tubing and dispensing needles, various tools such as polymer tweezers, and environmental monitoring consumables such as petri dishes.
- Suitable for ethylene oxide sterilization: this technique can be used for many applications but is less and less common due to rejection of highly toxic wastes.
- Suitable for vapor hydrogen peroxide (VHP) sterilization: VHP sterilization is very efficient but presents the disadvantage that residual hydrogen peroxide should be low enough to have no impact on the product and on the safety of the operators. VHP sterilization is frequently used for sterilization of the external part of wrapping bags and the inside of isolators.
- Suitable for on-line sterilization such as vials to be sterilized in depyrogenation tunnel or syringe tubs to be externally sterilized by e-beam. This last case has been voluntarily excluded as it is a bit out of scope of a pure specific transfer through a barrier.

What is the frequency of transfer operation?

- Single transfer operation (e.g., tubing installed at the beginning of a production batch)
- Few transfer operations (e.g., entry of a second set of containers for a small batch filling)
- Multiple and frequent transfer operations (e.g., primary packaging components such as plungers and sterile caps)

9.3 Methods for Material Transfer

Various methods for material transfer are available on the market according to the three characteristics described above. In this paragraph, the various methods are summarized with their key advantages and weaknesses.

9.3.1 Methods for Solid Transfer

The first group of materials to be transferred is made of the solid materials. The variety of materials to be transferred under solid state by far exceeds the liquid or suspension states. As the materials are more diversified, more solutions are available.

9.3.1.1 Transfer from Large Vessels

Large vessels are used mainly on high speed filling lines to transfer large quantities of material such as stopper and plungers. Vessel size can reach up to 300 L, representing capacities for few hundreds of thousands of components. These vessels are used to clean and sterilize the material before transfer through the barrier.

The principle of the use can be summarized in few steps:

- Unclean and unsterile material is loaded in the vessels.
- Vessels are mounted on the washing and sterilization equipment.
- The equipment is able to perform multiple operations such as washing with water for injection (WFI), steam sterilization, siliconization, drying, and cooling.
- After the material processing, the vessel is closed and transferred to the barrier system.
- The vessel is connected to the barrier either through a rapid transfer port (RTP) using the alpha-beta concept (see Sect. 9.4 for detailed explanation) system or through a pipe which can face clean-in-place (CIP) and/or steam-in-place (SIP) sterilization before transfer.
- Once the port being open, the material is transferred mainly by gravity.

This transfer technology is clearly not very flexible. In addition, it impose heavy investments (the vessel, the CIP-SIP device (when selected), a lift technology to bring the vessel to the port on the barrier, …) and operational activities are also more pronounced than for single-use technologies.

The key advantages of vessels are the limited amount of connection and the low expense per unit when very large quantities of material are required. For example, several pharmaceutical companies are using this technology to prepare and transfer batches of up to 250,000 stoppers, corresponding to the need for approximately 8 h of production on a 36,000 vials/h filling equipment.

Fig. 9.1 DTPE stainless steel containers for transfer of material through various sizes of DTPE RTP (courtesy from Getinge La Calhène)

9.3.1.2 Transfer from Smaller Vessels Through Rapid Transfer Ports

Smaller stainless steel vessels are used to transfer material which can be automatically connected to an RTP. This RTP technology has been initially developed for the nuclear industry which faces the issue to avoid any leakage of contaminating material during transfer. The most famous RTP is from LaCalhene (now Getinge), called DTPE, but several other suppliers are available on the market such as Central Research Laboratories.

These vessels connect to the RTP according to the alpha-beta principle (Lechiffre and Barbault 2010). The vessel (see Fig. 9.1) owns the beta part and connects to a flange with the alpha part. As this connecting technique is very frequently used, a specific paragraph is dedicated later to this concept (see Sect. 9.4).

As an alternative to stainless steel, rigid plastic containers can be used as well but the pharmaceutical industry strongly favors the stainless steel format for its resistance and the opportunity to clean it between multiple uses.

The principle of the transfer is that:

- Material to be transferred, previously cleaned if necessary, are placed in the stainless steel vessel.
- The entire vessel, thanks to its limited size, can be autoclaved for sterilization. To ensure optimal sterilization in the vessel, this one is equipped with vent filters allowing the steam to penetrate and circulate inside the container.
- After sterilization, the vessel is brought to the alpha flange located on the barrier system.
- The vessel is docked. On all systems, there are interlocks to prevent accidental opening of the RTP when the vessel is not properly in place.
- The RTP is open from the inside of the barrier and the material is transferred.

This technology can be used for a wide range of materials such as the needle-tubing assembly or tools to be used inside the barrier such as tweezers.

This technology presents the advantage of being very robust in terms of contamination avoidance during transfer systems. On the other hand, this technology requires investment in autoclave systems which are not only a source of investment expenses but also of operating and validation costs. In regard of the length of an autoclave sterilization cycle, this technology can only be used for planned introduction or for materials stored in sterilized containers.

9.3.1.3 Direct Introduction of Sterile Material

Several materials can be easily supplied as sterile materials. The use of gamma-irradiation offers a wide range of easiness and flexibility for materials which can be gamma-irradiated without facing damages. As an alternative to gamma-irradiation, obtained from cobalt sources most frequently, beta-irradiation is developing.

Beta-irradiation, a beam of electron sent at very high speed on the target to be sterilized, offers the advantage of the absence of radioactive waste as it is generated only from electricity. The weakness of beta-irradiation is its low penetration inside the material hence sterilization units must be extremely powerful to ensure sterilization of a box of material while gamma-irradiation can handle complete pallets.

Once the material is sterilized in protective bags (usually made of a double or triple bag of polyethylene), the sterile bag can be introduced inside the barrier during the setup of the filling equipment. After introduction, the bag will be treated as the rest of the equipment to ensure external sterility. For example, the bag can be wiped or it can face VHP sterilization in an isolator. After sanitization, the bags can be handled by gloves as any other part inside the barrier.

This process is clearly the most simple and the cheapest but it presents the disadvantage that it can only be used for limited quantities of material due to lack of space in the barrier. It is also only applicable to materials which are well defined in terms of quantities at the beginning of process because there will be no additional opportunity to perform such transfer once the line has been fully sanitized and the barrier has been definitively closed. Such transfer is used for materials such as tools and environment control consumables (e.g., contact and sedimentation plates).

9.3.1.4 Introduction by Beta-Bags

The use of RTP is a fast growing process which allows entering a wide range of materials in a fast and flexible way (Zandbergen and Monge 2006). As already briefly addressed in the large and small stainless steel vessel sections, the RTP system is an efficient one which facilitates transfer without imposing complex processing steps.

The RTP concept, as detailed later in Sect. 9.4, consists in a flange located on the barrier (called alpha part) and a docking part (called beta part) which connect to the

Fig. 9.2 Docking of a beta-bag to a BioSafe RTP (courtesy from Sartorius Stedim Biotech)

alpha part to be opened together. As its name indicates, a beta-bag is a beta part consisting in the docking part equipped with a bag. As this bag can be sterilized by irradiation, multiple parts can be introduced such as container components (stopper, plungers), tubing assembly or tools.

The process of such introduction is the following one:

- Prepare all materials to meet specific requirements in terms of cleanness, absence of endotoxins, etc., as required by specifications
- Introduce the material inside the beta-bag. This is usually made through the beta port as the bag is supplied sealed
- Close the port of the beta-bag
- Sterilize the beta-bag and its content by irradiation
- Bring the beta-bag to the barrier as all other materials and equipment
- Connect the beta-bag to the alpha part (see Fig. 9.2)
- Open, through glove ports, the RTP and introduce the content inside the barrier

The key advantage of this system is its flexibility. Transfer may occur at any time during the process according to requirements and as frequently as required. This solution is also applicable for materials which do not resist to steam sterilization but well to irradiation, on the contrary of the small vessel system.

There are limited amount of suppliers of beta-bag and corresponding RTPs. The most famous ones are Getinge, Central Research Laboratories, ACE, Ingenia, Ostermeier, and Sartorius Stedim Biotech. The five first companies offer RTP which functions on the basis of a rotation system engaging the interlock. This system is compatible with small stainless steel vessel connection and allows multiple opening–closing (Lechiffre and Barbault 2010). Sartorius Stedim Biotech system is based on an electromagnetic interaction which allows only a single connection and is not compatible with stainless steel vessel (Zandbergen and Monge 2006).

Fig. 9.3 VHP airlock located next to an isolator (courtesy from Skan AG)

The Sartorius Stedim Biotech beta-bag is significantly cheaper than other systems but the installation of the alpha flange is more expensive due to the electromagnetic system. Note that some suppliers of container components such as Becton-Dickinson and Aseptic Technologies offer them pre-packed and sterile in multiple types of beta-bags.

Several applications are also using beta-bags to exit material from the barrier such as waste and environmental monitoring consumables after sample collection. By this way, the exit is done in a safe way which does not put at risk the isolator integrity.

9.3.1.5 Transfer Through VHP Airlock

Recently, Skan has developed the concept of VHP airlock for transfer of material (Fig. 9.3). This airlock is equipped with two doors, one exiting in the clean room surrounding the filling equipment and the other opening to the inside of the barrier system. The concept is that the material, usually in sterile bag, is introduced inside the

airlock through the external door and then sterilized by VHP sanitization inside the airlock. After venting of the hydrogen peroxide, the airlock is opened at the level of the door going to the barrier to introduce the materials (Sigwarth and Vanhecke 2011).

The process to follow is the following one:

- The material is cleaned according to specifications
- The material is placed in polyethylene bag (optional)
- The bag is sterilized by gamma-irradiation or another technology
- The material is brought to the filling room
- The material is introduced in the airlock
- The sanitization cycle is activated
- The inside door is opened and the material is introduced inside the barrier

The key advantages of this technology are its flexibility as you can introduce multiple elements at the same time and its price as this solution is cheaper compared to RTP technology in terms of operating costs. You have also the flexibility to introduce only what is needed without having to connect a complete beta-bag of material. This technology is suitable for almost all types of materials whereas other technologies have limited applications regarding material to transfer.

Its weaknesses are its investment cost and the length of introduction process. Investments is significantly higher than RTP but can be rapidly compensated by lower operating costs in case of intensive use. The introduction process takes some time to be completed due to the VHP cycle and the venting phase taking place after ensuring the absence of too high concentration of oxidizing hydrogen peroxide when the internal door is opened. A complete cycle usually requires about 20 min, imposing efficient planning for introduction to avoid process stops. Some companies have optimized the cycle to be below 10 min, increasing the flexibility of the system.

9.3.2 Methods for Liquid Solution and Suspension Transfer

Liquid transfer is significantly riskier than transfer of solid material. The main reason is that a contaminant may be easily captured by the liquid and end inside the filled container, leading to contaminated product.

Until recently, the only robust liquid transfer technology was based on a CIP process followed by a SIP process. In the last years, technologies have been developed according to the alpha-beta principle and these will be detailed here after.

Another important aspect to keep in mind is that the best practices recommend filtering a liquid through a 0.22 μm filter the latest as possible before filling. Usually it is recommended placing that filter before the pump to avoid interferences with the pump precision. The best place for the filter, based on comments above, is to locate it inside the barrier between the connection passing across the barrier wall and the pump.

9.3.2.1 CIP-SIP Transfer

This method was designed for transfer of liquid when the first barrier systems have been designed. The concept is that a tube is passing across the barrier and connections are made on both sides. Before making the fluid passing through, the tubing and all connections are first cleaned with WFI and then sterilized with steam. By doing so, the risk of contamination in the tubing is eliminated.

This connection system is extremely demanding in terms of operational work and time, requires significant investment and imposes the presence of source of WFI and cleaned steam. All these complexities have created a demand from the pharmaceutical for more simple but still robust transfer systems.

9.3.2.2 RTP Transfer with Beta-Bag

To get rid of the complex CIP-SIP method, Sartorius Stedim Biotech developed the first easy connection system for fluid transfer called RAFT (rapid aseptic fluid transfer). RAFT is a beta-bag with a wrapped tubing entering inside through the polyethylene bag. The external part of the tubing is connected to the liquid container. Once being connected to the alpha flange, the port is open and the tubing is brought inside the barrier. At that time the tubing is connected to the manifold and the fluid transfer system is operational without need of a CIP-SIP process (Zandbergen and Monge 2006).

Recently a similar system has been developed by Getinge, based on the well-established DPTE RTP. They introduced the concept that the fully assembled tubing with needles can be supplied inside the bag. This new design eliminates the connection performed in class ISO5 environment which is of minimal risk. This fully assembled solution is also offered by Sartorius Stedim Biotech since then.

The two systems differ mainly by the fact that the Sartorius Stedim Biotech port is a pure single use system which does not allow disconnection and reconnection whereas the Getinge system allows multiple opening and closing.

9.3.2.3 RTP Transfer with Designed Connectors

The initial RAFT system and the similar Getinge systems present the disadvantages of being large, creating space and handling issues, and presenting the risk of contamination if an undetected piercing affects the bag, hence a risk of contamination. The third but much less important issue is that the ring of concern (see Sect. 9.4 for more in-depth explanation) is quite large with circumferences which can exceed 300 mm.

These three issues led Aseptic Technologies and GSK Biologicals to develop a new connector system commercialized by Sartorius-Stedim under the brand name SART. The principle is that a polymer connector is connected to the tubing at the end of the container. This connector is made of two parts: the body which allows the

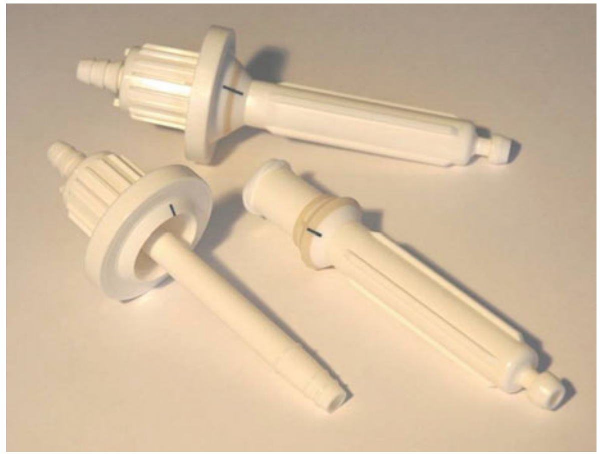

Fig. 9.4 SART connector presented as fully assembled (*top*) and with the cover withdrawn from the body and the tubing (*bottom*)

passage of the liquid through a tube and the cover which protects the tubing until use. Both molded parts are made of polybutylene terephthalate (PBT), the cover being equipped with an over-molded flexible joint of santoprene (Verjans et al. 2007).

The Fig. 9.4 illustrates the complete connectors and its two parts separated. This connector is also functioning on the alpha-beta principle which will be detailed in Sect. 9.4. The connector is assembled and closed very precisely and a pressure test is performed to verify closure integrity. To ensure sterility of the entire connector, including all internal surfaces which cannot be reached by other techniques, it must be sterilized by gamma-irradiation at a certain point of time before use. Two options are feasible: either the connector is assembled on a container to be sterilized by gamma-irradiation (e.g., a flexible pouch) or on a container to be sterilized by steam (e.g., a stainless steel vessel).

In the case of the container to be sterilized by gamma-irradiation, the simplest way of working is to assemble a non-sterilized connector and to sterilize the complete assembly.

In the case of the container to be steam-sterilized, the connector must be pre-sterilized by gamma-irradiation and then assembled. A second sterilization by steam can be run. To validate that steam is going properly to the end of the connector, a vent filter must be located close to the connector, allowing the steam to circulate in the tubing and pulse of overpressure and vacuum must be performed first to push the steam inside the connector and second to dry it.

Some companies using the connector in combination with container to be steam-sterilized have taken the decision to avoid steam-sterilization of the connector. Therefore, they combine both techniques by using a rapid connection system (e.g., Lynx from Millipore, Kleenpak from Pall, or Opta from Sartorius Stedim Biotech) or a tube-to-tube welding system (such as BioWelder from Sartorius Stedim Biotech).

The connection is very simple:

- The connector is introduced inside the port and secured with clamps. This introduction liberates the interlock which prevented port rotation and opening

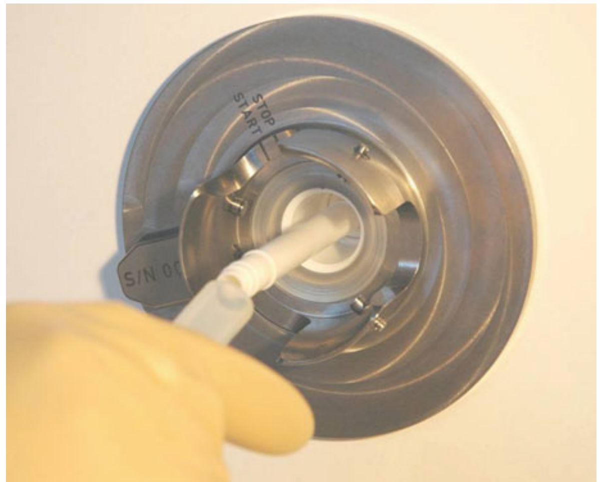

Fig. 9.5 Silicone tubing connection to a SART connector placed inside its RTP

- From the inside of the barrier system, a lever is moved to clamp the connector cover
- Then the port is open by rotation
- Once the connector tubing has been freed inside the ISO5 environment, the flexible tubing, already inside the barrier, is placed manually (Fig. 9.5)

The key advantages of the SART technology are:

- The connection is performed very rapidly (few seconds)
- The device is much less expensive than any other solution on the market
- The device is much lighter (only a bit more than 50 g) and take much less space than any other solution on the market
- The device can be closed and reopened several times if needed

The fact that the connector can be closed and opened several times has beneficial effect for multiple applications. Some examples are illustrated below:

- A complete batch of API has been connected to a filling line and after a short period of operation, the equipment faces a major breakdown. Thanks to the reclosure and future reopening capabilities, the bulk can be disconnected and stored back in the right conditions until the problem is fixed.
- Several products cannot be sterilized either by terminal sterilization or by filtration such as vaccines containing aluminum particles. In such case, it makes a lot of sense to perform the formulation work in barrier systems to avoid contamination. The formulated product must be transferred to containers until use for filling. Transfer through connectors which can be safely disassembled and closed is critical for such products, even if it is recommended to use a second connector later on for transfer to the filling line.

Fig. 9.6 Stainless steel transfer system for liquid (courtesy from Getinge La Calhène)

Similar technologies have been developed by Getinge, for example, based on their existing RTP alpha ports as discussed above for solid transfer. The connector is based on a stainless steel container as illustrated in Fig. 9.6. The advantage is that a single alpha port can be used to introduce multiple materials and liquids but the connector is much bigger, heavier, and more expensive as a cleaning procedure must be applied at the end of each transfer.

9.3.2.4 The Specific Case of Suspensions

Particle suspensions such as vaccines (frequently containing aluminum particles) have the additional issue that they can face sedimentation in case of interruption of liquid flow. Sedimentation may lead to variable distribution of particle content and therefore of administered dose to the patient. To avoid this, a circulation loop is set up to bring back to the main container all the suspension which has not been dispensed. To make this loop as efficient as possible, the collection of the dose to be dispensed should be made as close as possible to the filling needle. Nevertheless, this must be done before the dispensing pump. As the pump head is located inside the barrier, the loop should penetrate inside the barrier and exit to return back to the main container. To address this, two connections should be performed, one "IN" and one "OUT." Having a double connection system is significantly increasing the impact of various disadvantages such as the handling difficulties, the risk of piercing and the investment and operating costs. Therefore, a low cost and easy-to-handle solution such as the SART connector appears to be even more beneficial than for a single connection.

9.4 The Alpha-Beta Concept

In the above paragraphs, several transfer technologies have been referred as being based on the alpha-beta concept. This concept has been a radical change in the approach of transfer and have eliminated multiple of inefficient and risky transfer procedures which were used in the first ages of the pharmaceutical industry. Therefore it is highly valuable to address a complete paragraph to detail that concept further.

The alpha-beta concept was initially developed for the nuclear industry which is facing major issues to transfer highly radioactive materials from one area to another. The risk attached to radioactivity was and is still a concern for the safety of the operators and therefore any transfer performed should be robust and should not lead to loss of material during transfer.

This alpha-beta concept has been rapidly identified as highly valuable for the pharmaceutical industry because the objective is different but the need is the same. Instead of preventing loss during transfer, the pharmaceutical industry needs to prevent entry during transfer. Therefore, both industries require a closed tight system during transfer operation without exposing any internal part to the external environment and vice versa (Lechiffre and Barbault 2010).

The alpha-beta technology consists in four “V-points” coming into contact with each other at various stages of the process. As exposed schematically in the Fig. 9.7 using the SART connector, we can see that the “V-points” belong either to the port (=alpha part, the part fixed on the barrier system) or to the connector (=beta part, the part which is mobile and to be connected). Two of the “V-points” must be rigid and

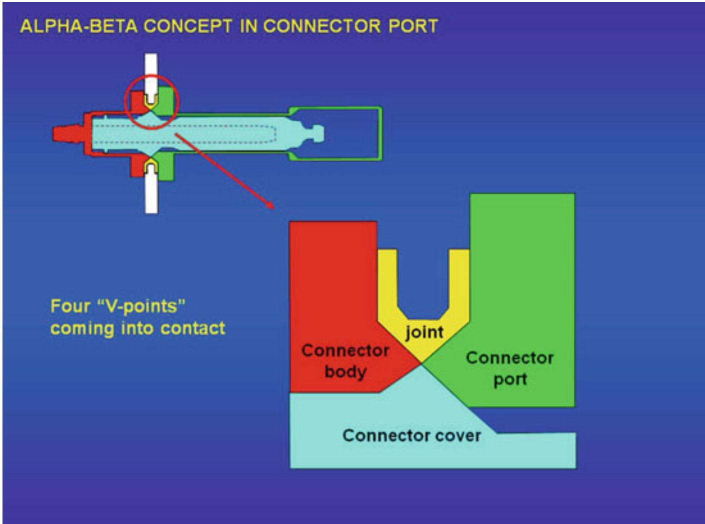

Fig. 9.7 Schematic design of the four “V-points” of an alpha-beta concept

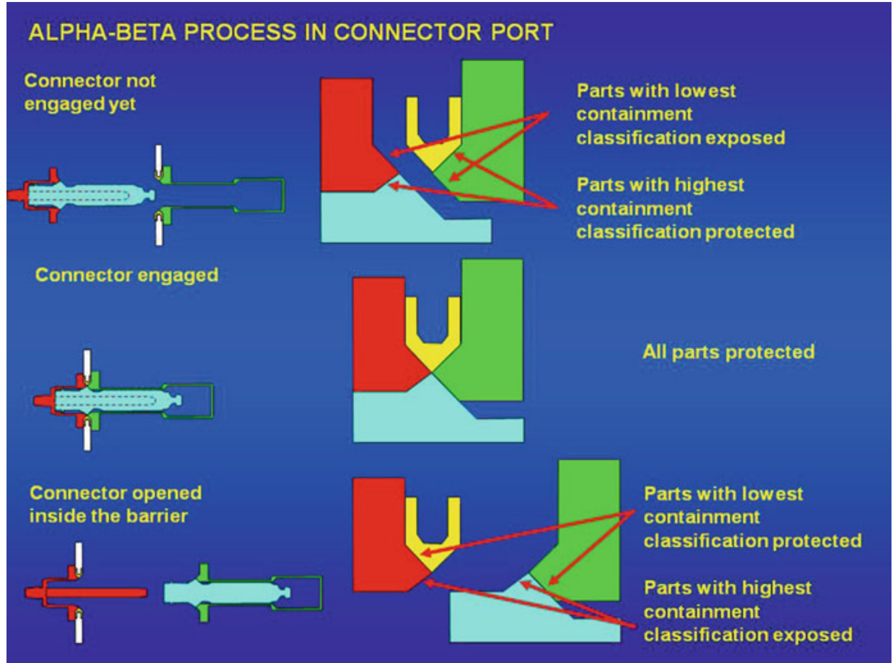

Fig. 9.8 Exposed surfaces during alpha-beta process

two must be flexible in order to obtained tight contact between the "V-points." In the Fig. 9.7, the flexible "V points" are the joint (made of silicone) and the connector cover (equipped with a santoprene joint) whereas the solid "V points" are the connector body (made of PBT) and the connector port (made of stainless steel).

The various steps of the process are illustrated in the Fig. 9.8. Before entering the connector inside the port, only the external surfaces of the connector and of the port are exposed to the environment with the lower quality (e.g., ISO8 environment). When the connector is engaged, all parts which have been exposed to that external environment are into tight contact. Therefore, all contaminants which can be on these parts are entrapped between both surfaces and cannot be released. When the port is opened, only the surfaces which were previously protected are exposed to the environment inside the barrier, i.e., the ISO5 environment. These surfaces were previously either sanitized during line sanitization or sterilized during connector gamma-irradiation.

This technology presents a weakness which is the line of confidence. This line, consisting of the tip of all "V-points" cannot be in a "so-perfect micrometric" tight contact that absence of bacteria is guaranteeing. Therefore it is critical to sanitize by wiping this line just before use to reduce the presence of bacteria and to minimize it by design. For example, if we compare a DTPE from Getinge which has a diameter of 105 mm for the smallest format, the SART connector (diameter of 30 mm) represents a reduction of the risk by a factor of 3.5.

This line of confidence is well accepted by all regulatory authorities because the alpha-beta technology brings such a quality improvement for the transfer of material compared to the classical technologies. All risk analyses performed have shown that alpha-beta transfer systems are very robust and minimize the impact of multiple sources of risks mainly linked to operator presence and mistakes.

9.5 Regulatory Constraints and Validation Process

All the transfers described above have already been approved by authorities for commercial production of pharmaceutical drug batches. Therefore, the risk of non-acceptance by the authorities should not be considered as a major element to reject a solution compared to the others. Nevertheless, to ensure a smooth approval process, the following approaches must be conducted by the pharmaceutical company:

- Perform a risk analysis on the transfer process in relation with the overall production process. The main risks must be addressed to reduce their impacts and/or occurrences.
- Set up clear procedures and perform appropriate training for the operators to ensure that the transfer process is well under control and that the risks have been minimized.
- Have full control of the quality of the transfer system. This is quite broad but include among others:
  - Maintenance of the transfer system (e.g., control of joints of an RTP port and preventive replacement according to a defined plan)
  - Full control of suppliers of consumables (e.g., audit plan to ensure that appropriate production processes are respected and under control)
  - Full quality control of the transfer process (e.g., quality control of the WFI used for a CIP-SIP process)
  - Integration in the quality assurance of a part dedicated to the transfer process (e.g., records of respected procedures)

The validation of a transfer system is highly variable as it depends on the transfer system itself. In case of the installation of an equipment such as an RTP port on the barrier, it is mandatory that a complete validation plan including IQ/OQ and PQ is performed. It is obvious that the transfer procedure must be covered in all media fill performed to validate the overall equipment.

9.6 How to Select the Most Appropriate Transfer System

In this last paragraph, various case studies have been elaborated to point out what could be the best solutions to be implemented. First, it is useful to have a good picture of the most common transfer systems with their application, strengths, and weaknesses as per the above paragraphs. This overview is provided in Tables 9.1 and 9.2.

Table 9.1 Overview of transfer systems for solid material

	Most suitable for				Sterilization technique			
Transfer system	Packaging components	Tubing assembly	Environmental monitoring	Tools	Autoclave	Irradiation	Key advantages	Key disadvantages
Large stainless steel vessels	X				X		Transfer of large quantities Compatible with cleaning systems	Very significant investment costs Use limited packaging components
Small stainless steel vessels	X	X		X	X		Flexible solution to introduce material to be sanitized by autoclave Limited operating expenses Robust and reliable technology approved by authorities	Investment costs in ports and containers Low flexibility for unplanned material
Direct introduction	X	X	X	X	X	X	Cheapest solution Applicable to all materials	Operator-dependent Significant risk of mistake Not feasible after closing of the doors
Beta-bag	X	X		X		X	Flexible solution to introduce material to be sanitized by irradiation Robust and reliable technology approved by authorities	Significant operating expenses Optimization of content to be done to minimize operating expenses

(continued)

Table 9.1 (continued)

Transfer system	Most suitable for				Sterilization technique		Key advantages	Key disadvantages
	Packaging components	Tubing assembly	Environmental monitoring	Tools	Autoclave	Irradiation		
VHP airlock	X	X	X	X	X	X	Very robust technique Highly flexible quantities of material to enter Applicable to all materials	Significant investment expenses Planning of introduction to be carefully prepared and checked due to process time

Table 9.2 Overview of transfer systems for liquid and suspension solution

Transfer system	Key advantage	Key disadvantage
CIP-SIP	No source of supply to be controlled after installation	High investment and operating expenses Validation work to be done continuously
Beta-bag RAFT	Suitable for fully assembled tubing and needles Robust technology Opportunity to use same port as for solid transfer	Risk of bag damage Expensive solution Volume of bag making handling difficult
Beta-bag DPTE	Suitable for fully assembled tubing and needles Robust technology Possibility to disconnect Opportunity to use same port as for solid transfer	Risk of bag damage More expensive solution Volume of bag making handling difficult
Designed connector: SART	Robust technology Less expensive solution Light and small solution Few opening/closing possible	Specific port required (not compatible with solid transfer) No opportunity to enter the fully assembled tubing without making a manual connection
Designed connector compatible with RTP for solid transfer	Robust technology Possibility to disconnect Opportunity to use same port as for solid transfer	No opportunity to enter the fully assembled tubing Expensive solution Very difficult to handle due to size and weight

These two tables provide a good overview of the factors which can drive a decision to select one system or another one. To go in more details, three case studies have been made and decision processes have been highlighted. For all of them, the proposed solution is one solution among many other possibilities. Each setup should be optimized according to multiple other constraints and capabilities (e.g., supply chain security) which were not addressed in this paragraph.

9.6.1 Case Study 1: Lab Scale Isolator to Fill Multiple Research Batches of Potent Drugs

Description: a research lab active in cytotoxic drugs performs frequently multiple filling of small batches (few hundreds of vials) of pharmaceutical products to be used for preliminary animal tests. The GMP must not be respected as it is for research purpose but there is a significant risk for the operator. High flexibility and low risk of cross-contamination are mandatory.

The characteristics of the process are:

- Size of barrier equipment: very small barrier system.
- Process design: not well defined, requires high flexibility.
- Solid material to introduce: multiple tubing, packaging components, and tools.
- Liquid material to introduce: multiple small quantities, almost no API should be lost during process, API is sensitive to VHP, cross-contamination must be avoided.
- Material to exit: many, including filled containers and waste after completion of each production.

The main issue in this case study is the flexibility for both entry and exit of material. In case of single batch, entry of all materials during sanitization could be envisaged but, in the present case, this option must be rejected.

A second issue is the fact that API is a scarce resource and therefore obliges to transfer all the liquid without losing it inside a connector or the tubing.

A potential solution could be:

- Use of a VHP airlock allowing entry of packaging components, assembled tubing and tools whenever needed. The VHP airlock will be used to exit the filled containers but of course without performing a VHP sanitization cycle as it is not necessary in such case.
- Use of a SART connector to transfer liquid completely to a pouch located inside the barrier and being part of the assembled tubing. By this way, all the liquid may be transferred inside the isolator without facing loss inside the tubing and the connector.
- Use of an RTP port to exit all waste in a closed and secured beta-bag. This bag can be directly incinerated to destroy the toxic waste.

In this case, three transfer systems have been selected to ensure that all transfer in or out of the isolator can be done properly.

9.6.2 Case Study 2: Small Production Scale Isolator to Fill a Niche Biological Drug in Prefilled Syringes

Description: a GMP facility performs filling of small batches (few thousands of syringes) of a single biological drug to be commercialized. The GMP must be respected and change-over is not an issue as each batch will be produced in complete independence from the previous and the next ones.

The characteristics of the process are:

- Size of barrier equipment: medium size with enough space to install multiple transfer systems.
- Process design: fully defined, no flexibility required.
- Solid material to introduce: single tubing, few defined tools, and significant quantities of plungers to be introduced.

- Liquid material to introduce: small quantities, almost no API should be lost during process, API is sensitive to VHP.
- Material to exit: none, except syringes exiting through a mouse hole. All the rest is taken out at the end of the process.

The main issue in this case study is the cost of the API and all precautions must be taken to avoid loss of API.

A potential solution could be:

- Use of a first RTP with stainless steel container for tools and needle assembly. This RTP is located close to the pump. This RTP should not be permanently occupied for any other application as a second tubing assembly transfer can be mandatory in case of damage to the first one.
- Use of a second RTP to bring the plungers. This RTP is located close to the sorting bowl of the plungers.
- Use of a SART connector to transfer liquid completely to a pouch located inside the barrier and being part of the assembled tubing for the same reasons as explained above.

Again, three transfer systems have been selected. The RTP may be different. If the first one should be compatible with small stainless steel vessel, the second one may be of any type.

9.6.3 Case Study 3: Large Production Scale Isolator to Fill Aluminum Particle Vaccine in Vials

Description: A GMP facility performs filling of large batches (several hundreds of thousands of vials) of a vaccine to be commercialized. This vaccine is a suspension and sedimentation must be avoided in case of short stop of the filling equipment. The GMP must be respected and change-over is not an issue as each batch will be produced in complete independence from the previous and the next ones. As it is a modern design to respect the latest authority requirements, the capping has been located inside the barrier. Therefore, transfers of materials are very frequent as hundreds of thousands of container parts are required (both stoppers and caps)

The characteristics of the process are:

- Size of barrier equipment: large size with enough space to install multiple transfer systems
- Process design: fully defined, no flexibility required
- Solid material to introduce: single tubing assembly, few defined tools but very large quantities of stoppers and caps
- Liquid material to introduce: large quantities, recirculation loop mandatory to avoid sedimentation in case of short stop
- Material to exit: none, vials exit through a mouse hole, all the rest is taken out at the end of the process

The main issue in this case study is that transfers are very consequent with hundreds of liters liquid suspension which impose a recirculation process. Hundreds of thousands of stoppers and caps must be transferred as well.

A potential solution could be:

- Use of a first RTP with stainless steel container for tools and needle assembly. This RTP is located close to the pump. This RTP should not be permanently occupied for any other application as a second tubing assembly transfer can be mandatory in case of damage to the first one.
- Use of a second RTP to bring the stoppers from large stainless steel vessels. This RTP is located close to the sorting bowl of the stoppers. As stopper transfer is mainly done by gravity, a lift is required to reach sufficient height.
- A third RTP will be used to bring caps which have been sterilized by ethylene oxide in beta-bags. This RTP will be located close to the cap sorting bowl.
- Use of two SART connectors (“IN” and “OUT”) to set up an API circulation loop. Coming as close as possible to the pumps.

In such case, the use of large vessels makes sense as the quantities of stoppers by far exceed what is financially reasonable to do with sterile material in beta-bags.

9.7 Conclusions

In this chapter, it has been highlighted that the setup of a transfer system is not something basic. First it is mandatory to define all the materials to be transferred, second the transfer conditions such as frequency must be carefully assessed and then the various solutions must be evaluated according to their respective advantages and disadvantages.

If this process is carefully respected, it is possible to set up the solution which will minimize the risk of mistake and therefore batch rejection, be the most cost-effective and ensure a smooth process for transfer before, during, and after production.

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Chapter 10
Challenges and Innovation in Aseptic Filling: Case Study with the Closed Vial Technology

Benoît Verjans

Abstract Aseptic filling of injectable drugs is one of the most critical manufacturing processes in the pharmaceutical manufacturing industry as it presents a significant risk for the patient in case of bacterial contamination. Therefore, a series of constraints are imposed to the manufacturers, leading to a very complex process requiring intensive validation, training and care during operation. To optimize this process, several improvements have been made, focusing on (1) the design of pharmaceutical facilities to ensure an optimal environment for the filling area, (2) optimal gowning of operator, and (3) separation of the operators from the filling area. Regarding containers, the pharmaceutical industry disposes of a wide range of solutions to aseptically fill injectable products. The most classical ones such as ampoules and vials are now challenged by new technologies which offer several advantages to the pharmaceutical manufacturer, to the healthcare practitioner and/or to the patient. Four recent technologies came to the market in the last decades, all of them with their own profile of advantages: on one side, the prefilled syringe and the cartridge which provide a ready-to-inject solution to the practitioner and the patient; on the other side, the blow-fill-seal container and the Closed Vial technology which offer a safer solution and an easier solution for the manufacturer. In this article, these new solutions are compared to the well-established ones and their profiles of advantages and disadvantages are detailed.

Abbreviations

API Active pharmaceutical ingredient
BFS Blow-fill-seal
cfu Colony forming unit

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CMO	Contract manufacturing organization
COC	Cycloolefin copolymer
COP	Cycloolefin polymer
EMA	European Medicine Agency
EPO	Erythropoietin
FDA	Food and Drug Administration
OEL	Overall exposure limit
PFS	Prefilled syringe
RABS	Restricted Access Barrier System
SOP	Standard operating procedures
TPE	Thermoplastic elastomer
VHP	Vapor hydrogen peroxide
WFI	Water for injection

10.1 Introduction

In the early ages of the pharmaceutical industry, the need to administer products by injection became rapidly obvious. Injection is critical for several drugs which cannot be absorbed through the classical administration path such as the gut or topically on the skin. Another critical need was the administration of drugs to patients in critical condition and being unable to swallow a pill or a liquid.

The initial fill and finish technology was based on the concept of terminal sterilization, consisting in the filling of the drug without major protection followed by a steam sterilization of the container and its content. In case of accidental introduction of bacteria, these would be killed during the sterilization process. This technology appeared quite rapidly inappropriate for many products which are susceptible to heat degradation. The aseptic filling is recently booming with the development of the biological drugs, vaccines, and some chemical product categories such as cytotoxic drugs (Agalloco and Ackers 2010; Lysfjord 2009a).

Aseptic filling presents a significant additional risk over terminal sterilization as any bacteria entering in the container cannot be killed by heat later and is a major source of potential contamination depending on growth speed. Authorities and pharmaceutical companies realized that this risk must be properly addressed and therefore a wide range of improvements have been implemented, including use of preservatives and improvement of filling operations, to prevent entry of a contaminant.

Preservatives are chemical molecules with bacteriostatic properties. In presence of such chemical, bacterial growth is inhibited and the patient does not face injection of a major quantity of bacteria. Until early 2000s, this chemical approach was widely used but recently preservatives, including thimerosal, have been suspected to generate side effects to patients despite being never clearly proven. As a precaution, in 2002, many governmental organizations, including the Center for Disease Control and the Food and Drug Administration (FDA), recommended to withdraw

thimerosal from injectable drugs to young children and pregnant women (http://www.cfsan.fda.gov/~lrd/tphgfish.html).

This initial recommendation triggered a trend to avoid preservatives from many products, in particular vaccines which are frequently administered to young children. It created also a need to significantly improve operations as the last safety barrier in case of contamination has been eliminated.

Among the most radical changes to improve quality are (1) new gowning procedures, (2) clean rooms with higher quality and more stringent environment monitoring, and (3) the separation of operators from the filling area by barriers.

An effort was also placed on container design to better meet the expectations of the users, i.e., mainly a better quality and an ease of use for administration. The initial container was the ampoule which appeared at that time to be the easiest container to fill and close. To address the risk of ampoule breakage, the vial developed quite rapidly but imposed the use of stoppers to close the container and to allow product collection.

More recently, several technologies have emerged to improve (1) the ease of administration such as the prefilled syringes (PFS) and cartridges, PFS being usually more dedicated to administration by a healthcare practitioner whereas cartridges being often self-administered by the patient with auto-injectors and (2) the quality and the ease of manufacturing such as the blow-fill-seal (BFS) containers and the Closed Vial technology.

In this article, the most important requirements to ensure optimal quality of aseptic filling and the various containers available will be addressed in detail. A specific case study addresses the Closed Vial technology as it is the most recent technology on the market (Verjans et al. 2005).

10.2 Aseptic Filling Requirements and Sources of Improvements

Aseptic filling is significantly risky for patients as a single bacteria inside a container may proliferate and therefore leads to a major contamination with frequent fatal issue. The main requirements from all pharmacopeias to minimize such dramatic effect can be summarized in one sentence: *A product should be filled in a high quality and monitored environment by properly trained and gowned operators*. The focus of the authorities has been permanent during the last decades to improve the fill and finish processes according to this sentence. The main outcomes are described below.

10.2.1 High Quality Environment

The environment required is variable according to the fill and finish equipment performances, especially regarding the separation of the filling area from the surrounding environment. The Sect. 10.3 is dedicated to the barrier systems used to separate the

operators from the filling area. Various denominations for room classification have been used by the FDA and by the European Medicine Agency (EMA). Some minor differences exist between both classifications, hence the creation of a Class ISO definition which starts to be used throughout the world.

Currently, the European requirements are more stringent because monitoring of living organism in the air should be performed according to a wider range of tests compared to US authority requirements. In the European definition, the concept of "at rest" and "in operation" is also used. Usually, the quality at rest should be much more stringent than the in operation mode. Table 10.1 illustrates a comparison between the European (Guide to Good Manufacturing Practices—Manufacture of Sterile Medicinal Products), the US (USP 797) and the ISO (14644–1) requirements (Mackler 2004).

Due to this highest stringency, the European example is used along this paragraph and the Annex 1 of the Eudralex is taken as main source of reference (Eudralex 2008). To facilitate the reading below, only the ISO definition will be used to avoid repeating the three classifications. It should be understood, unless stated as different, that it represents all three similar classifications.

The authorities agree usually on the following classification:

- ISO5 environment is mandatory for the filling area where are located the exposed containers, the exposed needle, and the exposed solution.
- In case of use of a barrier which can be crossed by the operator such as flexible walls or Restricted Access Barrier Systems (RABS), an ISO7 surrounding around the equipment is required. According to the filling technology used, this rule is not always applicable. For example, BFS is accepted by the European authorities to be located in a Grade C clean room as the safety of the filling is strongly increased compared to open containers.
- In case of overpressured barrier such as isolator, an ISO8 surrounding is sufficient. Some companies have successfully validated such equipment in ISO9 environment after conducting a robust risk analysis showing that the ISO5 filling area is not compromised by design.
- Recently, the European authorities have required performing capping of vials under Grade A air supply. Previously, it was done in ISO8 environment. The main driver for such a decision was that a stopper is not secured until being capped and therefore a bacteria may enter by accident inside the vial before capping. For the pharmaceutical industry, this is the most important difference between the European and most other authorities regarding vial fill and finish operations.
- All other operations such as washing, preparation, particle inspection, labeling, packaging, … are accepted in ISO9 clean room.

10.2.2 Monitored Environment

Environment must also be heavily monitored to ensure that the environment quality is not compromised during operation. These requirements are applicable for the

Table 10.1 Comparison between the different clean room classification standards, data correspond to acceptable limits without action required

		European standard	US standard	ISO standard
Grade A/Class 100/ISO5	Nonviable particle			
	• ≥0.5 μm	3,500 particles/m^3	100 particles/ft^3	3,520 particles/m^3
	• ≥5 μm	0 particles/m^3	NA	29 particles/m^3
	Viable particle in air volume	<1 cfu/m^3	<1 cfu/m^3	<1 cfu/m^3
	Contact plate (55 mm)	<1 cfu/contact plate	<3 cfu/contact plate	<3 cfu/m^3
	Settle plates (90 mm)	<1 cfu/plate/4 h	optional	optional
	Glove print (five fingers)	<1 cfu/glove print	<3 cfu/glove print	<3 cfu/glove print
Grade B/Class 10,000/ISO7	Nonviable particle in operation			
	• ≥0.5 μm	350,000 particles/m^3	10,000 particles/ft^3	352,000 particles/m^3
	• ≥5 μm	2,000 particles/m^3	70 particles/ft^3	2,930 particles/m^3
	Nonviable particle at rest			
	• ≥0.5 μm	3,500 particles/m^3		
	• ≥5 μm	0 particles/m^3		
	Viable particle in air volume	10 cfu/m^3	<10 cfu/m^3	<20 cfu/m^3
	Contact plate (55 mm)	5 cfu/contact plate	<5 cfu/contact plate	<10 cfu/m^3
	Settle plates (90 mm)	5 cfu/plate	Optional	Optional
	Glove print (five fingers)	5 cfu/glove print	NA	NA
Grade C/Class 100,000/ISO8	Nonviable particle in operation			
	• ≥0.5 μm	3,500,000 particles/m^3	100,000 particles/ft^3	3,520,000 particles/m^3
	• ≥5 μm	20,000 particles/m^3	700 particles/ft^3	29,300 particles/m^3
	Nonviable particle at rest			
	• ≥0.5 μm	350,000 particles/m^3		
	• ≥5 μm	2,000 particles/m^3		
	Viable particle in air volume	100 cfu/m^3	<100 cfu/m^3	<100 cfu/m^3
	Contact plate (55 mm)	25 cfu/contact plate	<100 cfu/contact plate	NA
	Settle plates (90 mm)	50 cfu/plate	NA	NA
	Glove print (five fingers)	NA	NA	NA

(continued)

Table 10.1 (continued)

		European standard	US standard	ISO standard
Grade D/ISO9	Nonviable particle in operation			
	• ≥0.5 µm	NA	NA	35,200,000 particles/m^3
	• ≥5 µm	NA		293,000 particles/m^3
	Nonviable particle at rest			
	• ≥0.5 µm	3,500,000 particles/m^3		
	• ≥5 µm	20,000 particles/m^3		
	Viable particle in air volume	200 cfu/m^3*	NA	NA
	Contact plate (55 mm)	50 cfu/contact plate*	NA	NA
	Settle plates (90 mm)	100 cfu/plate*	NA	NA
	Glove print (five fingers)	NA	NA	NA

filling area but also for the rooms surrounding the equipment and to all rooms which are classified. According to Eudralex, the following monitoring for a Grade A area must be conducted:

- Total particle monitoring: samples of one cubic feet must be collected every minute and the acceptable limit is less than 100 particles bigger than 0.5 μm and less than one particle bigger than 5 μm. The amount of sampling position is function of the size of the Grade A area and of the risk analysis conducted.
- Viable particle monitored per cubic meter of air: less than one colony forming unit (cfu) per collected sample.
- Viable particle collected by sedimentation on petri dishes (90 mm): less than 1 cfu per plate.
- Contact plates (55 mm): less than 1 cfu per plate.
- Glove print: less than 1 cfu per glove print (five fingers).

Performance of such monitoring activities implies that the fill and finish equipment must be designed to allow installation of all monitoring tools and devices. The location of the monitoring tools should be defined according to a risk analysis. Usually, the most critical areas are the filling area and the areas where contact parts are exposed (e.g., close to plunger or stopper bowls).

Monitoring is also required for other environment such as ISO7 and ISO8 environment but at a lower frequency and with less stringent requirement (see Table 10.1).

10.2.3 Properly Trained Operators

The operators must be well aware of the standard operating procedures (SOP), must understand the reasons behind these procedures and must follow them. Regular trainings are expected from the authorities to ensure that bad practices are corrected and do not become standard practices.

The SOP list must also be complete, meaning that there is no significant activity left at complete freedom of operators.

Not only the operators must be trained but also all the people who could be in contact with the production area. As an example, operators are usually well trained to avoid presence in the filling area because it is a key aspect of his final objective to produce clean products. Let's now imagine a maintenance technician who has been asked to realign parts which are misaligned. His only objective is to bring back the equipment in a production state as soon as possible. Therefore the risk of contamination of the filling area is higher because the technician will, e.g., puts his head inside the line to check visually the alignment. By doing so, the technician applies the best method to meet his objective (effective corrective action) but not to restart production in appropriate condition.

10.2.4 Properly Gowned Operators

Operator gowning was very rapidly a main focus for aseptic filling requirements. As almost all contamination accidents can be related to the operator, his/her behavior and his/her mistakes, it was critical to eliminate this source of contamination or at least to minimize the probability to release a contaminant. As it is unrealistic to reduce significantly the bacterial load on the operator, the concept was to isolate the operator and his/her bacterial load from the filling area. Initial filling were performed by operators with lab coats and not always gloves with the open container in direct contact with them. Now, the operators must be gowned according to the clean room where they operate. The following is applicable according to the Eudralex:

- Grade A in operation: as the operator could be in direct contact with elements of the container, the needle and the solution to be filled, it is mandatory that he is fully separated from the environment. Usually companies are using complete and closed gowning suits to get such separation.
- Grade B in operation: the operator must be completely covered. This means that he should wear a complete suit closed at extremities by stretching bands, complete overshoes, full hair cover, gloves coming on the suit, mask, and eyes must be covered.
- Grade C in operation: the operator must wear a lab coat tight closed at the neck and the wrestles, preferably with a full hair cover, mask, and gloves are usually recommended.
- Grade D in operation: the operator must wear a lab coat, simple overshoes, and hair cover.

10.3 Barrier Systems

As briefly addressed in previous paragraph, different barrier systems may surround the fill and finish equipment with the objective to separate the operators from the filling area.

The absence of barrier in case of aseptic filling is more and more challenged by the authorities according to the safety principle that an operator is a permanent risk and should be separated from the filling area.

Two main groups of barrier systems have been set up: the RABS and the isolators. According the Lysfjord (2009b, c), the RABS and the isolators are both booming with approximately 20–30 installations performed each year for each of them. Several pharmaceutical companies and contract manufacturing organizations (CMO) have installed many of them and it is frequent to see both types of barriers within the same company.

A RABS includes the following main characteristics (Lysfjord 2009c):

- Hard walls.
- HEPA filtered air supply generated either by a dedicated system (active RABS) as shown in Fig. 10.1 or by the clean room (passive RABS).

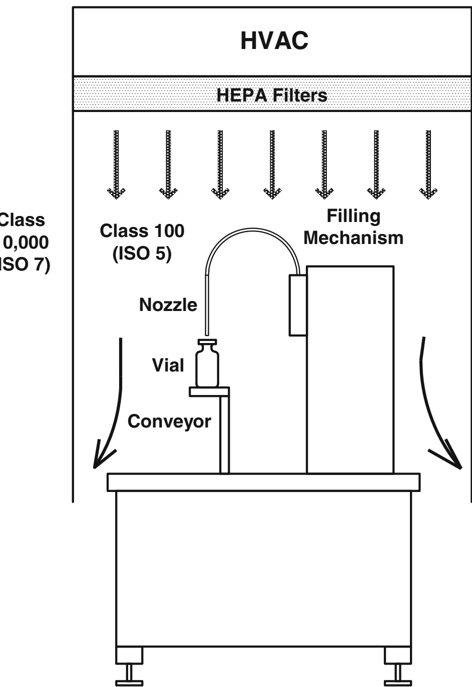

Fig. 10.1 Active RABS with its own airflow dispensing unit and its open bottom for air exit. Courtesy from Jack Lysfjord

- Open bottom for air exit.
- Sanitization by wiping with sporicidal agents.
- During operation, the doors can be opened to allow access for the operators. Nevertheless, most of classical operations should be done through glove ports. Door opening is allowed for exceptional interventions.

The two last points are highly debated as it is considered as the main weakness of the RABS because both imply that the doors can be opened and therefore a contaminant can enter in the equipment during operation. Therefore, RABS are required to be located in ISO7 clean room when open containers are used. In case of frequent RABS opening, it is recommended to add an ISO5 annex above the door opening to ensure that the environment quality inside the RABS is not compromised.

The isolator, as shown in Fig. 10.2, has the following main characteristics:

- Hard walls.
- HEPA filtered air supply generated by a dedicated system.
- A sucking system located at the bottom of the filling area to collect the air.
- The inside of the isolator is pressurized to prevent entry of contaminant through mouse holes for example. This pressure is obtained by modulating the speed of air supply and air suck.
- Sanitization is performed by gassing such as vapor hydrogen peroxide (VHP).

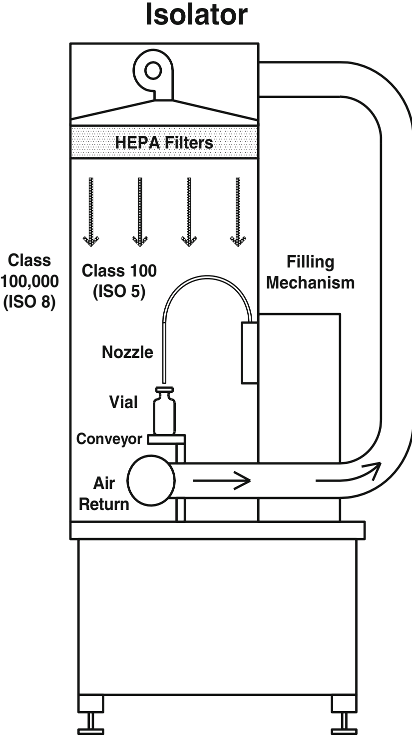

Fig. 10.2 Isolator with its own airflow dispensing unit, its closed bottom, and its air sucking system located at the bottom of the barrier. Courtesy from Jack Lysfjord

- Doors cannot be open once the equipment is sanitized.
- Access inside the isolator is done exclusively through glove ports.
- Entry of material is done exclusively through specifically designed systems such as Rapid Transfer Ports.

When comparing both systems, the main advantages of the RABS are its lower investment cost, its easier validation (only airflow validation), its faster change-over, and its lower cost of use (no VHP gassing, …). Nevertheless, most of these advantages are eliminated by the fact that it should be located in an ISO7 clean room vs. and ISO8 clean room for isolators. An ISO7 clean room means more gowning for operators (time and costs), additional airlock with their respective clean room space (investments and operating costs) and higher monitoring of the clean room environment (costs and validation). Several companies have performed cost comparison between RABS and isolators and came to the conclusion that RABS do not provide significant savings over isolators and sometimes are slightly more expensive (Lysfjord 2009c).

In case of equipment retrofit, the RABS technology is much simpler to add above an existing equipment thanks to its open bottom. For example, following the publication of Eudralex Annex 1 (Eudralex 2008) requiring capping under Grade A air supply, many RABS have been added above existing capping equipment.

Two specific designs, the closed RABS and the under-pressure isolator, are detailed hereafter because they differ from the classical barrier systems. Both are usually used for highly potent products such as cytotoxics and immuno-modulating drugs. The role of the barrier in such case is extended to the protection of the operators. For highly potent drugs, a protection is required according to an overall exposure limit (OEL). This OEL, expressed in microgram of pharmaceutical drug per cubic meter of air, depends on the toxicity of the product, its volatility (e.g., powder is much more susceptible to be in suspension in the air than liquid) and the exposure time for the operator. Lower is the OEL, higher must be the efficiency of the barrier system to retain any loss of drug (Lee 2007).

The closed RABS has a very similar design to isolators. The closed RABS shares all technical characteristics with isolators except that the sanitization is not done by gassing but by manual wiping as for RABS. The closed RABS can be either overpressured or under-pressured as explained for the isolators just below.

Isolators for highly potent drugs are usually under-pressured. This creates a flow of air entering through the mouse holes to prevent any loss of toxic product and therefore exposure of the operators. This slight vacuum is obtained by modulating the sucking system, collecting more air than the quantity delivered from the ceiling by the laminar airflow pulsing system. Such design reduces the quality for the patient as a contaminant may enter through the mouse holes, carried by the inflow of air. Several companies, to prevent this issue, have added pressurized airlock on the other side of each mouse hole. In such case, there are two flows exiting from this airlock: the first to the inside of the filling isolator to retain the highly potent drug and protect the operator, and the second to the outside to prevent entry of a contaminant.

10.4 Containers Available and Their Respective Filling Process

In this paragraph, the focus is put on small volume parenteral drugs. Large volumes are usually administered by infusion from flexible pouches or BFS from 250 to 1,000 mL usually.

10.4.1 The Ampoule

The initial container used of injectable drugs was the ampoule. This container is still widely used, especially in areas such as Asia, Latin America, and Russia. The ampoule, made of glass, presented the advantages of being easy to fill and to close but also to be an easy container to open for liquid collection.

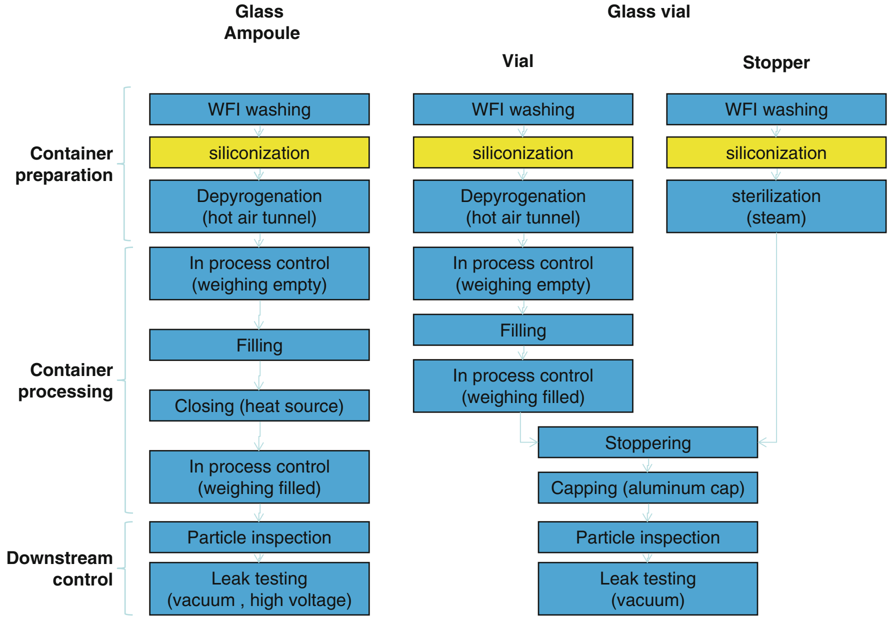

Fig. 10.3 Filling process for ampoules and open vials

The most frequent process to fill an ampoule (see Fig. 10.3) is to use open ampoules which are:

- Washed with water for injection (WFI)
- Siliconized by addition of liquid silicone if the product may interact with glass
- Sterilized and made pyrogen-free in a hot air tunnel (also called depyrogenation tunnel), this tunnel will also solidify the silicon as a layer on the glass
- Filled, after cooling, with the expected volume plus an overfill (the overfill covers the loss of liquid in the container and during handling of the syringe by the practitioner)
- Closed by heat, the most frequent source of heat is a flame but now new technologies such as lasers are used

After filling process, the container is submitted usually to two inspection processes: visible particle inspection (to ensure that big particles are not injected to the patient and generate thrombosis) and container closure integrity test (to ensure that the ampoule is properly closed and there is no entry for contaminant). This last test can be done by vacuum leak test to detect a gas leak through a pinhole or by high voltage leak test to detect abnormal conductivity through pinhole.

10.4.2 The Vial

The main weakness of ampoule is its susceptibility to breakage. More robust materials were investigated, leading to the development of the glass vial. This vial, due to a significantly ticker wall is more resistant but does not allow neither closing by flame nor opening by cut as it was for the ampoule. To address these two points, stoppers have been developed to close the vial and to allow needle penetration to collect the liquid. The vial is the most frequently used container across the world and is almost the only container used for lyophilized products.

The process to fill an open glass vial (see Fig. 10.3) consists in multiple steps:

- The vial body washing with WFI
- The vial body siliconization if interaction between glass and the product must be prevented (optional)
- The vial body sterilization and depyrogenation in hot air tunnel
- The stopper washing with WFI
- The stopper siliconization to ease stoppering (optional)
- The stopper sterilization by steam
- The introduction of the stopper, usually through a transfer port
- The filling of the vial body
- The stoppering to obtain a container closure integrity
- The capping the secure the closure integrity, achieved by crimping of an aluminum cap

After this process, particle inspection must be conducted and some pharmaceutical companies are performing as well a container closure integrity test by, e.g., leak test.

As the vials are still susceptible to breakage, some companies such as Schott and Gereishamer have launched vials made of polymer. Usually cycloolefin copolymer (COC) and cycloolefin polymer (COP) are used as these polymer materials have excellent properties in terms of clarity, resistance, and barrier to gas exchange.

10.4.3 Recent Advances in Containers

More recently, three new packaging have been launched with various objectives in mind: the PFS, the cartridge, and the BFS.

The PFS has been used initially by the biotech industry but is now endorsed by all pharmaceutical industry. Its main markets are clearly North America and Western Europe. This technology is widely used for two reasons: an easier administration path for the healthcare practitioners as no specific liquid collection should be performed and a reduction of drug loss due to noncollection from the initial container. PFS has attracted a lot of attention regarding its development and a wide range of solutions are available on the market. Among the most important development, various heads have been developed from the classical stacked needle up to multiple systems to protect the user from needle injury. A lot of work has been put as well on

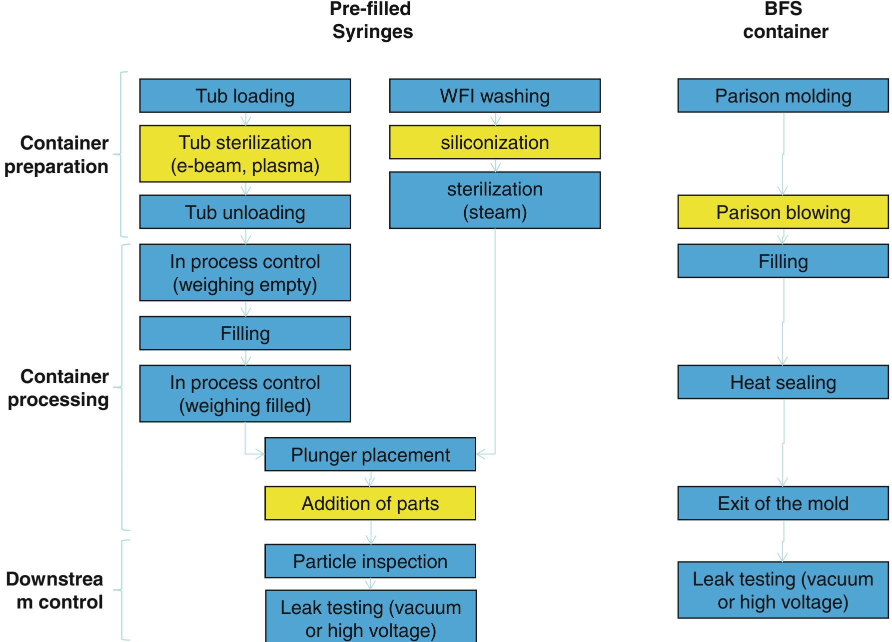

Fig. 10.4 Filling process for PFS and BFS

plungers to develop coated plungers and try to decrease the quantity of silicone required for plunger sliding during injection.

The cartridge is more designed for frequent injections or for injection with specific tools. The explosion of the use of the cartridge has been driven by the self-administration of insulin. Patients requiring one injection every day were facing the choice to visit frequently a healthcare practitioner or to perform their own injections. To address this issue, auto-injectors have been developed, frequently looking as a pen, to allow the patient to perform its injection with a simple technique, being a real improvement for the patient. To load these auto-injectors, the cartridge has been developed. A cartridge is a tube stoppered on both sides. One stopper is pierced to allow exit of the liquid whereas the other slides to push the liquid out.

The filling process for these two last containers is very similar. Usually presterilized tubs containing multiple containers are used. These tubs are closed by a sheet of Tyvek, allowing various sterilization such as gamma-irradiation and ethylene oxide sterilization. Therefore, the sterilization and depyrogenation process is not necessary on the contrary of the glass vial. The filling process for PFS (see Fig. 10.4) and cartridges consists in:

- Sterilization of the external part of the tubs, usually done by e-beam but recently a plasma sterilization has been installed by GSK in UK. Some companies such as Vetter have developed a cascade system which avoids expensive investments in sterilization equipment.

- Opening of the tub initially was done manually but several pharmaceutical companies have installed robotized units to avoid presence of operators.
- Unloading of the tub.
- Siliconization of the syringe to ease plunger placement and further sliding.
- Filling of the container.
- Closing with the plunger. As plungers are significantly smaller than vial stoppers, frequently they are supplied presterilized in beta-bags and are loaded through a rapid transfer port. For large quantities (over hundred thousands), equipment for plunger washing and sterilization (similar to equipment for vial stopper processing) combined with dedicated transfer systems may be used.
- Addition of container parts such as needle and plunger rod if relevant.

Inspection consists usually in particle inspection. Some companies perform leak tests to detect small cracks in syringes.

Dual chamber syringes are available for lyophilized products or products to be mixed just before injection. The principle is that a central plunger separates two chambers. The second chamber (the most away from the needle) contains the liquid. When the practitioner starts moving the end plunger, this one pushes both the liquid and the central plunger. At a moment, the central plunger reaches an area with a tiny channel which creates a bypass over the central plunger. The practitioner movement then has not more impact on the central plunger which stops sliding but well on the liquid which is transferred to the first chamber. As a result, the two products are mixed (e.g., the lyophilized powder is solubilized in the liquid solution). The further movement of the practitioner aims to inject the mix by sliding both plungers until the syringe is empty.

The BFS consists in a container made of polymer, usually polyethylene or polypropylene. This container is formed by blowing the polymer material inside a mold and the blown container is immediately filled and sealed, hence its name (Reed 2002). This technology was initially widely used for ophthalmic and nasal products of which administration benefits of the flexible container walls. This technology is used as well for large parenterals as semirigid containers present an easier handling compared to fully flexible poaches. Its use for small parenterals is more limited as the polymers used present the disadvantage of having a certain level of water vapor transmission rate which can results in a significant loss of water for small units due to a disadvantageous ratio between the surface of container wall and the volume of liquid. This problem is not noticeable for large parenterals as the volume-surface ratio is much more favorable.

The filling of a BFS container (see Fig. 10.4) takes a limit period of time (as low as approximately 12 s) and all operations take place at the same location:

- A parison of melted container material is injected in a mold.
- The parison is blown to form the container with either vacuum or over pressure.
- The liquid is filled.
- The container is heat-sealed.
- Then the filled container is taken out of the mold.

Particle inspection is hardly feasible due to the opacity of the polymer materials used; therefore particle tests are usually not conducted. Leak test can be conducted to detect a problem at the sealing process.

10.5 Challenges of Aseptic Filling

The biggest challenge of aseptic filling, whatever the container, is to prevent product contamination. On the contrary of terminal sterilization, there is no process to eliminate a contamination if it enters inside the vial. Some products contain preservatives but there is a higher and higher concern regarding the absence of side effects from these products, hence the recommendation from various authorities to withdraw preservatives for drugs to be administered to children and pregnant women (http://www.cfsan.fda.gov/~lrd/tphgfish.html). Other challenges such as ensuring that product specifications are respected (filled volume, temperature respect, …) will not be addressed.

To reduce occurrence of accidental contamination, in-depth risk analyses must be conducted to minimize the sources of contamination. In this paragraph, some of the main sources of contamination are identified and some ideas to reduce the risk of such occurrence are provided.

It is important also to keep in mind that almost all contaminations are due to the humans (Agalloco and Akers 2005) and we can probably find a human reason behind the few others. Therefore, as a general concept, we need to avoid as much as possible interaction between the operators and the product. As the operator being a source of contamination has been addressed in Sect. 10.2 regarding his/her gowning and training, these aspects will not be addressed again

The first source of contamination is the bulk itself. It can be contaminated by various means:

- Introduction of contaminated elements (excipients, …): all elements to be introduced and the bulk container (including tubing) must have been sterilized in an acceptable manner (steam sterilization, irradiation, or filtration) to ensure that no contaminant is accidentally introduced.
- Absence of or inadequate last sterilization step: the last sterilization must occur at the latest as possible. Usually a last product filtration through a 0.22 μm filter is done just before entering the dispensing pump.
- Entry of contaminant during formulation: in some cases, a filtration cannot be performed at such a late stage. For example, vaccines containing aluminum particles cannot be filtered. In such case, it is mandatory to implement a very robust formulation process. To reduce risk of bulk contamination, GSK Biologicals has set up a formulation process for its vaccines inside an isolator to reduce as much as possible the presence of a contaminant.
- Absence of integrity of the bulk container: the bulk container must be checked for absence of leak. Many new processes rely on ready-to-use containers such as pouches. Integrity is usually certified by the manufacturer but an additional check is certainly worth to be conducted just before use.

- Ineffective sterilization: it is mandatory to ensure that the sterilization procedure has been fully respected. Therefore, the irradiation dose must be controlled before release of a gamma-irradiated part, the autoclave reports must be checked before release of a steam sterilized part and filters must be checked for integrity before releasing the bulk product or the filled vials.

The second source of contamination is the product path. Sterilization must be ensured as for the formulated bulk so the above recommendations are applicable to the product path. The integrity of the product path is critical because usually it cannot be tested in an efficient way, especially if it is open at both extremities. Therefore, a robust design is mandatory to ensure that all connections should be resistant in worst case. It is also recommended to secure all the connections with cable-tie for example and to perform a visual check before using the product path.

The third source of contamination is the filling equipment. A filling equipment is made of a multitude of parts. Some of them are moving, raising the question of the efficiency of the sanitization, some others are in contact, leaving some interstices which could contain bacteria and finally, some of the surfaces can lose along the time their polish and therefore be more susceptible to protect bacteria during sanitization. The sanitization of the equipment must be carefully conducted if it is done manually. A special care must be given to the parts mentioned above as these have a higher probability to trigger contamination. Gassing with a sporicidal agent such as VHP is much more effective but is only applicable to equipment located inside an isolator or to a complete clean room. Due to size constraints, high efficiency can be achieved in isolators but overall clean room sanitization is less efficient. It is also important to validate the gassing time and conditions according to the equipment materials as the killing speed can be very variable according to the materials used and their polish.

To ensure the quality of the sanitization, a special care must be given to the training of the operators whereas the use of a gassing method must be supported by a complete validation plan including the effectiveness of the sanitization, the completeness of the sanitization (all areas have been well treated) and the detection of all defects which could lead to an ineffective gassing cycle.

Few parts, called the product contact parts, are very critical because they will be in contact with elements finishing inside the vial. Besides the product path, addressed above, the other contact parts are the vibrating bowls and the ramps which will be in contact with parts such as stoppers and plungers. If a bacteria is still present inside a vibrating bowl, there is a significant chance that this bacteria will be transferred to, e.g., a stopper and if it sticks on the internal part, it will end inside the vial and be in contact with the drug. Therefore, such contact parts must be fully sterilized either by gassing or by autoclave.

All what has been described above must be weighed with the exposure time and surfaces. If a contaminant is present inside the filling area, its probability to enter inside a container is directly proportional to the time of exposure of the internal parts of the container and to the surface exposed. Taking the example of an open glass vial, these factors can be easily evaluated:

- Surfaces:
  - A contaminant can enter inside the vial body through the neck, therefore the critical surface is the surface of the vial opening.
  - A contaminant can stick to a stopper. If it sticks to the external part, it will remain out of the vial. On the contrary, if it sticks to the internal part, it will end inside the vial and lead to contamination.
  - A contaminant can stick to the needle and be brought inside with the liquid flow.
- Time:
  - A vial is coming out of the hot air tunnel and is then exposed. At the beginning, the temperature is high enough to kill any bacteria sticking to the glass but after a short period of time, the temperature will decrease and lose its killing effect.
  - Stoppers are loaded inside the vibrating bowl and can stay there for very long period of time.
  - The needle is exposed during all the production time.
  - Any short stop has an immediate impact on the exposure time of all the above elements.

Based on this analysis, any action or procedure which allows reducing the exposed surfaces, the exposure time of components and the short stops of the equipment should be implemented as it has a direct impact on the exposure risk.

For example, the BFS technology has strongly reduced the exposure time of the components to a minimum as the overall cycle time may be limited to as low as 12 s. As an outcome, the European authorities have recognized the robustness of the BFS process and allow the installation of filling equipment surrounded by RABS in a Grade C clean room whereas a Grade B clean room is mandatory for vials, ampoules, syringes, and cartridges (Eudralex 2008).

10.6 Containers Available and Their Respective Profile of Advantages

The selection of a container is depending on multiple aspects. When a container is selected, several rationales are investigated by the pharmaceutical company. There are three main categories of rationale: the stability of the product, the manufacturing process, and the preference from the healthcare practitioner. The latest can increase the potential market acceptance for the product and therefore its sales.

10.6.1 Stability of the Product

Stability of the product is critical as a reasonable shelf-life is expected. A product with a shelf-life below 1 year has a significant risk to see its market acceptance

affected due to risk of reject. Most of products are stable but it has been observed several instability issues, especially with the most complex products such as biological drugs. Among the most frequent source of instability are:

- Adsorption on the container wall: the source of interaction is often electrostatic (e.g., with the multiple ions present in glass) or hydrophobic (e.g., with polymers).
- Oxidation due to the presence of oxygen in the container: this issue can be eliminated by using oxygen depleted containers (e.g., with nitrogen flush before closing).
- Loss of water: if too much water is lost by transfer across the container wall, the product characteristics may be affected. Water vapor transmission rate is higher with polymer materials compared to glass. Companies will have to prove that the effect on the product will not affect its therapeutic activity, including the administration of a sufficient dose.
- Effect of leachables: most of container materials release some tiny quantities of molecules inside the product solution. These products are called leachables and may have various side effects such as toxicity or effect on the product itself. Recently, some famous cases have highlighted this issue as several products have been affected. For example, tungsten released from stack needle was suspected to induce erythropoietin (EPO) aggregation which, once being injected, triggered an immune response against injected EPO but also endogen EPO. Several patients suffered from pure red cell aplasia, a serious anemia condition, after multiple EPO injections. Rubber stoppers and plungers are also known to release several chemical molecules and manufacturers are now offering coated container parts to reduce such impact.
- Degradation of the container: very recently, it has been shown that EPO and few other products such as methotrexate induce delamination of glass containers during aging, hence generation of glass particles. One of direct impact was the withdrawal of over 300 lots of EPO and the reduction of EPO shelf-life from 36 to 12 months.

10.6.2 Selection Criteria for Manufacturing Processes

The robustness and the simplicity of the manufacturing process can provide significant advantages to the pharmaceutical companies, especially regarding a cost reduction and a reliability of the supply chain to avoid stock-outs. For aseptic filling, the most impactful aspects are the following:

- The amount of components: higher is the amount of components (ranging from only pellets for BFS up to three components for glass vials, i.e., vial body, stopper, and cap), more complex will be the process because it is necessary to prepare each component (e.g., clean and sterilize) and to transfer each of them to the filling line.

- The use of presterilized components: preparation steps are usually highly demanding regarding equipment, human resources, and financial resources but are also a significant risk on the product quality. For example, the use of standard glass vials imposes to have a washing equipment, a hot air tunnel, and a cooling zone on the filling equipment. In addition, it is necessary to install, validate, maintain, and operate a source for WFI. In addition to operating expenses and investments, out-of-specification results during quality control may put a complete batch at risk.
- The size of the equipment: bigger is the equipment, bigger will be the barrier and the clean room to operate it.
- Overall reliability of the equipment: a highly complex equipment generates frequent short stops which can have significant impact on the productivity.
- The change-over time: several pieces of equipment are designed to run multiple size of containers. Some small scale equipment are even designed to process multiple types of containers such as glass vials and syringes. In such case, faster is the change-over, higher will be the productivity.
- The cost of the container: some containers are very expensive compared to others. For example, a non-sterile glass vial cost a few cents whereas a sterile glass vial supplied in its protective packaging can cost few dollars.
- The resistance of container to breakage: significant breakage may result in loss of productivity and above all loss of expensive active pharmaceutical ingredient (API). This effect is present during the production but also during entire supply chain until use for injection.
- The use of API: lower is the residual volume inside each container and lower is the loss of API during set up and operation, lower is the cost of filling.

10.6.3 Selection Criteria for Marketing Acceptance

End users, i.e., doctors and nurses mainly and to a lower extend pharmacists, have the tendency to prefer some containers vs. others for various reasons:

- Risk for the patient: as doctors are responsible for any accidental contamination which can affect their patient, their preference is going to containers which increase the safety. For example, the decrease of ampoule in western countries is partially driven by the risk that glass particles are generated during opening and can be injected to the patient. Among the biggest source of risk are (1) the presence of particles, (2) the presence of a contaminant, and (3) the counterfeited drugs which often do not deliver the right amount of products.
- Ease of use: being able to inject a drug rapidly and without excessive handling is very attractive for healthcare practitioners. This reason was a main driver for acceptance of PFS as handling is very limited compared to ampoules and vials which require container opening and liquid collection. With the same philosophy, the use of cartridges combined with auto-injectors provides a real facility for patients who have to make daily injections at home.

- Risk of breakage: with the development of expensive and/or highly potent drugs, the risk of breakage becomes a significant issue. Small cracks are also a source for product contamination as bacteria can enter through such cracks. Any solution which can reduce such risk of breakage is favored by end users.
- Cost: cost containment in healthcare is increasingly a focus of governments. Any container which succeeds to reduce healthcare cost of a product could therefore be favored. This issue is quite important in hospitals which have to buy their drugs and in some countries where benchmarks of prescription costs have been set up to force the doctors to reduce the quantities of drug prescribed.
- Size: aseptically filled drugs are usually heat sensitive and therefore must be kept at 2–8 °C. When large quantities of drugs are used such as in hospital settings, large storage capacities are required. Purchase managers may impose to buy drugs with the smaller size possible to reduce their need of storage requirements.

10.6.4 Exposure Risk Criteria

As described in Sect. 10.5, the exposure risk is variable. The most risky container is by far the open vial as both the vial body and the stoppers are exposed. This exposure can be for very long time (e.g., it can represent more than 30 min for a vial to move from the exit of the hot air tunnel up to the stoppering station). In addition, the exposed surfaces of a stopper are usually larger than others such as plungers and the stoppers are in contact with equipment parts such as the vibrating bowl and the ramps.

The ampoule is also exposed for long but there is no stopper exposed. This has a significant impact on the quantity of exposed surfaces and it eliminates to contact between a product part and the equipment.

PFS are exposing also two elements but for shorter period of time (no cooling time required) but also smaller surfaces as syringes are narrower than vial necks.

BFS, as mentioned above, have the shortest exposure time as a complete cycle for small containers is usually in the range of 12 s.

10.6.5 Comparison of the Containers Along the Criteria

The Table 10.2 illustrates the advantages of each container according to the list of criteria described above.

Based on this table, it appears that each container has its own profile of drugs for which its advantage profile outweighs the disadvantages:

- PFS: injectable drugs with a certain cost for which market acceptance is mainly driven by ease of use.
- Cartridge: drugs to be self-administered such as insulin.
- BFS: large drugs not sensitive to oxygen ingress and loss of water, especially well designed for infusion.

Table 10.2 Profile of advantages and disadvantages of containers

Container	Key advantages	Key disadvantages
Ampoule	Stability: only one components, no organic leachable, no gas exchange	Manufacturing: breakage
	Manufacturing: single component with limited equipment required	Market acceptance: difficult to handle, opening may generate glass particle, breakage
		Exposure risk: significant due to cooling time
Vial (glass)	Stability: very low glass exchange (water and oxygen)	Manufacturing: three components therefore operations are expensive and complex
	Manufacturing: more resistant container at low price	Market acceptance: difficult to handle (opening of aluminum cap and piercing), still breakable
	Market acceptance: preferred than glass ampoule	Exposure risk: very high due to long time and multiple elements
PFS (glass)	Manufacturing: use of presterilized containers and lower residual volume	Product stability: high contact surface increasing the leachable generation
	Market acceptance: very simple way to administer drugs	Manufacturing: breakage, more expensive container
	Exposure time: significantly better than open glass but not as good as BFS	Market acceptance: breakage, large size container
Vial and PFS (polymer)	Manufacturing and market acceptance: unbreakable container	Manufacturing: very expensive container, similar operations as for glass containers
Cartridge	Similar for PFS	Similar to PFS
	Market acceptance: very simple for self-administration	Market acceptance: very expensive container as an injection device is required (especially if single use)
BFS	Manufacturing: most simple production process, very safe manufacturing process as the open container is never exposed to the environment	Product stability: organic leachables, high gas exchange rate for both water and oxygen
	Market acceptance: unbreakable, easy to open	
	Exposure risk: by far the lowest exposure risk due to very short cycle time	

- Ampoule: low-cost drugs for which breakage is not a too significant issue, still the standard containers in many regions such as Asia.
- Vials: standard container used for most products in developed countries, is increasing in other markets. Is almost the only container used for lyophilized products.

10.7 Closed Vial Technology Case Study

To address the two main issues of aseptic filling, i.e., the risk of contamination and the complexity of operations, GlaxoSmithKline Biologicals decided in 2001 to develop a new form of vial and a new filling process. This technology, called Crystal technology, consists in a polymer vial supplied clean, sterile, and stoppered to the pharmaceutical manufacturer. To fill the vial, a needle pierces the stopper and the trace left by the needle is closed by laser resealing (Verjans et al. 2005; Thilly et al. 2006). This technology is suitable for filling of both liquid and lyophilized products.

The vial consists in a vial body made of COC and a stopper made of a thermoplastic elastomer (TPE). The two elements are assembled and secured by a top ring using the snap fit principle. The cap of the vial is made of polyethylene and is also assembled by snap fit after filling (see Fig. 10.5).

The manufacturing of the vial is critical to ensure cleanness. Therefore, multiple precautions have been taken to ensure that the particle content is low enough and that the vial is not contaminated neither by bacteria nor endotoxins:

- Particle content: to minimize particle content, the vial body and the stopper are molded in a class ISO5 clean room. They are picked out of the mold by robots and directly assembled, hence a closing in a low particle environment. These robots, initially designed for the microelectronic industry, are suitable for operations in class ISO4 and ISO3 environment. The molding process is also designed to minimize particle generation.
- Bacteria contamination: using an ISO5 environment for assembly should ensure asepsis and absence of bacteria. To ensure sterility, the fully packed vials are submitted to gamma-irradiation at a minimal dose of 25 kGy.

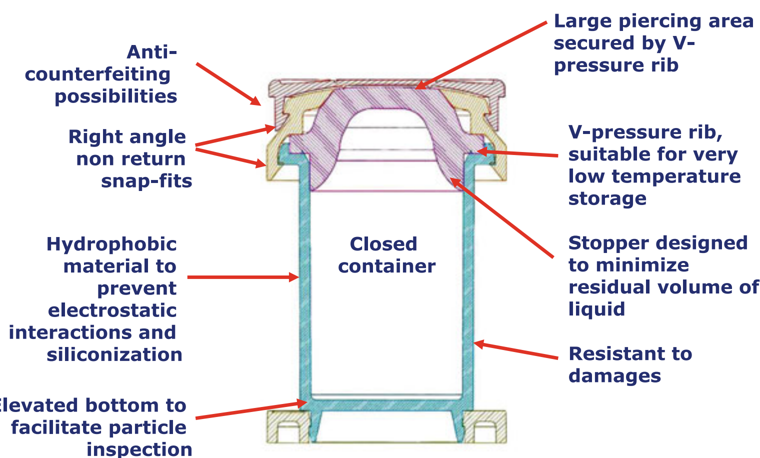

Fig. 10.5 Cut view of a Closed Vial with the stopper in place. The Closed Vial technology and the use of polymer materials offer multiple advantages compared to classical glass vials

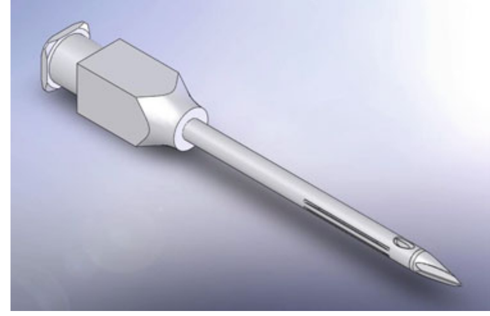

Fig. 10.6 specific needle shape to (1) minimize particle generation during piercing of Closed Vial stopper thanks to a pencil point and (2) evacuate gas from the vial during filling thanks to grooves located in the needle wall

- Endotoxin contamination: the raw materials (pellets of COC and TPE) are checked for endotoxin presence before molding. In addition, vials are randomly tested for endotoxin presence according to standard sampling rules.

The cleanness properties of the vials allow filling directly the vial without performing cleaning and sterilization of the components. As briefly explained above, the filling process consists in three main steps: filling with a needle piercing the stopper, laser resealing of the piercing trace and capping with polyethylene caps. To ensure a complete robustness of the process, the following precautions have been taken:

- Specific design of the needle: to minimize particle generation during piercing, the needle is a pencil point needle (Fig. 10.6). This needle has no coring effect to (1) reduce particle presence and (2) facilitate future laser resealing by avoiding loss of material. The result of using a pencil point is that needle holes are located on the side of the needle and the liquid is coming out with an angle of 30°. This side exit generates a smooth flow coming first in contact with the vial walls and not hitting the bottom of the vial. This effect is of particular interest for products sensitive to physical damages such as large proteins. In addition to the specific point, the needle is equipped with grooves which are located on the needle side to allow evacuation of the overpressure generated during filling of the liquid. Finally, the needle wall is thick enough to allow over than 25,000 piercing with the same needle without damage or bending.
- Tolerance and reliability of the laser resealing: to ensure that the laser resealing is effective, three specific properties are included in the laser. First, the piercing trace is significantly larger than the potential deviation of the piercing trace due to the needle tolerance. Second, thanks to a specific optical tool, the laser hits the vial stopper with similar intensity on all the hit area (this property is called flat top curve), to avoid low intensity (risk of absence of resealing) and high intensity (risk of burning) area. Third, a specific measurement of the laser intensity is done at the laser head, i.e., just before delivery to the stopper, to ensure that the right laser beam has been delivered.
- A second closure integrity generated by the cap: the cap is assembled by snap fit so the position and its tolerances are fully controlled by design of the vial and the cap. Thanks to a rib located on the inside face of the cap, a second closure

integrity is achieved to protect the piercing area until use by the healthcare practitioner. To fully benefit of this property, the capping must be done in a class ISO5 environment to ensure asepsis of the piercing trace.

The use of polymer materials must be carefully assessed regarding its barrier properties for gas exchange. For example, water vapor exchange rate has been measured in various storage conditions according to the International Conference on Harmonization rules. The request is that the water loss for liquid products should not be higher than 5 % on a period of 3 months in accelerated conditions. The verification has shown that the COC is an excellent barrier as the measures were always under these requirements. In special conditions, such as to maintain low concentration of oxygen or to prevent tiny water ingress for lyophilized products, the use of a secondary packaging such as aluminum pouch is required. Such secondary packaging provides a full isolation from the environment.

Another aspect of polymer materials is that the profile of extractables and leachables is expected to generate higher dose of organic compounds. Nevertheless, the purity of both COC and TPE generates less contamination than rubber materials used for stoppers or plungers according to specialists. As well, all leachables attached to glass process such as ions but also silicon oil are not present. The main leachables found with the Crystal technology are small organic acid such as formic and acetic acids. These acids are generated by gamma-irradiation of polymer materials and are in such low conditions that a tiny buffer eliminates any effect on the pH.

On the contrary, the use of polymer and the specific vial manufacturing and filling technologies bring multiple advantages according to the Sect. 10.6 above:

- Better asepsis for the patient: the vial being kept closed during all process, it can be considered as a mini-isolator at item level. This permanent closure prevents entry of nonviable but above all of viable particles from the environment. In relation with Sect. 10.6.4, the Closed Vial reduces the exposed surface to the surface touched by the needle. There is also no exposure of vial elements to equipment parts as the stopper is in place. In addition, the time inside the filling area is very limited as the vials are ready-to-fill and therefore there is no need to wait for e.g., cooling as it happens with glass vials. All these aspects made the exposure risk for the Closed Vial very similar to the one for BFS.
- Safer supply chain: the polymer resistance is much higher than glass, leading to a reduction of breakage and especially small cracks. These small cracks lead sometime to container contamination. For example, several newborn babies have been contaminated in 2010 in a German hospital, the cause being identified as a small crack in a glass container.
- An easier manufacturing process: thanks to the fact that assembled vial is provided ready-to-fill, all preparation steps are eliminated. As a consequence, utilities are strongly reduced, in particular there is no need of WFI for washing of vials as for open glass vials.
- A preferred solution for end users: a market study comparing the glass vial and the crystal vial showed that 87 % out of 246 end users (doctors, nurses, and hospital pharmacists) prefer the Crystal vial vs. 7 % for the glass vials. The main

drivers are its easier use (easy to handle, to open, to pierce, and to collect all the liquid), its resistance to breakage (very important for users of potent drugs as it was mentioned as a key driver by 75 % of people working in oncology) and its improved asepsis.

10.8 Conclusions

Aseptic filling processes have achieved tremendous improvements driven by quality for the patient. Most of them are linked to environment with lower presence of operators and better monitoring to ensure optimal quality.

The development of barrier systems has also brought a significant source of additional safety for the patient especially if the most advanced systems such as isolators are considered.

Regarding containers, several new designs have been launched recently with the objective to meet some demands driven by the product itself, the manufacturing complexity, the healthcare practitioners, and the risk of contamination. The latest objective is still collecting high scrutiny from authorities because an aseptic process aims to come close to a sterile process but will probably never be a sterile process.

The last innovation, the Closed Vial technology is bringing the quality of aseptic filling one step further compared to most technologies currently available to the pharmaceutical industry.

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Chapter 11
Contemporary Approaches to Development and Manufacturing of Lyophilized Parenterals

Edward H. Trappler

Abstract This chapter provides a historical reference, covers the progression in the scientific and technological development, highlights the contemporary aspects, and describes the application of the current USFDA guidance to the development through commercial life cycle for lyophilized products. Considerations of designing formulations, including the use of organic solvents, and influence of packaging are noted. Emphasis is placed on the engineering of the lyophilization process, establishing the critical process parameters, and defining of the critical quality attributes. Utility of applying the US FDA process analytical technology initiative, as well as the notion of applying design space principles to the lyophilization process is included, leading into discussions on applying the current USFDA guideline on process validation to the development and manufacturing. Current challenges and unique aspects in development of lyophilized products are also highlighted, including poorly soluble drug substances, novel delivery systems, improving manufacturing capabilities, and reducing unit costs for world wide product distribution. This presentation encompasses the progression of the technological developments, reviews current thinking on the science and technology, and highlights contemporary approaches to the development and manufacturing of lyophilized parenterals.

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11.1 Introduction

11.1.1 Historic Review of Lyophilized Products

Lyophilization came of interest as a novelty in a laboratory setting, later as a method of preservation in hospitals until acknowledged as being a method of commercial manufacturing. A comprehensive review that included a historical, contemporary, and future potential highlights the future growth of lyophilization for parenteral products (Trappler 2011). The preservation of yellow fever virus was reported in the Journal of Experimental Medicine in 1929. In 1938, the Journal of the American Medical Association highlighted developments in the preservation and concentration of human serum for clinical use. The first products to be preserved by this method were human plasma, vaccines, and antibiotics. Early work on developing manufacturing was in collaboration between Max Strumia of Bryn Mawr College and the Sharp and Dohme Company in Philadelphia (Stark 1998).

Naturally derived biological preparations were prominent. Early commercial products included hemin by Abbott and corticotrophin by Parke Davis and Rorer Pharmaceuticals. One of the first antibiotics was penicillin G procaine by Wyeth. Development of antibiotics grew through the 1970s, with aminoglycosides (Tobramycin, Lilly) and cephalosporins (Keflex, Lilly), and cefazolin (Ancef, SKB, Kefzol, Lilly). Other new antibiotics included β-lactams vancomycin HCl, (Vancocin, Lilly) and tetracycline (*doxycycline*, Vibramycin, Pfizer). Continued development of new vaccines included IBV H-52 and H-120 for infectious bronchitis. Dactinomycin (Cosmegen, Merck) and cisplatin (Platinol, BMS) were some of the first lyophilized oncolytics. The Upjohn Company marketed two lyophilized corticosteroids, hydrocortisone sodium succinate (Solu-Cortef) and methylprednisolone sodium succinate (Solu-Medrol).

In the period of the 1980s through the 1990s, new products continued to be introduced to the market, many being anti-infectives to include antivirals. BMS introduced a new β-lactam (Azactam) and Merck developed a combination of a β-lactam and cephalosporin to treat gram-positive and gram-negative infections (imipenem/cilastatin, Merck). Antiviral therapies were also introduced to the market through the 1980s, with acyclovir (Zovirax, GSK) and ganciclovir (Cytovene, Syntex/Roche) and interferons ra-2A (Roferon-A, Roche) and ra-2B (Intron A, Schering). Novel treatments for MS became available with Bayer's Betaseron (interferon rB1b) and treatment for heart attack patients with Genentech's Activase (alteplase). New vaccines continued to be on interest. Treatments for HPV (Cervarix, GSK), DPT + polio and hemophilus influenza b (Pentacel, Sanofi Pasteur), pneumococcal (Prevnar, bulk powder intermediate, Wyeth), and herpes zoster (Zostavax, Merck) were marketed.

Early on as an academic curiosity, the later use in research laboratories and hospitals, and now an important technology, the use of lyophilization has become more commonplace in processing active pharmaceutical ingredients (APIs) as well as finished drug products. Since the inception of lyophilization as a method of

preservation, the number of products and the expanded application to therapeutics, diagnostics, and medical devices has driven the growth as a commercial manufacturing method. The greatest growth has been spurred on by the growth of the biopharmaceuticals.

11.1.2 New Products of Key Interest

Three major product classes continue to be of interest: antibiotics to treat drug-resistant infections, specifically MRSA; oncolytics that also now include biopharmaceuticals such as monoclonal antibodies; and vaccines. Immunomodulators is also a therapeutic class of products that continues to gain attention. Lyophilization is also used for the preservation of conjugated chemical entities, nanoparticles, and liposomes. New promises for treatment using RNA interference (RNAi) for oncology and genetic conditions are a new class of therapeutics that will also likely be lyophilized preparations.

11.1.3 Science and Technology Advancements

Interests in measuring, manipulating, and controlling conditions during lyophilization have led to new directions and developments, and continued studies are warranted to gain a better understanding of the basic principles and mechanisms that apply to freezing along with primary and secondary drying.

Control of freezing, specifically the random nucleation of water and resulting stochastic growth of ice, has long been an ambition. Various potential methods and the interest in controlling freezing drew interest for process and product improvements (Bursac et al. 2009; Patel et al. 2009). More recently, methods suitable for inducing nucleation that promise to be scalable has recently been introduced and is currently being studied by a number of investigators (Konstantinidis et al. 2011). Control of the ice crystal size and employing Ostwald ripening, with the ambition of improving the mass transfer of water vapor through the dried layer above the sublimation front during primary drying, has also been studied (Searles et al. 2001). The use of annealing during freezing has also been investigated for inducing complete crystallization of solutes such as ionic species and amino acids, and may influence the polymorphic form of some solutes. Beyond impacting the physical structure on a macroscopic scale, conditions during freezing can also influence the morphology of the solid form, as illustrated with mannitol (Cannon and Trappler 2000). The effect of excipients on crystallization of active ingredients has also been shown to occur (Korey and Schwartz 1989).

Shelf temperature is a process parameter that has always been recognized to be the principal variable affecting the product temperature and processing rates. Chamber pressure, though currently recognized as a critical process parameter (CPP), received

little attention in the early application of the drying technology: one simply reduced the pressure to as low as the equipment could achieve any given day. Observations to the effect of pressure on processing were reported as early as 1954 at the American Vacuum Society annual meeting (Ginnette et al. 1958). Since 1980, control of the pressure during primary drying by introducing nitrogen into the product chamber has become the convention (Nail 1980). Defined as a CPP, contemporary process engineering approaches has also included pressure control during secondary drying. Still, there remain unanswered questions on the principles and effects involved, with a growing understanding of the mechanisms and direct influences on heat and mass transfer. Influences on the progression during primary drying have received the greatest level of attention.

Increased understanding and improvements in factors that influence secondary drying have been limited. Desorption of water is perceived to be straightforward. The influence of chamber pressure was investigated and, within the nominal pressure range studied, is reported to have little effect (Pikal et al. 1990). Measuring and understanding the factors that influence removal of absorbed vs. adsorbed water has received little attention and warrants further study.

Overall, the most significant area of research has been on methods for the nucleation of water to control uniformity of this stochastic event. It is well recognized that ice formation and solute solidification during freezing has the greatest influence on behavior during processing and for some products, finished product attributes. Endeavors to control nucleation draws significant interest (Bursac et al. 2009). This includes investigation into the use of an "Ice Fog" technique (Patel et al. 2009). Investigations and methods suitable for application in commercial manufacturing have been refined by Praxair (Konstantinidis et al. 2011). Control of the nucleation is gaining great interest, as it shows great promise for improvements in processing and finished product quality with implementation in a commercial manufacturing scale. The advancements in control of freezing promises to be the most significant since the control of chamber pressure was introduced by Nail in 1980.

Other advancements involve the use of organic solvents as adjuncts to formulations. Organic solvents in combination with water have been selected as an aid in improving the dissolution and solubility of a poorly soluble compound, for accelerating the rate of vaporization during primary drying, and for altering the characteristics of the finished product. The most common mixed solvent systems are water and methanol, ethanol, or *t*-butanol. Early investigations focused on using pure organic solvents such as ethanol for low-temperature vacuum drying (Flamberg et al. 1970). Other investigators exploited improvements in processing and the resulting product attributes obtained for combinations of various organic solvents and water (Seager et al. 1985). Interest in the use of combinations of *t*-butanol and water gained greater interest (DeLuca and Kasrain 1995). There are, however, concerns in using organic solvents in the level of residuals and their pharmacologic effect. Process control and safety during lyophilization and toxicologic effects of residual solvents in the finished product are the principal concerns in the use of organic solvents (Teagarden and Baker 2002).

11.1.4 Current Expectations and Drivers for Improvements

Interests in improved control of CPPs for better reproducibility and efficiency and assuring consistent finished product critical quality attributes (CQAs) throughout the batch and from batch to batch are the two key aspects pushing innovation in the field. Driven by economics as the market value of products continue to increase, reduction in losses because of a batch being rejected due to loss of control of the CPPs, or a significant reject rate during physical inspection is receiving more focus and attention.

Product and process understanding is a precursor to achieving greater control for commercial scale lyophilization. It is well accepted that the CPPs throughout the lyophilization process are the independent variables of shelf (inlet) temperature, chamber pressure, and time. Key process parameters are the dependent variables of product and condenser temperatures. Basing the process on product temperature is recognized as inadequate for commercial manufacturing due to influences of temperature probe placement and product location, both of which are directed by the need to follow proper aseptic processing technique. It is well known and accepted that product temperature measurements of the vials containing probes are atypical of the rest of the batch: they are the first to freeze and the first to dry. As well, automated material handling systems for transfer, loading, and unloading the lyophilizer have precluded monitoring product temperature in routine manufacturing, simply because the probes cannot be automatically positioned in a vial. This fosters a reduced reliance on product temperature as an indicator of adequate process control and places greater emphasis on precise, reproducible control of the independent parameters.

In particular, there is a quest for understanding of how different product behavior and finished product attributes result with using the same CPPs in a different lyophilizer when scaling up or transferring a product from one lyophilizer to another or to different manufacturing sites. Investigations into failures in product transfer lead to a greater understanding of gas and vapor flow. Mechanisms and models of flow through the restrictive connection from the product chamber to an external condenser have been identified as a significant influence and a major consideration.

Suitability for the intended use is the rationale by which quality attributes are established. For a lyophilized parenteral specifically, this encompasses dried state storage stability and the ability to readily revert to a parenteral solution for patient administration upon reconstitution. Improved stability for longer shelf life resulting in greater effectiveness of material management and inventory control is becoming more important as market quantities and batch sized continue to increase. Distribution networks are also more extensive as global markets develop. Alleviating returns for product beyond its expiry date can provide significant savings. A long shelf life is also imperative for the increasing number of products used in therapies treating rare conditions: products that have received orphan drug status. Inventory turnover is not as often as products used in therapies for more common conditions.

Improved dried state stability encompasses both an extended expiration date and more importantly suitability for storage at room temperature. As product manufacturing becomes more centralized and distribution becomes more global, product may reside in the distribution chain longer. Manufacturing larger batch sizes is reflected in the number of larger capacity lyophilizers. A common size commercial scale lyophilizer prevalent in the turn of the century may have had the capacity for 60,000 10 cc vials. It is now more common to install capacity for batch sizes of 130,000 10 cc vials. Larger batch sizes allow for a reduction in unit costs with higher throughput in manufacturing.

The need to distribute products that must be maintained at controlled temperature conditions of 5 or −20 °C is becoming more difficult and costly as distribution channels become more extensive and distances greater. This is an important consideration during development and becomes compelling for designing formulations that will stabilize the dried product at more elevated temperatures. Relative to the needs for distribution of commercial product, cold chain distribution for clinical studies is manageable, though not easy, particularly with global product distribution into a wide variety of countries. Product development is not complete when a product is sufficiently suitable for conducting clinical trials. Further work is warranted for developing a suitable commercial product that does not require cold chain distribution. Even in the rare case when cold chain distribution and storage is necessary, stability studies to establish an acceptable duration when the product may be beyond the intended storage conditions for some interval are warranted.

In addition to suitable stability for an extended shelf life, achieving desired finished product quality attributes has also focused on the absence of melt-back and collapse. Product exhibiting any incidence of melt-back or collapse is considered to be a product defect unless proven to have acceptable CQAs. Melt-back or collapse may be proven to be a cosmetic concern when shown to have no effect on dried product attributes, including storage stability, though melt-back and collapse are generally considered to be less desirable.

11.2 State of the Industry in Commercial Manufacturing

Increased market demands as the patient population and market size grows, and global distribution becomes more common, has led to increased batch sizes. As well, with an increase in specialized courses of therapies, treatments for less common conditions and specialized indications driven by development of orphan drugs require a fewer number of smaller batches necessary to supply the focused market. One might expect this to lead to segregation in manufacturing operations: high capacity for large market products and boutique operations for specialty products like orphan drugs.

Manufacturing capabilities and capacity continue to reside within an innovator's operations as well as being outsourced to a Contract Manufacturing Organization (CMO). For some pharmaceutical and biopharmaceutical companies, the

manufacturing may be solely outsourced. For others, in-house capacity may be supplemented using a CMO. The most significant differentiation between an innovator's in-house manufacturing operation and a CMO is that the innovator may have a dedicated manufacturing site while a CMO is a multi-product facility. Control and security of automated control systems and equipment cleaning are prime concerns with such multi-product operations. The increased separation of an operator for decrease exposure to the product widely applied to parenteral manufacturing has also been used for lyophilization operations as well.

Automated control of the lyophilization process has become common and includes lyophilization and support processes. Automated systems are comprised of control capabilities, process monitoring, and data acquisition, as well as batch report generation and historical data archiving. Process control and automation combines the necessary hardware in lyophilizer design and construction and the automated control system. This allows for complete automation of clean-in-place (CIP), steam-in-place (SIP), and nitrogen filter integrity test (FIT) along with the lyophilization process and has become common. These sophisticated automated control systems may stand alone, be able to be accessed remotely, or an integral part of an expansive network. Technical support of automated systems requires staff with knowledge of the control system hardware and software, as well as having a good understanding of the process requirements and how the lyophilizer operates. Control of the applications software as well as the lyophilization process recipe is critical. The operator interface is also important. Security for the system access and confidence that the correct recipe is initiated to begin the process for a specific product is imperative. As for any process step in manufacturing a pharmaceutical, documentation of the step, either manually or electronically, and verification by a second individual is expected.

Automation has also been effectively applied to cleaning of the lyophilizer interior. CIP systems for the product chamber and condenser are routinely included in new production lyophilizers. Although not a direct product contact surface, the lyophilizer interior may be treated as such (Johnson et al. 2012). Expectations for cleaning effectiveness and residual levels used for process equipment such as bulk solution tanks are often applied. Unique to lyophilizers is that the use of any cleaning agent is rare a, most often only as a decontamination agent. Rather, a rinse with purified water, USP, or water for injection, USP is all that is often used. The most effective cleaning verification consists of swabbing surfaces that have been shown to be difficult for the CIP system to effectively clean. This may be the location furthest from the cleaning nozzles or where the spray may be obstructed. CIP of both the product chamber and condenser should be verified.

The combination of increased batch size and the interest in minimizing product exposure to operators has driven continued improvements and frequency of application in high speed automated material handling systems. Approaches have varied between transfer and loading of the lyophilizer in a single row directly off of a conveyor to accumulating a large quantity, often an entire shelf of product at a single time for transport and loading of the lyophilizer. Both approaches have also been used for unloading the lyophilizer.

Restricted access barrier systems (RABS) have also become more common. Application of a passive system, where operator access is achieved by opening the barrier separating the operator from the product, or complete isolation where the enclosure is sealed and sanitized where access for operator activities is provided through gloves in the isolator wall.

11.3 Contemporary Technology

11.3.1 Formulated Product Characterization

Identifying the low-temperature characteristics is imperative for investigating the CPPs during process engineering studies. The shelf temperature intended during the freezing needs to be sufficient to assure adequate solidification. Product characterization and behavior while warming a frozen preparation is crucial for investigating conditions appropriate for primary drying. These temperatures can be determined using common methods of low-temperature thermal analysis (LT-TA). These methods include electrical resistance (ER), low-temperature differential scanning calorimetry (LT-DSC), and freeze-drying microscopy (FDM).

There are various methods available for characterizing the liquid preparation to be lyophilized. Many depend upon the change in physicochemical nature of the formulation. Specifically, it is the change in state that is of interest. Fundamental difference in physical properties including heat capacity and thermal and electrical conductivity are classical methods for determining when a material undergoes a change in state.

Electrical resistance measurements have been used to effectively determine when a material solidifies with cooling of an aqueous system containing a solute that crystallizes from a dilute aqueous solution. It is also effective to indicate at what temperature a melt occurs upon warming a frozen preparation. For an aqueous solution containing a solute that crystallizes, subsequent to nucleation of ice and ice crystal growth, continued cooling will show a sharp increase in electrical resistance when the solution undergoes nucleation and crystal growth of the solute with the coincident solidification of the remaining unfrozen water. Upon warming such a composition, a most highly concentrated solution will form at a distinct temperature as a result of melting. This behavior, occurring at a distinct and reproducible temperature and concentration, reflects the behavior of an eutectic material, referred to as eutectic behavior. The temperature at which the crystalline material and a small amount of ice melts to form a highly concentrated solution is the eutectic temperature. As many lyophilized pharmaceutical preparations solidify as amorphous rather than crystalline compositions, lyophilized preparations exhibiting eutectic behavior are rare and measurements of electrical resistance of limited value. In mixed preparations where there may be a solute, such as an ionized species of an organic compound, the solidification upon cooling and softening upon warming may be revealed by a change in the electrical resistance, though the results would not be considered definitive but rater supportive data when other methods of analysis are employed.

The evolution and consumption of the heat of fusion and a change in thermal conductivity and heat capacity are the basis of LT-DSC. For material that tends to crystallize, an exotherm occurs, reflected in a sudden increase of the sample temperature due to the heat of fusion when nucleation and crystallization occurs during cooling, relative to a reference that does not exhibit eutectic behavior. Upon warming the sample, an endotherm occurs when consuming the heat of fusion with the melt of crystalline material. For material that does not crystallize, but rather solidifies in an unstructured amorphous mass, there is a change in heat capacity, thermal, and of lesser magnitude, electrical conductivity. This change manifests in the different cooling and heating rates due to differences in heat capacity and thermoconductivity of the material in the liquid and solid state, with a sudden shift in that rate occurs when the material progresses from one state to another. It is the sudden shift in the rate that reflects the glass transition, denoted as T_g' for low-temperature analysis of a preparation to be frozen and lyophilized. A sensitive method with the ability to determine solidification of a solution to form ice and solidified solutes, and to distinguish a glass transition and crystalline melt upon warming, LT-DSC is a useful analytical tool. Most often, a glass transition of an amorphous complex occurs and the temperature at which this change occurs is of greatest interest and is the result reported. As many instruments report the change in enthalpy (ΔH), it may also have value as a quantitative method, though these values are seldom reported.

Like LT-DSC, differential thermal analysis (DTA) measures the difference in thermal conductivity and heat capacity relative to the material in the liquid state, and the temperature where there is a change in the heat capacity coincides with the change in state. DTA measures the difference in temperature relative to a reference material when the instrument is cooled and warmed at a constant rate. Measurement of the difference in dielectric measurements correlated to a change in state of a frozen system has also been explored (Evans et al. 1995). These methods are rarely used as commercial instruments are not readily available.

FDM is considered to be the gold standard of the industry. A sample is placed on a specially constructed stage contained within a vessel capable of exposing the sample to low temperatures at a reduced pressure. Commercially available units utilize piezoelectronics for accurate and precise control of the sample stage. Using conventional microscopic techniques, a drop of sample is placed on a coverslip and a second coverslip placed over the droplet to form a thin film of the sample solution. The sandwich of the coverslips and sample is then placed on the stage positioned under a standard microscope. An advantage to this method is that a polarizing microscope may be used to distinguish the development of a crystalline form of a solute upon freezing and the melt upon subsequent warming, along with sometimes vibrant colors. Changes in the physical appearance during cooling and warming may be correlated to the temperature of the instrument stage. Upon cooling, the nucleation of water to form ice and subsequent solute solidification may be observed. Subtle changes in state during cooling are often difficult. With the stage evacuated to a reduced pressure, the stage is warmed, ice begins to sublime, and observations made of the sample. The ice-vapor interface, termed the sublimation front, may be observed as it progresses from the outer edges of the coverslip towards the center of the film. One can observe the growth of a dried layer as sublimation

continues and the material dries with retention of the structure established during freezing. As the solute composition continues to warm, a change in the structure different than previously appearing at lower temperature can be observed: the material has warmed through it's glass transition, has softened, is no longer able to support its original structure, and is becoming sufficiently fluid and a change in the observed appearance occurs. Continued warming leads to catastrophic collapse observed as a complete loss of structure.

Though the common methods of LT-TA described utilize different techniques, all are based on a measurement associated with a change in state. The presence of an ionic species, where transfer of an electrical current with the species being ionized and can effectively carry an electrical current when in the liquid state, is the basis of ER measurements. This is an effective method with the presence of an ionized species and of no value when the composition is amorphous. Measuring the glass transition by LT-DSC is an effective method for many pharmaceutical preparations as the great majority of the products are amorphous. The limitations and the interpretation of the thermogram are somewhat subjective, as some thermal events are difficult to detect and interrupt on the thermogram. There is also no standard reference to reporting the results. For a crystalline melt, the temperature at the onset of the melt or the temperature at half the peak height, or the temperature at the peak of the exotherm may be reported. A glass transition may be difficult to determine, is open to interpretation, and varied in reporting. As there is a shift in the thermal conductivity and heat capacity, the onset of the shift or the point of inflection may be considered more significant. Regardless of the method when interpreting the analysis, the approach should be clear and consistent when reporting the results.

11.3.2 Understanding the Influence of Packaging

Unique in pharmaceutical product manufacturing, the packaging is an integral part and has a significant impact on lyophilization. The vial influences achievable processing rates during freezing and subsequent drying due to its influence on heat transfer during the process as suggested by Pikal et al. (1984). Tubing vials have been shown to be more effective in heat transfer compared to molded vials under the same set of processing conditions. This, however, causes the temperature around the bottom perimeter of the vial to become the warmest location and can lead to product exceeding the threshold temperature in the local region. Molded vials, though having poorer overall heat transfer, tend to provide more uniform heat transfer (Trappler et al. 2012). The bottom curvature, specifically the bottom radius and extent of contact between the vial and the lyophilizer shelf is the most significant factor in the vial geometry for heat transfer (Cannon and Shemeley 2004). For a given volume of product, the diameter of the vial dictates the depth of the product, which has an influence on mass transfer of water vapor from the sublimation of ice and therefore potential drying rates. Mass transfer through the dried layer is well recognized as one of the rate-limiting factors, with different model systems demonstrating varied resistances (Pikal et al. 1983).

Interactions between lyophilized product and glass vials would not be expected to be prevalent since the product is not in contact with the glass in the liquid state for any extended time and the product is in the solid state during storage. Therefore, concerns of leachable and extractable components from the glass vials have not been a significant issue for lyophilized products as compared to liquid preparations. There are instances where a lyophilized product at extremes in pH have undergone a significant pH shift when comparing the bulk solution to the reconstituted product after lyophilization due to interaction with the glass.

The unique stoppers used for lyophilized products are designed to allow water vapor to pass thought the opening provided when the stoppers are partially inserted into the vial during the filling operation. The geometry of the stopper and resulting opening has been shown to have little effect on passage of water vapor though the partially inserted stopper: single, two, and three vent stoppers are comparable. There have been stoppers with more numerous vents that become restrictive to water vapor flow, though such stoppers are not common (Bosch and Shultz 2008).

Improvements in stopper rubber formulations have been made to reduce water and gas permeation through the stopper and the tendency of the stopper to absorb moisture during steam sterilization to subsequently desorb the moisture during product storage. For low dry weight products where even small amounts of moisture remains in the stopper, contribution of moisture from the stopper may increase the moisture in the product, leading to collapse upon storage. Stopper drying processes are critical for some combinations of product and stopper types (Hora and Wolfe 2004). Leachable and extractable materials from the stopper are of growing interest, though more of a concern for liquid products exposed to the stopper during long-term storage.

There is a growing interest in cartridges and syringes as a lyophilized product presentation. Like many lyophilized preparations, cartridge and syringe presentations are often unit dose. The increase in products intended for self-administration makes such a product presentation attractive for ease of administration, improved convenience, and patient compliance. There are also suggestions that the total cost for delivery to the patient is a commercial advantage and self-administration of injectables offers advantages and challenges as they become more prominent (Kaifman et al. 2012). Generally, this presentation is limited to small product dosages for subcutaneous and intramuscular administration where volumes are often 2 mL or less, though bolus injections require larger volumes.

Significant challenges are associated with processing for material handling and lyophilization. Cartridges were first introduced by Wyeth under the Tubex® brand. Cartridge holders for administering the products were as common as stethoscopes for health care practitioners. Dental cartridges have been and continue to be manufactured in large quantities with the use of high speed manufacturing. Cartridges are also available in tubs as a ready-to-use presentation for manufacturing: they are washed, siliconized, and sterilized. Dual chamber cartridges and syringes have been developed and are available as a product presentation with unique designs suitable for reconstitution of a lyophilized preparation. These cartridges are unique in that there is a channel protruding from the side wall of the cartridge that allows the

diluent to pass by a center plunger, and with a unique plunger design, aid in reconstitution of the lyophilized cake upon activation of the cartridge. The manufacturing entails first dispensing the bulk solution into the cartridges held in a frame or magazine, orienting the cartridge vertically. After the product is dried, the product is placed on a filling line, the diluent dispensed into the open end of the cartridge and a second plunger inserted to seal the diluent. Vetter is the prominent CMO that has established commercial manufacturing. Merck manufactures its own cartridges and markets lyophilized polyethylene glycol (PEG) Intron in a dual chamber cartridge as a Redipen® for self-administration.

Limitations and challenges for dual chamber cartridges for lyophilized preparations include the unique material handling required in an aseptic environment. The number of manipulations of an open container increases the opportunity for contamination. As the geometry of the cartridges, complicated by the arrangement in tubs, does not lend to a significant contact with the lyophilizer shelf, heat transfer is a significant rate-limiting factor, particularly during freezing. Because of this, low temperatures sometimes required during freezing for solidifying the product are difficult to attain. With the product to be lyophilized positioned where there is no intimate contact with the shelf, as with vials, efficient and effective heat transfer is even more of a challenge.

The other limitation is the batch size within a given lyophilizer relative to those of products contained in vials. The number of containers for a given shelf surface when processing product in vials is relatively high since the vials can be close packed, referred to as a "nested" configuration, where each vial, except for the edge vials, has six neighbors. For example, approximately 355 of 3 cc vials can be placed within 1 ft^2 (100 cm^2) of the shelf surface. A magazine used for commercial manufacturing of a 3 cc dual chamber cartridge allows for processing only 146 units within the same shelf surface area, and the cartridges stand approximately ¾″ (9 mm) apart from each other. There is also a lack of intimate thermal contact between the product container and the shelf. A shelf clearance of 3″ (76 mm) is sufficient for lyophilizing in a 10 cc vial containing up to 3 cc. For the equivalent syringe or cartridge, 8″ (205 mm) is required to accommodate the cartridges vertically oriented within the magazine. All of these factors present new challenges in understanding the mechanisms for heat and mass transfer; different approaches to processing are warranted.

11.3.3 Identifying Critical Process Parameters

It is well accepted throughout the industry and the US FDA that shelf temperature, chamber pressure, and time are the CPPs as independent variables[1] for lyophilization (Food and Drug Administration 1993). The lyophilization process is described as a series of soaks and ramps of the shelf temperature and soaks of the chamber pressure.

[1] An independent variable is those that are under direct control and are not resulting from influencing factors or dependent upon other processing variables.

A soak is a predefined interval for which the parameters are controlled at the desired target condition. During these soaks a steady state condition can be achieved. A ramp is an average rate of change in temperature from one soak setpoint to another. Target CPPs reflect an ideal set of soaks and ramps for the shelf temperature and soaks for the chamber pressure as the CPPs.

Determining the CPPs suitable for a specific product is dependent upon the formulation characteristics and the product presentation. Formulation characteristics need to be determined and understood early in the development and precedes any process engineering studies. As the volume within the container and the size and type of container has an influence on processing, the final product presentation also needs to be defined.

Contemporary approaches in conducting process engineering explore variables in the CPPs and can commence once the formulation and product presentation are defined. The objective of process engineering is to investigate the effects of altering the CPPs on the resulting behavior during processing and the resulting finished product attributes. The goal of the process engineering is to establish the combination of shelf temperature, chamber pressure, and time for each step of the process that are safe, effective, and sufficiently robust. The preferred combination of the CPPs is verified by correlating the essential CQAs and desired finished product attributes, including sufficient stability during storage. A rigorous approach to development to gain an understanding of the product and process that may include empirical and mathematical data as outlined in the ICH Harmonised Tripartite Guideline on Pharmaceutical Development (Q8R2) (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use 2009).

The validity of the parameters in the process engineering studies during development is dependent on the ability to emulate commercial capabilities. Component preparation needs to yield attributes of cleanliness achieved in a commercial operation. The vials and stoppers should be of the same condition as when producing a product in a commercial operation: clean and essentially particulate free. The bulk solution should be sterilized by filtration. Assembly of the product with dispensing the sterilized bulk solution and transfer and loading of the lyophilizer should be in a class 100 environment: Class A is not required, Class B is sufficient. Each of these factors influences the cleanliness and particulate burden. The cleanliness of the components and product to be lyophilized affects the nucleation of water and the growth of ice. These events during freezing in an uncontrolled environment are different than that which occurs when processing commercial sterile product in an aseptic operation.

To gain confidence in the processing parameters and the lyophilizer is performing as expected, the equipment should be qualified and a preventative maintenance (PM) and calibration program should be in effect. Equipment qualification and proper PM are critical for confidence that the equipment used in engineering the process is operating properly and there is adequate control of the conditions during processing. As in a commercial operation, to have confidence in the measurements and in following sound scientific principles, the instrumentation for controlling and

Table 11.1 Operational qualification test function and parameters

Qualification test	Test parameters		
Shelf cooling	Maximum		Controlled
Shelf heating	Maximum		Controlled
Shelf control	Low temp	Intermediate	High
Condenser refrigeration	Maximum cooling rate		Ultimate low temperature
Vacuum system	Maximum evacuation rate		Ultimate low pressure
Leak rate	Threshold capabilities		
Pressure control	Low	Intermediate	High
Condenser capacity	Threshold capabilities		
Sublimation / Condensation Rate	Maintain CPPs		Maximum rates at predefined conditions

monitoring the process, as well as those used for monitoring the product should be calibrated. The steps in preparing the material under study should be well documented. Utilizing batch records for preparation and processing the batch in a study when conducting the process engineering provides an opportunity to record critical data and documenting observations. This provides many advantages over simply making notes in a laboratory notebook. Such formalized and detailed records provide valuable documentation as part of a development report. It also provides well organized, complete, and comprehensive data, becoming a valuable reference when preparing the chemistry, manufacturing, and controls (CMC) section of a regulatory submission.

In the clinical phase of development, establishing the CPPs need to focus on those suitable to prepare material for clinical studies. Parameters that are safe, effective, and sufficiently robust to accommodate unexpected influencing factors are most appropriate. Experience and expertise in the science and technology are paramount in obtaining this goal in a limited number of studies with a limited amount of API. The goals and expectations should be towards parameters necessary to prepare clinical material rather than those that may be desired for routine commercial operations. Optimal parameters for preparation of clinical material are those that are sufficiently robust and be suitable for differences in characteristics that may occur in the API. As well, it would be appropriate to verify the low-temperature thermal characteristics of each batch of API early in clinical development. This should be a routine practice until the upstream processing has been shown to be reproducible and able to yield API of consistent purity and potency.

Upon achieving favorable clinical results and in parallel to conducting later clinical studies, further process engineering studies to begin exploring process parameters for commercial manufacturing are appropriate. The goal for the studies at the later stages of clinical development is to establish the CPPs suitable for integrating into a commercial product manufacturing operation. Knowledge of the capabilities in the commercial operations as well as for the lyophilization equipment is imperative. A good source of information about such capabilities is the lyophilizer operational qualification study results. Test results listed in Table 11.1 would be appropriate to consider in conducting the studies in process engineering.

Clinical manufacturing and commercial lyophilizer performance provide the guidance in selecting the parameters used in the process engineering studies early in clinical development as well as in the later studies for a process suitable for commercial operations. Executing an engineering study in the clinical and commercial operations would be prudent to assure the lyophilizer can execute the intended parameters prior to processing actual product.

Different approaches may be employed to assess the impact of profiles in the parameters: shelf temperature for loading the lyophilizer and completing the freezing step and combinations of shelf temperature and chamber pressure for primary and secondary drying. The objective during the process engineering studies later in the clinical development phase of bringing a new product to market is to vary the CPPs in order to assess their impact on the resulting product temperature, processing rates, and residual moisture. Key to evaluating any combination of processing variables is knowledge of the product character and behavior during processing. Thermal analysis studies, as described in the earlier section, are the very first study and prior to undertaking any process engineering studies. Implementation of processing parameters based upon mathematical models can also be valuable as an initial trial, gaining experience on the behavior of the product under an initial set of processing conditions.

Shelf temperature has the greatest influence processing rates and the ultimate product temperature at the end of each step. The rate at which heat is removed during freezing, quantified as heat flux, dictates the initial cooling rate, rate at which the ice crystals grow, rate at which the highly concentrated solution cools, and rate at which the final solidification occurs. Annealing during freezing may alter the ice of initial freezing by inducing Ostwald ripening (Searles et al. 2001). During primary drying, the shelf temperature mostly dictates the rate of sublimation (Deluca and Lachman 1965). During primary drying, however, the heat flux is coupled with mass flux, the rate at which the water vapor can traverse through the dried layer above the sublimation front. This achievable mass flux has been studied to a great extent and a term of R_p assigned to the resistance of the dried layer (Pikal et al. 1983). Classically, the greatest direct influence of the chamber pressure is on the pressure differential that develops between the vapor pressure of ice at the sublimation front and the partial pressure of water above the sublimation front. This partial pressure is influenced by the flow of the water vapor through the dried layer above the sublimation front. The resistance, or more directly the pressure differential between the vapor pressure of ice and the chamber pressure dictates the flow rate, affecting the achievable rate of sublimation, ultimately and indirectly, the product temperature. It is important to recognize that the achievable rate of sublimation is dependent upon the relative partial pressure of water vapor above the sublimation front, above the top of the dried material, and in the atmosphere in the lyophilizer. Overall, the greater the difference in the ice vapor pressure in the product and the partial pressure of water that comprises the atmosphere, the greater the rate of sublimation that can be achieved.

Contemporary processing conditions entail control of the shelf temperature and chamber pressure during the secondary drying step. Principles and mechanisms that

effect desorption are the driving force in secondary drying. Classically, heat is the predominant influence to rates of desorption. Some studies have been conducted to evaluate the influence of chamber pressure up to 200 μmHg with no significant effect determined (Pikal et al. 1990).

Initial process engineering studies may focus on holding one variable constant while altering the second. The principal interest in these early studies is in the effect of the various combinations on the product temperature. A suitable and safe threshold temperature may be as close as 2–3° below the indicated collapse temperature measured during the LT-TA studies. Though a general guideline, a safe margin in temperature is selected empirically based on the character of the product gleamed from LT-TA, particularly FDM and during the early process engineering studies.

Though not intended to take the process to the point of causing the frozen material to melt back and form a liquid or the dried layer at and above the sublimation front to collapse, the CPPs should be varied to at least approach, though not necessarily exceed the critical threshold temperature. Results from studies that force the product to approach the critical threshold temperature can be used to identify both a target set of parameters as well as the combinations of parameters suitable for the process boundary conditions of the proven acceptable range (PAR) (Chapman 1984). Depending upon the character of the material and behavior during processing at the various conditions, the most effective combination of shelf temperature and chamber pressure can be identified. A study to evaluate the behavior during processing at the target conditions and the rates at the selected shelf temperature and chamber pressure would identify the time required to complete each step. It is important to consider that the time indicated for reaching the ultimate temperature during freezing and primary drying indicated by the containers monitored using a temperature sensor will be atypical of other containers, due in part to the stochastic nucleation event and resulting ice crystal growth. With uncontrolled freezing, the containers with the temperature sensors are the first to undergo nucleation of water to form ice, generally have larger ice crystals, and will therefore be the first containers to complete the sublimation of the ice. Recognizing this difference warrants consideration in the time selected to complete each process step. In freezing, additional time may be included to assure all the material in all the containers are near or at the same temperature and within an acceptable variation relative to the shelf temperature. During primary drying, all the containers should be near or at the shelf temperature for an amount of time so that the one is assured that all the material in each container and all the containers are at the same condition prior to progressing to secondary drying. The time necessary for secondary drying is easily determined by measuring the residual moisture as time progresses at the target shelf temperature and chamber pressure. Residual moisture can be plotted relative to time to determine how long is required at the target process conditions to achieve the level that correlates to long-term stability in the dried state.

An essential part of verifying the target CPPs is correlating long-term stability at the intended storage conditions for the dried product. Storage at accelerated conditions may also be useful. Dried state stability may utilize the application of Arrhenius equation for predicting stability upon storage. Note, that the Arrhenius

Table 11.2 CPPs for a mAb formulation, 4.1 mL fill, 30 mL type 1 tubing vial, and 20 mm single vent stopper

Process step	Soak (°C)	PAR value (°C)	Duration hours	Ramp rate (° per hour)	Pressure (μmHg)	PAR value (μmHg)
Loading	5	0–10	4		10 psia	
Freezing				15	10 psia	
	−50	−55 to −45	4		10 psia	
Primary drying				30	80	60–100
	−18	−23 to −13			80	60–100
Secondary drying				15	80	60–100
	25	10			80	60–100

activation energy and Arrhenius A factor are intended for calculating rate constants of reactions in solution, and should be considered a first approximation for reactions in the solid state. It is expected that, at a minimum, the ICH guidelines for conducting stability studies be followed. Use of the glass transition (T_g) of the dried material to evaluate the relative temperature difference for the storage condition has also been studied and may be applied to predict the dried state stability (Fitzpartick and Saklatvala 2003). If the product is stored at a temperature above the T_g, molecular mobility, a prerequisite to chemical reactions is possible, thus leading to potential degradation.

Once the target parameters of shelf temperature, chamber pressure, and time are established, data from the early studies in varying the CPPs can be used to identify the parameters for boundary studies to establish the PAR for the process. The boundary conditions are most often selected that they vary equally from the target CPPs. For example, a target parameter for the soak during freezing of −40 °C may have a PAR of ±5 °C, providing an acceptable range from as low as −45 °C to as warm as −35 °C. A description in the Master Batch Record would therefore describe the process in a series of soaks and ramps as in the example listed in Table 11.2.

Studies conducted to verify these conditions that form the PAR demonstrate the product temperatures remain below the threshold temperature and there is sufficient time in each step to take the process to completion. Conducting the Boundary Studies to establish parameters at the PAR creates a window for the process around a set of ideal CPPs. A set of parameters for a PAR for a set of target CPPs, creating the process window is illustrated in Fig. 11.1. This chart can be an effective tool in comparing the parameters achieved during product and process transfer as well as during routine commercial manufacturing.

11.3.4 Establishing Critical Quality Attributes

The goal in the vast majority of applications when lyophilization is used for preservation is the removal of water that may be involved in hydrolysis reactions leading to degradation of the active ingredient. Residual moisture is therefore a CQA as the

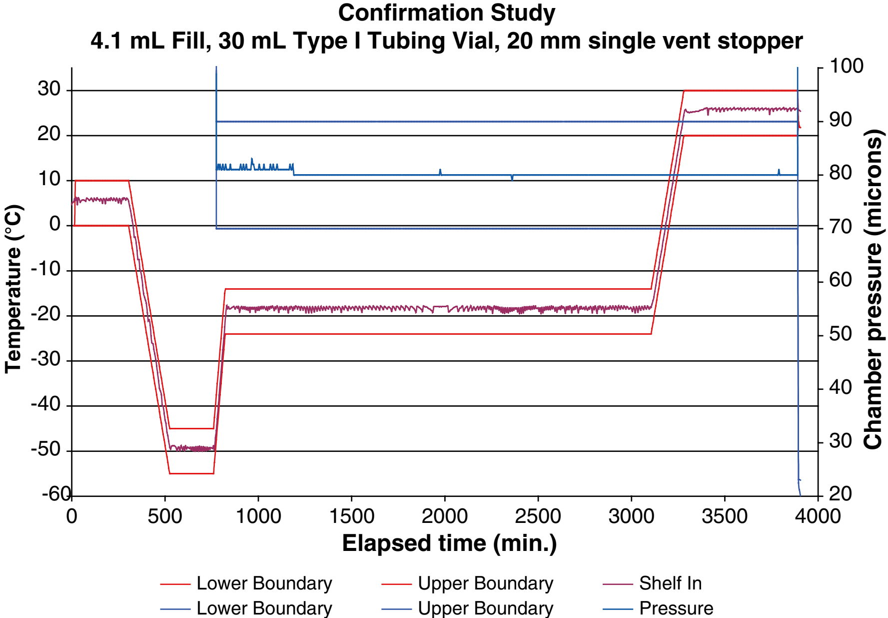

Fig. 11.1 Executed CPPs and process window of a set of conditions defined as the PAR, or boundary conditions

level of residual moisture has a direct correlation to dried state stability. Correlating the CPPs to this CQA is straightforward: drying with retention of the structure during freezing and sufficient time in secondary drying at elevated temperatures yields a product of predictable residual moisture and long-term stability. Later studies in process engineering can include preparation of multiple sublots stoppered at different times during secondary drying to correlate time, residual moisture, and long-term stability.

Complete dissolution is imperative when the product is to form a true solution suitable for parenteral administration. Establishing a specification for reconstitution time is influenced by the intended use of the product. For a product designed as a pharmacy bulk pack for reconstitution and dispensing into syringes for later direct administration addition into an IV solution at the bedside in a prophylactic course of therapy, a reasonably short reconstitution time is more a matter of convenience and is desirable, not a critical product design attribute. For a product designed for treatment in a critical course of therapy and may be on an emergency room crash cart such as tissue plasminogen activator or an operating room crash cart such as dantrolene, when complete dissolution in seconds rather than minutes is imperative, this dried product characteristic becomes a CQA.

Physical form may also be a CQA. Pharmaceutical products are more stable when crystallized during freezing. If so, then such an attribute needs to be characterized and monitored during development, through technology transfer and in routine

Fig. 11.2 A lyophilized cake having desirable level of "pharmaceutical elegance"

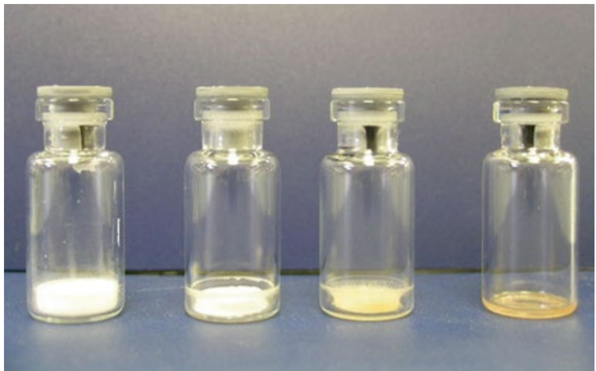

Fig. 11.3 An amorphous solid formulation exhibiting a range in the extent of collapse

manufacturing. If the formulation consists of one or more excipients that tend to and may not crystallize, being impeded because of other excipients, crystallization of these excipients should be monitored as well. For example, a formulation containing glycine, mannitol, phosphate buffer, or sodium chloride in combination with sucrose that do not readily crystallize during freezing may crystallize during storage in the dried state.

Physical appearance is a desirable attribute and not a CQA. A dense, uniform, and white lyophilized cake with the absence of cracking and an irregular cake surface reflects an attribute referred to as "pharmaceutical elegance" as depicted in the photograph of Fig. 11.2.

Such pharmaceutical elegance is strived for in product design and is a desirable finished product attribute, though not always achievable and not a CQA. A desirable physical appearance is also the structure and strength of the dried cake, when the cake structure remains intact without cracking and breaking into pieces or being friable and forming a powder. The retention of product structure established during freezing is also desirable: The presence of collapse of amorphous material, illustrated in Fig. 11.3, or melt-back of crystalline material in Fig. 11.4 is considered a product defect unless proven to have no effect on product stability or reconstitution.

Fig. 11.4 A crystalline formulation exhibiting a range in the extent of melt-back

Melt-back of a crystallized fraction or an amorphous product in the presence of ice with the product reverting to the liquid state is considered to be catastrophic. Melt-back results in a complete loss of structure to at least some portion of the cake and is associated with high residual moisture and inadequate stability, and poor dissolution with a prolonged reconstitution time. Collapse is associated with a loss of the structure achieved during freezing and may be less definitive. Some products may exhibit a slight loss of structure associated with collapse and have no distinguishable differences in their CQAs.

Focus is given to establishing a reproducible process and consistency of finished product quality; an essential aspect of achieving a high level of quality is batch uniformity. As each unit of product provided to the ultimate customer is never tested, verification of quality is based upon sampling of a batch to assess the product quality and the sample tested is expected to reflect the attributes of each unit within the batch. Sampling provides a level of confidence though is not often statistically significant. Sampling for uniformity of dosage form requires 30 units for a sample set, with ten tested and the remaining 20 sequestered for retesting. Assessment of sterility directs 20 units be tested. Residual moisture is commonly evaluated based on three samples. For assessment of each CQA, each test is grounded that each and every sample is representative of the rest of the batch.

Specific to a lyophilized product, sensitive to the time in the presence of water and, for some attributes, influenced by the presence of location in the lyophilizer, assessment of batch uniformity is of two relative perspectives. Evaluating the beginning, middle, and end of the batch is not an unfamiliar concept for assessing quality aspects of a pharmaceutical product. For a lyophilized preparation, it reflects the exposure of a reactant the leads to potential degradation. As a bulk solution the product experiences conditions of high volume and low surface area, perhaps exposed to metal surfaces of the stainless steel holding tank for the duration of the end of compounding and filtration through a sterilizing filter to the completion of the filling operation at the end of the day. Part of the batch is also exposed to glass surfaces with low volume and high surface area ratios, and is dependent on the length of the filling operation. Assessing finished product quality attributes for the beginning, middle, and end of the day encompasses the variability of conditions the product

experiences. Measure of potency and degradation products is prudent to monitor the influence of the time is within a specific environment in the presence of water.

As well, there may be an influence of location within the lyophilizer on critical quality and sometimes desirable quality attributes: residual moisture as it correlated to stability, reconstitution, and appearance. These attributes may be correlated to thermal history through the lyophilization process. Thermal history may be correlated to attributes of a lyophilized preparation and thermal history may be influenced by position within the lyophilizer. It would therefore be prudent to identify and discriminate which are indeed desirable and CQAs. Generally, each is considered desirable and may be useful attributes in assessing the influence of location within a lyophilizer. One approach is introduced as a statistically based critique comparing thermal history throughout the process to finished product attributes (Trappler 2004). The approach evaluates distribution of product temperature at the critical times during the process: the temperature profile within the batch at the completion of a stabilization period upon completing the loading operation, at the end of freezing, primary and secondary drying. These intervals are critical for the process to assure each and every vial is at the same condition prior to proceeding to the next step of the process where the processing conditions change significantly. At the completion of freezing, it is imperative that the product is adequately solidified with sufficient time at the final freezing temperature. Prior to proceeding to secondary drying, all the ice must be sublimed and the product should be within a reasonable range relative to the shelf temperature prior to progressing to warmer temperatures for secondary drying. Removal of residual moisture with the product at warmer temperatures is time and temperature dependent, and therefore should be close to the shelf temperature prior to stoppering the product. Evaluating the variation in product temperature at these critical times in the process can be achieved using a statistical analysis relative to the mean of the temperatures for the entire batch. One approach is by converting the temperature to a relative z-score and evaluating the z-score values at the critical time for each step of the process. If product at the most representative and at the most varying temperature location shows no difference in finished product attributes, then product distributes throughout the lyophilizer will also yield the same finished product properties. Correlation of thermal history to lyophilized product attributes builds a significant body of data for establishing a level of confidence in batch uniformity, independent of location within the lyophilizer.

11.3.5 Attempts in Applying the FDA PAT Initiative

Significant attention has been given to process analysis technology (PAT) since the issuance of the FDA PAT (process analytical technology) initiative. Historically, the progression of the lyophilization process was indicated by the product monitored using temperature sensors. It is well recognized that the presence of a temperature sensor influences processing: the containers with sensors are the first to freeze and the first in which sublimation of ice is complete. This makes these containers atypical of the rest of the batch, and therefore the data a relative indication.

Alternatives for monitoring the product temperature and the progression of the process have long been of interest. Various techniques in monitoring of the progress during primary drying have been investigated. A barometric method where the pressure rise in the chamber is correlated to the amount of ice remaining in the product interrupts the process by isolating the product chamber to the condenser and monitoring the increase in the chamber pressure. An alteration to the technique referred to as the manometric method, correlates the chamber pressure achieved to the product temperature. This technique, coupled with a mathematical modeling, became the basis for application to process engineering techniques in identifying the shelf temperature and chamber pressure during primary drying (Milton et al. 1997). Semiquantitative measurements for the progression of primary drying have also been of interest. Comparison of pressure measurements based on the presence of water vapor comparing the chamber pressure indicated by a thermoconductivity gauge to that of an absolute manometer has also been used to indicate the progression of ice sublimation. Measurement of the water vapor in the atmosphere using a moisture sensor was also explored (Pikal and Roy 1989). Use of mass spectroscopy in the analysis of the composition of the atmosphere during vacuum processes was first of interest in other industries (Landsberg et al. 1956). The technique was later applied to lyophilization, though there have been limited investigations. This semiquantitative method of gas and vapor analysis does, however, provide significant insight to the conditions during processing, principally during primary drying. Implementation of any of the various methods focusing on the progression of the sublimation of ice may lead to greater insight into primary drying (Nail and Johnson 1991). More recent investigations have been into monitoring the flow of water vapor from the product chamber to the condenser measuring relative velocities of nitrogen, and water vapor based on the Doppler effect using tunable diode laser absorption spectroscopy (TDLAS) has gained interest (Gieseler et al. 2007). As these process monitoring techniques have been considered for application in a PAT initiative, none of the technologies provide comprehensive process analysis and there has been no widespread use of any one method.

11.3.6 Incorporating Design Space Principles

Application of the principles of process and quality control entails defining the product quality attributes and identifying the influencing factors that affect the quality attributes. Successful execution of a process involves controlling the inputs reproducibly to yield consistent product attributes. Achieving adequate control and reproducibility requires a target and a defined range for an allowable variation for the inputs to the process. This entails consistent product components, including drug substance, added formulation components, and packaging components, as well as controlled and reproducible processing conditions. Based on this defined range, fixed target processing variables of the CPPs are required. Process design and executing adequate process control are based on allowable input variation; reproducibility of

Table 11.3 Component inputs to consider for design space

Component	Input/factor		Influence/attribute
API	Purity	Final concentration	Potency
Excipients	Assay	Final concentration	Stability
Acid/base/buffer	pH	Final concentration	Solubility/stability
Container: product	Composition	Leachable/extractable	Purity/stability
Container: process	Geometry	Construction	Heat/mass transfer
Closure: product	Composition	Permeability	Gas/moisture content
Closure: process	Geometry	Construction	CCI

the CPPs and consistency of CQAs can be achieved. This requires identifying the inputs and conducting studies to quantify the relationship of those inputs to the process output; the finished product quality. Once quantified, an allowable process input variation can be identified and a range of CPPs established. Such an approach requires greater efforts in development to generate the empirical data, leading to understanding the relationships of the multivariate inputs. During process engineering the inputs that affect the process and resulting CQAs are measured and the CPPs are adjusted accordingly. This approach can be readily implemented for processes with a single variant: It becomes more complex for multi-variant processes.

Product and process knowledge, along with predefined and controlled inputs to a process with allowable process variability around fixed CPPs that influences the CQAs establishes a PAR. Adjusted CPPs according to variation of process inputs to control the CQAs is referred to as the design space. Inputs for lyophilized preparations can be categorized as being components or process. Components include ingredients of the formulation and packaging. Processing entails preparation of the bulk solution, sterilization filtration, dispensing, as well as lyophilization. Interactions between components may be within the formulation components or between the formulation and packaging. The formulation and packaging components may influence behavior during processing, as well as stability during long-term storage. Processing factors encompass preparation of the bulk solution through the completion of lyophilization. Table 11.3 highlights some of the component inputs while Table 11.4 lists process inputs.

The influences of product and process variations need to be assessed during process engineering and development. Early during clinical development, many assumptions are often and need to be made, with potential for an interaction and impact on the final product evaluated. More knowledge and insight into the drug substance and product are gained as the product progresses through the clinical development: success and failures reveal whether the assumptions made earlier in development are correct.

Nominally, the conventional approaches to pre-formulation studies that entail measuring the pH solubility and pH stability characteristics for different acids or bases over a range of pH are necessary. Relative solubility in different solvents is also commonly studied. Consideration also needs to be given to compatibility and the risk of potential interaction of an excipient is also important to assess.

Table 11.4 Process inputs to consider for design space

Process step	Input/factor		Influence/attribute
Compounding	Assay	Purity	Purity/potency
Sterilization (filtration)	Sterility	Endotoxin	Microbiological purity
Bulk storage: chemical	Potency	Purity	Purity/potency
Bulk storage: micro	Sterility	Endotoxin	Microbiological purity
Container preparation	Cleanliness	Residuals	Purity
Closure preparation	Cleanliness	Residuals	Purity
Dispensing	Accuracy	Precision	Potency
Loading	Temperature duration	Thermal history	Purity/uniformity
Freezing	Cooling rate	Thermal history	Stability/purity
Freezing	Temperature duration	Solidification	Stability/purity
Primary drying	Pressure	Product temperature	Retention of structure
Primary drying	Temperature duration	Process rate	Dissolution/purity
Secondary drying	Pressure	Product temperature	Retention of structure
Secondary drying	Temperature duration	Process rate Residual moisture	Stability/purity
Stoppering	Pressure		CCI/dissolution

For example, the amine or carboxylic group, or the R group of an amino acid, may be reactive with part of an active compound. Effects of combinations of excipients also need to be explored as they have been shown to have an effect on physic-chemical aspects of the finished product (Byron et al. 1990; Fang et al. 2012). For solutes in solution, any interaction between the excipients and the API and the excipients with each other should be studied.

Packaging components also need to be selected to be appropriate for their effect during processing as well as post processing. For example, a stopper needs to seat properly during processing in order to provide an adequate opening for the water vapor to travel through during primary and secondary drying. Upon stoppering in the lyophilizer and throughout handling during unloading the lyophilizer, transfer to the capping operation, and until the over-seal is in place, the stopper needs to remain in its fully inserted position and not unseat itself. The geometry and construction of the plunger for a cartridge is also critical for the product and final use. The plunger needs to provide an adequate seal to separate the diluent from the dried product. As well, the geometry and construction can influence the extrusion force required for activation, reconstitution, and administration.

Preparation of the bulk solution and components going into the lyophilization process can have an influence on the product behavior during processing as well as the finished product attributes, initially and during longer-term storage. The chemical and microbiological qualities directly influence the suitability of a sterile lyophilized preparation for its intended use. Conditions during lyophilization, including the thermal history prior to and during solidification in the loading and freezing steps, achieving the required rates while retaining the original structure during primary drying, and achieving the level of residual moisture necessary for

long-term stability, all directly influence the behavior during subsequent processing and finished product attributes.

In considering the use of a design space approach to development, it is important to identify and discriminate which may be likely to vary, which are readily detected, and the magnitude of the impact on finished product quality attributes. In the case of a biological preparation where the ratio of the total protein content relative to the active protein varies, and the concentration of an excipient such as an amino acid is dependent upon the total protein, resulting in a variation in the relative molar ratios, and if that variation may be significant, then a range should be studied and established. For a product where an excipient is weighed according to specifications in the Master Batch Record, and any difference in concentration a rare event, is easily detected, and there is no need to study and establish a range.

For a formulation that does not require a buffer and the Master Batch Record that allows for a pH adjustment within a range, it would be prudent to assess the influence of the extremes in pH on physicochemical properties and behavior during processing, at a minimum the low-temperature thermal properties. Recognizing that pH units are a log function of the hydronium ion molar concentration, if the range is more than fractions of pH unit, it may be prudent to monitor the effect of the range on processing, finished product, and any effect during long-term storage.

Other factors may have less of a direct correlation. An example is the level of residual moisture, dictated by stopper drying as part of the processing method, and residual moisture levels upon storage. The long-term stability would be affected by higher residual moisture, where the moisture is desorbing directly or permeating through the stopper. Such a circumstance can also link to initial product design. Formulations with low total solids after lyophilization are more significantly impacted by small amounts of moisture that may be desorbed from the stopper over the product shelf life.

The amount of active for a lyophilized product is claimed as a quantity per vial basis and the dispensing accuracy and precision needs to be within a narrow range. For products which the fill volume is calculated based on an in-process assay, the range of dispensed solution may have a significant range that times during each step of the lyophilization process needs to accommodate the greatest dispensed volume. This unique combination of multi-variant factors is a set of conditions for which pursuing studies to establish a design space is appropriate, as the factors of dispensed volume and process parameters in this case are linked. The process needs to be engineered such that times during freezing and primary drying would be appropriate for the greatest volume that may be dispensed. For a minimum dispensed volume, the product would be expected to reach a low residual moisture content earlier in secondary drying, resulting in a potentially over-dried product. If a range of dispensed volume, or the concentration of the solutes would vary, the minimum and maximum allowable for the range needs to be taken into consideration in establishing the appropriate CPPs.

Control of the inputs into the process listed in Table 11.3 and the outcomes of the processing steps preceding lyophilization in Table 11.4 is essential to achieving the CQAs at the conclusion of the lyophilization process. Each of the lyophilization

process steps has a distinct influence on the CQAs. If the degradation kinetics are such that the product needs to be maintained at a reduced temperature when the product is a liquid; then conditions of temperature and time for loading product are critical and influence the finished product purity and potency. The resulting thermal history may also influence solidification and therefore the finished product, and as importantly, batch uniformity.

11.4 Application of Current Principles of Process Validation

11.4.1 New Essentials of Process Validation

The FDA process validation guideline "Process Validation: General Principles and Practices" issued January 24, 2011 is a paradigm shift for the industry (Food and Drug Administration 2011). In the guideline published in 1987, the perspective was to generate documented evidence that a process does what it purports to do. In the current guideline, the focus is on process and product knowledge gleamed during development, experience, and empirical data during the technology transfer when integrating the product and process into commercial manufacturing, and concurrent collection and evaluation of adequate data during routine manufacturing. The stated intent is for scientific evidence in demonstrating a reproducible process that consistently delivers product of predefined and consistent quality. The guideline goes on to define process validation in three stages: Stage 1 is process design, Stage 2 is process qualification, and Stage 3 is continuous process verification. The goal is achieving a high degree of assurance that the manufacturing process will produce finished product of known "identity, strength, quality, purity, and potency." Objective information and data from development, pilot scale, and commercial scale studies in order to establish that a commercial manufacturing would yield product of the quality attributes suitable for the products intended use. Two key considerations are apparent in the guidelines: uniformity (homogeneity) and reproducibility (consistency). Emphasis is placed on the lifecycle of the product. This lifecycle begins with development, progresses though integration into a manufacturing environment, and continually assessed throughout commercial manufacturing. The proposed approach is for decisions to be made upon perceived risk based on criticality of a quality attribute. A continual evaluation is also a key part of the proposed approach.

Monitoring and trending the equipment performance and control of the CPPs is also a key part in achieving a high level of process control. At a minimum, the shelf temperature, chamber pressure, and time parameters for the process need to be monitored and trended. Certainly trending these parameters throughout the process for each batch is crucial. There is also value in trending the level of control of these CPPs among batches over time. Other performance indicators including the condenser temperature, shelf outlet, refrigeration units, and vacuum system can also be useful to monitor and trend. Each of these monitored conditions need to be evaluated for potential use in designing an appropriate process control strategy.

As the development studies in Stage 1 are a valuable body of knowledge and become an important resource for future reference, good documentation practices are important. Documenting the objectives, rationale, and study design along with recording the experimental data, compiling the results, and justifying the conclusions provide a useful reference in transferring the product and process to a commercial manufacturing operation in Stage 2. It is also an important reference during process performance qualification and process validation, as well as continued process monitoring in Stage 3.

11.4.1.1 Stage 1: Process Design

Early development activities focus on the product design, quality attributes, and identifying requirements for manufacturing, including the parameters necessary for processing. Sound scientific methods and principles are the benchmark of development activities. The studies need to be conducted in accordance with good documentation principles, consistent with ICH Q10, Pharmaceutical Quality Systems. The guideline recognizes the value of development data as a historical reference for use in commercial manufacturing. Though a process validation guideline, product design of the intended dosage form, CQAs, and manufacturing requirements identifying the CPPs are to be considered in "Building and Capturing Process Knowledge and Understanding" (Food and Drug Administration 2011).

The intended dosage form for a lyophilized preparation, including the product design and formulation dictate the parameters for processing. A product comprised of 0.5 mL in a 3 cc vial for a product consisting of a formulation containing excipients such as sucrose and an amino acid for which the solidified composition is amorphous and has a corresponding low glass transition temperature (T_g'), parameters of a relatively low shelf temperature and chamber pressure during primary drying would be appropriate and the lyophilization process may require 2 to 3 days to complete. Conversely, a product that consists of mannitol and an API that crystallizes may exhibit a eutectic melt at a relatively high temperature. Such product characteristics may warrant a high shelf temperature and high chamber pressure, and for such a product that is a 2.1 mL fill volume in a 10 cc vial the lyophilization process may be completed overnight.

Product design entails considering the intended dosing regimen, API stabilization, and manufacturability. The dosing regimen directs route, volume, and frequency of administration, as well as formulation design. Needs of stabilizing the API include the liquid and dried state. Realizing that the majority of the product life cycle will be in manufacturing and distributing, product stability in the dried state requires and warrants the most extensive study in terms of time, effort, and attention. Development objectives are to explore and establish product design and processing parameters for routine manufacturing of cost effective commercial products having consistent high quality. Knowledge and understanding of these perspectives are key for product design in lyophilized parenteral development.

An essential aspect of product design is also the assignment of CQAs. This too is based on the intended use of the product. Quality attributes of potency and purity are imperative for any product and are established through product knowledge from clinical and chemical/biochemical studies early in development. Attributes unique to lyophilized preparations are established later in development. Critical product attributes are residual moisture and reconstitution. Residual moisture correlates to stability in the dried state during distribution and storage. Reconstitution consists of two aspects: time to achieve complete dissolution and attributes of the constituted solution.

The needs for the addition of excipients for stabilizing the API and that are suitable for the intended route of administration dictates the formulation. This stabilization encompasses the product as a bulk solution during manufacturing, the lyophilized product in the dried state during distribution and storage, and the constituted solution in preparation for administration. Pre-formulation studies to ascertain the effect of pH on the solubility and stability are critical in constructing potential formulations and should be included in the development report. Constructs of a formulation consider the API chemistry and known mechanisms and pathways in order to inhibit or minimize degradation. This knowledge and understanding is crucial for justifying the presence and concentration of an excipient used in the formulation.

A unique aspect of lyophilization is the interrelationship of the finished product attributes and the process. It is widely accepted that the CPPs are shelf temperature, chamber pressure, and time. Developing a process and identifying the specific CPPs correlates manufacturing conditions to finished product attributes, giving the highest priority to the impact of the process on the predefined CQAs. Focused process engineering assesses the impact of varying the CPPs on finished product attributes. It is also well accepted that the formulation and packaging components may influence the behavior during processing and finished product attributes. The magnitude of such variability and impact on the finished product need to be identified and quantified as part of the process engineering in order to establish the level of control necessary during routine manufacturing.

Sound scientific principles to establish a reasonable rationale along with appropriate and controlled methods in generating data that support the conclusions are imperative. This needs to be coupled with a knowledge and understanding of the capabilities in manufacturing.

In order to effectively study and identify the target and allowable ranges for CPPs when engineering a process, the laboratory and pilot plant environment needs to be representative of the commercial unit operations. Packaging and formulation components, methods and procedures, environmental conditions, and the measurement and control during the process engineering studies should emulate those in the intended commercial production operations.

Packaging and formulation components can be a significant variant with an impact on behavior during processing and finished product attributes. Vials of different types and specifications have different heat transfer capabilities that effect product temperature and processing rates. It is well understood that packaging and formulation components may vary within the allotted range of specifications within

a batch and from batch to batch. Excipients may be different in their purity levels and influence behavior during processing and dried state stability. Treatment of the components and handling the bulk solution should also emulate treatment during routine manufacturing. Care should be exercised to achieve the same level of cleanliness of the packaging components and the bulk solution, particularly with respect to particulates. Washing the packaging components, filtering the bulk solution, and processing in a controlled environment are influencing factors. Dispensing methods with desired accuracy and precision are important. Some products may be sensitive to sheer, and fill volume can influence rate-limiting factors such as dried product resistance during primary drying.

Success of process engineering the lyophilizer performance and capabilities, method of monitoring and control of the CPPs and associated process conditions, instrumentation quality and calibration, and equipment preventative maintenance, as in a manufacturing environment. Parameters that can be achieved during studies when engineering the process in a development setting and are not achievable in routine manufacturing requires further engineering studies when integrating a product and process into a commercial product production operation. Instrumentation needs to directly measure processing conditions, be sufficiently accurate and precise. It is well understood that the shelf temperature for a commercial production lyophilizer refers to the temperature of the heat transfer fluid measured by an RTD in the fluid path going to the manifold that supplies the fluid to all of the shelves. Chamber pressure is measured using an instrument that measures the pressure in the product chamber directly and is not influenced by composition of the atmosphere. The equipment needs to be able to execute the critical parameters of shelf temperature, chamber pressure, and time as parameters that are independent of any other process and product variables. The shelf temperature should be able to be controlled in an acceptable range, as shelf temperature dictates processing rates. The chamber pressure, and specifically the composition of the atmosphere within the product, needs to be within a predicted and acceptable range, as it has an influence on product temperature as well as processing rate. A laboratory and pilot unit should utilize the same type of measurements and achieve the same level of control of the CPPs in order to be comparable to a unit used for commercial scale manufacturing.

Consideration has sometimes been given to pushing the process to extremes in order to reach conditions that lead to process and product failure. There are numerous interrelationships between variables that could be used to reach the point of failure, though altering the CPPs is the most direct and controllable. Though possible to execute, the value and benefit has not warranted the widespread pursuit throughout the industry to conduct such studies. Even with well-understood influences such as the formulation and packaging components, and recognized CPPs of shelf temperature, chamber pressure, and time, the use of design of experiment (DoE) principles may be warranted where there are special relationships that need to be explored. DoE is an effective research tool to identify variables that are suspected to have an influence or assess the impact or quantifying a variable known to have an influence. With multiple known variables that may have an impact, a risk assessment can be useful for evaluating the variable's significance. Such evaluation may be an ongoing

activity during development and process engineering. For example, upon concluding the product design, defining CQAs, establishing the target CPPs, the significance of the curvature of the vial bottom radius that influences heat transfer can be evaluated using risk analysis tools in deciding if further studies, perhaps using DoE techniques, are suitable and provide significant data.

The product and process knowledge and understanding are critical for establishing the strategy for verifying process control. Risk assessment used for identifying the variables when evaluating the need and creating approaches to DoE can be an effective tool for establishing approaches to process control. Controlling variation of components that can influence behavior during processing and finished product attributes is an important aspect for achieving a high level of process control. Testing of incoming packaging components for characteristics may need to include measurements of the bottom radius of the vial that influences heat transfer. The quality and purity of formulation components and API which may influence the thermal behavior during freezing and drying are also important. Any materials that can influence the product behavior during processing and comprise the integral parts of the finished product are critical to identify and monitor, and can be useful for predicting and to correlate to processing results and finished product quality.

11.4.1.2 Stage 2: Process Qualification

Technology transfer to commercial manufacturing entails verifying that the process can be integrated into commercial scale operations. Evaluating the capabilities of the lyophilizer to execute the CPPs and achieve the performance required to control the processing conditions are a prudent first step. Data from the operational qualification of the lyophilizer for commercial scale manufacturing can be compared to the process requirements established in Stage 1. Comparison of performance capabilities to the process parameters in the example listed Table 11.2 should be conducted as the first step in qualifying the lyophilizer as being capable of executing the process conditions and adequately controlling the process.

The change control program for the lyophilizer to assess any impact of modifications to the equipment since the execution of the operational qualification should be evaluated for the impact on achieving the process control necessary for the product intended to be manufactured.

A satisfactory comparison and with the confidence that the lyophilizer will implement and control the required process parameters provides that assurance that the integration of the product and process into the commercial operation will be successful. The next step in the sequence in integrating a new product and process into a manufacturing operation to produce commercial product is to conduct process performance qualification studies. The PPQ studies verify the suitability of combined effects and abilities of the components, equipment, procedures, and operations intended for use in manufacturing commercial product. It is the final step in the development pathway of bringing a product to commercial status in a new manufacturing operation. Historically, this is the step in which three successful subsequent batches were produced, upon which the process was deemed to be validated.

Using the established CPPs and assessing the success of processing with quantifying predefined CQAs based on the data, results and conclusions from development in Stage 1, the PPQ studies can be designed. The PPQ is executed with the intent of conducting the studies following the procedures identified in the Master Batch Record, controlling the CPPs established during development, assessing the predefined CQAs, conducting the sampling and finished product testing, and meeting the acceptance criteria identified in the PPQ protocol. This study brings all the aspects, demands, and challenges of commercial product operations together to demonstrate capability of manufacturing the product with assured, consistent quality.

It is intended that process validation is not the period of discovery; results and outcome of the validation study should be known prior to conducting PPQ studies. It is therefore prudent to design a study and execute an engineering batch to confirm the anticipated equipment performance and verify the expected level of process control, as well as assess the attributes of the lyophilized material. The design of the engineering batch should follow the procedures intended to be described in the Master Batch Record, the CPPs controlled as established during development, and the relevant CQAs assessed. The sampling and finished product testing should be according to that intended during execution the PPQ study. This engineering batch may consist of actual product or may use a surrogate specifically developed to emulate the attributes of the actual product.

Designing a surrogate is based on the knowledge and understanding of the product characteristics during processing and the finished product attributes as a lyophilized preparation. Packaging components are to be the same as that for the actual product. Formulation components may be identical and substitutions made if necessary, depending upon the actual formulation for the product. Substitutions necessary for replacing the API are suitable when it has been verified that the surrogate behaves similarly as compared to the actual product during processing. Undertaking the design and development of a surrogate is similar to that of designing and developing a product. Critical characteristics and behavior during processing need to be known and understood. Dried material attributes need to be quantified in order to be useful when evaluating commercial manufacturing operations. In essence, the designed surrogate needs to be qualified as a suitable substitution for the actual product.

Critical characteristics and behavior include total weight of solute and solvent in the container, influence of the solutes on resistance to water vapor transport through the dried layer, and rates of the solvent sublimation. The total weight of the solute and solvent effect the load conditions on the lyophilizer and challenges the systems performance and capacity in implementing the CPPs and achieving the required performance. Influence of the container is on the heat transfer achieved from the shelf to the product, thereby influencing the achievable rates during freezing and sublimation. The total amount of solute and solvent creates a challenge during freezing as the heat load of the total mass, particularly when the heat of fusion is liberated during nucleation and growth of the solvent crystals. This heat load again presents a challenge while the solvent is subliming during primary drying. The effect of the solutes is on the ease of the water vapor traversing through the dried

layer to leave the product. This resistance influences the amount of total water vapor transport through the lyophilizer to be converted back to a solid on the condenser. The effect of this resistance can also be observed for its impact on the ability of the system to control the CPPs and reflected in the resulting product temperature. The overall rates of sublimation and the associated vapor transport through the lyophilizer for the solvent vapor to be condensed and collected on the condenser are reflected in the control of the CPPs and time required for the solvent to be sublimed reflected in the break of the product temperature.

It is also desirable, though not essential that the physical appearance, residual moisture, and reconstitution of the surrogate emulate those of the dried product in order to assess the effect of processing. It is important to recognize that physical appearance may well be different, simply due to the nature of the solutes used in the surrogate formulation. Acknowledging that the physical appearance is subjective, it would be useful in order to discriminate product that may have undergone collapse. Design of the surrogate should, however, possess a similar threshold temperature in order to be indicative of the possibility of melt-back or collapse during processing. This threshold temperature is established based on the T_g' of the surrogate. Though not needing to be identical, it needs to be suitable to justify the same threshold temperature and be predictive of the presence of collapse in the actual product. The residual moisture is useful in evaluating the rate of desorption achieved with the conditions for secondary drying. Though the residual moisture may be different due to the nature of the solutes, it would be useful if the desorption rates for the surrogate relative to the actual product were known in order to compare the effects during processing. Like physical appearance and residual moisture, reconstitution time is dependent on the nature of the surrogate formulation. More quantitative than physical appearance and more subjective than residual moisture, it is useful information when compared to the values established during the qualification of the surrogate.

Replicating the attributes unique to lyophilized preparations also allows for the evaluation of batch uniformity relative to the location within the lyophilizer. Based on adequate knowledge and understanding of the product and surrogate, the surrogate can be used to assess the influence of position within the lyophilizer and predict the batch uniformity relative to the unique attributes of a lyophilized product: physical appearance, residual moisture, and reconstitution. Temperature during processing may be correlated to finished product attributes at selected locations within the lyophilizer to determine the most representative and extreme locations within the lyophilizer. This may be accomplished by monitoring product temperature and sampling in proximity of the monitored material for evaluation of the dried material attributes for the corners and center of each shelf. Analysis of the temperature data at the end of each process step of loading, freezing, primary and secondary drying, and assessment of the unique attributes of a lyophilized preparation can be used to justify two locations for sampling product during the process performance qualification studies for the product.

Processing an engineering batch provides an opportunity to evaluate the performance of the equipment, control of the CPPs, and assess the attributes of the lyophilized material. Successful results during the engineering batch provide an increased level of assurance that the equipment is capable of controlling the CPPs and the product will meet the CQAs relative to the influences of the equipment and process. It also reduces the risk of loss of product when conducting the subsequent PPQ studies.

With the experience of the engineering batch, any final refinements to the procedures and process parameters for the PPQ studies using actual product can be implemented. Additional sampling would be warranted for evaluating the variation that may occur due to the processing of the actual product. This may include the locations identified as being the most representative and most extreme during the engineering study. In addition, batch uniformity and any influence of lyophilization may be assessed when comparing the bulk solution to the final lyophilized material. The potential for variation of the bulk solution and filled product over the time interval required for the filling operations may be evaluated. This sampling may entail the beginning, middle, and end of the batch. If material is sampled and tested as a solution prior to lyophilization and shown to be no different when compared to lyophilized material from the beginning, middle, and end of the batch, it is shown that there is no change during the course of filling and product difference due to lyophilization.

The more extensive sampling of the PPQ batches provides the data that supports the conclusion of assured batch uniformity. Data from the sampling liquid product at the beginning, middle, and end quantifies any change in potency and purity over the duration of the filling operation. Sampling the lyophilized material at the beginning, middle, and end of the batch quantifies any change in potency and purity, and the effect such changes may have on the behavior during processing and attributes of the lyophilized product attributes of appearance, residual moisture, and reconstitution. Sampling at the most representative and extreme locations in the lyophilizer identified during the engineering study quantifies any variation in the appearance, residual moisture, and reconstitution attributes of the lyophilized product.

Completing the PPQ studies using the sampling plan with eight sample sets of data for each quality attribute described above for three samples for each sample set provides 24 data points that can be compared for each batch. Statistical analysis of the 24 data points can be a useful tool in evaluating batch uniformity, process reproducibility, and finished product consistency. A statistically sound analysis is more easily accomplished for attributes where quantifiable values are measured, such as potency, purity, and residual moisture. Evaluation of semiquantitative and subjective attributes such as reconstitution time, completeness of dissolution, and physical appearance is less rigorous. For physical appearance, a catalog of photographs as visual evidence is very effective in recording results and supporting conclusions of the PPQ studies. Such a catalog can be created during the later stages of product and process development. The catalog of expected product appearance is also valuable for future reference during routine manufacturing.

11.4.1.3 Stage 3: Continuous Process Verification

Historically, process and product is assessed through a commercial product stability study for a single batch and during an annual product review. Assurance of product quality is also supported through the change control program. Stage 3 of the process validation guideline consists of continuous and more rigorous monitoring. Trending of process data can be an effective tool in assessing the level of control of the CPPs in routine processing. Trending may entail a comparison of the actual and or range of CPPs of shelf temperature, chamber pressure, and time for each batch. A statistical analysis may be comprised of the variation from the target setpoints of the shelf temperature, chamber pressure, and time throughout the process or the minimum, maximum, and average CPP for each part of the process or the entire process. Alternatively, analysis may be based on the trend of instances where alarm levels or action levels have been exceeded. Multiple batches may be identified and scheduled for periodic evaluation of the process data and include additional sampling. This sampling program may duplicate that used in the PPQ studies and the finished product assessed and compared to the finished product results for the PPQ studies. Data from these selected batches may also be trended. The number of batches selected for increased sampling and analysis may be based on frequency of product manufacturing and may be adjusted based on results of such sampling and the historical manufacturing experience. For any approach selected, development and assessment of the trending program warrants an interdisciplinary team consisting of representatives from development, manufacturing, quality, and a statistician.

11.5 Current Challenges in Development of Lyophilized Products

Opportunities for providing lyophilized preparations have created new challenges in product development and commercial product manufacturing. Investigations into use of cosolvent and aqueous/organic solvent systems have led to reconsideration of compounds that have limited solubility. Following the trend for liquid, ready-to-use preparations in product presentations not limited to an ampule or vial, lyophilized products are available in more diverse delivery presentations, including cartridges and syringes. In addition, there has been more attention to expanded capabilities and gaining efficiencies in commercial manufacturing.

11.5.1 Poorly Soluble Drug Substances

The classical approach to converting a poorly soluble chemical entity to a more soluble form is to create a salt form of the compound: Converting a free acid or base to a salt improves the solubility. The salt form also impacts stability of the compound.

Lyophilized preparations being administered by injection, bioavailability of various salt forms is less of a concern compared to other dosage forms. Other techniques for designing an injectable have included the use of a cosolvent, encapsulating the active in a liposome, and creating a conjugated form.

Cosolvents can be categorized into two main types: solvents and those that will vaporize and be removed with the water during the process and those that will remain as part of the formulation to also solubilize the API upon reconstitution. Those utilized to increase API concentration and are removed during lyophilization are the organic solvents ethanol, methanol, and tertiary butyl alcohol. Solubility in the various molar ratios of the organic and aqueous solvents warrants a specific preformulation study, as the solubility may be different than in each solvent alone. Testing as part of product batch release and specifications for a residual organic solvent are warranted.

In combination with water, ethanol and methanol remain as a liquid through the freezing step and vaporize directly from the liquid state at high rates during primary drying. Use of any organic solvent should be kept to a minimum, as they create challenges during processing because of the high rate of vaporization from the liquid state. The design of lyophilizers is based on requirements for processing purely aqueous-based formulations and not well suited to accommodate high levels of such organic solvents.

Tertiary butyl alcohol has received growing interest as it solidifies during freezing when in combination with water. Although solid at room temperature, the solidification for aqueous compositions occurs at temperatures commonly used for lyophilization. The unique characteristic of forming multiple eutectic solutions and the behavior during processing has been studied and reported in the literature (DeLuca and Kasrain 1995). There have also been circumstances where product has been lyophilized directly from tertiary butyl alcohol alone, as in the case of alprostadil for injection (prostaglandin E1) by Pfizer.

Agents used to solubilize the API and the final drug product has been used since the early 1970s. Amphotericin B was combined with desoxycholic acid to yield the desoxycholate form and a colloidal dispersion, initially developed by Squibb Institute of Medical Research. Amphotericin B for injection has also been prepared as a liposome and lipid dispersion. Though lyophilized lipid preparations are rare, liposomes encapsulating lipophilic compounds have provided an alternative for drug entities having poor solubility in water and mixed organic solvents. Lupron Depot® is formulated as a dispersion consisting of the leuprolide acetate salt as lyophilized microspheres to form a suspension upon reconstitution, having the benefit of sustained release of the active.

Conjugating a drug entity has been pursued where an active is combined with a polymer or protein for improving the solubility. PEG has proven to be an effective agent. Covalently connecting an API to PEG is known as PEGylation. PEGylation combines PEG to a small molecule, peptide, protein, antibody, or oligonucleotide with the use of a linker. The new entity exhibits different characteristics, including solubility of the active. Vaccines such as hemophilus b protein conjugate have been shown to be effective.

11.5.2 Delivery Systems for Parenteral Products

Classic product design approaches have been to package a lyophilized product in a vial. This approach continues for market entry of a new entity, partly because commercial manufacturing operations are geared towards a vial packaging system. Innovations in delivery systems include dual chamber syringes. As home health care becomes more common, there will be a growing need for self-administration of lyophilized products. Opportunities for errors associated with the multiple steps required for reconstitution is a driving factor for development of new delivery systems. Auto-injectors using a syringe are currently available for liquid, ready-to-use preparations such as Humira, a leading treatment for rheumatoid arthritis (RA) with nearly 1.5 million prescriptions across all its indications, and predominantly a self-administered therapy. Such systems for lyophilized preparations would improve the safety and aid in patient compliance. Implantable devices where the active ingredient is embedded in a polymer, allowing sustained release would also provide benefits for therapy regimens in the treatment of chronic conditions.

11.5.3 Improving Manufacturing Capabilities

Increased volume of lyophilized preparations requiring leading to larger batch sizes has also resulted in reducing unit costs. A larger batch size requires higher speed filling lines and larger lyophilizers. Typical commercial manufacturing lyophilizers are 450–570 ft^2 (42–53 m^2) of usable shelf surface area, processing up to 200,000 of 3cc vials. High speed filling systems and automated material handling for loading and unloading the lyophilizer have also provided increased efficiencies and the potential for greater yield. Reduced unit costs are realized through greater utilization of personnel, facilities, and capital equipment. The number of samples and overall batch release testing costs are also lower, decreasing the costs on a per vial basis for each batch.

There have also been improvements in the understanding of lyophilization science and technology. This leads to more safe, effective, and robust processes used in manufacturing. There is no doubt that the time required to complete the lyophilization process is important, influencing the number of batches necessary to meet market demand. It is also important to acknowledge that there is less likelihood of a batch failure with a robust process. There is a balance between the processing time conducive to high throughput in a manufacturing operation and a robust process making a product less susceptible to slight variations in processing conditions and providing greater assurance that all batches produced can be released. A batch failure has an impact on throughput of an operation as well as the direct and indirect costs. These costs are attributed to material consumed to manufacturing the batch, lost revenue from the sale of the batch, and the costs of conducting an investigation into the failure.

11.5.4 Reducing Unit Costs of Products for World Distribution

Realizing impact on reduced unit costs from increased manufacturing capacities contributes to greater global accessibility to lyophilized pharmaceuticals. As well, focusing on the actual cost to deliver a product to the patient is also imperative. Certainly, reducing the costs to manufacture a lyophilization preparation, such as increased batch sizes and more robust processes is beneficial. Reducing the cost of the lyophilized preparation alone will be insufficient. The cost to deliver a product to a patient also includes the cost of the diluent, syringe, needles, and alcohol wipes. In addition, there is also the cost of the clinician in assembling the product, diluent, and components; completing the reconstitution and administering the product to the patient. In markets where labor is not a significant factor, there may be a nominal cost for the preparation and administration, though there are still the material costs. Cost of product and administration needs to be compared to the cost of a delivery system with all the materials combined, minimizing the need for all the individual components and product preparation for administration.

Achieving reduced costs, greater safety, and improved patient compliance requires innovation in packaging systems paralleling the innovations made in auto-injectors for liquid injectables. These systems will have an influence on packaging design and the formulation. These will all drive the needs for manufacturing of lyophilized preparations. Vials and special stoppers, now conventional packaging systems for lyophilized preparations will someday be replaced by improved packaging having reduced component and manufacturing costs along with benefits of improved patient safety and compliance.

11.6 Summary

The initial interest in lyophilization was as a method of preserving products known to be unstable upon storage as a liquid ready-to-use presentation. This technology, not well understood and cumbersome in integrating into a commercial manufacturing operation, was initially used in a hospital setting and then expanded into larger scale commercial manufacturing for a limited number of products, principally antibiotics, blood products, and vaccines. With an increased number of products and the development of biopharmaceuticals that require preservation for suitable long-term storage, the utilization of lyophilization in manufacturing has grown. Greater knowledge and understanding of lyophilization science and technology has paralleled the growth in its application. Current interests are in commercializing cost effective and user friendly product presentations, efficient and robust processes, and greater level of control. All of these are driven by the continued expansion in the application of the science and technology to meet the needs of future generations of new products.

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Chapter 12
Advances in Container Closure Integrity Testing

Lei Li

Abstract Container closure integrity (CCI) testing, along with other engineering and administrative controls, must be incorporated into a holistic approach to ensure overall integrity of a container closure system during the package design and development phases. CCI testing plays an essential role in providing feedback to package design and ultimately demonstrating CCI. To ensure they are suitable for the intended use, the testing methods must be appropriately selected, developed, and validated for the specific drug product-package configuration. This chapter addresses key considerations during packaging design and development phases with focus on CCI testing strategy development and appropriate CCI testing applications. It provides an overview of various commonly used testing technologies and proposes a guideline for method selection, development, and validation.

12.1 Introduction

A primary container closure system provides the critical barrier that protects drug product contained therein. For sterile products, container closure integrity (CCI) is an inherent and critical component of the overall sterility assurance program. In addition to serving as a microbial barrier, for moisture sensitive drug products (such as lyophilized products) and oxygen sensitive products, the primary container closure system also must provide adequate barrier property against migration of the undesired gaseous species from external environment. The integrity of the container closure systems is essential for maintaining drug product stability and sterility throughout its entire shelf life.

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CCI assurance and testing should be integrated throughout the life cycle of the drug product. Critical CCI requirements are usually defined and addressed during the initial development phase when packaging components and systems are designed and qualified, and packaging processes are developed. Drug product-package specific CCI testing methods should be developed in this phase and subsequently used to support the design and development activities and ensure all CCI design requirements are met. For example, CCI testing should be performed to verify the integrity of the container closure system when it is exposed to anticipated extreme conditions in manufacturing and sterilization processes, storage, shipment, and distribution. The design and development phase output will ultimately drive implementation of a comprehensive CCI control strategy in the continuum of pharmaceutical manufacturing processes, ranging from receipt and inspection of components, primary and secondary packaging, to shipping and distribution. The CCI testing methods will eventually be fully validated and applied to monitor and demonstrate the effectiveness of the CCI assurance program in routine manufacturing and marketed product stability phases.

This chapter addresses key CCI considerations during packaging design and development phases with focus on applying appropriate CCI testing to support package and drug product development. It provides an overview of various commonly used testing technologies and proposes a guideline for method selection, development, and validation.

12.2 Regulatory Requirements and Expectations

Regulatory requirements and expectations have been well established and documented in a series of global regulatory requirements and guidance. US FDA (1999) requires suitability of the selected container closure system be sufficiently established in four key aspects: protection, safety, compatibility, and performance. CCI is considered an essential part of suitability, especially in the aspect of protection against microbial contamination, reactive gases (e.g., oxygen), and moisture. A container closure system that permits penetration of microorganisms is unsuitable for a sterile product.

In order to ensure CCI, appropriate product-package integrity testing should be integrated into the entire product life cycle, ranging from package development, routine manufacturing, and marketed product stability (USP Chapter <1207>). During drug product development phases, it is required to substantially demonstrate that the container closure system is capable of maintaining integrity of its microbial barrier, and, hence, the sterility of drug product throughout its claimed shelf life. Study designs should simulate the stresses of the sterilization process, handling, and storage of the drug and their effects on the container closure system. Container closure integrity should be demonstrated on product units that have been exposed to the maximum sterilization cycle(s). If a product is exposed to more than one process, then exposure to the maximum cycle of all processes should be

incorporated into the study design. The studies must be described and included in the submission to gain regulatory approval (ICH 2009; US State Food and Drug Administration 1994).

Drug and biological products manufactured by sterilization and aseptic processing must be adequately validated under current Good Manufacturing Practice (cGMP). For initial validation of microbiological integrity of container closure systems, product sterility testing is not normally considered sufficient. CCI testing methods and results should be summarized to demonstrate the integrity of the microbiological barrier. This should include testing for initial validation as well as the procedures used for the stability protocol (US State Food and Drug Administration 1994). During routine manufacturing, sterility of a product lot must be initially established by validated sterility testing methods (USP Chapter <1211>; USP Chapter <71>). However, the initial time point sterility testing is not considered sufficient to demonstrate the microbial integrity of a container closure system. Additional CCI testing may be required. For example, EU guideline annex 1 (EU Guideline to Good Manufacturing Practice 2008) requires containers closed by fusion (e.g., glass and plastic ampoules) should be subject to 100 % integrity testing and other containers should also be checked appropriately. For drug products produced by aseptic processing, US FDA cGMP guidance requires any damaged or defective units should be detected, and removed, during inspection of the final sealed product (US State Food and Drug Administration 2004).

In 2008, US FDA promoted container and closure system integrity (CCI) testing as a component of the stability protocol for sterile products (US State Food and Drug Administration 2008). The guidance recommended CCI testing on stability in lieu of traditional end-of-shelf-life sterility testing for better sterility assurance, especially continued sterility of a drug product. ICH (1995) requires sterility testing or alternatives (e.g., CCI testing) should be performed at a minimum initially and at the end of the proposed shelf life.

12.3 CCI Testing Strategy Development

A large variety of failure modes can affect CCI, including material and component defects of various shapes, forms, and sizes, package design faults, and issues related to packaging processes and subsequent shipping and storage conditions. Many of the defects and failure modes are drug product-package specific. Driven by novel patient-centered designs and new drug product formulations, pharmaceutical packaging material selection and system designs have been increasingly innovative, diverse, and complex. Many of the package systems, such as cartridges and prefilled syringes, have become more integrated with medical device designs. These design trends present increasing needs as well as technical challenges for effective CCI testing. Therefore, CCI assurance and testing must take a holistic and comprehensive approach and an overarching testing strategy needs to be developed and implemented during the drug product and package design and development phases.

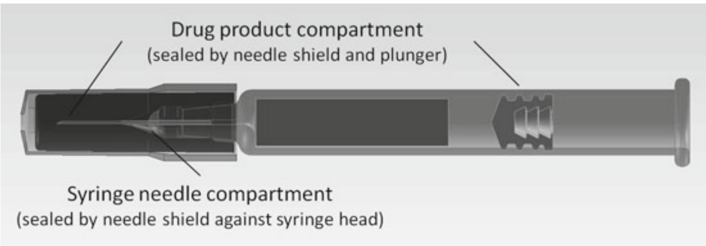

Fig. 12.1 Illustration of a prefilled stake needle glass syringe

A risk-based approach is usually used to drive CCI testing strategy development. The technical foundation for the strategy must be based on thorough understanding of materials, design, and manufacturing processes of the container closure system and the drug product. The focus is to identify the critical risk factors for potential loss of integrity due to defects and failure modes in materials (both package component and drug product), designs (container closure system design, product formulation design, and device design if applicable), and processes (ranging from package component manufacturing to distribution and storage of the drug product). Upon being identified, the risk levels and mitigation plans, including utilization of CCI testing, should be assessed within the context of the overall CCI assurance including existing engineering and administrative controls.

A best practice for risk assessments is to perform a two-step process: CCI-related failure mode and effect analysis for the design of the container closure system followed by a process-focused analysis. During the design-focused assessment, the potential failure modes associated with each packaging component, containment compartment, and seal interface should be identified and analyzed. An extensive evaluation is essential for understanding complex packaging designs. For example, stake needle prefilled syringes (Fig. 12.1) provide a syringe barrel compartment for drug product containment and a separate needle shield compartment for needle protection. The syringe barrel compartment is sealed by the plunger on one end and by the needle on the other with the needle tip embedded in the needle shield. The needle shield compartment, sealed by the syringe barrel head, protects the needle exterior surfaces from potential contamination. Each compartment and seal interface involves different materials and sealing mechanisms and consequently features a distinct set of risks.

In the subsequent process-focused risk assessment steps, the design elements will be further assessed at each processing step to ensure the key processing parameters are taken into considerations. Many distinct CCI failure modes can occur throughout the life cycle of a container closure system, ranging from component manufacturing, drug product filling and sealing, device assembling, to subsequent shipping and storage. Some package designs may have unique interactions with the processes, causing additional risks. Using a prefilled syringe as an example, its plunger is allowed to move within a range along the syringe barrel. When

experiencing pressure variations during shipping, plunger movements in response to pressure variations may potentially affect seal integrity. It is therefore essential to evaluate plunger seal integrity following these stress conditions in the shipping process.

In addition to the design and process of the container closure system, the drug products contained therein should also be taken into consideration. Some drug products require unique process and storage conditions that may potentially affect risks associated with CCI. For example, many biological products could require extremely low temperature storage (e.g., −70 °C). Since the seal property of syringe components, especially elastomers (e.g., stoppers and plungers), is temperature dependent, CCI testing under extremely low temperatures could be required if theoretical justifications based on elastomer property are not adequate (Brigitte 2012). Moreover, drug products may interact with CCI defects, which may subsequently affect CCI testing method sensitivity and selection. For example, proteinaceous products could prevent mass transfer through CCI defects and reduce the sensitivity of a vacuum decay method (Orosz and Guazzo 2010). Therefore, a CCI testing method must be selected and developed to ensure its applicability to the specific drug product-package.

The risk assessment output, along with the regulatory expectations, will drive CCI testing strategy development and decision making on when and how CCI testing should be performed. A comprehensive CCI testing strategy usually consists of a series of CCI tests intended to address the critical risks identified in the risk assessment. Each CCI test may serve a distinct and well defined purpose, such as demonstrating package design integrity or monitoring stability, while collectively the entire testing strategy ensures the holistic approach to CCI assurance. The well developed and documented strategy will be used to drive CCI testing method selection, development, and ultimate implementation in support of drug product and package development and eventually manufacturing controls.

12.4 CCI Testing Method Overview

In response to the increasing regulatory expectations, the pharmaceutical industry has driven and witnessed significant technical advancements in CCI testing technologies (Guazzo 2010; Li 2013). Conventional testing methods, such as dye ingress and microbial ingress tests, have been used in the industry for decades. Recent development focused on instrumentation-based technologies, such as high voltage leak detection (HVLD) (Möll et al. 1998), vacuum/pressure decay (Wolf 2009a), mass extraction (Yoon et al. 2012), and tracer gas detection (helium leak detection (Kirsch et al. 1997), headspace oxygen testing (Brigitte 2012), etc.) has successfully demonstrated improved detection capabilities. Many of the emerging technologies have been used for drug product stability CCI testing, in-process control, and even online 100 % inspection.

Table 12.1 Characteristics of major CCI testing methods

CCI testing	Merits	Disadvantages
Vacuum decay	Nondestructive 100 % testing feasible	Proteinaceous products may interfere with defect detection
Mass extraction	Nondestructive 100 % testing feasible Highly sensitive (2 μm demonstrated)	Proteinaceous products may interfere with defect detection
High voltage leak detection	Testing under normal atmospheric pressure Applicable to high-concentration proteinaceous products Nondestructive 100 % testing feasible	Requires conductive liquid fills May cause drug product degradation
Helium leak detection (with mass spectrometric detection)	Highly sensitive; can readily detect submicron size defects Quantitative leak size/rate determination feasible Nondestructive 100 % testing feasible	Requires helium-containing headspace May not be able to detect large defects Proteinaceous products may interfere with defect detection
Headspace oxygen testing (e.g., frequency modulated spectroscopy)	Highly sensitive detection feasible; can potentially detect submicron size defects when time allows May detect past transient CCI failures (as it detects the cumulative effect of CCI failures) 100 % testing feasible	Usually requires modified atmosphere packaging Proteinaceous products may interfere with defect detection Longer turnaround-time (days) or a oxygen "bombing" procedure may be needed for sensitive detection
Dye ingress	Widely used for decades Industry and regulatory familiarity	Less sensitive Detection is probabilistic for small-size defects (i.e., <approx. 10 μm) Destructive
Microbial ingress	Widely used for decades Industry and regulatory familiarity Readily incorporated into media fill runs	Less sensitive Detection is probabilistic for small-size defects (i.e., <approx. 10 μm) Destructive Not applicable to drug product-filled syringes

However, it is important to point out that current technologies do not offer an ideal "one-size-fits-all" solution. It is important to understand the detection principle, capability, and major advantages and limitations of each technique to ensure selection of appropriate method(s) that are suitable for the intended uses. Table 12.1 provides an overview of major CCI testing techniques with detailed discussions given below.

12.4.1 Microbial Ingress Testing

Microbial ingress testing utilizes physical appearance changes caused by microbial population growth in a media solution filled container to detect breaches that allow for microbial penetration. Containers with media fills are immersed in a dense suspension or aerosol of bacteria while applying vacuum or/and overpressure challenges. The containers are then incubated for several days before inspection for visible microbial growth.

Microbial testing is still a benchmark method for understanding microbial ingress behaviors through a container closure breach. Extensive research has been performed to characterize the minimum defect size allowing microbial ingress. The findings showed large variability, ranging from 0.3 to 10 μm (Kirsch et al. 1997; Keller 1998; Burrell et al. 2000). The disagreements may be attributed to many factors, such as different microorganisms, CCI defects, and vacuum challenge conditions used in the studies. In a frequently cited study (Kirsch et al. 1997), Kirsch et al. reported that microbial ingress failure rates increased with increasing defect nominal leak diameters: although microbial ingress was observed in some defects as small as 0.3 μm, it only became probable around approximately 1 μm and near 100 % probability at approximately 8 μm levels. Also note these studies were performed under "stressed" conditions (i.e., extremely high microorganism population density and vacuum/over-pressurization conditions) and may not reflect the exact microbial ingress behaviors under normal product storage conditions.

Microbial ingress testing can be readily implemented for media fill process simulation to demonstrate the containers are appropriately closed during sealing processes. However, the studies listed above also demonstrated the key limitation of microbial ingress testing as a definitive CCI test: detection of small integrity breaches (e.g., <8 μm) is probabilistic and unreliable. In addition, microbial ingress tests can only be used with media filled containers and may not suitable for drug product-filled samples, limiting its applicability in stability studies that focus on evaluation of potential interactions between the drug products and packaging components.

12.4.2 Dye Ingress Testing

Dye ingress methods are similar to microbial ingress testing. Instead of using microbial suspension or aerosol, a dye solution is used to challenge the container samples under vacuum/overpressure conditions. Upon testing, container content discoloration indicates presence of CCI defects. Since it does not require microorganism growth and therefore incubation periods, dye ingress testing does not require media filled containers (i.e., can be readily performed on drug product-filled container samples) and provides much quicker turnaround of testing results. For these reasons, dye ingress methods have been widely used by the pharmaceutical industry for decades and are familiar to regulatory agencies.

Because both are based on similar testing principles and procedures, dye ingress and microbial ingress methods share the same major limitation in terms of lack of reliable detection for small defects (Wolf 2009a). For product-filled containers, dye ingress testing is usually considered destructive and therefore it cannot be used for 100 % testing.

12.4.3 Vacuum Decay Testing

When a container sample with CCI defects is placed in an enclosed evacuated chamber, the vacuum extracts headspace gas or liquid vapor content from interior of the samples, causing significant pressure rise (vacuum loss) in the enclosed chamber. Vacuum decay testing detects package leaks by measuring the pressure increases (or vacuum decays). By utilizing an absolute pressure transducer for large leak detection and a more sensitive differential pressure transducer for small leak detection, this technique is capable of reliably detecting leaks as small as 5 μm while maintaining a large detection range (Wolf 2009b). In addition, vacuum decay testing for intact samples is considered nondestructive, making it a feasible candidate for 100 % CCI testing.

A few limitations should be noted while selecting vacuum decay methods. First, large defects in containers with limited headspace or liquid content (e.g., vials with lyophilized product) may not be effectively detected. Large defects in the samples may allow all interior volatile content to completely escape during the evacuation stage prior to leak detection, and therefore cannot be detected. The large leak detection limit should be characterized during the method development stage. Second, a recent study (Orosz and Guazzo 2010) revealed that some drug products, especially proteinaceous products, can clog leak channels and cause false negative detections (i.e., failure to detect defects). Such "clogging" issues may worsen over time, which significantly limits the applicability of this technique to proteinaceous products, especially in stability studies.

12.4.4 Mass Extraction Testing

Mass extraction testing (Yoon et al. 2012) is closely related to vacuum decay technology. Instead of measuring the pressure increases, mass extraction directly measures the mass flow rate of the volatile content (i.e., headspace gas and/or liquid vapor) extracted from the container samples using a flow sensor connected to a vacuum reservoir. Similar to vacuum decay testing, mass extraction technology shares the drawback of being prone to false negative detections due to "clogging" issues caused by proteinaceous drug products. However, thanks to the superior precision and sensitivity of mass flow measurement, it demonstrated higher sensitivity

with established detection limit of 2 μm level, making it a preferred method for powder fill and lyophilized product containers. A large leak check procedure can be built in the method to extend its detection range for CCI defect sizes while maintaining its high sensitivity.

12.4.5 Helium Leak Detection

Helium leak detection usually utilizes a mass spectrometer to quantitatively measure trace amount of helium gas that leaks out of a helium filled container sample. It is one of the most sensitive CCI testing techniques owing to the excellent sensitivity of mass spectrometric helium detection combined with extremely low helium background variations in atmosphere. Kirsch et al. (1997) reported a minimum observed absolute leak rate was $10^{-6.6}$ standard cc/s, which likely corresponds to helium permeation. Leaks of 0.5 μm can be readily detected by the technology. The high sensitivity and qualitative absolute leak rate measurement capability make it a preferred method to verify container closure system design and confirm good seal integrity. However, in order to apply helium detection to product-filled containers, additional engineering measures need to be implemented to allow "bombing" of helium gas into the containers (which may not be feasible for containers without significant amount of headspace). Since usually there is a time lapse between helium filling and detection, large defects allowing complete escape of helium gas would not be detected.

12.4.6 Headspace Oxygen Analysis

For containers sealed under modified atmosphere or vacuum (e.g., lyophilized vials), headspace oxygen analysis has been well established as an indirect CCI testing method. Usually a nondestructive oxygen analysis, for example, frequency modulated spectroscopy (FMS) (Lighthouse Instruments Inc.), is applied to monitor oxygen concentration changes over a period of time (days, weeks, or even years). Significant oxygen increases indicates substantial oxygen ingress from ambient atmosphere, which can be further correlated to presence or sizes of CCI defects. Thanks to its nondestructive nature, headspace oxygen testing can be readily implemented in stability studies. When long testing intervals are allowed or a high pressure oxygen "bombing" procedure is used (Hirotaka 2012), the total amount of oxygen ingressed through even minuscule defects can reach a level that is readily detectable; therefore the method is sensitive and capable of detecting submicron size defects. In addition, FMS features high throughput and is well suited for online 100 % testing.

Use of oxygen as the tracer gas renders another significant advantage: the oxygen concentration increase in a container sample reflects the cumulative effect of its atmospheric exposure. Therefore, CCI defects under special storage conditions (such as −80 °C) can be readily detected even after samples were removed from these conditions and allowed to equilibrate with laboratory ambient condition (Brigitte 2012).

Its major limitations include limited applicability to containers without headspace volume. For containers with atmospheric headspace, sample may need to be stored in special storage conditions (such as nitrogen rich environment) to allow for oxygen exchange. In addition, for containers with liquid content, the potential "clogging" issues as described above may prevent oxygen exchange and cause false negative detections.

12.4.7 High Voltage Leak Detection

HVLD (Möll et al. 1998) subjects samples to a strong alternating electric field. Intact containers provide extremely high electrical impedance and only allow a negligible current to pass through. However, CCI defects present in a nonintegral container can significantly lower the sample's electrical impedance, resulting in electrical discharges with substantially higher currents. Therefore, CCI defects can be detected by measuring currents passing through the samples and abnormally high currents indicate CCI defects.

The detection principle of HVLD is fundamentally different from the CCI testing techniques described above. The aforementioned techniques rely on some form of mass transfer and exchange through the defects in the container closure system while HVLD relies on differences in electrical resistive and capacitive conductivity of electrical current. This fundamental difference provides HVLD a unique advantage to overcome drug product "clogging" issues mentioned above. Clogged defects, although they may prevent mass transfer, can still allow electrical current to flow through and thus enable effective detection by HVLD. This advantage was demonstrated in a side-by-side comparison study between HVLD and vacuum decay testing (Orosz and Guazzo 2010). Furthermore, HVLD's high throughput detection makes it a perfect selection for online 100 % inspection.

It should be noted that HVLD can only be applied to test container closure systems containing conductive content (e.g., conductive liquid formulation). Nonconductive containers, such as powder fill or lyophilized product containers, cannot be detected by HVLD. In addition, the electrodes must be placed within close proximity (approximately 3 mm) of the testing sample; for container closure samples assembled into devices, such close placement of electrodes may be challenging.

12.5 CCI Testing Method Selection, Development, and Validation

12.5.1 Method Selection

The method discussions above clearly demonstrated that, for CCI testing, there are no one-size-fit-all testing solutions. None of the CCI methods can meet all desired testing needs and are readily applicable to all product-package configurations. Appropriate CCI testing methods should be prudently selected based on thorough technical understanding of testing method characteristics as well as drug product-package properties. The key method selection considerations are as follows:

- *The method selected must be suitable for the intended use*. The selected method(s) must be suitable for its intended use as defined in the CCI testing strategy and can effectively detect and help mitigate CCI-related risks. If a single method cannot meet all the testing needs, complementary methods may be applied in tandem to achieve definitive and comprehensive testing conclusions. For example, one may encounter a situation where the most sensitive method available for testing drug product-filled containers (e.g., stability testing) is only capable of achieving 5 μm detection limit. To mitigate risks associated with smaller defects, nitrogen-filled "empty" containers can be placed in a development stability study and more sensitive oxygen headspace detection methods may be utilized to test for defects below 5 μm. The combined results would substantially increase the overall confidence in the integrity and stability of the container closure system.
- *The methods selected must be applicable to the specific drug product-package*. As previously mentioned, drug product formulation (liquid vs. lyophilized products) and package design (e.g., headspace volume) directly affect method applicability. In addition, drug product (such as proteinaceous products) can interact with CCI defects, further affecting detection effectiveness of the CCI testing methods. Therefore, method selection must be drug product-package specific and method effectiveness must be evaluated and adequately demonstrated in the method development and validation phases.
- *The methods selected should provide sufficient detection sensitivity and reliability*. Recent technologies utilizing mass extraction (Yoon et al. 2012), HVLD (Möll et al. 1998), and vacuum decay (Wolf 2009b) have demonstrated reliable detection of CCI defects of 5–10 μm or better. These technologies are based on quantitative measurement of certain sample characteristics that can be further correlated to presence and/or sizes of CCI defects; therefore, they can be readily validated with quantitative results. The superior sensitivity and reliability made them preferred CCI testing methods over conventional dye or microbial ingress tests.
- *Nondestructive CCI testing methods are preferred*. Nondestructive methods enable 100 % CCI testing. In addition, the defect samples can be further analyzed to determine the failure modes and root causes, which in-turn provides valuable feedback for continuous package design and packaging process improvement.

Table 12.2 Commonly used CCI defect standards

Types	Advantages	Disadvantages
Micropipettes (Kirsch et al. 1997)	Easy sample preparation	Fragile, and broken tips may not be easily detected
Laser-drilled defects (Wolf 2009b)	Sample geometry can remain unchanged Better resemble natural defects in glass (cracks) and polymer (pinholes)	Cost "Hole" size of laser-drilled effects needs to be calibrated using flow models
Microtubes (Keller 1998)	Easy sample preparation Robust, easy to use	When incorporated in to a container sample, the length of the microtube defects is usually longer than that of typical "real-world" defects (e.g., container wall thickness)

12.5.2 Method Development

Method development activities should focus on optimizing testing parameters and determining the appropriate pass-fail threshold for the specific drug product-package system. In this stage, various leak standards of known sizes (Table 12.2) can be incorporated into the samples and used as known defects samples. The correlations were thoroughly explored between key method parameters and instrument responses to both intact and defect samples, aiming to identify a set of parameters that yields optimized separation between defect and intact samples (i.e., signal-to-noise ratio). Variations in packaging components (e.g., different sources/lots) and the drug products contained (e.g., various batches, packaging sites/lines) may also affect instrument responses, therefore it is critical to evaluate multiple lots of materials and defect standard samples should be randomly inserted into the intact sample population for testing. The results should be statistically evaluated to establish the appropriate pass-fail threshold to achieve the desired detection sensitivity while keeping false positive detection probability (i.e., intact samples detected as defects) within an acceptable level.

It should be noted that, although leak standards are essential for initial method development and optimization, they do not necessarily fully represent natural CCI defects. Natural CCI defects are of a large variety and most of them are not simple orifices or tubes. Therefore, the method performance should be further evaluated using "real-world" CCI defects that represent all major probable CCI failure modes identified in the prior risk assessment. Actual CCI defects obtained from various sources, such as reject samples from incoming or in-process controls, as well as lab-made simulated defects can be used. For methods used for stability testing, additional studies should be performed to verify the methods are capable of detecting defects in "aged" samples. This can be demonstrated by placing a set of product-filled samples with known defects on a stability study and testing the defect samples at various time points.

Because the drug product formulation and package design may change during early development phases, a phase-appropriate approach is usually taken to ensure CCI testing method development and validation stays in concert with product development phases. In early phases, scientifically sound methods can be used to support packaging system selection, qualification, and development stability studies. Once the formulation and packaging design are finalized, the methods can be fully validated prior to being used for late-phase and manufacturing testing (e.g., process validation CCI testing, routine manufacturing lot testing).

12.5.3 Method Validation

It is desired to follow ICH analytical method validation guideline (ICH 1996), where applicable, to validate CCI testing methods. The key method characteristics, as detailed below, may be evaluated and demonstrated during the method validation stage.

1. Accuracy: false detection probability for pass-fail detection methods; bias for quantitative testing methods.
2. Detection limit: smallest defect size the method can reliably detect. For quantitative methods, method limit of detection (LoD) and limit of quantitation (LoQ) should be characterized.
3. Range: method capability of detecting gross/large defects.
4. Repeatability & intermediate precision: variations within/between analysts, instruments, labs, etc.
5. Specificity: method specificity to the drug product-package of interest, including potential interfering factors, if any, that may cause false detection.
6. Robustness: method performance consistency against various instruments, environment variations.

In order to demonstrate detection sensitivity, micropipettes, microtubes, and laser-drilled standards of known sizes are usually used, which also allows direct comparison of testing capability of various methods. Method effectiveness studies using natural and simulated defects may be included in the robustness studies.

12.6 Summary

CCI testing plays an essential role in providing feedback to package design and ultimately demonstrating CCI. However, current CCI testing technologies do not offer an ideal method that can satisfy all CCI testing needs. A risk-based approach should be implemented to ensure CCI-related risks are fully understood and effectively mitigated. To ensure the testing methods are suitable for the intended use, the methods must be appropriately selected, developed, and validated for the specific

drug product-package configuration. CCI testing, along with other engineering and administrative controls, must be incorporated into a holistic approach to ensure overall integrity of a container closure system during the package design and development phases.

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Chapter 13
Pen and Autoinjector Drug Delivery Devices

Ian Thompson and Jakob Lange

Abstract Pens and autoinjectors are mainly used for the subcutaneous delivery of biopharmaceuticals, primarily for self-administration by the patient. The ability to self-inject has been the core driver for their development and made them such an important part of the world of drug delivery devices for more than 25 years. With the worldwide increase in diabetes as well as the trend towards biological drugs which cannot be administered orally, their importance continues to grow.

This chapter provides an overview of the different types of injection devices as well as what the development of such a device entails. Firstly, the landscape of injection devices is presented including a brief review of the different primary containers around which the devices are designed. The different types of pens and autoinjectors are described in some detail before reviewing the development process. The development sections outline the applicable regulatory requirements, provide examples of useful development tools, and then describe the different steps involved in injection device development and industrialization.

13.1 Introduction

Pens and autoinjectors are mainly used for the subcutaneous delivery of biopharmaceuticals, primarily for self-administration by the patient. The ability to self-inject has been the core driver for their development and made them such an important part of the world of drug delivery devices for more than 25 years. With the worldwide increase in diabetes as well as the trend towards biological drugs which cannot be administered orally, their importance continues to grow (Thompson 2006; French and Collins 2010).

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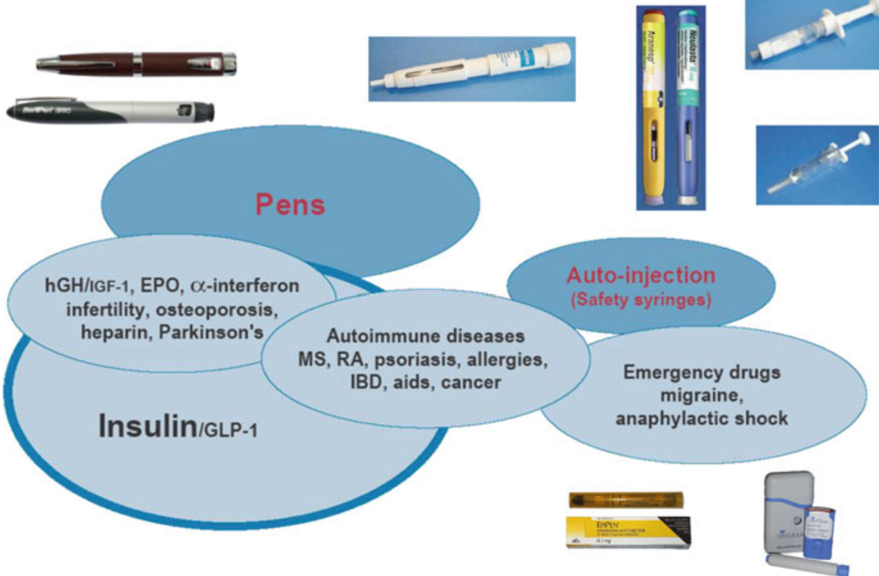

Fig. 13.1 Overview of self-injection therapies and devices

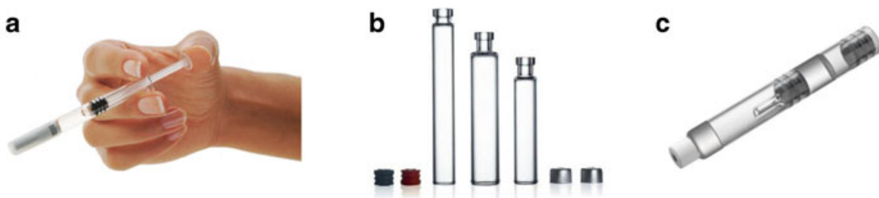

Fig. 13.2 Primary drug reservoirs: (**a**) prefilled syringe, (**b**) 3 mL insulin cartridge, and (**c**) 1 mL dual-chamber cartridge

Pens and autoinjectors have continuously been developed to meet the needs of patients in the key areas of diabetes (insulin and glucagon-like peptide), growth hormone and other hormone replacement therapies, hepatitis C, multiple sclerosis, cancer treatment, autoimmune diseases, and emergency injections for treating anaphylactic shock and migraine as represented in Fig. 13.1.

The differences between pens and autoinjectors are firstly related to the primary drug reservoirs as shown in Fig. 13.2, where pens are usually designed to work with cartridges and pen needles and autoinjectors with prefilled syringes, and secondly the mode of injection, with pens mainly being manual dosing systems and autoinjectors automatic injection devices (Thompson 2006). In fact, today there is a degree of overlap between the technologies which is described later in this chapter.

This chapter provides an overview of the different types of injection devices as well as what the development of such a device entails. Firstly, the landscape of injection devices is presented including a brief review of the different primary containers around which the devices are designed. The different types of pens and autoinjectors are described in some detail before reviewing the development process. The development sections outline the applicable regulatory requirements, provide examples of useful development tools and then describe the different steps involved in injection device development and industrialization.

13.2 Pen and Autoinjector Overview

13.2.1 Autoinjectors

Autoinjectors, as their name implies, automatically insert the needle and perform the injection—typically spring driven—and are usually designed for use with prefilled syringes. A key requirement for autoinjection is the need for liquid-stable formulations in a prefilled syringe or cartridge-based drug reservoir. Some drugs are injected daily, but many long-acting therapeutics are now injected weekly or less frequently, particularly those for treating autoimmune diseases such as rheumatoid arthritis (RA) and psoriasis. Most of these newer drugs do not contain preservative and have injection volumes of 0.5–1.0 mL.

While the market for traditional reusable autoinjectors is limited to frequently injected multiple sclerosis therapies and emergency injections for migraine, the market for disposable autoinjectors continues to grow driven by the demand for less frequently injected drugs, particularly biologics.

13.2.1.1 Monodose Injections

Autoinjectors are typically used to deliver monodose injections, generally from a prefilled syringe or a dual-chamber cartridge. Monodose formulations are single doses where the dose is fixed and fully injected or can be varied and partially injected. In either case only a single dose is given and the drug container/injection device is disposed of after the injection.

Ideally the drug is liquid-stable and the full dose is injected. The need to inject a partial dose using a prefilled syringe is not very common. If different doses are needed, then providing syringes with different fill volumes is preferred.

If the drug is lyophilized, the preference is to use a dual-chamber cartridge and to inject the full dose after reconstitution. Manufacturing different fill volumes of freeze-dried drugs is costly, and there are examples of devices on the market today where a partial dose is injected from a dual-chamber cartridge, e.g., Pegintron® (Peginterferon alfa-2b). The Pegintron® device is shown in Fig. 13.3.

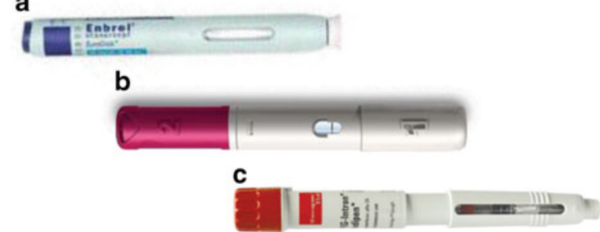

Fig. 13.3 (**a**) Enbrel® and (**b**) Humira® autoinjectors and (**c**) Pegintron® dual-chamber monodose pen

For the prefilled syringe, the device may simply be the standard syringe without any additional injection aid. More typically a safety syringe or fully disposable autoinjector is offered. The choice of device depends on factors such as:

1. Proportion of patients self-injecting
2. Frequency of administration and duration of therapy
3. Need for needle safety to prevent needlestick injury
4. Necessary level of convenience (i.e., patients with motor disabilities)
5. Competitive situation

For example, heparins were traditionally provided in prefilled syringes and are now supplied in safety syringes to protect hospital staff from needlestick injuries as most of the injections are performed in the clinical setting. Another example is the TNF inhibitors which are predominantly self-injected at home by patients who may have motor disabilities and are increasingly offered in a disposable autoinjector presentation, e.g., Enbrel® (Etanercept) device and Humira® (Adalimumab); see Fig. 13.3.

13.2.1.2 Autoinjector History

The first autoinjectors were developed in the 1970s for military emergency drugs such as atropine and antidotes injected intramuscularly (i.m.). Similar devices are provided to patients for the emergency treatment of anaphylactic shock using epinephrine. The primary container used in such i.m. devices is the pen cartridge, because the drug is preferably filled bubble-free and without contact to the needle.

A key feature of the original EpiPen® (see Fig. 13.4) and similar devices is the integrated safety mechanism to prevent inadvertent activation. This means that a safety mechanism has to be removed before the injection can be performed by pressing the autoinjector against the skin. The EpiPen® contains an "interlock" which means that pushing the device against the skin activates the injection "power pack" to start the injection process.

The next real milestone in the development of autoinjectors and the first subcutaneous system was the introduction of the semi-disposable Imitrex® (sumatriptan succinate) autoinjector for treating migraine; see Fig. 13.4. The reusable power pack and disposable dual-syringe pack provides a compact and cost-efficient autoinjector.

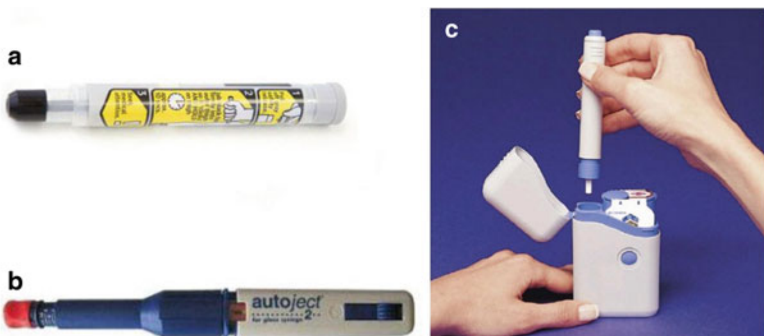

Fig. 13.4 (**a**) EpiPen®, (**b**) Imitrex® emergency autoinjectors, and (**c**) Autoject reusable autoinjector

In this device the “interlock” requires the patient to press on a button at the same time as pressing the device on the skin.

During the 1990s the first reusable autoinjectors were introduced with the Autoject family of devices; see Fig. 13.4. The main application was for the frequent injection of beta-interferons (IFN-beta) and Copaxone® (glatiramer acetate) for treating multiple sclerosis.

None of the above autoinjector devices contained immediate needle safety which is a requirement today in the clinical and home environments. When disposable autoinjectors were introduced in the 2000s, they all included integrated needle safety. Today, the newest epinephrine autoinjectors also include needle safety.

In the 2000s demand for disposable autoinjectors increased with the introduction of biotech drugs such as TNF inhibitors in prefilled syringe-based liquid-stable formulations requiring weekly or less frequent dosing.

13.2.1.3 Disposable Autoinjector Developments

Disposable autoinjectors have come a long way over the last few years. In order to provide the maximum amount of convenience, they have a certain level of internal complexity to provide the following key features and handling benefits:

1. Large viewing window for clear visualization of the syringe and drug before injection
2. Safety mechanism to prevent inadvertent activation
3. Audible and tactile start and end of injection feedback
4. Needle hiding to reduce fear of needles
5. Needle safety after injection to prevent needlestick injury using needle shielding or needle retraction

Depending on the autoinjector, these technical features are achieved in different ways, e.g., the geometry/location of the injection spring may have an impact on the

size of the autoinjector, different safety/activation mechanisms, and needle shielding or needle retraction.

The arms race to develop full-feature autoinjectors, many of which are button activated, has been largely achieved. Devices with push-on-skin activation for prefilled syringes are now available, and simpler devices with manual needle insertion are generating interest as they are smaller and more discreet.

13.2.1.4 Autoinjector-Compatible Syringes and Needles

Approximately 2.5 billion prefillable syringes are sold globally each year (Ypsomed 2011). The majority are used for heparin and vaccines and as diluent syringes. Approximately 10 % are used for biotech drugs and drugs that could possibly be self-injected. Approximately 15 % (40–50 million) of these are incorporated into disposable autoinjectors for drugs such as epinephrine (cartridges and syringes), sumatriptan, and TNF inhibitors.

Prefilled syringes with pre-attached needle are the preferred primary drug container for autoinjectors. The main advantage is the pre-attached needle meaning that the patient does not need to attach a needle during preparation of the injection.

Prefilled syringes are now being specified and manufactured for use with disposable autoinjectors. Ideally the syringe should be held on the front syringe shoulder rather than the finger flange as this may break. If the syringe has a rigid needle shield with a larger diameter than the syringe, this makes assembly into the autoinjector syringe holder more difficult, but there are a number of systems available to accommodate this.

A key improvement of the prefilled syringe is the availability of different diameter thin-wall cannulas. By providing needle sizes in the range 29–25G, a range of drug viscosities up to 30–40 cp can be covered by a standard autoinjector device.

13.2.2 Pen Injectors

Pen injectors are essentially sophisticated "cartridge-based" syringes. The first pens were introduced for the reliable and accurate self-administration of the first wave of biotech molecules, mainly insulin and human growth hormone (hGH) (Selam 2010; Jorgensen et al. 1989). Today, insulin still dominates the market for self-injection devices, followed by hGH, glucagon-like peptide (GLP-1), follicle-stimulating hormone (FSH), and parathyroid hormone (PTH) (Dumas et al. 2006; Devonshire et al. 2010; Hey-Hadavi et al. 2010).

These therapies require frequent, often daily, manual injection with weight-based or fixed dosing and injections are repeated until the cartridge is empty—usually after 1–2 weeks or up to 1 month. The drugs in the multiple-dose cartridges are formulated with preservatives, while individual doses are typically in the range 0.02–0.60 mL. Pen injector patients are accustomed to injecting themselves

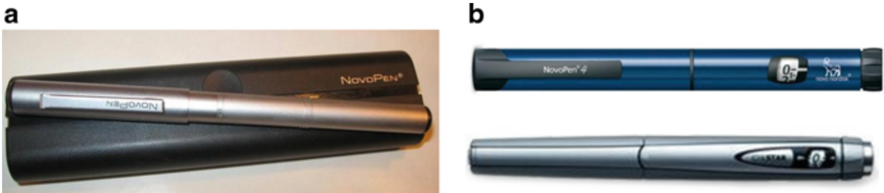

Fig. 13.5 (**a**) Original NovoPen and (**b**) latest reusable insulin pens

manually with 29–32G pen needles, and the need for automated needle insertion or injection has traditionally been outweighed by the patient's desire for discreet and easy-to-use devices.

13.2.2.1 Insulin Pens

Pen injectors were developed and first commercialized by Novo Nordisk with the launch of the first *reusable* insulin pen in 1984 (Rex et al. 2006), depicted in Fig. 13.5. The very first insulin pen was based on a 1.5 mL insulin cartridge which allowed for a very slim "pen-like" device. The need to include more sophisticated mechanisms to fulfill international ISO standards, as well the use of the 3 mL insulin cartridge, has increased the size of insulin pens but made them easier to use (Dumas et al. 2006; Perfetti 2010; Pearson 2010).

Various reusable pen technologies have been established with varying levels of success including automated needle insertion, electronic displays, and spring-driven injection. Today, the most successful and widespread technologies are based on geared "dial and dose" mechanisms including a clutch which allows doses of up to 60 or 80 insulin units to be dialed/corrected and then manually injected; see Fig. 13.5. All insulin pens also communicate to the patient how much insulin remains in the cartridge either before the final dose is given or what remains to be injected if the dialed dose cannot be injected.

The first *disposable* insulin pens were simple devices—without any form of gearing and comprising few plastic parts—and were introduced in the 1990s initially by Novo and Lilly and later by Sanofi. Second-generation disposable insulin pens including dial and dose gearing have been successfully introduced by all three companies during the 2000s (Perfetti 2010). Second-generation disposable pens are shown in Fig. 13.6.

Currently approximately 1.3 billion insulin cartridges are filled each year with half being assembled into disposable pens and the other half used with reusable pens (Ypsomed 2011). An in-depth understanding of pen gearing mechanisms, material selection, and the patent situation is necessary to be able to develop and manufacture both reusable and disposable pens in the large quantities required by insulin manufacturers. The most recent development trend has been towards spring-driven pens covering a large dose range and including dose correction both for reusable and disposable insulin pens thus further simplifying the injection process for the patient.

Fig. 13.6 Latest disposable insulin pens, (**a**) FlexPen from Novo, (**b**) SoloStar® from Sanofi, and (**c**) Kwikpen from Lilly

13.2.2.2 Current Pen Technologies and Demands

Pen injectors today provide a high level of convenience and provide the following key handling features for patients:

1. Large display and dose correction for easy dose selection
2. Geared or spring-driven injection mechanics for ease of injection
3. Simple cartridge exchange for reusable pens

The move to spring-driven injection technology is ideal for patient populations which may have problems injecting themselves with manual geared pen technology such as multiple sclerosis patients or children injecting hGH. Accessories and options offered to diabetics such as needle hiders, auto-inserters, and electronic dose history are also available for certain patient groups.

Some therapies require weight-based dosing, but once this dose is defined, the patient does not have to change the dose, e.g., for hGH. Dose-memory pens simplify handling so that the patient only needs to set the required dose once. For all subsequent injections, the patient only needs to pull and push the dosing knob until the cartridge is empty. An example of a dose-memory pen for Genotropin™ is provided in Fig. 13.7. Other therapies require very small fixed doses, and the pen must incorporate mechanisms to clearly communicate that the dose has been set and injected such as for Forteo™; see Fig. 13.7.

Frequent large doses are often injected from prefilled syringes. As part of the life cycle management of these therapies, there is also the opportunity to develop preserved formulations that can be injected from pen-based cartridges. An example of this is Rebif® (IFN beta-1α) for treating multiple sclerosis which is now available in cartridges and injected using a motor-driven device, depicted in Fig. 13.7.

13.2.2.3 Pens Based on Dual-Chamber Cartridges

A dual-chamber cartridge cannot be used on its own as it requires a Luer or pen-type needle and some form of reconstitution and injection mechanism. The simplest version is Lyo-Ject®, shown in Fig. 13.8, which is used in the clinical setting with a

Fig. 13.7 Examples of (**a**) dose-memory, (**b**) fixed-dose, and (**c**) motor-driven pens

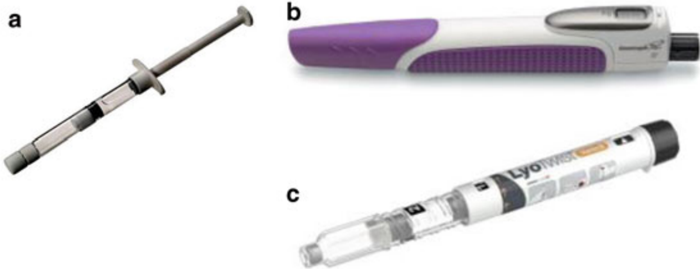

Fig. 13.8 Examples of (**a**) Lyo-Ject®, (**b**) dual-chamber multidose, and (**c**) dual-chamber monodose pens

Luer connection. For home use patients require more convenience in the form of pen-type systems.

Dual-chamber-based devices designed for the simple reconstitution of lyophilized drug and diluent have been on the market for over 20 years. Examples include multidose pens for therapies such as hGH. All insulin pen technologies can be modified to accommodate a dual-chamber cartridge allowing simple reconstitution and priming prior to use; see Fig. 13.8. It is very important for the patient that these steps are therefore easy to learn and always performed in the correct order.

Today, the dual-chamber cartridge is also used for monodose therapies where it is difficult to develop a liquid-stable drug formulation. This requires disposable monodose pen devices which are essentially the equivalent of the disposable autoinjector for dual-chamber cartridges. Monodose dual-chamber-based injectors may include needle safety, or the needle safety is provided by a dedicated safety pen needle; see Fig. 13.8.

Manual twist motion reconstitution of a dual-chamber cartridge and priming is easy to visualize and easy to perform for patients. Automating these steps may help patients with motor disabilities but adds complexity and cost to the device. Regardless, the device must always be held in the correct position during reconstitution to prevent incomplete mixing or inadvertent expelling of the drug. Injection may be performed manually or automatically depending on the needs of the patient.

13.2.2.4 Cartridge Requirements

The original 1.5 mL pen cartridges were based on a shortened version of the 1.8 mL dental cartridge. During the 1980s the 3 mL pen cartridge became the international standard. Although the cartridges are standardized, there are subtle differences in the key dimensions, e.g., the internal diameter between Novo, Sanofi, and Lilly. In fact the "Lilly" cartridge tolerances are closest to the international ISO standard for pen cartridges (International Standards Organisation 2000).

Different cartridge sizes based on the standard 1.5 and 3 mL cartridges have been used for different therapies, but more and more the 3 mL cartridge is selected even if the required fill volume is significantly lower than 3 mL. This means that standard pen technologies can be selected avoiding the development costs associated with a new pen device.

Both standard and dual-chamber cartridges must be overfilled to guarantee that the specified number of doses or dose volume can be injected. Understanding the cartridge system and filling tolerances and not just the individual cartridge components is critical. The cartridge is overfilled by as much as 7–10 % of the 3 ml liquid volume.

13.2.2.5 Pen Needles

Cartridges are mainly used for multiple injections with insulin pen needles which are available in different needle gauges and lengths; see Fig. 13.9. Pen needles started with the advent of the insulin pen in 1984, and today over five billion pen needles are manufactured worldwide mainly for injecting insulin.

Original pen needles were 27–29G and up to 8–12 mm long. Today, most patients, particularly diabetics, use much finer (30–32G) and shorter (4–6 mm) pen needles. The only limiting factor being that the small orifice of the finer gauge needles mean that high-volume injections require more force to perform the injections.

Safety pen needles (see Fig. 13.9) are now available for healthcare professionals performing insulin injections with pens in the clinical setting. They are also used with dual-chamber pen devices where needle safety is provided by the needle rather than the device.

13.2.3 Development Trends and Drivers

The market for self-injection devices—pens and autoinjectors—continues to show above-average growth based on a number of factors:

1. More protein-based therapies are being developed.
2. The increased incidence of diseases such as diabetes.
3. Overall economic growth worldwide.
4. More self-administration due to pressure on healthcare systems to reduce costs.

Fig. 13.9 Examples of (**a**) pen needles and (**b**) safety pen needles

Many existing injectables are biotech drugs which are being reformulated and improved. Improvements include liquid-stable formulations, long-acting formulations for less frequent dosing, and multidose preserved formulations which help differentiate against generic/biosimilar competition. Another trend is that antibody cancer therapies that are currently infused are being reformulated to allow subcutaneous self-injection. In addition, self-injectable therapies in new therapeutic areas such as Alzheimer's and cardiovascular diseases are in development. Some injectable therapies are facing competition from substitution technologies; examples include DPP-4 inhibitors vs. GLP-1s in diabetes and new oral drugs for treating multiple sclerosis.

Pens and autoinjectors have reached a high level of patient-friendly functionality, and there is a clear demand for disposable devices instead of reusable devices as this provides a higher level of convenience for patients, outweighing the greater cost of the disposable device and the issues related to disposal. The main area of demand for reusable devices is for reusable insulin pens in developing markets that are moving away from vials to cartridge-based insulin injections and where disposable pens are not yet affordable.

13.2.3.1 Pharma Needs

As self-injection device technology has matured, the drive to customize platform products rather than develop completely new devices has intensified. Due to infrequent dosing or multidose drug presentations, many therapies require nominal

device quantities even for relatively large patient populations. Apart from diabetes and certain autoimmune disease treatments, most injectable therapies require no more than hundreds thousands to a few million disposable devices per year. This means that the use of standardized device platforms that can be used for a number of therapies is part of each pharma company's strategy, in order to speed up time to market, minimize risks, reduce costs, and maintain quality.

In addition, through consolidation and experience, the pharma industry has acquired a level of knowledge about complex medical devices including injection devices. There is general awareness of which devices are best to use for which therapies. This combined with cost pressures on big pharma means that they are looking for off-the-shelf solutions which reduce investment during phase 3 clinical trials until it is clear that the drug is going to be launched. Pharma companies want to be able to move into the clinic with a device which can be manufactured in the required volume with a minimum of modifications. It is therefore important for device suppliers to leverage platform products and minimize costs based on the use of common tooling, assembly, and printing systems throughout the manufacturing process.

13.3 Development of Pens and Autoinjectors

The development of a pen or autoinjector device is an extensive process, comprising a number of steps and typically lasting for several years. Table 13.1 provides an overview of the different steps included in a typical development program. Figure 13.10 provides an example of a project timeline, describing the main activities over time and their interdependencies. Injection device development is also complex, in that it involves the interaction of a number of companies and organizations. Furthermore, there are significant formal and regulatory requirements which have to be taken into account (French and Collins 2010).

13.3.1 Regulatory Requirements

Pens and autoinjectors are, depending on their primary mode of action, often regulated as combination products, which means that they have to meet the safety and performance-related requirements for medical devices as well as certain conditions of drug regulations (French and Collins 2010). The GMP requirements for injection devices are defined through the International Standards Organization (ISO) 13485 standard (International Standards Organisation 2003) and the Food and Drug Administration (FDA) Code of Federal Regulations (CFR) 21 Part 820 (US Code of Federal Regulations). These two sets of requirements are quite similar, the main difference between them being the level of detail in certain areas (French and Collins 2010).

Table 13.1 Overview of the steps typically covered in an injection device development project

Step	Activity	Input	Output
1. Initial considerations	Assessment of: Drug formulation and primary container Patient population and dosing requirements Available time to market and financials Competitive landscape within therapeutic field	Drug, indication and patient information Pharma company position/strategy	Framework, scope, and setup for the device project
2. Choice of device type	Evaluation of vendors and technology User handling studies	Available platforms/technologies	Selected device type/platform
3. Device customization	Fine-tuning of the device design: Technically, i.e., primary container, dose steps Look and feel, i.e., industrial design	Device type/platform Specification of primary container and dose steps User preference, patient characteristics, marketing aspects	Detailed device design Final prototypes for handling studies
4. Manufacturing strategy	Definition of tooling concepts, printing, and assembly processes incl. manufacturing capacity and ramp-up/scale-up steps	Required quantities for development, launch, and commercial Commercial strategy	Device industrialization and scale-up strategy
5. Design freeze	Compilation of output from steps 3 and 4, preparation for industrialization	Detailed device design Device manufacturing and scale-up strategy	Lockdown on device design, documentation, and manufacturing strategy
6. Device industrialization and capacity buildup	Buildup of manufacturing infrastructure incl. process validation Device design verification and validation	Frozen device design and manufacturing strategy	Verified and validated device design Validated device manufacturing process
7. Filing and launch	Submission of regulatory filing for approval	Verified and validated device design (for filing)	Approved device
	Market launch	Validated device manufacturing process (for launch)	Production of commercial quantities

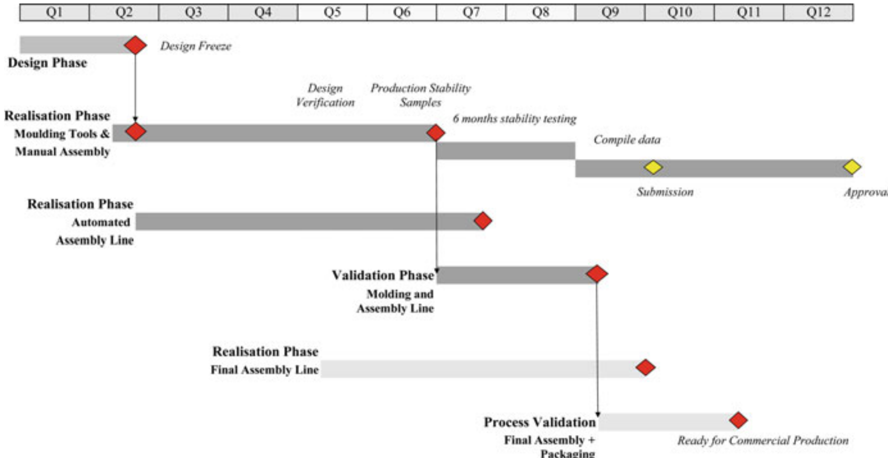

Fig. 13.10 Example timeline for a pen injector project (combination product, minimal approval time)

A pen or autoinjector will usually require premarket approval by a regulatory authority before it is allowed onto the market. Reusable devices are the exceptions which are certified via the CE-marking process in Europe and the 510(k) registration in the USA. In general, the requirements on the type and amount of information to be provided in regulatory filings for combination products such as pens and autoinjectors are increasing. In practice, this means that information, e.g., detailed real-time stability data, has to be generated for the device combination product and that the timeline for regulatory approval typically extends beyond that for manufacturing readiness in the device development project (see Fig. 13.10).

13.3.2 Development Tools

One important factor contributing to a successful development of a pen or autoinjector is the use of appropriate and up-to-date tools and techniques. Throughout all stages of development, from early concept definition to detailed design as well as formal design verification, deployment of the right tools and methods will allow time savings, enable improved device performance, eliminate technical risk, and help in ensuring that regulatory design requirements are met.

13.3.2.1 Finite Element Analysis

Finite element analysis and other computer-based modeling techniques are used to develop, evaluate, and test functional designs as well as manufacturing processes (e.g., injection molding). Such modeling is performed both prior to and in parallel with the building and testing of physical prototypes.

Finite element analysis involves building a mathematical model of one or several device parts, representing the mechanical properties of the part or parts, through the use of dedicated software. Using the model, the parts can be subjected to expected deformation and movement, and the part design as well as choice of material can be varied to provide the optimum design solution. Since the turnaround time is much shorter with modeling compared to the preparation and testing of physical prototype parts, the use of such techniques allows significant time savings.

Similar modeling approaches are used to evaluate and optimize other aspects of device design, e.g., the relationship between material properties, part design, friction coefficients, and system efficiency with respect to injection force. Such modeling enables the rapid screening of multiple material combinations and the selection of optimized material pairs, e.g., with respect to tribological properties.
Simulation of the injection molding process for a part is employed to evaluate if a proposed part design can be manufactured reliably and cost-efficiently. This approach is used to optimize part design in early development as well as to aid in injection molding tool design in preparation for industrialization.

13.3.2.2 Rapid Prototyping

The ability to quickly prepare prototype parts and devices at different stages during a project is central to injection device development. As a design progresses, the requirements on number and quality of prototypes evolves as well. Depending on the stage of development and the requirements on the parts to be produced, different technologies are typically employed.

In the early concept phases, parts are often prepared using stereo-lithographic processes. Such techniques allow rapid production of prototype single parts which can be assembled and used for demonstrational and initial functional evaluations. Turnaround times can be shorter than 24 h. However, the materials do not have the same properties as those employed in serial production which precludes more in-depth testing and mechanical evaluation on such prototype devices.

In later phases, scaled-down production tooling, also known as rapid or prototype tooling, is employed to produce devices for verification, to confirm manufacturability, as well as to provide qualified devices, e.g., for clinical studies. Such tools are similar to commercial scale production tooling, using the same technology, but simply smaller in size and often using inserts for the part-shaping sections of the tools to provide flexibility and shorten lead times. Turnaround times here are typically of the order of weeks. The parts are manufactured using the same materials as in serial production, thus enabling full testing and evaluation of properties and performance.

13.3.2.3 Design for Manufacture

Design for manufacture refers to the practice of systematically considering the manufacturing process throughout the device design process. By thinking already from the outset about not only how parts function and perform but also how they can be

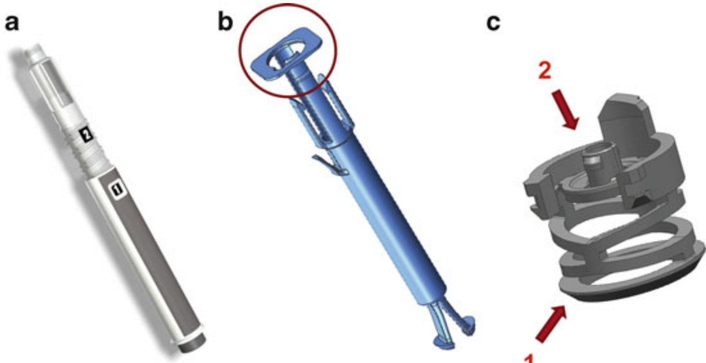

Fig. 13.11 Two examples of design for manufacture for a pen injector. (**a**) Dual-chamber pen injector. (**b**) Example 1. The original design of the plunger rod did not allow for bulk handling due to the risk of part damage, and the orientation of the part for assembly was difficult. The solution was to add a protective ring (indicated with a *circle*) to the part, thus enabling bulk handling and straightforward orientation of the part. In spite of the additional step required to remove the ring, the overall result was a reduction in cost and increase in process stability. (**c**) Example 2. Combining the cartridge spring (1) and flange (2) into one part saves one tool and reduces the number of assembly steps. The parts are separated during final assembly of the pen with the cartridge

produced and assembled, potential problems can be solved and significant benefits obtained. The benefits can be in the form of savings, e.g., through a reduction in number of parts, reduction in the number of assembly steps, or part handling in bulk as opposed to individually, as well as in terms of process robustness and stability. Two examples of what can be achieved with the design for manufacture approach are provided in Fig. 13.11.

13.3.2.4 Human Factors Engineering

Usability engineering, or human factors engineering, are formal and systematic methods to ensure that use errors and use-related risks are minimized (International Standards Organisation 2007; FDA 2000; Shah et al. 2009). The use of such methods as part of medical device development has recently become mandatory. The systematic deployment of usability and human factors engineering throughout development strives to ensure the device is optimally user-designed and avoids user-related risk.

The usability method involves the steps of defining the application, analyzing hazards and hazardous situations during use, defining measures to improve usability, and implementing these measures (e.g., design of a user interface), followed by technical verification and user validation of the design. Regular user or handling studies with relevant users are part of the concept, providing input and direction in early stages of development and confirmation that the design solutions perform as intended in later stages of the project.

13.3.2.5 Testing and Evaluation

The physical testing and evaluation of device properties and behavior is central in injection device development. Such methods are used to evaluate and confirm performance of early concepts, often as a next step following the modeling results. Physical testing is also employed for the formal design verification which takes place at the end of the design process.

For needle-based injection devices, detailed technical requirements regarding function and performance are described in the latest version of the ISO 11608-1 standard (International Standards Organisation 2010). This standard provides a list of tests, including practical details for conducting the experiments as well as evaluating the results, and forms the basis for the verification of injection devices. The main focus is on dose accuracy and reliability of device function under various environmental conditions.

Dose Accuracy and Robustness

Providing the desired dose in a reliable fashion is the primary function of an injection device. The evaluation of dose accuracy is performed according to the ISO 11608-1 standard (ASTM International 2010). The test procedure is based on a gravimetric determination of the expelled amount and prescribes the testing of a certain number of devices with a number of injections per device so as to enable a statistical evaluation of performance. The standard also provides statistically based acceptance criteria for different device categories.

Device robustness is assessed by subjecting devices to various pretreatments followed by evaluation of the dose accuracy (ASTM International 2010). The pretreatments include increased temperature and humidity, free fall as well as vibration, and, for devices with electronics, electromagnetic radiation.

Stability and Shelf Life

In order to assess the stability over time and assign a shelf life to the device, different testing approaches can be used. In principle, stability can be evaluated either under real conditions or through accelerated aging. Whereas the former, as the name implies, simply means testing devices after storage under the expected conditions of temperature and humidity, the latter involves accelerating the aging process by storage at elevated temperatures prior to testing.

The accelerated aging approach is based on certain assumptions regarding the influence of temperature on the degradation mechanism of polymeric materials (ASTM International 2010). This approach makes it possible to assess a desired shelf life of years with tests lasting only a couple of months. However, the results typically have to be confirmed by real-time testing. Real-time shelf life testing of the final drug-device combination is also a regulatory requirement and as such an obligatory part of any disposable pen or autoinjector development project.

Mechanical Testing

Mechanical testing methods are much used in development, providing information the integrity of devices and device parts, as well as objective data on user handling forces. Since handling forces are becoming increasingly important as device differentiators and device performance indicators, and handling studies generally only provide subjective data, the need for physical measurement techniques is increasing (Toraishi et al. 2005).

Although the assessment of handling forces such as injection force and operating torques appears relatively straightforward from a measurement point of view as standard equipment can be used, care has to be taken in terms of test setup and testing parameters so as to ensure that results are repeatable as well as comparable across devices and test series.

13.3.3 Initial Considerations and Device Choice

Before an injection device development project can begin, the pharma company has to consider a set of basic questions, the answers to which will provide the scope and framework for the project. With the outline of the project defined, the pharma company then has to go through a process of device, technology, and vendor selection.

13.3.3.1 Basic Questions

The basic questions which have to be answered fall into four categories:

1. Drug formulation and primary container. Is the drug in a liquid or lyophilized form? Will the drug be filled into a prefilled syringe or a cartridge?
2. Patient population and dosing requirements. Are the users naive or experienced with injection devices? How often will the drug be dosed, e.g., daily, weekly, or less frequently? What is the duration of the therapy? Is the dose constant, i.e., the same for all users, or variable? What is the size (volume) of a typical dose?
3. Is IP ownership and/or exclusivity on the technology/device required? What level of involvement in the development and manufacturing is desired?
4. Available time to market and financial limitations. How much time is available for bringing the device to the market? What is the preparedness to invest in the development of a bespoke device?

13.3.3.2 Device Technologies/Platforms

It is often preferred to start a specific development project with an injection device technology which has already been developed up to a certain level. The main

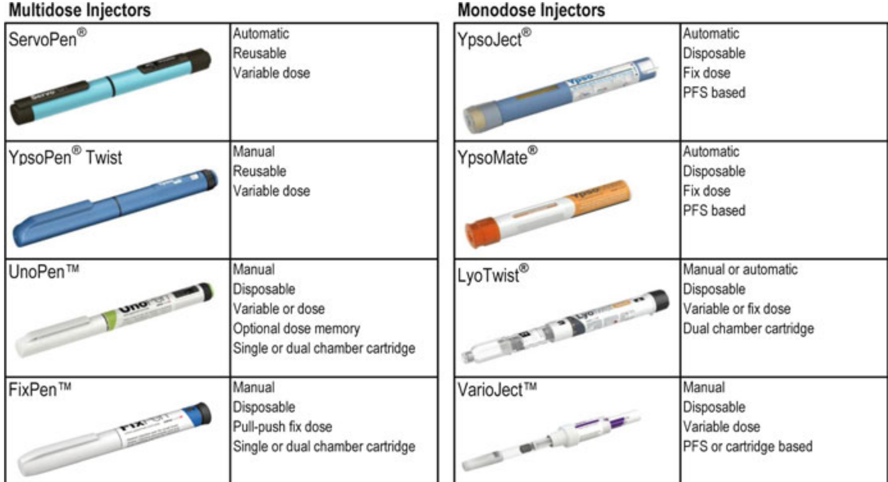

Fig. 13.12 Example of a device platform portfolio, structured around the primary container (single- or dual-chamber cartridges and prefilled syringes)

benefits in starting with a platform device are a reduced technical risk in development, shorter timelines, and the possibility of sharing production equipment between different products based on the same platform. Most vendors will have at least some platforms on offer. An example of a platform portfolio is presented in Fig. 13.12. Typically, a platform device will have been developed to proof of concept status, which means that prototypes have been manufactured and tested to ensure their functionality. The mechanical device design will have been defined, and considerations for possible manufacturing processes made.

13.3.3.3 Choice of Device

The answers to the basic questions above provide a first direction as to which injection device technology can be considered for the drug and indication in question. In some cases, the decision could be to develop something entirely new. In most cases, however, the decision will be to start with a platform device such as illustrated in Fig. 13.12. Many technical aspects of the device may be given by the primary container and dosing regimen, and thus limit the choice of technologies being considered. Aspects related to the user may be more open, and here handling or user preference studies can provide useful guidance as to which technology is best suited. Finally, the capabilities of the vendor providing the technology, as well as the financial aspects of a possible development and supply of the injection device, have to be considered when making the final choice.

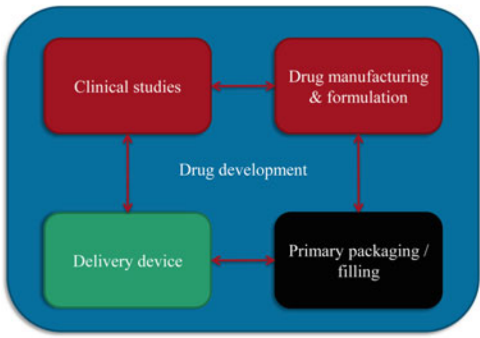

Fig. 13.13 Context for an injection device development project

13.3.3.4 Project Context

Before initiating an injection device development project, it is worth noting that the device project typically has to be seen in the context of a wider drug development program, involving drug manufacturing and formulation, primary container and filling, as well as clinical studies. This context is illustrated in Fig. 13.13. To be ultimately successful, the device development activities have to be conducted in concert with the other parts of the drug development program. The device project provides input to the other parts (e.g., devices for clinical studies) and at the same time depends on output from the other parts for its progress (e.g., primary packaging specifications and samples). A close coordination between the different parts of a drug development program is thus required.

13.3.3.5 Project Partners

In setting up an injection device development project, it is important to consider that in addition to the pharma company and device vendor, a number of further players and partners are involved. A schematic overview of the different kinds of entities involved in a drug delivery device project and how they interact is provided in Fig. 13.14. The pharma company often has activities such as industrial design, primary packaging/filling, and final assembly of the device performed by external parties who then have to interact not only with the pharma company but also with the device vendor. Most device vendors will outsource manufacture of the production equipment and often procure some of the single parts for the device from sub-suppliers. All in all this means that there are a number of interfaces and interactions which have to be defined and managed throughout the project.

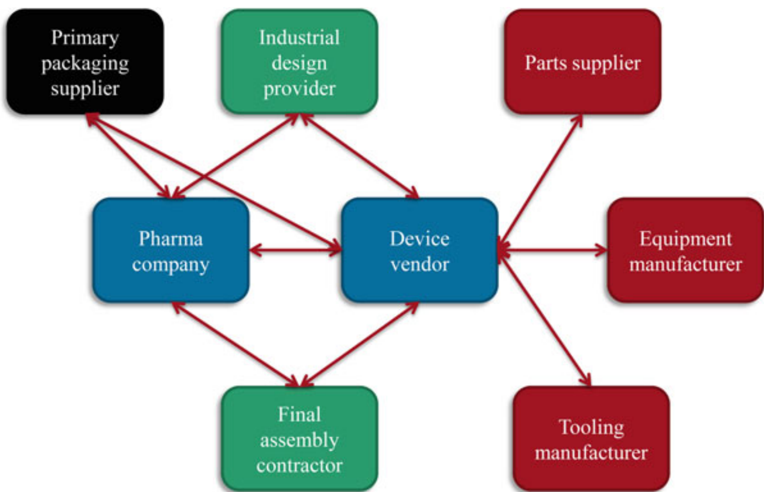

Fig. 13.14 Typical partners in an injection device development project

13.3.4 Device Customization, Manufacturing Strategy, and Design Freeze

Once the basic choices have been made, the device technology defined and the vendor selected, the actual device development project can begin. The first phase of the device development project includes the activities of adapting the selected design for the specific needs of the drug and indication, as well as elaborating an industrial design (look and feel) suitable for the user and market. In parallel, the manufacturing strategy, i.e., the plan for how the device is to be produced and manufacturing capacity scaled up, is defined. Finally, the two parts are brought together in the form of a design freeze to form the basis for the industrialization project.

13.3.4.1 Device Customization

To obtain the required functionality and performance for the specific intended use, the injection device technology has to be adapted to the primary container (cartridge or prefilled syringe) it is to be used with. This involves adjusting the technical design according to the specifications of the primary container, including dimensions, stopper position(s), and fill volume. Furthermore, depending on the dosing requirements, the number and size of the dose steps may have to be adjusted. Typically, the pharma company also wishes to provide a distinct “look and feel,” i.e., industrial design, to the device. This is to render the device as attractive as possible to the users and on the other hand to set it apart from competition and bring it in line with company and drug’s visual identity and branding. Industrial design work generally

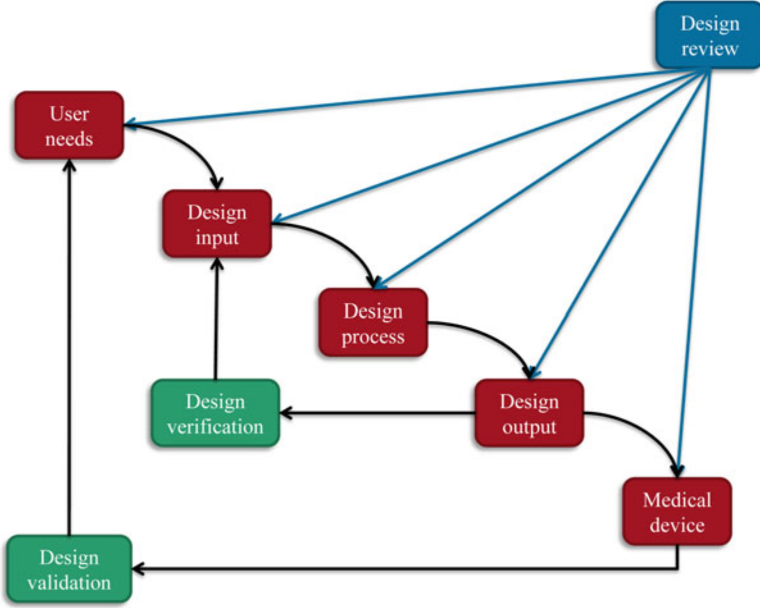

Fig. 13.15 Illustration of the design control concept (Food and Drug Administration 1997)

involves iterative steps where alternative designs are evaluated by users and is typically integrated with human factors engineering so as to maximize the usability of the design (Guerlain et al. 2010). Industrial designs are commonly elaborated together with a design house which has experience in the injection device field.

The device customization activities form an important part of the design controls procedure for the injection device. Design controls are defined as a set of practices and procedures incorporated into the development process, acting as a system of checks and balances to ensure that a systematic assessment of the design remains an integral part of development (Food and Drug Administration 1997). The concept of design controls is depicted in Fig. 13.15. The procedure involves the systematic, stepwise definition of requirements, starting with the user needs, as input for the device design process, and the equally systematic and stepwise verification of the design output against the requirements. Regular design reviews, conducted at strategic points in the design process, are an essential part of the procedure. Design validation, which encompasses verification, extends the assessment to whether the device fulfils the user needs.

13.3.4.2 Scale-Up Strategies

The definition of an industrialization and scale-up strategy is a pivotal part of an injection device development project, with a major impact on the timing and cost of the project activities. Manufacturing of an injection device typically involves the

individual operations of injection molding single (plastic) parts, followed by printing and assembly of the parts. Injection molding can be conducted with small (low-cavity) or large (high-cavity) tools, whereas printing and assembly operations can be manual, semi-automatic, or fully automated. Manufacturing capacity can be built up gradually, i.e., in several smaller steps, or directly, i.e., in a single large step. Generally speaking, building capacity in smaller steps is quicker and carries less technical risk, but is more costly and gives higher production unit cost.

As input for the elaboration of the industrialization and scale-up strategy, the required quantities of devices throughout development (including clinical), launch, and commercial phases first have to be defined. Thereafter, the often conflicting requirements on lead time, unit cost, and acceptable level of investment in manufacturing equipment have to be considered. In many cases a stepwise approach is selected, e.g., starting with low-cavity tooling and manual assembly for clinical and filing purposes, followed by high-cavity tooling and automated processes for commercial production.

13.3.4.3 Usability, Risk Management, and Specifications

In parallel with the detailed definition of device design and manufacturing processes, the procedures for risk management and usability are conducted. The risk management procedure includes going through a series of failure mode and effects analyses (FMEAs) on the use, design, and manufacturing process of the device. In the FMEAs, possible device-related patient hazards are systematically identified, their likelihood and potential consequences evaluated, and the ensuing risk estimated. Where required, risk control measures are then defined.

The usability procedure combines the human factors engineering work, including user studies, performed as part of device customization, with a systematic usability hazard evaluation and control approach similar to the one employed in risk management. All device design/customization work, the definition of manufacturing strategy as well as the design controls activities is documented in the design history file. The output of the work at this stage, i.e., the description of the detailed requirements on the final device design, is captured in a product specification document, which forms the basis against which device verification later will be performed.

13.3.4.4 Design and Process Lockdown

When the detailed device design has been finalized, the manufacturing strategy elaborated and the design controls documentation prepared, the documentation is subjected to thorough reviews, both from a technical and formal (completeness) point of view. On successful completion of the reviews, the device design (including engineering, documentation, usability, and risk management) and the manufacturing setup (including design for manufacture) are frozen in preparation for the next phase of the project.

13.3.5 Industrialization, Scale-Up, Filing, and Launch

With a defined injection device design and manufacturing strategy in place, the next phase of the development project can begin. It covers performing the steps of device industrialization and capacity scale-up, including device design controls (verification, validation), manufacturing process validation (equipment qualification, process validation), and finally the regulatory submission and launch.

13.3.5.1 Buildup of Manufacturing Infrastructure

Using the device design and manufacturing setup information as input, the manufacturing equipment, e.g., injection molding tooling, printing machinery, and assembly equipment, is specified and procured, typically from external sub-suppliers although in some cases it may be prepared in-house. Manufacturing documentation, i.e., the device master record, is drafted. In parallel, the process validation plans for the different equipment components are prepared, and the verification plan for the injection device is defined. Once the equipment has been built, it goes through a process of equipment qualification followed by operational qualification, where the appropriate production process parameters are determined.

Using the process parameters defined in the operational qualification, the manufacturing documentation is finalized, and a set of devices is built and subjected to formal design verification testing, with the aim of demonstrating that the device as produced fulfils all the requirements in the product specification.

Once device design verification has been successfully passed, the manufacturing process can go through process qualification, thus ensuring that the process is capable of consistently producing the product within certain acceptance criteria. Employing the final device design, design validation, normally in the form of user studies, is performed to confirm that the device meets its design intent. The risk management and usability processes are concluded, the development documentation is finalized, and the design history file subjected to review before the product can be transferred from development to production.

In the case of a stepwise buildup of manufacturing capacity, the whole process is repeated at least partially for each industrialization scale-up step. As a minimum, all equipment has to go through the qualification process, whereas design verification has to be repeated insofar as the manufacturing process is changed in such a way that it may impact device performance. Design validation is typically not repeated.

13.3.5.2 Filing and Launch

In order to submit a regulatory filing for market approval, the design verification as well as design validation of the device has to be completed. In most cases, stability data for the final packaged drug-device product also has to be provided.

However, validation of the manufacturing process does not generally have to be completed until the product actually is put on the market. As soon as the development project has reached the stage where the necessary data is available, regulatory filings can be prepared and submitted. Once the approval is received and the manufacturing process has been fully validated, the injection device can be launched. In many markets, however, successful launch requires negotiation of reimbursement with the health authorities, further adding to the launch timeline.

13.4 Concluding Remarks

An injection device development project is a complex and extensive activity, involving a number of steps and requiring interaction between many different partners. After having defined the basic device needs and the scope of the development activity, a project typically starts with the selection of a technology platform, which is adapted (customized) to the needs of the drug and users (patients). The industrialization and scale-up strategy is defined, and the project continues with the buildup of manufacturing infrastructure including verification and validation of the device design as well as manufacturing process validation. After transfer from development to production and regulatory approval, the device can then be brought to market. Regulatory requirements are central to device development and have significant influence on the conduct of the project, as well as the timeline to market.

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Part III
Regulatory and Quality Aspects

Chapter 14
Particulate Matter in Sterile Parenteral Products

Satish K. Singh

Abstract Particulate matter, visible or subvisible, in sterile parenteral products is regarded as a critical quality attribute, impacting safety of the product. Particles can arise from many sources foreign, intrinsic, or inherent to the product, the latter having particular emphasis for biopharmaceuticals. This chapter discusses the nature of these particles, the safety concerns behind the need to control them, and the various techniques available to monitor them. The concern with inherent proteinaceous particles in biopharmaceuticals has led to a large amount of research in this area and the development of a number of novel techniques and applications. The chapter also covers some special topics of current interest including the definition of "essentially free," topics related to the measurement and control of subvisible particles under 10 μm in biopharmaceuticals, the new USP<787> chapter, as well as guidance for addressing particles related regulatory queries.

14.1 Particulate Matter in Sterile Parenteral Products

Particulate matter in sterile parenteral products is an undesirable characteristic, except in the case of sterile suspensions. However, even in suspensions, the presence of foreign or contaminating particles is undesirable. This chapter examines the nature of particulate matter, the requirements for its control, measurement of particles, and some special contemporary issues around this topic. The focus is on sterile parenterals, not including suspensions, with special emphasis on biopharmaceuticals. We will use the term biopharmaceuticals to refer to products covered under USP<1045>.

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Particulate matter in the context of sterile parenterals is a broad term that covers all manners of particles that may be found in the product. The US Pharmacopeia (USP35<788>) definition is "particulate matter in injections and parenteral infusions consists of mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions." The European Pharmacopeia (Ph.Eur.) 7th ed. 2.9.19 definition is very similar but specifically refers to the particulates as "contamination" and "extraneous"—"Particulate contamination of injections and infusions, consists of extraneous mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions." The Japanese Pharmacopeia (JP General Tests 11) refers to "… foreign insoluble matter." The particles may be categorized on the basis of their source or origin, their nature, their size (distribution), and their number, as discussed in further detail in the subsequent sections of this chapter.

14.1.1 Source or Origin and Nature of Particulate Matter

Particulate matter in parenterals can arise from many sources, from within and outside the product. A discussion on some sources of particulate matter in parenteral is provided in USP<1788>.

14.1.1.1 Foreign or Extrinsic Particulates

When particles exist as a contamination, the source is likely the package, the manufacturing process, the use/administration step, and, more likely, a combination of the above. Insufficient cleanliness or preparation of the containers, closures, administration sets, manufacturing equipment, as well as inadequate maintenance of a suitable manufacturing environment can lead to such contamination (see, e.g., Dungan 1968; Jamet 1988; Signoretti et al. 1988; Pavanetto et al. 1989; Ball et al. 2001; Wen et al. 2007; Tyagi et al. 2009; Nayak et al. 2011). Given properly qualified and maintained equipment and suitably designed processes, these sources should not be significant contributors. Nevertheless, surprises can occur in the form of shedding from worn gaskets, ill-fitting valves, inadequately rinsed filters, shedding from piston fillers, or, in as one case, condensation from volatiles evolving from gaskets in dry heat ovens. Containers may carry contaminants such as residues of lubrication oil or contact material used in the fabrication of vials and syringes. Glass ampoules are notorious for shedding glass particles during the opening step (Lee et al. 2011b). Another major source of contaminant particulates is personnel activity, shedding from the body, e.g., hair and skin cells, and from clothing worn during manufacturing. Such particles can be considered "foreign" or "extrinsic," and are likely to be cellulosic, glass, rubber, man-made fibers, silastic, mineral, paint or pain components, metal, or biological debris such as hair, human epithelial cells, or even bacteria. By definition, these particles would be invariant over time

and likely to be non-uniformly distributed among containers in a batch of product. A good comprehensive review of this type of particulate contamination and its detection and mitigation was provided by Borchert et al. (1986).

14.1.1.2 Intrinsic Particulates

Another source of particulates may be the product, the formulation components, the production process, and the interaction of the product with the package. Presence of such particles may be indicative of systemic problems with process and/or package. Silicone oil used to lubricate surfaces of syringes or stoppers can be sloughed off, especially over time. Degradation or interactions of product or components may lead to the formation of insoluble substances that would precipitate out of solution (see, e.g., Piecoro et al. 1975; Rubino et al. 1999; Iacocca and Allgeier 2007; Newton and Driscoll 2008; Kahook et al. 2010). Incompatibilities between packaging and contents may lead to the formation of particles, for example, formation of glass flakes through delamination (Iacocca and Allgeier 2007; Iacocca et al. 2010; http://www.fda.gov/Safety/Recalls/ArchiveRecalls/2010/ucm227202.htm). Particles arising in these situations can be considered "intrinsic," and are likely to be related to the product or its components, such as glass flakes, silicone oil, and organics or inorganics from degradation products. By definition, these particles may change over time, and depending upon their origin may or may not be uniformly distributed within the containers of a batch of product.

14.1.1.3 Inherent Particulates

A final source of particulates is the so-called inherent particles that are an unintended but integral part of the product due to the physico-chemical characteristics of the product itself. The primary examples are found in biotherapeutic products containing particles generated from the degradation (generally aggregation) of the biotherapeutic itself. These particles may be composed of the protein alone, or protein in conjunction with formulation excipients, and are also referred to as proteinaceous particles. When protein is associated with foreign or intrinsic particulates such as stainless steel, glass, or silicone oil, we prefer to classify them as foreign (extrinsic) or intrinsic, even though they carry a greater degree of risk compared to traditional foreign (extrinsic) or intrinsic particles (see Sect. 14.1.3.3). Such "proteinaceous" particles may be present in increasing amounts over time and the visible fractions of these particles are also mentioned on the package inserts of certain products (see Sect. 14.2.1.3).

In summary, particulates in parenterals can comprise a wide range of species, all present unintentionally in the product. It is illustrative to compare the current USP definition of particulates (given above) with an earlier version (USP 30<788>) "consisting of mobile, randomly-sourced, extraneous substances, other than gas bubbles that cannot be quantitated by chemical analysis due to small amount of material that

it represents and to its heterogeneous composition." The qualification of the definition with the "nature, composition and inability to quantitate" has been removed to simply encompass "all matter that is unintentionally present." This reflects the growing concern about particulates in parenteral products, and the importance of this attribute as an indicator, not just of quality of product but also of the quality of its design and manufacturing process. Concern about harm to the patient has led to a move to develop and recommend the use of in-line filtration systems during infusion therapy (Ball et al. 2001; Ball 2003; Kuramoto et al. 2006; Anonymous 2011).

14.1.2 Size and Number Distribution of Particulates

Given the presence of particulate matter in parenteral products, it clearly is important to be able to quantify both its size and number. Particles can be broadly classified as being visible (to the naked eye) or not (also called subvisible). Although there is no strong consensus on what is visible, a cut-off at 100 μm is useful for most discussions, i.e., particles may be called visible if >100 μm, or subvisible (~1–100 μm). For most parenterals, this general classification has historically been sufficient. However, with the increasing importance of biotherapeutics and the specific concerns and difficulties posed by inherent proteinaceous particles, this definition has been augmented by further subclassification of the subvisible particles into a submicron (0.1 to ~1 μm) (Carpenter et al. 2010a; Narhi et al. 2012). A brief discussion about the limits and current state of affairs is presented here.

14.1.2.1 Visible Particles

The USP description of Injections (USP35<1> Injections) states "Each final container of all parenteral preparations shall be inspected to the extent possible for the presence of observable foreign and particulate matter (hereafter termed 'visible particulates') in its contents. The inspection process shall be designed and qualified to ensure that every lot of all parenteral preparations is essentially free from visible particulates." The Ph.Eur. (Ph.Eur. 7th ed. 01/2008:0520 Parenteral Preparations) requires "Solutions for injection, examined under suitable conditions of visibility, are clear and practically free from particles." The Japanese Pharmacopeia (JP 17th ed. 6.06) also specifies inspection conditions and states "… Injections must be clear and free from readily detectable foreign insoluble matter" and "… The solution thus constituted must be clear and free from foreign insoluble matter that is clearly detectable when inspected with the unaided eyes at a position of light intensity of approximately 1,000 lux, right under an incandescent lamp."

USP<1151> Ophthalmic Preparations requires "Ophthalmic solutions are … essentially free from foreign particles." Ph.Eur. (01/2008:1163) requires that ophthalmic solutions are "practically free from particles."

The requirements of "essentially free" and "practically free" in the USP and Ph.Eur. will be discussed in more detail later (see Sect. 14.3.1), and also create some dilemmas for biotherapeutics (see Sect. 14.3.2). For now, it suffices to say that the ability to detect a "visible" particle is dependent on a number of factors such as its size, contrast with surroundings, color, reflectivity, and buoyancy, along with the lighting conditions, visual acuity of the inspector, etc. Thus the ability to see a "visible" particle is highly probabilistic, and the probability generally increases with the size. The threshold for human vision in the literature is defined to be about 50 μm, with the probability of detecting a single such particle in clear solution in a 10-mL vial under appropriate diffuse illumination being slightly greater than 0 %. The probability for detection of a 100 μm particle is around 40 %, and is greater than 95 % when the size reaches 200 μm or larger (cited in Madsen et al. 2009). A brief discussion about detection of visible particles in products is provided in Sect. 14.2.1.

14.1.2.2 Subvisible Particles

Parenteral Products

Requirements for subvisible particles in parenteral products are more definitively specified by the compendia (compared to visible particles). The chapters are harmonized except for a slight difference in the definition of small-volume vs. large-volume injectable in the Japanese Pharmacopeia. Upper limits are provided for acceptable number of particles of sizes ≥10 and ≥25 μm (see Table 14.1). The chapters also specify methods for measurement of these particles (see Sects. 14.2.2.1 and 14.2.2.2).

Although data on specific products and their subvisible particle content is not available, Nath et al. (2004) published a stimuli article where they summarized and analyzed data from (regulatory filings) batch analysis for 406 lots in 295 drug applications over 1998–2002. They showed that the actual particle count values for ≥10 and ≥25 μm sizes were significantly below the specified compendial upper limits, indicating that modern injectable manufacturing processes are highly capable when it comes to this characteristic. The data also showed that terminally sterilized products had significantly lower particle counts than aseptically processed products. There were differences also in the counts by container-type with glass vials being somewhat worse than ampoules, plastic vials, and glass syringes. It must be noted that the data analysis presented by Nath et al. (2004) was from ANDAs and thus did not include any biotherapeutic products. The conclusion about capability thus concerns primarily foreign (extrinsic) and intrinsic particles in products.

Ophthalmic Products

Products intended for ocular administration have significantly more restrictive requirements for subvisible particle content, specified in USP<789> (Table 14.2). These limits were set by the USP based on manufacturing capabilities for topical

Table 14.1 Summary of compendial requirements for visible and subvisible particulates in sterile parenterals

Attributes		USP*35*	Ph.Eur.7.0	JP 16th Ed.
		General chapter <1> *Injections*	General chapter 01/2008:0520 *Parenteral preparations*	General rules for preparations 11 *Injections*
			General chapter 2.9.19 *Particulate contamination: subvisible particles*	General tests, processes, and apparatus
		General chapter <788> *Particulate matter in injections*	General chapter 2.9.20 *Particulate contamination: visible particles*	*6.06: Foreign insoluble matter test for injections* *6.07: Insoluble particulate matter test for injections*
		Visible particulates		
Appearance	Visual or machine inspection	Each final container of all parenteral preparations shall be inspected to the extent possible for the presence of observable foreign and particulate matter (hereafter termed "visible particulates") in its contents. The inspection process shall be designed and qualified to ensure that every lot of all parenteral preparations is essentially free from visible particulates. Qualification of the inspection process shall be performed with reference to particulates in the visible range of a type that might emanate from the manufacturing or filling process USP <1>	Ph.Eur. 2.9.20: Light box with black and white panels, and illumination at the viewing point of between 2,000 and 3,750 lux. Gently swirl or invert container. Observe for 5 s in front of white panel Repeat in front of black panel Acceptance criteria Solutions for injection examined under suitable conditions are clear and practically free from particles (Ph. Eur. 01/2008:0520) Monoclonal Antibodies: Free of visible particulates (Ph.Eur.6.6 2031E)	JP6.06 (Method 1) Inspect with the unaided eyes at a position of light intensity of approximately 1,000 lux under an incandescent lamp. For Injections in plastic containers for aqueous injections, the inspection should be performed with unaided eye at a position of light intensity of 8,000–10,000 lux, with an incandescent lamp at appropriate distances above and below the container JP6.06 (Method 2) Inspect with the unaided eyes at a position of light intensity of approximately 1,000 lux, right under an incandescent lamp Acceptance criteria: Injections either in solutions or in solution constituted from sterile drug solids (Method 1): Clear and free from readily detectable foreign insoluble matters Injections with constituted solution (Method 2): Clear and free from foreign insoluble matters that are clearly detectable

Clarity	Turbidity compared to reference suspension	Not mentioned	Turbidity is the same as that of water R or of the solvent used, or not more than that of reference suspension 1 (Ph.Eur. 2.2.1)	Not mentioned
	Visual examination compared to reference suspension	Not mentioned	Turbidity is the same as that of water R or of the solvent used, or not more than that of reference suspension 1 (Ph.Eur. 2.2.1)	Not mentioned
Subvisible particulates				
		Unit product volume ≤100 mL = small volume parenteral (SVP) >100 mL = large volume parenteral (LVP)		Unit product volume <100 mL = small volume parenteral (SVP) ≥100 mL = large volume parenteral (LVP)
Limits: Light obscuration (LO) (preferred method)	SVP	≥10 µm: ≤6,000 counts/container ≥25 µm: ≤600 counts/container	≥10 µm: ≤6,000 counts/container ≥25 µm: ≤600 counts/container	≥10 µm: ≤6,000 counts/container ≥25 µm: ≤600 counts/container
	LVP[a]	≥10 µm: ≤25 counts/mL ≥25 µm: ≤3 counts/mL	≥10 µm: ≤25 counts/mL ≥25 µm: ≤3 counts/mL	≥10 µm: ≤25 counts/mL ≥25 µm: ≤3 counts/mL
Limits: Membrane microscopy (MM) (second stage to LO or in case LO cannot be used)	SVP	≥10 µm: ≤3,000 counts/container ≥25 µm: ≤300 counts/container	≥10 µm: ≤3,000 counts/container ≥25 µm: ≤300 counts/container	≥10 µm: ≤3,000 counts/container ≥25 µm: ≤300 counts/container
	LVP	≥10 µm: ≤12 counts/mL ≥25 µm: ≤2 counts/mL	≥10 µm: ≤12 counts/mL ≥25 µm: ≤2 counts/mL	≥10 µm: ≤12 counts/mL ≥25 µm: ≤2 counts/mL
Intravenous injections		Limits apply	Limits apply	Limits apply
Injections solely for intramuscular (IM) or subcutaneous (SC) dosing		USP*34*: Limits to apply for IM and SC products also	Limits apply. Higher limits may be appropriate	Not mentioned separately

(continued)

Table 14.1 (continued)

Attributes		USP*35*	Ph.Eur.7.0	JP 16th Ed.
		General chapter <1> *Injections*	General chapter 01/2008:0520 *Parenteral preparations*	General rules for preparations 11 *Injections*
			General chapter 2.9.19 *Particulate contamination: subvisible particles*	
		General chapter <788> *Particulate matter in injections*	General chapter 2.9.20 *Particulate contamination: visible particles*	General tests, processes, and apparatus *6.06: Foreign insoluble matter test for injections* *6.07: Insoluble particulate matter test for injections*
Non-IM/SC dispersed systems		MM (no special procedure described)	MM (no special procedure described)	MM (no special procedure described) Suspension particles ≤150 μm Emulsion droplets ≤7 μm
Non-IM/SC solutions or powders for injection	With final filter before injection	USP*34*: Parenteral products for which the labeling specifies the use of a final filter prior to administration are exempt from the requirements provided that scientific data are available to justify the exemption	Exempt from requirements, providing it has been demonstrated that the filter delivers a solution that complies	Not mentioned
	High viscosity	Dilution followed by LO	Dilution followed by LO	Dilution followed by LO
	Other solutions	LO alone or followed by MM	LO alone or followed by MM	LO alone or followed by MM

Test protocol	Statistically sound sampling plan	Required for <25 mL/unit For ≥25 mL/unit, 10 units acceptable	Required for <25 mL/unit For ≥25 mL/unit, 10 units acceptable	Required for <25 mL/unit For ≥25 mL/unit, 10 units acceptable
	<25 mL/unit	Pool ≥10 units to obtain >25 mL, Test 4 × NLT 5 mL aliquots, discard first result	Pool ≥10 units, Test 4 × NLT 5 mL aliquots, discard first result	Pool ≥10 units to obtain >25 mL, Test 4 × NLT 5 mL aliquots, discard first result
	≥25 mL/unit[b]	No pooling, Tested individually, Test 4 × NLT 5 mL aliquots, discard first result	No pooling, Tested individually, Test 4 × NLT 5 mL aliquots, discard first result	No pooling, Tested individually, Test 4 × NLT 5 mL aliquots, discard first result

[a]Refer to Pharmacoepial Forum 39(2) 2013. Proposed USP<787> also specifies "total particle load should not exceed 6,000 per container equal to or greater than 10 μm and should not exceed 600 per container equal to or greater than 25 μm"

[b]Revision in USP36 (2013): For pharmacy bulk packages for parenteral use labeled "Not for Direct Infusion," proceed as directed for small-volume parenteral when the volume is 25 mL or more. Calculate the test result on a portion that is equivalent to the maximum dose given in the labeling. For example, if the total bulk package volume is 100 mL and the maximum dose volume is 10 mL, then the average particle count per milliliter would be multiplied by 10 to obtain the test result based on the 10-mL maximum dose. For calculation of test results, consider this maximum dose portion to be equivalent to the contents of one full container

Table 14.2 Summary of compendial requirements for ophthalmic products

Attributes	USP*35*	Ph.Eur.7.0	JP 16th Ed.
			General rules for preparations 18 *Ophthalmic solutions*
	General chapter <789> *Particulate matter in ophthalmic solutions*	General chapter 01/2008:1163 *Eye preparations*	General tests, processes, and apparatus *6.08: Insoluble particulate matter test for ophthalmic solutions* *6.11: Foreign insoluble matter test for ophthalmic solutions*
Ophthalmic injections (intra-ocular injections)	Not mentioned separately. (author's comment: The US FDA generally requires the application of stricter limits from USP<789> instead of USP<788>)	Not mentioned separately	Not mentioned separately
Ophthalmic solutions (topical; eye drops) Limits: light obscuration (LO) (preferred method)	≥10 μm: ≤50 counts/mL ≥25 μm: ≤5 counts/mL ≥50 μm: Not mentioned separately	Appearance: Eye drops that are solutions, examined under suitable conditions of visibility, are practically clear and practically free from particles Subvisible particulate matter: not mentioned separately	(JP6.11) Appearance: The solution must be clear and free from foreign insoluble matters that are clearly detectable when inspected with the unaided eye at a position of light intensity of approximately 3,000–5,000 lux, right under an incandescent lamp
Ophthalmic solutions (topical) Limits: membrane microscopy (MM) (second stage to LO or in case LO cannot be used)	≥10 μm: ≤50 counts/mL ≥25 μm: ≤5 counts/mL ≥50 μm: ≤2 counts/mL		(JP6.08) Membrane microscopy >300 μm: Maximum 1 count/mL Particle to be sized on the longest axis

Ophthalmic suspensions (topical)		Not mentioned separately	Appearance: Eye drops that are suspensions may show sediment that is readily redispersed on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be delivered	(JP6.08) Membrane microscopy
			Subvisible particulate matter: Per 10 μg of suspended API Maximum dimension >25 μm: ≤20 counts per 10 μg Maximum dimension >50 μm: ≤2 counts per 10 μg Maximum dimension >90 μm: None per 10 μg	Specified in 18. Ophthalmic solutions: Usual particle size observed in suspensions for Ophthalmic Solutions is not larger than 75 μm
Test protocol	Statistically sound sampling plan	Required for <25 mL/unit For ≥25 mL/unit, 10 units acceptable	Subvisible particulate matter: Not mentioned separately	(JP6.08) Membrane microscopy Not specified for Aqueous Ophthalmic solutions, Ophthalmic solutions which are dissolved before use, Suspension type ophthalmic solution
Ophthalmic solutions (topical)	<25 mL/unit	Pool ≥10 units to obtain >25 mL, Test 4 × NLT 5 mL aliquots, discard first result		Pool 10 samples for Ophthalmic solutions contained in a single-dose container
	≥25 mL/unit	No pooling, Tested individually, Test 4 × NLT 5 mL aliquots, discard first result		
Test protocol		Not mentioned separately	Spread gently a quantity of the preparation corresponding to at least 10 μg of solid active substance as a thin layer. Scan under a microscope the whole area of the sample	Membrane microscopy
Ophthalmic suspensions (topical)				Specified in JP6.08

ophthalmic products from various companies and the FDA considers these limits as crucial for ensuring the safety of small-volume solutions for dosing in the eye. There are currently no specific limits for subvisible particulates in the Ph.Eur., and Japan specifies a NMT 1/mL for particulates ≥300 μm (see Table 14.2). The Ph.Eur., however, does specify limits for ophthalmic suspensions. It should be noted, however, that the above-mentioned limits are intended for "topically" administered ophthalmic products. The US FDA invariably applies the USP<789> limits to ophthalmic injectables (e.g., intravitreal injections), although slightly higher limits may be negotiable. A somewhat similar situation may be expected by sponsors in the EU and Japan.

Biotherapeutic Products

Inherent proteinaceous particles in biopharmaceuticals span a wide range of sizes from the so-called soluble (<0.1 μm) to insoluble or visible. This distinction has been based on analytical methods used to detect and quantify these species in protein solutions (see Sects. 14.2.1.3 and 14.2.2) (Narhi et al. 2012). The current compendial requirements, as applied to biotherapeutics, do not distinguish between the three types of particles, and thus the subvisible particles (≥10, ≥25 μm) have to meet the same requirements as listed in Table 14.1. However, an increasing degree of concern about potential for safety and immunogenicity risks associated with proteinaceous subvisible particles (see Sect. 14.1.3.3) in biotherapeutic products has led the US FDA to require sponsors to provide data on <10 μm particle size ranges also (Carpenter et al. 2009). Some specific but typical regulatory correspondence is excerpted below.

> … USP <788> testing results are critical to mitigate the risk associated with occlusion of small blood vessels and small subvisible particles may pose an immunogenicity risk. Provide USP <788> particulate testing data for in-use stability studies and an analysis of particulates between 2 and 10 microns.
>
> … in addition to measuring particulates that are ≥10 μm in size, subvisible particulates in the 2–10 μm range should also be characterized and quantified using technique(s) that can accurately estimate the amount of subvisible protein particulates present. Sub-visible particulates in the 0.1–1 μm range should be qualitatively assessed.
>
> … additionally characterize the types and amounts of subvisible particles (2–10 μm) in the drug product under stress conditions, at release, and throughout the shelf-life, and also propose an appropriate control strategy based on the risk to product quality.

The requirement to assess particles (sizes, number, characteristics) below 10 μm, including qualitative assessment in the 0.1–1 μm, has led to a significant amount of research in this field with biotherapeutics. A number of new technologies have become available, although primarily for the range ~1–10 μm, and a great deal of knowledge has been generated on the mechanism of formation of these inherent particles, the ability to distinguish them from other foreign (extrinsic) and intrinsic particles, the advantages and limitations of the technologies, and good measurement techniques. This has led to the development of a revised version of USP<788>

specifically addressed towards biotherapeutics. Chapter USP<787> is currently in draft form and a companion guidance chapter USP<1787> is currently being drafted. Further discussion of this special aspect of subvisible particles is provided in Sects. 14.3.3 and 14.3.4.

14.1.3 Safety Concerns of Subvisible Particles in Parenterals

The safety concerns related to particulate matter contamination in parenteral products are strongly dependent on the route of administration involved. The following are possible routes of administration for a parenteral product.

1. *Intrathecal* (spinal or lumbar replacement injections)
2. *Subcutaneous* (below the epidermal layer)
3. *Intramuscular* (into the muscle, generally gluteal)
4. *Intravenous* (into the venous blood supply; large volumes)
5. *Intra-articular* (for injection into joints)
6. *Intracutaneous* (just below the outer skin surface)
7. *Intracardial* (injection into the heart)
8. *Intraperitoneal* (into the peritoneal lumen of the abdominal cavity)
9. *Intracisternal* (into the intracranial cistern)
10. *Peridural* (into the space surrounding the dura mater of the spinal cord)

Particulate matter is of concern for all the listed routes, but of greatest concern for #1, 4, 5, 7, 9, and 10. There is a large amount of information about risks from particulates in intravenous (IV) therapy, since there is significant experience in dosing large volumes of liquids via this route. This is discussed in more detail below (see Sect. 14.1.3.1). There is much less published information about concerns with other routes of administration. The subcutaneous (SC) and intramuscular (IM) routes are common, but often involve relatively small volumes. Until recently, products intended for these routes were specifically excluded from USP<788> limits by USP<1>. This has changed in the USP35 and SC and IM products now need to comply with USP<788>. The Ph.Eur. 01/2008:0520 allows higher limits to be set for such products. A recent publication on the impact of suspension particle sizes on IM dosing-induced muscle damage suggests that (apart from the impact of drug itself), for neutral or negatively charged inert polystyrene particles, reduction in size from 3 μm to 400–500 nm reduced injection site irritation or myotoxicity. However, further reduction in size to 80–100 nm significantly enhanced the myotoxicity, suggesting that reducing particle size reduces irritation until the size reaches a point where (the increasing) contact surface area begins to enhance the damaging effects (Brazeau et al. 2011). In the case of biopharmaceuticals, the safety concerns for inherent particles in products intended for SC or IM injection may actually be higher than IV (see Sect. 14.1.3.3).

Other relevant routes of administration are

11. *Ocular* (*topical*) (on the corneal surface of the eye)
12. *Intra-ocular* (*intravitreal*) (into the posterior compartment of the eye; vitreous humor)

The intra-ocular route above is a special case in point. The US FDA has, in these cases, held the products to the more stringent USP<789> requirement even though it may be argued that the risk of corneal abrasion, etc., posed by particles dosed topically does not apply to the intra-ocular route (see Sect. 14.1.3.2).

14.1.3.1 Intravenous Administration of Particulates

The primary safety risk posed by particulates in products dosed into the systemic circulation arises from their direct ability to block blood vessels, triggering a cascade of downstream adverse events. Animal studies show that the tissue distribution of infused particles is related to their size. Particles in the size range 10–12 μm lodged in pulmonary capillaries, 3–6 μm sizes were found in the spleen and hepatic lymph nodes, and smaller ones in the liver. Liver and spleen retain particles for prolonged periods possibly due to phagocytosis by the reticuloendothelial system (Ball 2003). Some pathological conditions that may be produced include phlebitis, platelet agglutination leading to formation of emboli, local inflammatory reactions caused by impaction of particles in tissues, and antigenic reactions with subsequent allergenic consequences. The physiological effect of injected particles is related to size, number, rate of introduction, and the chemical composition of the particles. The condition of the patient may also be a factor in the pathology since the ability of the body to address the insult would depend on the health and age of the patient as well as the size and ability of the various organs. The chemistry of the particles would determine the strength of inflammatory or allergic response, and thus the damage caused by the particulates dosed (Russel 1970; Turco and Davis 1973; Kirkpatrick et al. 1999; Barber 2000). Generally, particles greater than 10 μm in diameter can lead to vasoconstriction (Munsch et al. 1991). Particulate matter contamination has been shown to be responsible for plugging coronary capillaries, adherent to the endothelial layer, and also responsible for activation of the polymorphonuclear granulocytes. In a study comparing the effects of IV dosage of an antibiotic from three different manufacturers, it was found that the higher particulate load resulted in significant loss of functional capillary density in traumatized striated muscles (Lehr et al. 2002). Teflon (90 % <40 μm) and silicone (100–150 μm) particles were found to migrate after IV injection in rats, and were associated with mild chronic granulomatous response, 2 years after injection. Both large and small diameter particles were found within the perivascular interstitium and within lumina (Dewan et al. 1995).

Although the nature of particulates varies significantly, a large amount of the IV data and adverse events are related to the hard non-pliable nature of the contaminant particles. Not much is known about impact of softer pliable particles. Since this level of characterization is usually not available for particle contaminants in products,

the higher risk factor associated with hard particles should be applied to all particles. The one exception seems to be silicone oil-based intrinsic particles, which arise from surface coatings applied to stoppers, etc. Silicone oil particles are allowed to be discounted from the total particle counts in products where such a distinction can be made (see Sect. 14.2.2.2).

In conclusion, a survey of the literature suggests that the human body can absorb large amounts of particulate matter and continue to function, but adverse effects, tissue damage, and loss of function are likely to occur in the long-term (Ball 2003).

14.1.3.2 Ocular and Intra-ocular Administration of Particulates

In a seminal report by Uemera et al. (1998), the damage caused by plastic particles dosed topically into the eyes of rabbits was studied by a fluorescein dye tracer and optical examination. Damage was defined as depth of dye penetration into the squamous layer. In all cases, only the 425–500 μm range and higher particle load produced superficial corneal damage, all of which healed within 24 h. The investigators reported healing within 72 h even after total corneal peeling. Nictation (blinking) played a significant role in the observed epithelial damage. The study showed that 2–3 particles/mL, which were <300 μm in size, did not cause significant corneal damage, and forms the basis for the Japanese Pharmacopeia limits for ocular products.

There is no other significant literature showing direct correlation of the USP<789> limits to corneal safety. [USP<789> first appeared in the Pharmacopeial Forum in Sept./Oct. 2001, with particulate limits proposed based on an industry survey (Fagan et al. 2001). An in-process revision appeared in the Pharmacopeial Forum in Sept./Oct. 2002 with the tighter limits that are currently in force.] In discussions with the FDA, however, it is apparent that the rabbit model is not considered appropriate for doing nonclinical corneal safety evaluations with respect to particulates because their recovery time is stated to be faster, and the anatomy physiology is considered to be significantly different from humans due to the presence of an additional nictating membrane.

From an intra-ocular (e.g., intravitreal) perspective it may be argued that this route resembles a normal parenteral injection more than a topical ocular administration. Nevertheless, although the eye is generally considered an immune-privileged organ and volumes involved in intra-ocular injections are small (usually ≤100 μL), the more stringent requirements of USP<789> have been applied to such products by the FDA. Particulate matter placed into the eye can result in serious undesirable consequences (Algvere et al. 1988). However, response is dependent on particle characteristics such as size and shape, state of dispersion, physical and chemical properties, surface area, and surface chemistry. As in the case of all injected particles, the chemical characteristics of the particles, including morphology, can allow an active interaction with certain components of the body, triggering a biologic response. Unfortunately, the criteria that differentiate benign vs. pathogenic particles are not well understood. Ultimately, the potentially devastating nature of the risk posed by particles in the intra-ocular space including infections (see, e.g., http://

www.fda.gov/Drugs/DrugSafety/ucm270296.htm), and the inability to clear easily particles once deposited in the vitreal compartment, requires that such products be held to a higher standard.

14.1.3.3 Particulates in Biopharmaceutical Products

As discussed in Sect. 14.1.2.2, biotherapeutic products carry all the same requirements of other parenterals, and also share the same risks when it comes to foreign (extrinsic) and intrinsic particles as discussed above in Sect. 14.1.3.1. However, inherent particulates in biopharmaceuticals carry another risk not encountered with other (small molecule) sterile parenterals.

Protein aggregates in biotherapeutics, starting from dimers, can grow and become large enough (>0.1 μm) to be classified as particles (submicron, subvisible, or visible). Such particulates arising from protein aggregation (inherent proteinaceous particles) have been implicated as a risk factor for generation of immunogenicity in patients (Rosenberg 2006; Carpenter et al. 2009). The ability of repetitive motifs to activate Toll-like receptors and thus enhance the immunogenicity of antigens is well established. Proteinaceous particles made up of large numbers of (native) protein molecules may present similar arrays of repeat structures, and thus lead to the generation of antibodies against the therapeutic, although direct clinical evidence is not equivocal and the detailed mechanism remains to be elucidated (Sauerborn et al. 2010; Singh et al. 2010; Singh 2011). It has now been shown that all aggregates are not equal with wide differences in the size, shape, as well as structure of protein in the aggregates (Joubert et al. 2011; Zhang et al. 2011, 2012). In animal models, aggregates generated as a consequence of different stresses have shown differing abilities to trigger an immunogenic reaction (Fradkin et al. 2009; van Beers et al. 2011; Pisal et al. 2012). The presence of foreign particles, such as glass, stainless steel, and plastics, which results in adsorbed and aggregated/denatured protein molecules on their surfaces, can even enhance the immunogenicity through an adjuvant-like effect (Fradkin et al. 2011; van Beers et al. 2012). Therefore, regardless of the cause and nature of the aggregate or proteinaceous particle, the risk they pose is real. Consequently aggregation, and by extension, inherent particles must be considered a critical quality attribute for biotherapeutic products. Among the common routes of administration, the probability of an immune response is generally considered highest after SC injection, followed by IM, intranasal, and IV routes although there exist examples to the contrary (Singh 2011). Therefore, biopharmaceutical products dosed by the SC route would carry a higher degree of concern compared to products dosed IV. However, until recently, the USP<788> limits were not applicable to SC and IM products. The Ph.Eur. does not exempt such products but higher limits may be set.

Clearly, biotherapeutic products can have (sub)visible particles of all three types—foreign (extrinsic), intrinsic, and inherent. Since the risks with these different categories and the ways to address/eliminate them are different, it is important to be able to distinguish between the various types of particles. The ability of the various methods to do this is included in the discussions in Sect. 14.2.2.

14.2 Measurement and Quantification of Particulates

Methods for measurement and quantification of particulate matter in sterile parenteral products have been detailed in many publications (Borchert et al. 1986; Chrai et al. 1987; Barber 2000; Sharma et al. 2010; Toler and Nema 2010; Singh and Toler 2012; Zolls et al. 2012). We will therefore only review these methods critically for their measurement principle, utility, advantages, and limitations, with particular emphasis on their utility for biopharmaceuticals. As mentioned earlier, particles of interest in the case of biopharmaceuticals product range over 5–6 orders of magnitude in size from “soluble” or nanosized (<0.1 μm) to “insoluble” or visible (>100 μm). Aggregates above 0.1 μm in size may be roughly viewed as “particles.” Separation techniques that measure “concentration” (e.g., size exclusion chromatography, field flow fractionation [FFF], analytical ultracentrifugation [AUC]) are generally used for the “soluble” or nanosized aggregates. Counting techniques, based primarily on interaction of the particles with light (obscuration, scattering), have been used for the subvisible and visible ranges above ~1 μm. The capabilities of the various techniques are broadly depicted in Fig. 14.1. In the transition region between concentration (<~0.1 μm) and counting techniques (~1 μm) is the so-called measurement gap. Particles can be detected , e.g., by light scattering techniques but cannot be accurately sized or counted (Das and Nema 2008; Singh et al. 2010).

As seen in the queries from the FDA (Sect. 14.1.2.2), apart from sizing and counting the inherent proteinaceous particles in a product, there is also a great deal of interest in “characterizing” them. Finally, it is important to recall that particles may not be uniformly distributed within a batch of product so that appropriate sampling to obtain material representative of the batch is an extremely critical aspect of the exercise.

14.2.1 Visible Particles

Sterile parenterals are inspected prior to labeling to ensure that the product and container meet predetermined criteria for integrity and particulate matter. Inspection serves as an indicator of not only product quality but also the quality of the overall process and of its level of GMP compliance. Inspection for visible particles is generally conducted either manually or by machine.

14.2.1.1 Manual Inspection

Manual visible inspection of 100 % of the batch is commonly practiced in the industry, and more so when dealing with clinical batches during development. This inspection is critically dependent on a number of factors that results in a high degree of variability in outcomes. As mentioned earlier, the ability to detect a “visible” particle is dependent on factors such as size, contrast with surroundings, color, reflectivity, and buoyancy, along with the lighting conditions, visual acuity of the inspector, etc.

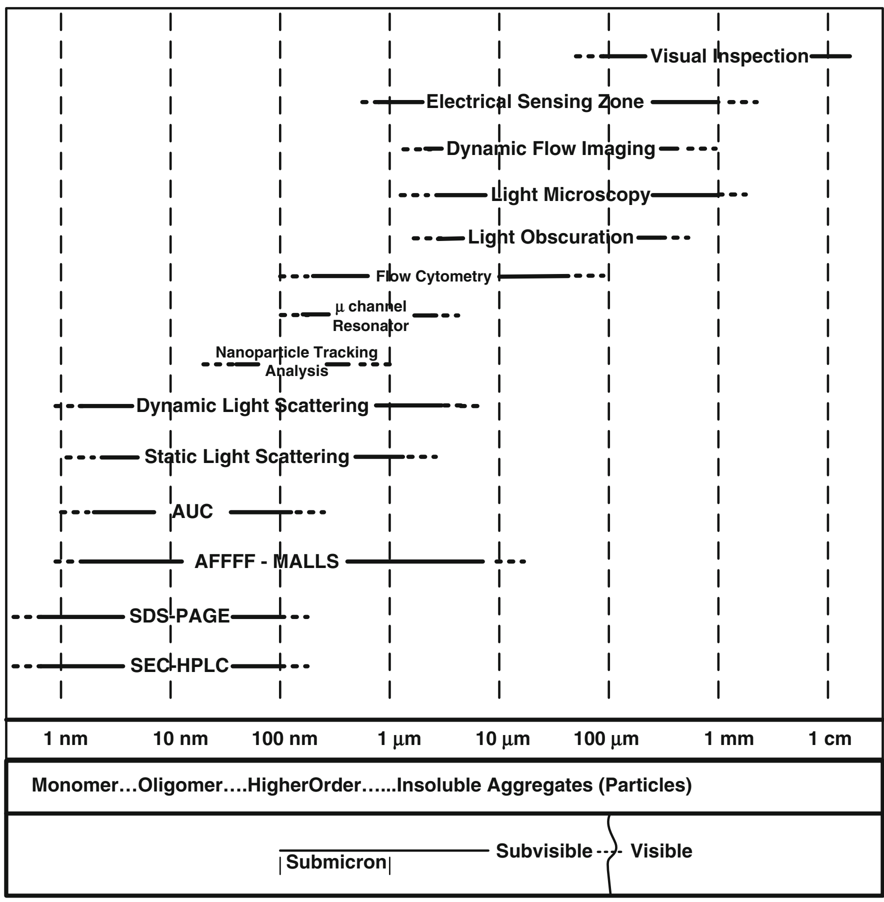

Fig. 14.1 Particle detection and measurement techniques and their operational ranges (adapted from Singh et al. 2010)

Thus the ability of an inspector to see a "visible" particle is highly probabilistic, and the probability generally increases with the size. A large body of work by Knapp and coworkers formalized this probabilistic process. A full discussion of their work is outside the scope of this review, and the reader is referred to Borchert et al. (1986), Knapp (1999a, b) as well as Barber (2000) (see Chap. 7 for more details.)

Manual detection of visible particles is a consequence of light scattering and thus factors that enhance this optical effect will improve detectability. Thus product-related factors that play a role are:

- Volume of solution in container
- Transparency or opacity of container
- Optical defects on wall of container
- Curvature of container
- Ratio of height of liquid to diameter of container

- Solution background color and/or opalescence
- Viscosity and foaming tendency of solution

The technique factors are:

- Type and intensity of lighting in inspection booth
- Background illumination in inspection area
- Background and contrast with particle
- Physical configuration of inspection station (angle of eye, distance, illumination at point of inspection)
- Time spent on inspection
- Use of magnification
- Motion of container relative to suspend particles

The human factors are:

- Visual acuity of inspector
- Color blindness of inspector
- Training of inspector, including use of challenge sets
- Inspector fatigue

The Ph.Eur. and JP provide guidance on lighting conditions for manual inspection (see Table 14.1). Ph.Eur. 2.9.20 specifies a black and white viewing station with illumination specified (2,000–3,750 lux) at the point of inspection. The method specifies a general procedure as well as time "Gently swirl or invert container and observe for about 5 s in front of the white panel. Repeat the procedure in front of the black panel." The JP6.06 specifies in Method 1 and Method 2 "… inspection with unaided eye under an incandescent lamp of light intensity of 1000 lux …" and a higher intensity of 8,000–10,000 lux for plastic containers.

Apart from the above guidance on lighting, the process of manual inspection has to be developed and put into place with a rigorous program for training, qualification, and requalification. Representative training sets are often created from culled samples for particular products. Considering the long list of factors provided above that can impact the outcome, it becomes clear that manual inspection is one of the most problematic routine analyses performed on products. With proper development and structure, this nominally simple, but in practice complex, process can provide significant value (Melchore 2010; Toler and Nema 2010).

A number of machine-aided manual inspection stations have been developed that perform the mechanical manipulations (e.g., swirling, inverting, braking, flagging to reject) required. Some include an imaging system for the aid of the operator. However, the final decision to accept or reject remains with the human operator.

The primary objective of visual inspection is to cull vials with particles from the product batch, so that the "essentially free" of "practically free" requirements can be met (see Sect. 14.3.1). The visual inspection process does not aim to count, identify (except bubbles), or generate any other information about the particles. There may be follow-on consequences of finding foreign particles, such as identifying the source, cause, and corrective actions. Such forensic examination of particles is briefly covered in Sect. 14.2.4.

14.2.1.2 Automated or Machine Inspection

Fully automated inspection machines have been developed to remove some of the subjectivity of the manual inspection. These machines can provide higher throughput and a more consistent evaluation of all product containers, especially in comparison with large batches inspected by multiple operators. Detection in these machines is by a photodetector or a camera that captures the presence of the particles by a change in intensity of either transmitted light or reflected light. An algorithm then determines if the image is a particle (i.e., mobile) or a surface defect (i.e., immobile) or a bubble (rises) and, using predefined criteria, decides to accept or reject a container. Proper selection of the machine parameters (e.g., spin rate, brake time, illumination, sensor sensitivity, dwell time) is important for each product type since product-related factors (e.g., fill volume, fill height to diameter, head-space, solution viscosity, surface tension, propensity to form bubbles, container curvature) determine the outcome (Rathore et al. 2009). It must be noted that human inspection remains the benchmark against which the automated systems are qualified and validated.

Inspection of large-volume parenteral units, whether manual or automated, is associated with technical difficulties due to inherent limitation of depth of focus and three-dimensional volume that can be critically examined in a specific amount of time. Large volumes will require longer inspection times and as the volume increases, the ability of the human inspector to be able to reproducibly handle such containers over a period of time decreases. Semi-automated stations and automated inspections become important, but limitations may be imposed by the common use of plastics as container material for large volume parenterals.

For more information on capabilities of machine-based inspections, the reader is referred to literature at www.eisaiusa.com and www.seidenader.com.

14.2.1.3 Visible Particles and Biopharmaceuticals

Inspection processes and techniques have traditionally been focused towards detecting foreign (extrinsic) and intrinsic particles in products. Foreign particles by definition should be invariant and in principle should be inspected out of the product batch. Intrinsic particles may however change over time, and therefore visual inspection must be a part of the stability program of all products. In the case of small-molecule parenteral products, the appearance of intrinsic particles (except silicone oil) is an indicator of potential instabilities in the product, and thus leaves no ambiguity about subsequent actions.

On the other hand, inherent particles in biotherapeutics present a dilemma. By definition, they may be an inherent part of the product (however unwelcome) and therefore acceptable (after proper qualification in safety and efficacy studies). In the inspection process, these particles must be distinguished from foreign (and intrinsic) particles and the product accepted or rejected on that basis. This must be done, not only over the shelf-life of the product but also over the lifetime of the product as

manufacturing process, etc., evolve as part of product life-cycle management. Sufficient numbers of units must be maintained to obtain representative statistics. Although in many cases, products are recommended to be filtered just prior to dosing, it is important to show that these inherent particles have been present and consistent through preclinical, toxicology, and clinical studies, and hence the accumulated safety database is applicable even during commercialization. This requires an ability to define “what is normal” with these inherent proteinaceous particles, and may include photo records, videos, reference samples, training sets, etc. Apart from qualitative descriptors, quantitation by number and size would be useful in this respect. Machine-based inspection has the ability to store databases of images that can be used as references, and thus helps to differentiate between foreign, intrinsic, and inherent particles, while helping to define “what is normal.”

A compilation of descriptions of such inherent visible particles in commercial biologics is provided in Table 14.3. The description of visible particles in these products varies but some common elements include “white,” “translucent,” “amorphous” or “gel-like” or “fiber.” The package inserts also instruct the end-user to discard the product if a foreign particle is observed, and accept these inherent particles. While a black fleck or piece of rubber may be easy to differentiate, an untrained observer may have a harder time trying to distinguish between a cellulose fiber or flake and an amorphous protein particle or gel. In a number of cases, the product solutions are also opalescent simply because of the colloidal nature of the protein and resultant light scattering, especially at high concentrations. Opalescence may also be mistaken as a manifestation of particulate contamination by the lay person. Examination of products becomes difficult as the concentration rises and as the curvature of the container increases, as in syringes. Viscosity of high concentration products also makes visual examination difficult, and even more so in syringes. For lyophilized products, long dissolution times, undissolved protein-gels, and bubbles can all add to the difficulty of distinguishing between types of particles. All of these factors have to be addressed during the development of the inspection process. A robust formulation and process, appropriate GMP controls and then a well-designed inspection procedure, should ensure that the product reaching the end-user is free of foreign and intrinsic particles, so that the safe and efficacious use of the product is not contingent upon the ability of the end-user to identify and reject product that contains these undesirable particles.

A number of the products recommend use of filtration prior to dosing, either via an in-line filter in case of infusions, or via needle or syringe filters (Table 14.3). [There are a few products that explicitly disallow the use of filters.] The use of filters helps to eliminate the risk of infusing proteinaceous (and other) particulates. The recommended filtration procedure and filters have to be qualified to ensure no drop in potency. The amount of protein in proteinaceous particulates is often quite small, so that the potency is generally not impacted, except possibly in the case of low concentration products. Actually, the use of filters should be qualified in all cases, since it is quite likely that internal procedures at many institutions mandate the use of filters, even if not specifically recommended in the product package insert. A variety of filter options in infusion systems are available (see, e.g., Anonymous 2011).

Table 14.3 Description of visible particulates in biotherapeutic products and recommendation about filtration[a]

Product name	Description of particles if present	Recommendation about filtration in conjunction with administration
Monoclonal antibodies, recombinant		
Arzerra (ofatumumab)	Arzerra should be a colorless solution and may contain a small amount of visible translucent-to-white, amorphous, ofatumumab particles. The solution should not be used if discolored or cloudy, or if foreign particulate matter is present	Administer using an infusion pump, the in-line filter provided with the product, and polyvinyl chloride (PVC) administration sets. Filters are supplied (*details not provided*)
Bexxar (tositumomab and I131 tositumomab)	Tositumomab is supplied as a sterile, pyrogen-free, clear to opalescent colorless to slightly yellow, preservative-free liquid concentrate	The Bexxar therapeutic regimen is administered via an IV tubing set with an in-line 0.22 μm filter. The same IV tubing set and filter must be used throughout the entire dosimetric or therapeutic step. A change in filter can result in loss of drug
Erbitux (cetuximab)	The solution should be clear and colorless and may contain a small amount of easily visible, white amorphous cetuximab particles	Administer through a low protein-binding 0.22-μm in-line filter
Lucentis (ranibizumab)		Using aseptic technique, all (0.2 mL) of the Lucentis vial contents are withdrawn through a 5-μm 19-gauge filter needle attached to a 1-cc tuberculin syringe. The filter needle should be discarded after withdrawal of the vial contents and should not be used for intravitreal injection
Mylotarg (gemtuzumab ozogamicin)		An in-line, low protein-binding filter must be used for the infusion of Mylotarg. The following filter membranes are qualified: 0.22 or 1.2 μm polyether sulfone (PES); 1.2 μm acrylic copolymer hydrophilic filter; 0.8 μm cellulose mixed ester (acetate and nitrate) membrane; 0.2 μm cellulose acetate membrane
Orthoclone OKT3 (muromonab-CD3)	Because Orthoclone OKT3 is a protein solution, it may develop fine translucent particles (shown not to affect potency)	Prepare Orthoclone OKT3 for injection by drawing solution into a syringe through a low protein-binding 0.2 or 0.22 μm (μm) filter

Prolia (denosumab)	Prolia is a clear, colorless to pale yellow solution that may contain trace amounts of translucent-to-white proteinaceous particles. Do not use if the solution is discolored or cloudy or if the solution contains many particles or foreign particulate matter	
Remicade (infliximab)	Allow the reconstituted solution to stand for 5 min. The solution should be colorless to light yellow and opalescent, and the solution may develop a few translucent particles as infliximab is a protein. Do not use if opaque particles, discoloration, or other foreign particles are present	The solution must be administered over a period of not less than 2 h and must use an infusion set with an in-line, sterile, non-pyrogenic, low protein-binding filter (pore size of 1.2 µm or less)
Reopro (abciximab)		Withdraw the necessary amount of abciximab for bolus injection into a syringe. Filter the bolus injection using a sterile, non-pyrogenic, low protein-binding 0.2 or 0.22 µm filter (Millipore SLGV025LS or equivalent) Withdraw the necessary amount of abciximab for the continuous infusion into a syringe. Inject into an appropriate container of sterile 0.9 % saline or 5 % dextrose and infuse at the calculated rate via a continuous infusion pump. The continuous infusion should be filtered either upon admixture using a sterile, non-pyrogenic, low protein-binding 0.2 or 0.22 pm syringe filter (Millipore SLGV025LS or equivalent) or upon administration using an in-line, sterile, non-pyrogenic, low protein-binding 0.2 or 0.22 µm filter (Abbott #4524 or equivalent)
Stelara (ustekinumab)	Stelara is colorless to light yellow and may contain a few small translucent or white particles. Stelara should not be used if it is discolored or cloudy, or if other particulate matter is present	
Tysabri (natalizumab)		Use of filtration devices during administration has not been evaluated
Vectibix (panitumumab)	Although Vectibix should be colorless, the solution may contain a small amount of visible translucent-to-white, amorphous, proteinaceous, panitumumab particulates (which will be removed by filtration)	Administer using a low protein-binding 0.2 or 0.22 µm in-line filter

(continued)

Table 14.3 (continued)

Product name	Description of particles if present	Recommendation about filtration in conjunction with administration
Yervoy (ipilimumab)	Discard vial if solution is cloudy, there is pronounced discoloration (solution may have pale yellow color), or there is foreign particulate matter other than translucent-to-white, amorphous particles	Administer diluted solution over 90 min through an intravenous line containing a sterile, non-pyrogenic, low protein-binding in-line filter
Zevalin (ibritumomab tiuxetan)	Ibritumomab tiuxetan is a clear, colorless, sterile, pyrogen-free, preservative-free solution that may contain translucent particles	Use a 0.22 μm low protein-binding in-line filter between the syringe and the infusion port
Other proteins, recombinant		
Amevive (alefacept)		Do not filter reconstituted solution during preparation or administration
Atryn (antithrombin alfa) (EU)		The reconstituted product should be administered by intravenous infusion using a sterile disposable syringe or an infusion bag with a 0.22 μm pore size in-line filter
Ceredase (alglucerase)		The use of an in-line particulate filter is recommended for the infusion apparatus
Cerezyme (imiglucerase)	After reconstitution, Cerezyme should be inspected visually before use. Because this is a protein solution, slight flocculation (described as thin translucent fibers) occurs occasionally after dilution. The diluted solution may be filtered through an in-line low protein-binding 0.2 μm filter during administration. Any vials exhibiting opaque particles or discoloration should not be used	
Elaprase (idursulfase)		Use of an infusion set equipped with a 0.2 μm (μm) filter is recommended
Elitek (rasburicase)		No filter should be used for infusion
Elspar (asparaginase)	Occasionally, a very small number of gelatinous fiber-like particles may develop on standing	Filtration through a 5.0 μm filter during administration will remove the particles with no resultant loss in potency. Some loss of potency has been observed with the use of a 0.2 μm filter

Enbrel (etanercept) PFS	There may be small white particles of protein in the solution. This is not unusual for proteinaceous solutions. The solution should not be used if discolored or cloudy, or if foreign particulate matter is present	
Eylea (aflibercept)		Use a 5 μm filer needle to withdraw product into syringe
Fabrazyme (agalsidase beta)		Do not use filter needles during the preparation of the infusion. The diluted solution may be filtered through an in-line low protein-binding 0.2 μm filter during administration
Helixate (rFactor VIII) (EU)		After reconstitution, the solution is drawn through the Mix2Vial filter transfer device into the sterile disposable syringe (both supplied)
Kepivance (palifermin)		Do not filter the reconstituted solution during preparation or administration
Kogenate (antihemophilic factor)		After the concentrate powder is completely dissolved, withdraw the solution into the syringe through the filter needle that is supplied in the package
Lumizyme (alglucosidase alfa) (see also Myozyme)	Perform an immediate visual inspection on the reconstituted vials for particulate matter and discoloration. If upon immediate inspection opaque particles are observed or if the solution is discolored do not use. The reconstituted solution may occasionally contain some alglucosidase alfa particles (typically less than 10 in a vial) in the form of thin white strands or translucent fibers subsequent to the initial inspection. This may also happen following dilution for infusion. These particles have been shown to contain alglucosidase alfa and may appear after the initial reconstitution step and increase over time. Studies have shown that these particles are removed via in-line filtration without having a detectable effect on the purity or strength	Administer Lumizyme using an in-line low protein-binding 0.2 μm filter

(continued)

Table 14.3 (continued)

Product name	Description of particles if present	Recommendation about filtration in conjunction with administration
Myozyme (alglucosidas alfa) (see also Lumizyme)	If upon immediate inspection opaque particles are observed or if the solution is discolored do not use. The reconstituted solution may occasionally contain some alglucosidase alfa particles (typically less than 10 in a vial) in the form of thin white strands or translucent fibers subsequent to the initial inspection. This may also happen following dilution for infusion. These particles have been shown to contain alglucosidase alfa and may appear after the initial reconstitution step and increase over time. Studies have shown that these particles are removed via in-line filtration without having a detectable effect on the purity or strength	The diluted solution should be filtered through a 0.2 μm, low protein-binding, in-line filter during administration to remove any visible particles
Naglazyme (galsulfase)	Naglazyme, for intravenous infusion, is supplied as a sterile, non-pyrogenic, colorless to pale yellow, clear to slightly opalescent solution	Naglazyme should be prepared using PVC containers and administered with a PVC infusion set equipped with an in-line, low protein-binding 0.2 μm (μm) filter
Nulojix (belatacept)	Reconstitute the contents of each vial of Nulojix with 10.5 mL of a suitable diluent using the silicone-free disposable syringe provided with each vial and an 18- to 21-gauge needle. Suitable diluents include: sterile water for injection (SWFI), 0.9 % sodium chloride (NS), or 5 % dextrose in water (D5W). Note: If the Nulojix powder is accidentally reconstituted using a different syringe than the one provided, the solution may develop a few translucent particles. Discard any solutions prepared using siliconized syringes	The entire Nulojix infusion … must be administered with an infusion set and a sterile, non-pyrogenic, low protein-binding filter (with a pore size of 0.2–1.2 μm)
Ontak (denileukin diftitox)		Do not administer Ontak through an in-line filter
Orencia (abatacept)	The Orencia powder in each vial must be reconstituted with 10 mL of Sterile Water for Injection, USP, using only the silicone-free disposable syringe provided with each vial and an 18–21 gauge needle. If the Orencia powder is accidentally reconstituted using a siliconized syringe, the solution may develop a few translucent particles. Discard any solutions prepared using siliconized syringes	… must be administered with an infusion set and a sterile non-pyrogenic, low protein-binding filter (pore size 0.2–1.2 μm)

Proleukin (aldesleukin)		In-line filters should not be used when administering Proleukin
Refacto (antihemophilic factor)		Solution should be withdrawn from vial using plastic syringe and sterile filter needle. Use separate unused filter needles if pooling from multiple vials
Replagal (agalsidase alfa) (EU)		Administer the infusion solution over a period of 40 min using an intravenous line with an integral filter
Saizen, Serostim (somatropin)	The Saizen solution should be clear immediately after reconstitution. Do not inject Saizen if the reconstituted product is cloudy immediately after reconstitution or refrigeration. Occasionally, after refrigeration, small colorless particles may be present in the Saizen/Serostim solution. This is not unusual for proteins like Saizen/Serostim	
Tev-Tropin (somatropin)	Occasionally, after refrigeration, some cloudiness may occur. This is not unusual for proteins like Tev-Tropin growth hormone. Allow the product to warm to room temperature. If cloudiness persists or particulate matter is noted, the contents must not be used	
Other proteins, human- and animal-derived		
Aralast (proteinase inhibitor, human)	When reconstitution procedure is strictly followed, a few small particles may occasionally remain. These will be removed by the microaggregate filter	Reconstituted product from several vials may be pooled into an empty, sterile IV solution container by using aseptic technique. A sterile, 20 μm filter spike is provided for this purpose
ATGAM (lymphocyte immune globulin, anti-thymocyte globulin, equine)	ATGAM (lymphocyte immune globulin) is a transparent to slightly opalescent aqueous protein solution. It may appear colorless to faintly pink or brown and is nearly odorless. It may develop a slight granular or flaky deposit during storage	ATGAM is appropriately administered into a vascular shunt, arterial venous fistula, or a high-flow central vein through an in-line filter with a pore size of 0.2–1.0 μm. The in-line filter should be used with all infusions of ATGAM to prevent the administration of any insoluble material that may develop in the product during storage. The use of high-flow veins will minimize the occurrence of phlebitis and thrombosis

(continued)

Table 14.3 (continued)

Product name	Description of particles if present	Recommendation about filtration in conjunction with administration
Buminate (albumin, human)		Make sure administration set contains an adequate filter
Cinryze (c1 inhibitor, human)	The reconstituted solution should be colorless to slightly blue, and free from visible particles. Do not use if turbid or discolored	Use filter needle to pull reconstituted solution into syringe
Cytogam (cytomegalovirus immune globulin, human)		Cytogam should be administered through an intravenous line using an administration set that contains an in-line filter (pore size 15 μm) and a constant infusion pump (i.e., IVAC pump or equivalent). A smaller in-line filter (0.2 μm) is also acceptable
Gammagard (immune globulin infusion, human)		An in-line filter is optional
Gammagard (SD immune globulin intravenous, human)		Administration set contains an integral airway and 15 μm filter
Glassia (alpha1-antitrypsin, human)	The solution should be clear and colorless to yellow-green and may contain a few protein particles. Do not use if the product is cloudy	When infusing directly from the vial, use a vented spike adapter and a 5 μm in-line filter (neither is supplied) When infusing from a sterile intravenous container, attach an appropriate intravenous administration set to the intravenous container. Use a vent filter (not supplied) to withdraw the material from the vial and then use the supplied 5 μm filter needle to transfer the product into the infusion container. In addition, during infusion, it is recommended to use a 5 μm in-line filter (not supplied)
Hemofil M Antihemophilic factor (human)		Use filter needle to draw reconstituted solution into plastic syringe. Each filter needle is intended to filter the contents of single bottle of Hemofil M only

Koate_DVI (antihemophilic factor, human)		Attach the filter needle (from the package) to a sterile syringe. Withdraw the Koate-DVI solution into the syringe through the filter needle
Prolastin-C (alpha1 proteinase inhibitor, human)	A few small particles may occasionally remain after reconstitution	If particles are visible, remove by passage through a sterile filter (e.g., 15 μm filter) used for administering blood products. Reconstituted product form several vials may be pooled into an empty sterile IV container by using aseptic technique. A sterile filter needle is provided for this purpose
Thrombate III (antithrombin III, human)		Withdraw the reconstituted solution in to syringe through filter needle provided
Thymoglobulin (anti-thymocyte globulin, rabbit)		Thymoglobulin should b e administered through an in-line 0.22 μm filter
Zemaira (alpha1 proteinase inhibitor, human)		Filter the reconstituted solution during administration. To ensure proper filtration of Zemaira, use an IV administration set with a suitable 5 μm infusion filter (not supplied)

[a]Information extracted from the latest version of Prescribing Information (US) or Summary of Product Characteristics (EU; where indicated)

14.2.2 Subvisible Particles

Several techniques are available to detect, measure, and count subvisible particles in solutions, making this size range (~1–100 μm) less subjective and more quantitative than the visible range. The pharmacopeias specify light obscuration (LO) and membrane microscopy (MM) as the two techniques to measure compliance against the subvisible particle requirements (Tables 14.1 and 14.2). For sterile parenterals other than biopharmaceuticals, light obscuration and membrane microscopy techniques have sufficed since the objective has been to detect and quantify foreign (extrinsic) and intrinsic particles. With the growing importance of biopharmaceutical products and the need to monitor inherent particles, a number of other techniques have been brought into use, to not only count and size but also extract morphological and (protein) structural information.

14.2.2.1 Light Obscuration

Light obscuration (LO) detects and counts particles on the basis of the light they block as they pass in front of a detector, while the change in intensity is correlated to their size. The blockage is due to both scattering and absorption. More information on the principles of the method can be found in Allen (1990). Details about implementation of the LO method are provided in USP<1788>. All of the pharmacopeias specify the use of LO as Method 1 in their (harmonized) chapters for subvisible particulates (USP<788>; Ph.Eur. 2.9.19, JP6.06), and thus LO is the current gold standard in evaluating sterile parenterals.

The advantages of LO include widely available instrumentation, familiarity in the industry, and ease of use. Although the pharmacopeias specify measurements in the ≥10 and ≥25 μm ranges, the instrument is easily recalibrated to allow measurements to be made from ~2 μm and upwards. One commonly available LO instrument (HIAC by Hach, Loveland, CO, USA; www.hach.com) has a detector coincidence limitation of approximately 18,000 particles/mL. [PAMAS offers instruments with counting limits of 24,000 or 120,000 particles/mL (PAMAS GmbH, Stuttgart, Germany; www.PAMAS.de).] Accuracy and reproducibility in LO drop for counts below ~10 particles/mL (Narhi et al. 2009). Artifacts may arise if the flow channel gets physically blocked or if the saturation limit is reached. Saturation in one measurement channel can cause nonlinear effects in other channels also.

Obscuration of light is a function of the difference in refractive index of the particle and the aqueous background, which provides the optical contrast required for the “shadow” to be cast. Foreign (extrinsic) (e.g., fibers, metal, plastic that arise from the manufacturing environment) or intrinsic (precipitates of buffer salts or of degradation products in case of small molecules) particles generally have good contrast and are easily counted. Similarly, silicone oil droplets (e.g., from stopper coatings or in prefilled syringes) have a refractive index that allows them to be counted against an aqueous background, but the same holds true for bubbles. On the other

hand, inherent proteinaceous particles tend to have low contrast (high transparency) and are generally undercounted by this method, especially as the size gets smaller. The relevance of standards and the lack of appropriate ones for proteinaceous particles are discussed in Sect. 14.2.5.

LO cannot distinguish between types of particles. Proper sample handling procedures must be used to ensure that bubbles are not created or counted (see, e.g., Hickey et al. 2011). While the pharmacopeia offers sonication as a way to eliminate bubbles, this is generally not suitable for biopharmaceuticals, for which vacuum degassing is preferred. Silicone oil droplets will however be counted by this method and, in certain products (e.g., from prefilled syringes or cartridges), could contribute significantly to the total count. Lack of ability to distinguish between particles also means that no further characterization information is available from this destructive test.

The current pharmacopeial methods also require a large sample volume which can be expensive and difficult for products with small fill volume per container and many biopharmaceuticals. High concentration biopharmaceutical products can also be problematic to test in LO both due to higher viscosity and due to an increase in the background refractive index (Demeule et al. 2010). Such products are also likely to be opalescent, adding to the difficulty of use of LO. However, with suitable qualification, it is permissible to use small sample volumes or product dilution when performing LO measurements. Qualification of dilution requires careful consideration of the diluent medium and extent of dilution. Intrinsic particle counts may change due to concentration/equilibrium effects and/or particle detection may be impacted by change in refractive index. It is recommended to use the minimum dilution level that gives a linear response.

USP<789> also specifies the use of LO for ophthalmic products. Placing intravitreal injection products in this category, it becomes clear that testing per the monograph can be quite onerous. For example, the intravitreal biopharmaceutical product Lucentis™ (ranibizumab) contains 200 μL in a 2-mL vial with a dose volume of 50 μL. Similarly other intravitreal injection products, Macugen® (pegaptanib sodium), contains a nominal deliverable 90 μL of solution in a 1-mL prefilled syringe, and Vitravene™ (fomivirsen; now withdrawn) had a fill volume of 250 μL in a vial (size not known) with a dose volume of 50 μL. Test protocols will require pooling of multiple units, while preventing environmental contamination and eliminating bubbles. Very stringent controls must be put into place in order to ensure that false-positive results are eliminated. Methods with smaller test volumes are generally qualified for such products.

14.2.2.2 Membrane Microscopy

Membrane microscopy (MM) is Method 2 or the secondary method to test for subvisible particulates endorsed by the pharmacopeia. This method is to be used in case of limit failure with Method 1 (LO) or if Method 1 is not suitable, e.g., viscous solutions, or products that have reduced clarity or are opaque due to light scattering such as emulsions and liposomal preparations. MM involves filtering a known volume of

product through a 1 μm or tighter pore membrane, and then counting and sizing the particles on the basis of a prescribed graticule under a microscope. Details of the method are provided in the pharmacopeia but a much more critical and useful discussion is available (Aldrich 2010). The minimum particle dimension that can be resolved by optical microscopy depends on the optical properties of the material (as well as instrument quality), but is generally considered to be in the range of ~1 μm (Toler and Nema 2010).

As mentioned above, Method 2 can be resorted to if the product fails the limits with Method 1. This flexibility allows counting artifacts in LO from bubbles and silicone oil droplets to be eliminated, and thus verify the counts of subvisible particles of concern only. It may however be prudent in case of failure by Method 1, that an investigation is conducted to identify the root cause, even if subsequent testing by Method 2 leads to a passing result. Note that the pharmacopeial acceptance limits for Method 2 are tighter than for Method 1. This is because MM significantly underestimates the number of subvisible particles present. The method also requires a trained operator, is exhaustive, subject to operator fatigue, and has a degree of subjectivity in estimation of size. USP<1788> instructs the operator "… transforming mentally, the image of each particle into a circle."

Microscopy offers the advantage of visualizing the particles and allows further in situ forensic examination using polarized light, fluorescence, and staining by dyes. Isolation, identification, and characterization, especially with visible particles, have been discussed briefly later (see Sect. 14.2.4), and the techniques may be applied to subvisible particles too depending on the size of the particles (Toler and Nema 2010).

The utility of membrane microscopy for biopharmaceuticals is considered to be limited, when it pertains to counting and sizing the inherent particles. The MM method in the pharmacopeia also instructs "… do not attempt to size or enumerate amorphous, semi-liquid, or otherwise morphologically indistinct materials …." Proteinaceous particles can be fragile, clear to translucent, not well retained, and not easily visualized on the membrane. Their soft nature makes size estimation difficult. Also, these particles can dry out rapidly on the filter, rendering a detailed analysis difficult. However, protein stains can be used to an advantage in this mode to identify proteinaceous compared to other particles (Li et al. 2007). The membrane microscopy method for biopharmaceutical is therefore recommended primarily for determination of foreign (extrinsic) and intrinsic particles only. For inherent particles, its utility is mainly in a diagnostic mode where add-ons such as dye staining, fluorescence, birefringence, and FTIR can be used to obtain characterization information.

14.2.2.3 Dynamic Flow Imaging

Dynamic flow imaging (DFI) is a variation of microscopy where the sample is passed through a specially designed cell in front of a microscope and imaged with a camera. The image can then be analyzed to count, size, and characterize the particles. Instruments based on this principle are available from at least three manufacturers that differ in their detailed specifications such as cell design, test sample volume,

illumination strategy, image field, imaged volume, image quality, image analysis algorithm, and user interface (Narhi et al. 2009; Singh et al. 2010; Zolls et al. 2012). Besides number and size, the flow imaging techniques also assess parameters such as transparency, intensity, and circularity/shape, thus potentially helping to differentiate between, for example, silicone droplets or air bubbles and foreign (extrinsic) particles. These latter analyses, while very useful, are a function of the algorithm employed and the results therefore subject to operator judgment. However, the ability to generate morphological data on the particles as well as access to the images after completion of the testing is an important advantage of this technique (Sharma et al. 2007; Strehl et al. 2012).

The algorithms that examine the image and discern whether a region represents a particle and assess its characteristics are based upon an assessment of the grayscale over the pixels occupied by the image. The smaller the particle, the fewer pixels it occupies and thus the lesser the information that can be gleaned from the image. It is well accepted that significant loss of information occurs for sizes below 4 μm wherein only the presence or absence of a particle can be detected (Brown 2009). The lower size limit for the technique is stated to be 1 μm but general experience suggests that 2 μm is the likely lower limit for good quantitation, especially for low contrast (proteinaceous) particles. Counting limits are quite a bit higher compared to LO, in the range of several hundred thousand per milliliter, depending on the instrument and settings employed.

This technology has seen its greatest utility in the analysis of biopharmaceutical products where the ability to count as well as image inherent proteinaceous particles has been valuable. The imaging sensitivity of these instruments, especially at the low sizes, is superior to the sensors in LO, and thus low contrast (refractive index difference) particles can be better captured and counted. Results on biopharmaceutical samples measured by both LO and DFI consistently report higher numbers with DFI, especially in the lower size ranges (Huang et al. 2008; Demeule et al. 2010; Sharma et al. 2010; Singh et al. 2010; Barnard et al. 2011). The differences between LO and DFI results are explained by the hypothesis that the smaller the particles, the closer their refractive indices are to that of the formulation buffer. In this situation, the DFI detectors, which capture an "image" as opposed to the LO sensor which captures a "shadow," are purported to give a more accurate count. Furthermore, it is proposed that particles with noncircular morphologies like those of inherent proteinaceous aggregates are not accurately counted by LO.

As the use of DFI has expanded, a number of publications have appeared showing the utility of this technology in formulation and process development, as well as product characterization (see, e.g., Wuchner et al. 2010; Southall et al. 2011). DFI for measuring and counting subvisible particulates in parenterals is not part of the pharmacopeial test methods (Oma et al. 2010). It is therefore currently utilized in the biopharmaceutical industry mainly as a developmental and (orthogonal) characterization tool, and will be discussed in the forthcoming guidance chapter USP<1787>. However, it is possible that a sponsor, after proper qualification and validation, could use this method from a regulatory perspective also. Some comparison with data obtained by LO would very likely be required.

14.2.2.4 Electrical Sensing Zone

Electrical sensing zone (ESZ), better known as the Coulter Principle, utilizes the phenomenon that particles placed in an electrical field will alter the current flow in that field (Graham 2003). Instruments based on this principle called the Coulter Counter® require the flow of the sample though a small cylindrical tube with a defined opening (called the aperture tube) that separates two electrodes. An electric current flows between the electrodes. As each particle passes through the aperture, it displaces its volume of the electrolyte and causes a transient change in the impedance across the aperture, leading to a voltage pulse. The amplitude of this pulse is directly proportional to the volume of the particle that produced it, with each pulse originating from a single particle. When a known volume of suspension is drawn through the aperture, the number of pulses can be converted to yield a concentration of particles per unit volume of the test suspension. If a constant particle density is assumed, the pulse height is also proportional to the particle mass. Since the pulse height is determined by the volume of electrolyte displaced, porous particles and particles carrying a large fraction of enclosed electrolyte will thus be sensed as being smaller than their geometrical/volumetric size. As the particles must pass through the sensing zone one at a time, the optimal concentration must be determined for each sample. In addition, the aperture tube has a defined opening, which will only allow a certain size range of particles (within 2–80 % of its nominal diameter) to pass. A range of aperture sizes is available in the instruments. If the sample particle size distribution is very broad, some particles may be excluded from analysis or possibly plug the aperture opening. The overall particle size measurement range that is obtainable by combining data from several apertures is between 0.4 and 1,600 μm.

For the Coulter principle to apply, the sample must be presented in a conducting media. This may in certain cases require that the product be "diluted" into an electrolytic solution, generally saline. A higher conductivity is required when using smaller apertures for smaller particles. Additional guidance on the Coulter principle can be found in the International Standard ISO 13319 "Determination of Particle Size Distributions-Electrical Sensing Zone Methods." This technique is often used as a reference for qualifying other particle sizing instruments.

The Coulter principle has found applicability in a diverse array of fields, and has lately also been proposed as a sensitive alternative for measuring inherent proteinaceous particles in biotherapeutics (Rhyner 2011). Since there is no dependence on optical properties, such particulates can be readily counted, and the ability to measure down to ~0.4 μm in the latest version of instruments means that the aforementioned "measurement gap" with regard to size exclusion chromatography can be narrowed. Biotherapeutics are generally formulated in a buffer solution with a tonicity modifier such as NaCl or a disaccharide. If dilution into an electrolyte solution is needed to use the Coulter Counter®, care must be taken to confirm that such a change in the media does not impact the proteinaceous aggregates already present and does not lead to further formation of such aggregates. Any impact of this manipulation on the material to be measured is likely dependent on the protein and

the nature of the aggregate constituting the proteinaceous particles. Foreign (extrinsic) particles should in general not be impacted by this manipulation. Loose aggregate morphologies carrying a large fraction of enclosed electrolyte, and/or particulate geometries differing significantly from spherical, can also lead to difficulties in sizing by the Coulter principle.

Some good assessments of the utility of this technology for biotherapeutics have been recently published (Demeule et al. 2010; Rhyner 2011; Barnard et al. 2012). The method detects more particles than light-based techniques because it does not depend on the optical properties of the particles. [This was also noted for particle detection in water quality measurements (van Gelder et al. 1999).] ESZ has been shown to function well over a wide range of protein concentrations and can be used as an orthogonal method to the LO or DFI techniques. Limitations include need for a conductive solution and limited size range of each aperture, potentially requiring use of multiple apertures and multiple filtration steps in case of a wide particle size distribution.

14.2.2.5 Other Counting Techniques

Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA) or submicron particle tracking involves analyzing the motion of particles, tracked by a laser, as they move in solution under Brownian motion. Particle motions are recorded by a camera as small points of (scattered) light under a microscope objective. The path taken by the particle over an appropriate period of time (generally 30 s) is tracked and the particle size profile is determined using an analytical software program. Particles with overlapping paths are eliminated from the analysis. This technology has been commercialized under the trade name NanoSight (NanoSight Ltd, Wiltshire, UK).

A large number of areas of application have been described (www.nanosight.com). For the purpose of this chapter, the application to subvisible particles in biotherapeutics is relevant. The lower size limit of detection depends in part on the refractive index of the particle. For proteinaceous particle samples, the lower limit is approximately 30 nm. The upper size limit depends upon whether the particle Brownian movement can be tracked accurately in spite of sedimentation during analysis. The stated limit is 1,000 nm. Optimal sample concentration is 10^8–10^9 particles/mL, while the minimum concentration is 10^7 particles/mL and maximum concentration is 5×10^9 particles/mL. At these high concentrations, individual particles cannot be resolved accurately. Stated accuracy of particle sizing is within 5 % of the diameter (for standard spherical reference materials, such as polystyrene standards). Reproducibility of sizing is approximately 3 % at optimal concentration (for standard spherical reference materials, such as polystyrene standards). The particle concentration is estimated from the number of particles detected and thus a particle size distribution can also be calculated. The accuracy of the concentration measurement depends on the number of particles detected. It should be noted that

NTA measures the diffusion coefficient of a particle and uses the Stokes–Einstein equation, assuming a spherical particle, to assign a (hydrodynamic) radius to that particle. This model applies to most spherical or near spherical particles. However, if the aspect ratio is >3, the Stokes–Einstein equation will no longer be applicable.

Submicron particle tracking analysis can be very useful as a developmental and (orthogonal) characterization tool. It requires a trained operator and manipulation of instrument settings depending upon the sample characteristics (Filipe et al. 2010).

Flow Cytometry

A couple of recent reports have appeared on the novel use of flow cytometry to count proteinaceous particles using protein dyes to label and thus facilitate sorting (Ludwig et al. 2011; Mach et al. 2011). The technique appears to hold promise in counting particles in a high throughput setting with the possibility of resolving only proteinaceous particles since other kind of particles would not be stained by the protein dye. Counting of particles down to ~1 μm seems feasible although sizing is not possible in the current set-up. Further work is required to fully identify the potential of this technique. Its primary utility may lie in rapid analysis of small volumes of solutions as part of pharmaceutical development, although the need for labeling is a drawback.

Microchannel Resonators

This is a novel technique coming out of the nanotechnology field called the Archimedes (Affinity Biosensors, Santa Barbara, CA; www.affinitybio.com). It utilizes a fluid-filled cantilever microfluidic channel within which the sample solution flows. The resonance frequency of this cantilever changes with the mass of material inside the channel (Burg et al. 2007). Thus, when a particle or aggregate of a density different than the solution flows into the channel, the shift in its resonant frequency is converted to mass and from there to size. These shifts are measured as each particle passes through the channel one-by-one and a statistical view of the mass and size distribution is created. Due to the high resolution of mass (at the femtogram level), sizes down to 0.1 μm are claimed to be measurable. The upper limit is stated at 5 μm. Particle concentrations up to 10^8/mL and viscosities up to 50 cP can be accommodated. The sensor requires a volume of only 100 μL, suggesting applications in high throughput screening formats. The sensitivity to density means that the technique can distinguish between different types of particles; e.g., silicone oil droplets have lower density while proteinaceous particles have higher density than the aqueous medium. The direction of change of the resonant frequency reflects this difference. The technique holds promise due to its sensitivity and ability to count and size particles as well as differentiate between particles to some extent. Further research is needed to explore the utility of this technique.

14.2.2.6 Other Non-counting Techniques

Several other techniques may be used to obtain orthogonal information about subvisible particles in sterile parenterals. However, the interest in utilization of these techniques for subvisible particle characterization is primarily for inherent particles in biopharmaceuticals.

Turbidity or Nephelometry

Turbidity of a solution is a function of ratio of incident and transmitted light and is thus a measure of the particles in the solution. However, it is a complex measure, dependent on size distribution, number, and nature of the particles (i.e., refractive index, absorption behavior), and a higher turbidity value cannot be unequivocally correlated to higher numbers or larger sizes. Information on particle concentration cannot be derived from turbidity measurements without knowledge of the nature of the particles. Turbidity, when measured by scattered light, is strongly instrument-dependent (Gregory 1998). The Ph.Eur. has a chapter on the use of Nephelometry to measure the opalescence of solutions. Ph.Eur. 2.2.1 is thus commonly used as a measure of parenteral product clarity and is a required part of the specifications in the EU. Nephelometry is a measure of turbidity through capture of light scattered at 90° by the combination of all particles and colloids in the solution. It can therefore be considered a surrogate measurement of the particulate content in the product, albeit with the reservations mentioned above. The formazin reference suspension against which the Nephelometric Turbidity Units are determined contains a wide range of particle sizes and shapes of the formazin polymer ranging from <0.1 to >10 µm (Sadar 1998). The light scattering response of the suspension is therefore a combination of scattering from all the particle sizes, although the complex nature of the scattering effect makes it difficult to attribute dominance to any specific sized population. However, within a set of samples from the same product measured on the same instrument, it may be assumed that scattering from the colloidal protein molecules is similar and the contribution from large particles (>50 µm) will likely be small due to the generally few particles and low likelihood of them passing through the light scattering zone. Under such circumstances a general conclusion, that the greater the turbidity, the higher the count of subvisible particles, may be possible.

Dynamic Light Scattering

Dynamic light scattering (DLS), also known as Photon Correlation Spectroscopy, is a well established technique that analyzes time-dependent intensity fluctuations (Doppler shift) in scattered light due to diffusive motion of particles, and thus reports hydrodynamic radius. Deconvolution of the correlation function gives information about the size distribution of particles. Particle sizes have to be greater than 3–5 fold different before they can be resolved by this technique. Also, the technique

reports size distribution based on intensity and is thus impacted strongly by scattering from large particles. It does not give a count of the particles. Conversion of intensity-based distribution to volume, weight, or number is possible but depends on a number of assumptions about the particles. DLS measurements can however cover a wide size range (from ~0.1 nm to ~5 μm), and can be performed in moderately concentrated solutions as long as viscosity corrections are applied properly. Low volume instrumentation is now available. While useful, readily available, and simple to use, the information generated by DLS can only be used for rapid measurements in a development mode for applications such as formulation screening (Philo 2006; Garidel and Kebbel 2010).

Taylor Dispersion Analysis

This is a newly introduced technique that utilizes the dispersion of solute in a plug as it flows through a uniform (capillary) cylindrical tube in laminar flow. A plug of solute injected into a mobile solvent stream disperses and broadens due to diffusion. This band-broadening is detected by a UV-detector and used to calculate the hydrodynamic radius of the solute. While the principles of the technique have been described in the 1950s, the technology and instrumentation required have only recently been developed (Hulse and Forbes 2011). It has been evaluated to size proteins as is and after thermal stress (Hawe et al. 2011). While able to detect changes in size as a consequence of stress, the lack of ability to separate monomeric and aggregated species means the technique has limited utility in quantifying particulates in a product solution.

Microscopy: Other

Rap.ID (Rap.ID Particle Systems GmbH, Berlin, Germany; www.rap-id.com) filters sample though a gold-plated filter and can be used to count as well as identify particles using Raman spectroscopy. The technique has a wide size range but suffers from the same limitations described earlier for membrane microscopy as it relates to proteinaceous particles. In the authors' experience, it is also not always unequivocal in identification of particles. The quality of the database and statistics of scanned particles as well as quality of spectral overlays impacts the analytical output significantly. Particles in protein solutions can be heterogeneous, and this also confounds the spectral identification.

A variety of high resolution techniques such as Atomic Force Microscopy, Scanning Tunneling Microscopy, and Scanning Electron Microcopy can be used to visualize and characterize proteinaceous particulates. The extremely small sample volume makes these techniques highly unsuitable for particle counting since it would be almost impossible to obtain a representative sample of a product in most cases. Some applications in the characterization of biopharmaceuticals have been published (Demeule et al. 2009; Lee et al. 2011a).

14.2.3 Submicron Particles (0.1 to ~1 μm)

Inherent proteinaceous particles in the submicron range lie in the measurement gap. The US FDA has requested qualitative characterization information for particles in this size range for biopharmaceuticals (see Sect. 14.1.2.2). DLS technique can in principle cover this size range. NTA and Microchannel Resonators described earlier can also cover a good part of the gap, proving some ability to assess this size range.

FFF-based separation and AUC are currently used as orthogonal techniques to characterize particles/aggregates. AUC can be applied in the 0.01–0.1 μm range, while FFF offers a broader dynamic range from 0.01 to several micrometers. The sensitivity of these techniques is limited by the sensitivity of the detectors used. A limitation of these methods is the difficulty of use of instrumentation as well as complexity of data analysis. These techniques, especially AUC, are therefore very useful for development and characterization, but not for lot release or stability (Philo 2006; Arakawa et al. 2007a, b; Philo 2009).

There are no commercially available techniques or instruments that can be used for quantitation of particles in this size range on a routine basis for quality control applications.

14.2.4 Particle Identification

The ability to identify particles is necessary to be able to address the root cause of their presence. This is particularly important in the case of foreign (extrinsic) and intrinsic particles for all products. Additionally, in the case of biopharmaceuticals, the ability to distinguish inherent particles from other sources such as silicone oil or from counting artifacts such as bubbles is critical as part of maintaining product consistency or performing risk assessments, etc. (see Sects. 14.2.1.3, 14.3.4, and 14.3.5). A good review of these techniques is available in Toler and Nema (2010). The general steps involve a first thorough examination in situ in the container, followed by isolation where possible and further examination by a variety of light microscopy and spectroscopic techniques for identification.

14.2.5 Particle Standards

An important aspect of particle size measurement is the standard used to calibrate instruments. Absolute calibration using qualified, traceable particle standards ensures that the instrument is operating within defined limits of accuracy. Most particle sizing techniques considered here report a single dimension corresponding to the equivalent circular diameter (ECD reported by LO, MM, DFI) or equivalent spherical diameter (ESD reported by ESZ, NTA, DLS). These equivalent "dimensions"

are defined as the diameter of a circle (ECD) or sphere (ESD) having the same area or volume respectively, as the particle being studied. [Imaging techniques such as DFI can in principle also report multiple other shape parameters such as area, perimeter, circularity, longest dimension, and aspect ratio. However, while these other measurements can be very useful for characterization, the most commonly reported parameter is the ECD.] Reference standards are used either for calibration (LO, ESZ) or for performance verification (MM, DFI, NTA, DLS). Calibration is required for instruments where the sensor response has to be correlated to the size of the particle. Performance verification is required where the measurements can be made independently based on fundamental principles. For these purposes, the standard must be well specified and the most critical dimension is size. The standards therefore have to be spherical, since particle size (= diameter) can be unambiguously defined only for a sphere (Mitchell 2000).

Latex (polystyrene)-based particle standards in various unimodal sizes are used for particle standards for the calibration and verification tasks discussed above. The pharmacopeia recommends calibration of the LO instrument with "dispersions of spherical particles of known sizes between 10 and 25 μm." Latex standards of 10, 15, and 25 μm are commonly used. When using the LO instrument for sizes below 10 μm, further calibration with multiple size standards in the size range 1–10 μm is recommended. The response of LO is not linear over the full range from ~1 to say 100 μm, and when using multiple channels on the instrument, a dense calibration scheme is preferable.

The response of the particle size analysis instrumentation to actual samples is impacted by factors other than size, such as shape (all), refractive index (LO, MM, DFI), reflectivity (LO, MM, DFI), bulk density, and porosity (ESZ, NTA, DLS), depending upon the principle of operation. Silicon-based cube/cuboid shape standards are available (www.lgcstandards.com) as suspensions, intended for use in characterizing aerosols. Their use, for example, in LO, would however be an academic exercise since, ultimately, the subvisible particle measurement results for any product are whatever the instrument reports. Furthermore, for most sterile parenterals, with the focus on foreign (extrinsic) and intrinsic particles, the refractive index is sufficiently different from the background medium (generally aqueous) that detection (LO) is not an issue. Therefore, the current spherical latex particle standards are adequate.

Intrinsic proteinaceous particles in biotherapeutics, on the other hand, tend to be gel-like and have refractive index close to that of the aqueous background. Their shapes are highly irregular and fibrous, and they lack rigidity. All of these attributes make counting and sizing of these particles by optical techniques highly error-prone. There open/flexible nature and irregular shape also mean that they are sensed as being smaller than their geometric ESD in the ESZ technique. The reported ECD is also likely to be smaller than the longest (ferret) dimension as the particle shape deviates from spherical. Spherical latex standards are not representative of these materials, differing in their refractive index, shape, and transparency. Development of standards that are more representative of proteinaceous particles has been called

for (Singh et al. 2010). It is likely that reporting of size may always have to rely on the universal spherical latex standards which enable a single unambiguous result (whose meaningfulness may be questioned!). However, standards that are more representative of proteinaceous particles may be useful for comparing responses across different techniques. Such standards may also be useful for demonstrating that instruments may be reliably validated with nonprotein particles that mimic the properties of proteinaceous particles (Ripple et al. 2011; Scherer et al. 2012).

14.3 Special Topics

In this section, we will focus on certain current topics related to particulates in sterile parenterals.

14.3.1 Essentially Free of Visible Particles

The USP<1> requirement of "every lot of parenteral preparations is essentially free of visible particles" (EFVP) and similar requirements in Ph.Eur. 01/2008:0520 have always created confusion in their interpretation [JP6.06 requires "free from readily detectable"—a statement in which the ambiguity lies in the "readily detectable" part of the phrase] (Table 14.1). While zero-defect is the desired state, current practical manufacturing capabilities preclude the ability to guarantee a 100 % particle-free batch. Post-manufacturing inspection aims to remove all containers that have visible particles classified as defects. However, a quantitative measure for this quality attribute has been lacking.

A recent stimuli article has been published that proposes to revise USP<1> with a sampling plan and test, as well as criteria by which a product can be considered EFVP (Madsen et al. 2009). For batch release purposes, the proposal specifies General Inspection Level II sampling plan as described in ANSI/ASQ Z1.4 with an AQL of 0.65 %. [The AQL of 0.65 % was identified as the median value for AQL for major defects (most often associated with particulate matter) from a PDA survey of visual inspection practices in 2008.] The test would be applied to products that have been 100 % inspected as part of the manufacturing process. A batch would be considered to meet the EFVP requirement when not more than the specified number of units contains visible particulates. On the other hand, a fixed sample size of 60 units would be used when a batch, that has already been released and distributed, needs to be reevaluated. A batch would be considered to meet the EFVP requirement when not more than one unit contains visible particulates. [This sampling plan has an AQL of 0.60 %, acceptably close to the ANSI/ASQ Z1.4 AQL of 0.65 %, which would accept a batch with 1.3 defect units per hundred, 50 % of the time.]

14.3.2 Ph.Eur. 01/2008:2031 Monoclonal Antibodies for Human Use

The Ph.Eur. (Edition 7.1) monograph on Monoclonal Antibodies for Human Use sets a requirement for appearance given below.

Characters

Liquid preparations are clear or slightly opalescent, colourless or slightly yellow liquids, without visible particles.

The test for *Appearance* states:

Liquid or reconstituted freeze-dried preparations are clear or slightly opalescent and colourless or slightly yellow, without visible particles.

The test for *Solubility* states:

Freeze-dried preparations dissolve completely in the prescribed volume of reconstituting liquid, within a defined time, giving a clear or slightly opalescent solution without visible particles.

The requirement of "… without visible particles" does not reconcile with Ph.Eur. 01/2008:0520 Parenteral Preparations, wherein Injections "… examined under suitable conditions of visibility, are clear and practically free from particles." More importantly, it does not reconcile with the presence of inherent proteinaceous particles in (commercial) biotherapeutic products as discussed earlier (see Table 14.3). Even with the best efforts at formulation and process development, it is considered difficult if not impossible to ensure that no such proteinaceous visible particles would be present at release or form over the shelf-life of the product. When present, such particles would be included in product tested in nonclinical and clinical studies. Assuming the results are supportive, such inherent proteinaceous particles would be a normal part of the product and should not present quality or safety concerns.

Based on comments from various industry and professional organizations, a revision has been proposed (Anonymous 2010).

The text from 01/2008:0520 regarding "… practically free …" has been added to *Definition.*

The section on CHARACTERS is to be deleted.

The proposed test for *Appearance* states: *Liquid or reconstituted freeze-dried preparations comply with the limits approved for the particular product with regard to degree of opalescence (2.2.1) and degree of coloration (2.2.2). They are without visible particles, unless otherwise justified and authorised.*

The proposed test for *Solubility* has been modified to conform to the requirements of *Appearance* removing the statement about particulates: *Freeze-dried preparations dissolve completely in the prescribed volume of reconstituting liquid, within a defined time, as approved for the particular product.*

The phrase "… without visible particles …" has been retained intentionally to emphasize that all efforts must be made to find an optimal formulation. The escape clause "… unless otherwise justified" has been added to allow products where it has been demonstrated that it is not possible to remove all visible particles. It is specifically noted that "practically free" cannot be a pass/fail criteria in a test.

14.3.3 Measuring Subvisible Particulates in Biopharmaceuticals Per the USP

The importance of being able to measure (count and size) the inherent proteinaceous particles in biopharmaceuticals has been mentioned earlier, as well as the limitations of the current methods, especially those in the pharmacopeia. This limitation was recognized and acknowledged at several discussion forums (see, e.g., Carpenter et al. 2010a, b; Greb 2011; http://www.usp.org/meetings/workshops/particleSizeAnalysis.html), and a stimuli article published by authors from Amgen (Cao et al. 2010). As a follow-up to the discussions, a revised version of USP<788>, specifically for therapeutic protein injections, has been drafted (as USP<787>) for public comment, and appeared in the Pharmacopeial Forum in early 2012 (Anonymous 2012). Based on comments received, a new version has subsequently been published in the Pharmacopeial Forum in early 2013 (Anonymous 2013). The main adaptations in USP<787> include:

- Identification that the chapter is applicable to therapeutic protein injection products, i.e., biotechnology-derived products as defined in USP<1045> *Biotechnology Derived Articles*, including their infusion preparations.
- Allowance for alternative analytical methodologies with adequately developed subvisible particulate limits.
- Recognition of inherent proteinaceous particles as a separate species to monitor.
- Introduction of concept of method verification.
- Recognition that standards over a wider range than 10 or 25 µm should be used for calibration, in anticipation of the need to monitor sizes below 10 µm.
- Acknowledgement that LO may not be suitable for many products, and MM is unlikely to be useful for such particles and that guidance for alternative methods will be forthcoming in a new guidance chapter USP<1787>.
- Introduction of a System Suitability blank (particle-free water and a USP particle reference standard handled the same as test article).
- Guidance on sample preparation, e.g., dilution, degassing (by vacuum, not sonication).
- Removal of specific requirements to pool 10 or more units or obtain at least 25 mL of product solution. The required pooled volume will instead be guided by the test aliquot volume.
- Reduction of minimum test aliquot volume drawn by instrument for measurement down to 0.2 mL.
- Clarification that for lyophilized products, the reconstitution or dilution must be performed with specified diluent and the particle count contribution from diluent itself cannot be subtracted.
- Elucidation of the need for dilution in case of high concentration/viscosity products, and the requirement to verify the suitability of dilution procedure using orthogonal methods.

- Specific statement that the limits do not apply to products that use final filtration during administration but do apply to the filtrate.
- Recognition that for proteinaceous particles, the MM test may not be entirely suitable so that MM and LO cannot be considered interchangeable.

The proposed USP<787> chapter retains the current reporting requirements and limits as in USP<788>. The intention is to allow flexibility, and the sponsor can choose to apply USP<788> or USP<787>. It must be noted, however, that for biotherapeutic solutions for parenteral infusion or injection supplied in containers with a nominal content of more than 100 mL, a second set of limits is applied to the total particle load in the container (≤6,000 per container for ≥10 μm; ≤600 per container for ≥25 μm). Current recombinant biotherapeutic products do not fall in the large volume parenteral (>100 mL) category, but for products intended for dilution prior to infusion, the infusion product volume can exceed 100 mL, and the above secondary limits would apply (also see Sect. 14.3.5).

Although the particle acceptance limits in USP<787> remain unchanged from USP<788>, the discussion in the text is instructive of the current thinking on this topic. The text in USP<787> states that applying universal limits to the wide range of biotherapeutic products is difficult, and that limits for specific product (and manufacturing process) should take into account the available clinical safety and efficacy data, as well as the dose and route of administration. The text also states that "setting limits is most appropriately done on a case-by-case basis during drug development and licensure." Therefore, the current limits, "derived historically from USP<788> … should be considered provisional until appropriate limits are specified in the approved regulatory application ...." Based on the language in USP<787>, it is clear that the US FDA will eventually (e.g., as post-marketing commitment) expect product-specific limits for subvisible particles (including for particles between ~1 and 10 μm; see Sect. 14.3.4). This is likely to occur post-licensure as increased experience is gained from the manufacturing of commercial product along with greater clinical exposure and thus a bigger safety database.

The ability to use smaller test aliquot volumes and thereby smaller pool and fewer pooled containers would be a benefit for many biopharmaceuticals since the products are expensive, and in many cases, have small fill volumes. It is the opinion of this author that in cases where the product fill volume is not a serious limitation, the largest viable test aliquot be used. Counting by LO especially at low particle counts is inherently error-prone. Counting methods applied on small samples provide an inaccurate representation of the population, especially when the counts are low—accuracy of counting increases with counts. Furthermore, taking a small aliquot from a larger fill vial may not provide a representative sample. If subsequently, the results from a small aliquot are multiplied by a factor to report the values per container as required by the pharmacopeia, the error can be magnified and may even lead to failure to meet the limits. For example, if a 0.5 mL test aliquot is used for a 20-mL product, the result from the aliquot will be multiplied by 40. A 15 count error in the 0.5 mL aliquot for the ≥25 μm particle (say 15 instead of 0) would lead to failure of the 600 particle limit. Considering that the resolution of LO is around 10 particles, a 15 count error is not very unlikely.

14.3.4 Subvisible Particulates Under 10 μm in Size in Biopharmaceuticals: Specifications

The importance of monitoring subvisible particulates smaller than 10 μm (primarily inherent proteinaceous particles) in biopharmaceutical has been acknowledged. Multiple publications have discussed the advantages and limitations of the various techniques available for this purpose (see, e.g., Mahler et al. 2009; Narhi et al. 2009; Demeule et al. 2010; Singh et al. 2010; Wuchner et al. 2010; Scherer et al. 2012). Irrespective of the technique used, it is now well accepted that the monitoring of (proteinaceous) particles in the ~1–10 μm size range is a valuable tool in formulation and process development of a biotherapeutic. Proteinaceous particles are a result of aggregate formation, which represent a continuum in size ranging from small dimers and oligomers to larger visible aggregates (Philo and Arakawa 2009). In the current understanding, aggregates above ~0.1 μm may be designated as "particulates." Among the main degradation pathways for proteins, aggregation is the least predictable and often the most difficult to control. There are multiple stresses that can result in aggregation through multiple mechanisms, and the aggregates formed differ in their structure even though they are all called by the same generic name (Mahler et al. 2009; Weiss et al. 2009; den Engelsman et al. 2011; Zhang et al. 2011, 2012). Monitoring of subvisible particulates in the under-10 μm range has been found to be an early marker for aggregation-related instability compared to other techniques such as size exclusion chromatography (Barnard et al. 2011). It therefore makes perfect sense to integrate the measurement of this attribute into the product development program (see, e.g., Tyagi et al. 2009; Wuchner et al. 2010; Southall et al. 2011).

From the same perspective, subvisible particulates should be monitored during stability and possibly for comparability exercises also. However, this position automatically leads to the question whether specifications should be placed on particulates in the ~1–10 μm range. It is clear that no universal limits along the lines of USP<788> are likely since the safety concern around inherent proteinaceous particles is primarily immunogenicity-related and is therefore product-specific. Product-specific limits could therefore potentially be developed by the sponsor, based upon nonclinical, clinical, and manufacturing experience. However, there are aspects to specification setting that need careful thought (Singh et al. 2010). Particulate counts below 10 μm have been found to be highly variable and dependent on technique, sample properties, etc. (Demeule et al. 2010; Scherer et al. 2012). Lack of ability to differentiate proteinaceous from foreign (extrinsic) particles, and variability introduced as a consequence of factors such as container/closure changes and product history, implies that the results could differ considerably between phases of the development cycle. Variability would also be introduced by factors such as silicone oil in case of prefilled syringes/cartridges, bubbles, environmental contaminants, impact of container/closure, handling, and time–temperature history. In order to accommodate this variability, product history would need to be acquired over multiple DS and DP lots. In the case of prefilled syringe or cartridge products, this need

will be magnified to cover various silicone loads. If such a path is taken, it is likely that specifications for each size range will need to be uniquely defined and qualified for each product/container configuration.

14.3.5 Addressing Regulatory Queries Related to Subvisible Particulates in Biopharmaceuticals

Some specific queries from the US FDA for data on the <10 μm particle size ranges have been listed in Sect. 14.1.2.2. Such queries have not been received from the EMA to date, to the best of the author's knowledge. The queries generally require measurement, characterization, risk assessment, and risk mitigation. The first step in addressing such queries is the use of data collected during the development phase, including on material that goes into nonclinical and clinical studies, release, as well as stability storage. It is advantageous to have a data set collected with the same technique (generally LO), supplemented where possible by orthogonal techniques such as DFI. Secondly, data can be generated as part of forced degradation studies (heat, light, agitation, freeze/thaw, oxidative, acidic, basic) to provide a picture of the particulate generation under worse-case scenarios. Finally, subvisible particulate data under in-use conditions should be collected, e.g., after dilution into infusion bags with specified diluent. Risk can then be assessed on particle generation under the various scenarios in relation to the nonclinical, clinical, as well as manufacturing experience. Identification of the risks can then lead to risk mitigation or control strategies. For example, a product susceptible to freeze/thaw-induced particulate formation may require extra controls in the shipping procedures to prevent freezing, more detailed studies to delineate impact of various freeze/thaw scenarios, and specific instructions about actions in case of such events. Recommendation for filtration (after proper qualification) prior to use is another risk mitigation strategy. Clearly, this type of exercise would be profitably done during development, ahead of regulatory filing, so that surprises in the form of serious risk scenarios are avoided.

14.4 Summary

Great strides have been made in improving the quality of sterile parenterals vis-a-vis particulate matter as a quality parameter. Optimized formulation, appropriate selected container/closures, and a well-designed manufacturing process are the key to reducing the numbers of foreign and intrinsic particles. With the increasing importance of biopharmaceuticals, a new challenge in the form of inherent proteinaceous particles has emerged in the particles field. Measurement (counting, sizing) of these particles in a diverse array of biopharmaceutical products is challenging the capabilities of the current techniques. Revisions to pharmacopeia have been proposed to address some of the challenges with biopharmaceuticals.

The push from the regulators and the pull from academic and product development scientists are leading to rapid advances in the field.

Note added in proof: The field of subvisible particulates around biotherapeutics has evolved rapidly since the preparation of this chapter. The reader is referred to the PF 2013 version of USP<787>, as well as forthcoming guidance chapter USP<1787> in the Pharmacopeial Forum (target late 2013) for the latest regulatory developments.

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Chapter 15
Appearance Evaluation of Parenteral Pharmaceutical Products

Erwin Freund

Abstract This chapter will describe the current state of the art and expectations of performing inspections of drug product with a focus on those defects that are visible to the eye. Visible attributes cover cosmetic and functional defects using both manual and automated techniques that use appearance as the key characteristic. The section will cover inspection attributes, compare and contrast manual and machine-based inspection with regulatory expectations, limitations, technologies, and provide several examples through case studies.

15.1 Introduction

In the process of manufacturing and releasing the final drug product (DP) presentation, a number of tests are required that verify the container/closure system is intact and the chemical and physical properties of the API (active pharmaceutical ingredient) meet all preset CQAs (critical quality attributes). In addition to these tests (based on sampling and destructive testing), there is an unique category that is both an unit operation and a quality verification test, also known as the 100 % nondestructive testing (NDT) based on visual inspection of the final DP in vials, syringes, or cartridges.

Visual inspection is executed multiple times during production runs starting with incoming inspection, followed by assessment of stability and retention samples as the basis for setting specifications, shelf life, and general monitoring, respectively. The stability assessment is limited to those visual attributes that may change over time as opposed to cosmetic inspection.

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NDT testing does not alter the item being inspected and is very useful as an in-process check or as part of release testing. Among many NDT methods, visual inspection is the oldest technique. The purpose of this step is to remove those units that fail specific appearance attributes and to confirm that the percentage rejects falls within historical limits as part of the control strategy. The inspection process serves as the final step to assure that the DP is free of flaws in appearance, no missing components, and confirms the container/device/packaging/label combination is intact and fully functional. The presence of particulate matter as part of the appearance inspection stands out, as particulates are currently one of the top ten reasons for recall events (Doessegger et al. 2012). In terms of DP functionality, i.e., the container in combination with a delivery device, the inspection does play a role but it is limited to what is visible on the surface.

Inspection is an activity that spans the continuum from OEM (original equipment manufacturer) to the DP, with incoming and final visual inspections as the key events. Controls are expected to be in place regarding sampling, defining defect criteria, and accept/reject limits, maximum allowable reject rate (triggers for investigations) and acceptable quality limits as a function of the type of defect.

15.2 Inspection and Regulatory Expectations

Inspection here is not referred to as the inspection as part of the auditing process by the regulators but the process of visual assessment executed by the manufacturer. The appearance specifications reflect a predefined description of expected attributes as a function of the QTPP (quality target product profile) and the manufacturing capability of both the container/closure systems and their content. Inspection processes start with incoming inspection for determining visual and dimensional defects for components using certified inspectors and calibrated inspection stations or fully automated and validated machine vision platforms. The ability to perform 100 % online inspection for CQA can be viewed as part of PAT (process analytical technologies) which is a system to understand, monitor, and control pharmaceutical manufacturing within the context of QbD (Quality by Design per ICH guideline Q8). This includes enabling continuous improvement as well as process verification and executing the control strategy. PAT is a system for designing, analyzing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality.

Sometimes vendors provide in advance samples (“tailgate”) from the same lot as an alternative to the buyer pulling samples. The sample size must satisfy a statistical justification and the defect classification (minor, major A/B, or critical) controlled per guidance document as published by the PDA (PDA, 1998).

Attributes (PDA, 2007) cover correct components, stains, blisters, cracks, marred finish, malformation, air bubbles/air lines, tool marks, deposits, bruises, etc. Some of these defects are called cosmetic which will be covered later in this chapter.

Technical reports by the PDA on defect nomenclature for syringes, stoppers, and plungers are still in progress as of this writing in 2013.

Particle matter in containers is another CQA attribute which raises concerns on risks based on immunogenicity and physiological/toxic effects (Rosenberg et al. 2012; Bee et al. 2012a; Carpenter et al. 2010). Container component handling (washing, etc.) process is expected to mitigate all particle matter and their occurrence must be under control as part of the incoming expectations and quality agreement. The type of particle is defined by its origin which is an important aspect of understanding the potential impact to the patient as well as in applying corrective and/or preventive action.

The major compendia have harmonized testing methodology and pass/fail limit criteria for subvisible particles but not for visible particles which continues to be a source for confusion in per recordings on the PDA. Org website interpretation of various guidance documents. Particulate matter continues to be a major cause for recalls and "483" observations (PDA/FDA Joint Regulatory Conference 2012). Future USP (United States Pharmacopeia) chapters (<790> and <1790>) are being drafted (Madsen et al. 2012) to provide further guidance on visible particle policies as described below.

The in-process visual inspection method is part of process control verification to detect errors or deviations to permit timely corrective action as basis for preventive action. They must be based on SOP's (standard operating procedures) with alert and action limits. The specifics are a function of the manufacturing process and serves to continue or stop of manufacturing as a function of the defect classification.

At the other end of the manufacturing process is the 100 % inspection process which is performed either manually or automatically through the application of mechanical handling and machine vision techniques including the use of multiple image sensors employed throughout the inspection program.

A human inspector is expected to be certified to detect and evaluate appearance attributes after training and certification processes that include passing a blind challenge set (Melchore et al. 2012) that contains a specific set of defects. Likewise automated equipment is validated (Melchore 2010; Rathore et al. 2009) to do the same, though expected to deliver greater consistency regarding sensitivity as human performance is variable.

Though desired, a 100 % flawless visual appearance test is neither achievable nor practical (PDA/FDA Joint Regulatory Conference 2012). Reflecting this reality inspection guidance documents in the United States and EU adopted the description of "essentially or practically free from particles," which is defined as being free from visible, intrinsic/extrinsic foreign particles to the maximum extent possible through both manufacturing controls and visual inspection.

The final DP must reflect both visual elegance and absence of visible flaws that poorly reflect on the manufacturing process that could raise concerns about those product quality attributes that are not readily detectable. As visual attributes consist of a mix of visual qualitative presentations, it is no surprise that as a test it is more complex and less robust than typical chemical tests that result in quantitative data on a single attribute. In terms of cosmetic and functional defects, the expectation is the application of multiple (sometimes 100 %) inspection steps performed by the

manufacturer of the container/closure before shipment to the fill manufacturing site. The DP manufacturing site incoming inspection covers only a small fraction of each component lot and which is no substitute for the final end of DP manufacturing 100 % quality verification among all appearance tests and specifications.

There is one category which is most controversial with respect to industry standardization and understanding of clinical impact (Doessegger et al. 2012). This relates to undissolved matter in parenteral injectables which is undesired in any size category unless it is part of the formulation design as for instance insulin crystals for controlled release or with suspensions. There is zero tolerance for visible matter in contrast to subvisible matter where generic compendial criteria exists covering the upper limit of particle numbers in a size range of ≥10 to >25 μm in small and large volume parenterals (USP 35 NF 30, Chapter <788>) that are aligned between the USP, EP (European Pharmacopeia), and the JP (Japanese Phamacopeia). Recently it was reported that the FDA desires particle quantitation (type and amount) between 2 and 10 μm and characterization between 0.1 and 1 μm (Hawa et al. 2012) including silicone particles.

15.3 Types of Visual Defects

Visual defects cover a range of attributes that cover a number of categories including the drug content, container, medical device or combination product (prefilled syringe), secondary or dispensing packaging, and artwork/text. Defects categories include cosmetic (visual elegance) or functional defects. The types of possible defects are described in detail in the Appendix. Those defects that possibly impact safety or efficacy are in the CQA or CQA category. There is no noncritical attribute though there is a range of criticality rankings within the CQA based on risk assessments.

15.3.1 Particle Types

There are many types and come in multiple shapes, colors, sizes. It helps to sort them by origin as follows:

- Foreign matter
  - Intrinsic matter: associated with the container/closure system including those derived from materials inherently present in the processing equipment and container such as glass, stainless steel, rubber, silicone lubricant (glass syringes), and filter housing plastics, adapted from USP 35-NF 30 Chapter <1788>.

    A unique subset of particles are silicone oil spray/droplets used as lubricant in prefilled syringes which can detach partially from the interior barrel surface as a function of time, vibration, shock, and storage position. Certain

excipients in the formulation such as nonionic detergents can influence the partition of the oil droplets between the bulk liquid and glass surface. Silicone particles are typically subvisible and partially detected by the light obscuration method.

Glass lamellae, another unique category mainly occurring sometimes in glass vials as a function of its glass surface chemistry, forming temperature history, and excipients. A combination of excipient (e.g., chelators), alkaline surface, and pH values at near neutral of basic accelerate this time-dependant phenomena. This makes it stand out among all other particles as delamination is a kinetic event in contrast to other nonprotein static particles. This dynamic event makes removal by inspection a challenge and results in recalls (http://www.rx-360.org/Alerts/GlassDelamination/tabid/234/Default.aspx).

- Extrinsic matter: foreign matter that is unexpected and atypical as it is derived from outside the controlled container and filling-contact material processes such as insect parts, hair, paint chips, depyrogenation oven materials, gaskets, oil droplets, etc. It is non-changing and additive to the contents and not part of the formulation, packaging, or assembly process (adapted from USP 35-NF 30 Chapter <1788>). Additional guidance will be available in the future (2013 or later) in USP <1790> chapter describing visible defect types and inspection technologies.

- Inherent matter related to the API which in the case of therapeutic proteins consists of visible protein aggregates (Das 2012; Mahler and Jiskoot 2011; Singh et al. 2009). There are several unique protein properties to be discussed briefly.
  - These aggregates can as a function of morphology, size, and number impact the potency, efficacy, and safety of the DP. Concerns include occlusion of blood vessels and potential immunogenicity (Carpenter et al. 2009, 2010; Cordoba-Rodriguez 2008).
  - These aggregates typically vary in size and number for each container.
  - The amount of aggregate is typically extremely small (~ or <0.01 %, Bee et al. 2012b). The aggregates are typically neutrally buoyant or settle very slowly as a function of the density. The density of pure protein is ~1.4 g/cm^3 (Quillin et al. 2000) which explains their ready precipitation in a typical isotonic parenteral liquid with a density of ~1.01 g/mL. The aggregates are composed of mostly liquid which explains the translucency.
  - These aggregates are at the individual level often nonhomogeneous in density/refractive index, gel-like, mostly liquid, semitranslucent, though on occasion have a more structured morphology in semicrystalline or fibers (Joubert et al. 2011).
  - Their formation is typically a function of chemical degradation, time (kinetics), and temperature, shear (vibration and shock), and foaming history experienced during the manufacturing and distribution process (Ripple et al. 2012).
  - The aggregation process can be reversible as a function of temperature and time and the combination of the inherent protein properties within the container materials and formulation design.

- A specific category of "immobile" particles are
  - Embedded in the container materials and could be exposed to the surface and can be referred to as "specs."
  - Located or loosely deposited on the surface.
  - Air pockets in container walls specifically in plastic injection molded containers or glass-based containers.
- Apparent particles: micro-bubbles caused by air entrapment present free in solution or adhered to the inner wall or polymer closure.
- They are the basis for false rejects and interfere in efficient inspection and require optimizing inspection parameters for automated systems (Melchore 2010; Rathore et al. 2009).
- *Functional inspection*
  - This area represents those defects that are much more than cosmetic, such as missing components, crimped vials with uneven seal compression, and deviations in critical dimensions. All of these compromise CCI (container closure integrity) or the ability to deliver the entire dose as indicated on the package inset. Expected is a guidance document that clearly assigns different defect categories as a function of risk assessments and functional requirements. The visual inspection for potential failures of CCI by cracks, chips, missing stoppers, is gross leak detection and no substitute for a true leak test (down to 5 μm) which is not in scope.

15.3.2 Limitations of Manual Inspection of Particles

Knapp et al. (1980) published a new inspection methodology data assessment that minimized manual inspection variability through the use of statistical means and controls. Their application reduces variability but nevertheless constraints exist.

The threshold for human unaided visibility of a single particle in a container under optimized condition is ~30 μm (http://www.physics.mcgill.ca/~moore/P101/Lectures/Lecture-16.pdf) with a probability of detection less than 1 %. A single particle at ~200 μm has a near 100 % probability of detection (Melchore 2011). When using a probability of 70 % as criterion (detecting a single particle) for what is practically visible, the size range is approximately 100 μm which is a function of the container fill volume/diameter, particle location/contrast/density/transparency, and properties of the liquid such as refractive index and opacity. It is expected that the DP manufacturer has in place a statistically justified definition of what is visible using traceable particle standards based on the specific DP presentation and using optimized inspection conditions. Machine-based inspection is expected to at least meet and ideally exceed human capability in terms of particle detection both in terms of probability, e.g., 90 % detection of a single visible particle per container and in terms of robustness. Increasing sensitivity to near 100 % typically results in significant increases of false positives, possibly requiring a second tier manual inspection that is discussed later.

Current size standards do not reflect typical protein aggregates in appearance or behavior. The NIST (National Institute of Standards and Technology, Ripple et al. 2012) is working on this and aims to develop standards that meet the typical protein aggregates faithfully.

Inspections performed by humans are variable when comparing inspectors with one another and when comparing performance of a single inspector over time. Humans have however superior interpretation skills that outperform machines in judgment which is important for certain types of particles that are expected and inherently part of the DP presentation.

15.3.3 Particle Identification, Impact, and Next Steps

It is good practice to build up as library of typical (inherent or intrinsic) particles specific to the manufacturing process and facility as a tool to monitor their presence and to rapidly investigate the nature of their observation within the context of assessing the potential impact to the safety and efficacy of the therapeutic proteins or risk to patient. Very useful is Pareto chart analysis comparing the number of defects for each type of defect. Providing inspectors with real size images and properties (morphology, color, buoyancy, typical numbers, size) of these particles, the inspectors can selectively trigger a follow up with forensic identification if extrinsic or atypical particles are suspected. The forensic techniques to achieve that start with isolation of the particles followed by a range of tests. The forensic outcome dictates the next steps in terms of issuing a nonconformance followed by CAPA (corrective action/ preventive action) steps. Key questions that are raised:

- Does the number of containers with particles exceed that as established by the historical record?
- Is the number or size of particles atypical?
- Are the particles intrinsic or extrinsic?
- Can particle matter leach chemicals over time?
- Are these chemicals toxic and compromise safety?
- Impact the stability of the DP and compromise efficacy?
- Can they function as an adjuvant and stimulate an immune response?
- Is the root cause known?
- Was the mitigation through a CAPA (corrective action preventive) completed?
- Can manufacturing continue without a risk of repeated particle contaminations?
- Are these particles a rare event and be detected during the 100 % inspection?

The level of NC (nonconformance) classification is associated with the type of particle, where extrinsic particles typically raising larger concerns than intrinsic particles. It is recommended that the manufacturing sites set up action and alert limits on different categories of cosmetic defects or particles based on product and SKU (stock keeping units) and established their specific historical trends to trigger an evaluation or investigation. This practice will also apply to intrinsic particles as part of a holistic approach to exclude any visible particle presence. Failure to

follow up (identify and mitigate) on particle findings is a frequent cause for observations by regulatory inspectors (PDA/FDA Joint Regulatory Conference Baltimore 2012).

15.3.4 Cosmetic Defects

Cosmetic is defined by the Mirriam-Webster dictionary as "not substantive", which can be applied to beauty or changes lacking a significant impact which is in the category of minor defects such as a small external removable stain or a Major B, such as partially round bottom of a vial. Cosmetic visual appearance evaluations recognize the limits of NDT by visual assessment in terms of defining the infinite number of possible states of appearance followed by interpretation of the findings per the intended application. Cosmetic defects in consumer products (other than vials or syringes) are often small errors that do not prevent or hinder functionality or impact safety, though their presence suggests a certain lack of controls in handling which is undesired. In the case of pharmaceuticals packaged in glass, even minor imperfections or scratches can cause after mechanical or temperature stress catastrophic failure resulting in fractures. A method to quantitatively define and assess the risk of cosmetic glass vial defects is described in Loui (2011). This example illustrates that contrary to the dictionary definition, not all cosmetic defects are minor in the case of DP.

The presence of cosmetic damage is not always caused by the manufacturer of components because mechanical handling by the DP manufacturing site can contribute during all unit operations including washing, drying, sterilization, and handling like conveyor belts, accumulation tables or during pick place and (un)-packaging motions. Areas to pay attention to are container to container contact as well as metal container contact during handling and storage. Examples based on real cases point to vials getting scratched on accumulation tables and compression fractures on syringes during labeling. Glass to glass or metal contact must be avoided or at least controlled.

15.3.5 Dimensional Defects

Dimensional measurements can be performed visually using contact-based go/no-go gauges, machine vision (camera based) tools or the Nikon™ systems using an array of optics or 3D tactile coordinate metrology sensors or the J&L optical comparator. Tactile technology is suboptimal to inspect parameters like needle deflection or soft rubber components, in which case cameras must be used. These contact-based inspections are out of scope. Camera-based inspections followed by digitization are extremely useful for this application but limited by the optical chip pixel size.

15.3.6 Functional Defects

These defects relate to the ability of the container or combination product to maintain sterility and deliver the intended dose to the desired target within the defined time limit. Visual inspection is limited in that regard. For instance, the visual inspection on CCI (cracks, chips, missing stoppers) is gross leak detection and no substitute for a true leak test (down to 5 μm) which is not in scope. X-ray inspection is best for combination products but the energy levels required could compromise the DP. It is an excellent tool for forensic troubleshooting of returned defective combination devices.

15.4 Inspection Technology

The focus is here on noncontact and nondestructive inspection. The inspection solutions range from manual to semi and fully automated vision equipment using a range of different illumination types, duration, container manipulation, camera's, background formats, and size calibration standards, all augmented with human judgment and computer-based image analysis.

15.4.1 Inspection Equipment

Nondestructive particle and cosmetic inspection practices can utilize three types of categories:

- A manual visual inspection (MVI) procedure employing human certified operators seated in front of an inspection booth that provide calibrated white light against a black and white background (Madsen et al. 2012). The operator typically agitates or inverts the container to set the liquid and contents in motion. Additional controls can be added such as position sensors, timers, light sensors, etc. Eisai/Bosch makes a visual vial or ampoule inspection tool with rotation capability that is operated manually and observed manually ("APK" unit) or with a camera ("ETAC easy view"). By pushing the start/stop button, the container is spun and abruptly stopped, leaving the particles swirling in the solution for a brief time. The bottom lighting permits easy detection. Color and turbidity are determined using standard references per USP compendia.
- Semi-automated systems minimize or eliminate the need to manually handle containers by conveying and then spinning the containers in order to set their contents into motion, and positioning them at optimum viewing angles to the inspector. This removes the variability inherent with manual agitation of containers, and allows the inspector to focus only on visual inspection, allowing increased inspection accuracy and throughput while reducing fatigue.

- Automated vision inspection (AVI) is generally selected over manual and semi-automated visual systems in order to increase output and improve inspection accuracy. Automated inspection machines can be categorized according to the manner in which defects are detected using reflected light for particle inspection and cosmetic inspection and transmitted light for particle inspection. Speeds are up to 600 U/h. AVI uses a range of sensors (camera imaging, light obscuration, etc.) in separate modules to inspect containers from multiple angles. The outfeed takes place in several areas permitting segregation of rejected and accepted containers at a minimum. Ideally the rejects are further classified and enumerated in certain categories as part of the inspection control strategy. Image sensors come in different types:
  - Two dimensional (2D) imaging cameras using reflected light as either monochrome or color (less resolution) based on a matrix of cameras (mostly CCD or charge coupled devices) taking snapshots used by Optrel (Stevanoto) and Seidenader (Korber MediPak).
  - Line scan cameras (e.g., used by Innoscan) using 1D chips providing speed and high resolution. Cognex, Keyence are examples of providers of cosmetic and functional inspection solutions using camera technology and image processing software.
  - Light obscuration as employed in the SD (static division: divides stationary shadows from moving particles) technology by Eisai Machinery USA (acquired by Bosch Packaging Technology in 2012) using transmitted LED light for detection of moving shadows (on a 1D diode array), caused by passing particles (Melchore 2010; Rathore et al. 2009). SD is claimed to be superior in size cut-off and with less impact by the particle reflective properties or extraneous particles. Note that light obscuration is also the basis for the Hiac-Royco particle (10–100 μm) analyzer (USP 35 NF 30, Chapter <788>) for injectable fluids in accordance with the USP 788 (USP 35 NF 30, Chapter <788>). Eisai/Bosch is evaluating cameras for biotech products as an alternative or in combination with SD (Hybrid).

Color and turbidity assessment also requires specific technology. Sensor technology covers both color (reflected or transmitted light using a photodiode sensor, camera or off line instrument) and turbidity. Turbidity or clarity is typically based on transmitted light attenuation.

Since the two attributes of color and turbidity/clarity are typically homogeneous across a lot, a single representative sample can be sufficient unless occasional color or clarity defects are a known defect in the specific DP. Some exceptions are known from actual cases such as the occasional Tungsten particle findings in prefilled syringes (Liu et al. 2010) and yellow colors as a result of oxidation of iron particles (derived from iron tools in glass forming) embedded in glass vials and oxidizing.

Opacity is best viewed with a light source using a wavelength as a function of the container material or DP sensitivity. This applies only to a container material that is deliberately not clear.

15.4.2 Techniques to Ensure Visualization of Particles

Any type of inspection must include a manipulation to resuspend settled particles (e.g., glass, metal) within the field of view. This is achieved either by inversion or rapid rotation followed by a stop resulting in the liquid angular rotation decreasing and the meniscus being reestablished in combination with an up flow of liquid within the center axis. The uplifting technique is a function of container geometry, liquid density/viscosity, and headspace volume ration. The process must be validated to employ a combination of manipulations and inspection angles to expose all visible particles where practically possible. The optimal inspection equipment setting is achieved by spiking containers with a range (including size) of possible (anticipated or by history) particles and track inspection performance as a function of settings that can include inversion, rotation (RPM and time), deceleration, and defining the timing windows of inspection.

Inspection of particles in a lyophilized product is limited to detect only those near the surface of the solid lyocake. Efforts are underway (e.g., WILCO AG) to use X-ray analysis using focused low energy beams that do not ionize the API. The current size limit is a function of the particle density and greater than lower limits for visual detection.

The visual "sampling" volume is less than 100 % due the meniscus shape, refraction blind spots (Smith et al. 2012) and bulge formation on the vial bottom. The introduction of additional cameras and lights at multiple angles will address that concern to some extent. The tendency in biotechnology has been the gradual introduction of more highly concentrated DP resulting in a viscosity increase which is a major barrier to desired state of >90 % detection of visible dense matter. This complicates the equipment setup validation process as the probability of detection of heavy foreign particles must remain high. The use of a mimic solution reflecting the rheological properties of the drug product is recommended to verify the detection capability or optimize settings.

The choices of light sources (wavelength spectrum) and illumination angles, possibly aided by mirrors, etc. are another variable to be optimized in the equipment set up. Typically white light is used covering violet (~420 nm) to red (~750 nm). UV light is detrimental to certain chemicals including therapeutic proteins. Light sources cover incandescent, halogen, electronic ballasted (minimizes 240 Hz flicker) fluorescent tubes or more recently white LED lights. Light intensities are typically 2,000–2,750 lx per EP but even greater (8,000–10,000 lx) for plastic containers per JP (Japanese Pharmacopeia, 15th edition).

Regarding inspection of text, art and 2D codes, or embossed data (e.g., Braille), a wide range of generic machine-based inspection solutions exists as automated proofreading and is not covered in this chapter. Neither is brand protection inspection as overt attributes are very brand specific and covert features are "hidden" and proprietary, and use micro text, color shifting, temperature responsive dyes, and UV fluorescence to name a few examples.

15.4.3 Human vs. Machine Inspection

The final inspection is performed by the human end user or healthcare provider. Naturally inspection started with humans in spite of its many deficiencies and lack of robustness. Machine vision is replacing human inspection with the expectation is for it to meet and slightly exceed human capability, except for one area. Machine vision cannot detect new types of defects unless preprogrammed to do so which is a contradiction. Therefore human inspection is the reference standard on which to base machine acceptance criteria for validation. In addition human inspection will be required for new products to ensure the machine vision programming is adequate. Finally, humans far exceed any artificial intelligence in its ability to interpret visual findings especially within a forensic context. This very relevant to place a defect within the "what is known" concept based on prior knowledge. A practical application is the use of high quality images or videos as basis for human inspection and study rather than rely on eyesight alone.

15.4.4 Opportunities for Improvements and Technology Trends

Manual inspection leverages the far superior capabilities that humans have in evaluating difficult to inspect containers (e.g., with air bubbles) in comparison with machine vision. Machine vision benefits from greater efficiency, contrast sensitivity, and robustness.

Its implementation within a cross-site integrated data management network will improve OEE (overall equipment effectiveness) and allow faster multisite data sharing and permit remote visual monitoring and intervention by experts. Rather than providing a high level of expertise at all manufacturing sites, a data sharing network enables a centralized expert team to rapidly and consistently interpret and evaluate appearance deviations.

The vision technology will benefit from continuous improvements in resolution optics, faster computing, faster and denser chip sensors, sophisticated algorithms, and improved or innovative mechanical engineering which can improve line speed. The image processing industry (e.g., textile pattern defects) is applying neuron network computing for pattern recognition across a wide range of images to recognize and classify visual defects. This applies mostly to appearance including the Lyo cake (Veillon 2012) and excludes particle type classification thus far.

Currently the installed infrastructure typically focuses on high speed. A trend is taking place with the emerging market to locate final packaging and inspection locally. This means that line speed becomes second to the ability to rapidly clear the line and inspect relatively small lots. In those cases, the inspection equipment must require less setup time and accommodate more SKU's with the least number of

change parts. In this scenario the inspection could be improved by a more extensive examination per container.

The lack of protein-like standards as of 2012 leaves synthetic (polystyrene, glass, etc.) beads as the only option for validation testing. The optical properties of proteins (scatter light) are very different from traceable microspheres (polystyrene) or glass beads that reflect light. Initiatives are underway to mitigate this gap (Ripple et al. 2011).

15.5 Inspection Strategy

Inspection is a complex and laborious time consuming procedure. Therefore it should be executed within an environment of clear rules based on sampling statistics, expectations, allowing for consistency and efficiency. Access to a well-equipped forensic lab is essential as part of the root cause analysis and mitigation.

15.5.1 In Practice

The visual inspection processes and interpretation require an upfront risk assessment per ICH Q9 guideline of all attributes with descriptions that guide the inspection process and related decisions regarding defect classification. Defect categories are critical, major A/B, or minor, the latter being acceptable. In contrast, a critical or major defect can result in a NC (nonconformance) per established quality system procedures.

A single appearance release test alone is insufficient for batch release testing and a complete program for the control and monitoring of particulate matter remains an essential prerequisite (Madsen et al. 2012). This is the basis for the 100 % inspection requirement (typically owned by manufacturing) and acknowledges the probabilistic nature of visual defects across a lot. The next step is a test to validate through acceptance sampling (acceptable quality level [AQL] based by the Quality unit) that the 100 % inspection met the preset target which is based on statistics within the context of realizing that producing units 100 % free of any major or critical defect is not practical, even though it is desired.

The effectiveness of 100 % the inspection is a function of its sensitivity which, when pushed to extremes, will result in false rejects. There are specific cases known to occur which cause false rejects during automated inspection. For instance, the presence of small air bubbles that either adhere to the inner surface or move within the liquid column interfere with machine inspection. Another example is based on incorrect particle differentiation and identification. An example is based on turbulent flow during the inspection process where it is possible for dense particles to ascend and light “particles” (like air bubbles) to descend momentarily. The

implication is that manual particle tracking over several seconds or more can be required for proper interpretation.

These examples are often associated with therapeutic proteins or proteinaceous excipients (e.g., human serum albumin) that can act like nonionic detergents and lower the surface tension resulting in surface foam and/or micro-air bubbles.

Automated inspection equipment technology as it currently stands lacks the algorithms to track particles and correctly interpret that certain "defects" like air bubbles which are acceptable. The mitigation of false rejects has been addressed by some manufacturers as follows in case of automated 100 % inspection and specific susceptible products:

- The out-feed of particle containing units is directed to an "eject" area.
- These "ejects" are reinspected by a different method which is the certified manual process and is thus not defined as rework.

The eject reinspection must be justified on a product and SKU basis. This practice is referred to as a two-tiered inspection approach which can work well if

- The line speed is high for the first tier and the eject rate relatively low, e.g., 5 %.
- The line speed of the second tier can, in this case, be nearly 20 times as slow and still keep up with the processing speed.

15.5.2 Interpretation

The verification of the effectiveness of the 100 % inspection process to define what is acceptable or not, is referred to as the AQL test also referred to as ASP (acceptable sampling planning), a procedure to define the acceptable level of risk to the consumer in receiving a defective product. The PQL (product quality level) reflects the statistical risk to the producer rejecting an acceptable product.

The 100 % inspection unit operation is accepted if the number of defects observed during the inspection by certified inspectors in the Quality unit falls below the AQL as a function of the number of units sampled randomly taken from the previously passed containers. AQL inspection is typically performed manually (MVI) using the inspection booth as described above with the exception that no time limit is applied, enabling the inspectors sufficient time.

There are several scenarios in this process:

1. The AQL is exceeded and the validity of the 100 % inspection is in question. Action is to tighten the statistics by inspecting additional containers and compare the results against the tightened AQL. The pass/fail criteria are based both on preset guidelines that are based on sound and defendable statistic and include the data from the first round of AQL testing, which cannot be ignored.

2. The AQL process by manual inspection can on occasion reveal the presence of a subvisible particle based on probability of detection and due to the higher inspection time. Particles below e.g., 100 μm can on occasion be observed but are defined as subvisible because the probability is <70 %. Technically speaking this is not an AQL failure. Yet this observation must be followed up by an evaluation if the particle is or could be atypical (extrinsic) based on its appearance. A NC (nonconformance) can be issued if the number of subvisible intrinsic particles is atypical or if the subvisible particle is extrinsic. The only firm test to determine that is by particle isolation followed by filtration and forensic techniques (Shearer 2003; Narhi et al. 2009).
3. If the AQL inspection reveals possible glass lamellae, a forensic confirmation must take place and the entire lot put on quarantine as the presence of glass lamellae is a dynamic process and could grow more intense over time.

Note that in the Quality world, AQL (risk to producer) is used in combination with RQL or UQL (rejectable or unacceptable quality levels, respectively) representing risk to consumer. A detailed discussion of these concepts is outside the scope of this chapter.

15.5.3 Specification Setting of Product Appearance

Specifications for appearance are based on a combination of process capability and assurance of safety and efficacy combined with a sufficient number of test results that form the basis of a statistically justified sampling plan and number of lots. The typical definition on particle appearance states "essentially free from particles" in the United States or "practically free from particles" in the EU. The USP 35 chapter <790> (USP Pharmacopeial Forum 2012) provides specific guidelines as to the inspection methods referring to ISO standards (2859-1) with an AQL (ANSI/ASQ Z 1.4) of ≤0.65 % with 95 % confidence, with intrinsic particles being in the Major A visual category. AQL levels for critical defects are in the range of 0–1 % (Leversee and Shabusnig 2008).

As the regulations evolve, the most current USP section applies while anticipating future changes in the direction of gathering more quantitative data. Detecting no more than a specified number of drug product units containing visible particles, as specified in the sampling plan, means that the lot is "essentially free from particles." The definition does not apply to a single unit. The current draft <790> describes a two-stage process. Pass if zero rejects in 20 samples. If one reject, no particles may be observed in an additional 80 samples. A new USP chapter <1790> will be added as a companion chapter to <790>.

For therapeutic proteins, additional information on inherent protein particles can be provided in protein specific monographs, using (Narhi et al. 2012) for nomenclature guidance.

For critical defects (e.g., glass lamellae and extrinsic visible particles) the AQL values are less than 0.65 % and must be defined by the Quality System.

15.6 Case Study Examples

Glass vials were rejected based on black spots, not seen previously. The investigation shows that the black spots are deposits rather than black particles in solution. Next the deposits are analyzed for their location and found to be present on the outer surface and not embedded in the glass. Pictures are taken and all reject vials are evaluated for number, size, and location of the black spots. The forensic results show the black deposits to be carbonized material present in the exterior several % of the vials with each vial containing 1–3 spots ~1 mm in diameter located near the neck. The impact assessment shows no risk to drug product quality so classified as a major B defect. The next step is to put in place amendment to the inspection process until the root cause has been mitigated. Specifically a "tactile" tool will be used to determine if the black spots if seen in the reject bin are located on the surface and removable. Any container with an interior black spot will be segregated for forensic analysis. The root cause analysis in collaboration with the vendor demonstrated traces of lubricant that was carbonized in the depyrogenation oven. The situation was corrected and the inspection amendment terminated.

False rejects impact yield as described by Nielsen (2012) who presented a range of causes related to foaming, condensation, adhesion, droplets, micro-bubbles, etc. An example of this was observed during machine-based inspection of injectables where a high number of defects (~14 %) were observed in prefilled syringes of which only ~4.4 % were confirmed, mostly with minor cosmetic non-particle-based observations (Frantz 2011). The investigation revealed presence of air and was related to the stopper insertion process and timing of inspection after the fill. The camera could not distinguish efficiently between a defect and a bubble. The mitigation was a manual inspection on the ejects or reclassified rejects. Implementation resulted in only rejecting true defects and savings in case where the eject rate is high.

An example of equipment contributing to mostly subvisible and a few visible particles was published (Tyagi et al. 2009) on a study involving vial fills with 20 mg/mL of an IgG molecule using a positive displacement pump equipped with a stainless steel rotary piston pump. The data show that nanoparticles (mostly 1.5–3 μm) of stainless material can serve as heterogeneous nuclei for the formation of hybrid protein aggregates of which a very small subset was visible. The release of stainless steel particles occurred also with pumping a placebo solution (Tyagi et al. 2009).

Similar observations were made by Nayak et al. (2011) who confirmed the conclusion and extended the findings by comparing particle shedding using different types of pumps. In case of direct steel to steel sliding friction and lubricated by the liquid drug product solution, the shedding was higher than for other pump types

These cases are important as the filling operation takes place at the end of the manufacturing operations and no further filtration takes place beyond the final sterile filtration. This illustrates the importance particle identification within the frame work of selecting any type of technology as in this case the filling pump working principle.

Finally a case of disposable materials shedding particles lists syringe filters as a source (Liu et al. 2012). In all these cases the foreign material was subvisible but in combination with proteins, some hybrid particles can form of which a small population can become visible as a function of additional processing like agitation or storage time in the case of solutions containing proteins.

15.7 Conclusion and Discussion

The appearance attributes are considered a CQA of the drug product. Visual inspection is a complex unit operation that stands as the last step before the DP is shipped to the end user. The goal is to deliver DP free of visible defects which can only be achieved by 100 % visual inspection realizing that it is not 100 % effective, which is why the regulatory compendia refer to particle levels as being “essentially free” or “practically free” of particulate matter, in recognition of the fact that “free of particles” is not a practical meaningful specification. Note that the expectation is to track and control reject-numbers (defined as maximum allowable % rejects per lot) per each defect category (GMP Trends 2012).

The inspection process provides continuous feedback on the effectiveness of the manufacturing unit operations verifying they remain in the validated state and furthermore provide opportunities for improvement.

The inspection itself applies multiple techniques to survey a range of attributes which are often more qualitative than quantitative. Of all visual attributes, particulates are most difficult to assess, after micro-cracks in the container, possibly compromising sterility. The introduction of therapeutic proteins added a new level of complexity associated with aggregation which is only partially understood. The inspection process is part of the expected control strategy as long as the process is not used to inspect quality in but to validate the absence of atypical defects and a control over the amount of typical, though undesired, defects. The documented characterization of typical particles including those associated with proteins is recommended through the establishment of a library including chemical identification.

The previous information touched on foreign matter (e.g., paint flecks, hair), false defects (air bubbles), color defects, intrinsic issues (glass lamellae), inherent particles (protein particles) as examples of real cases. Other examples of intrinsic matter are glass chips, rubber fragments (plungers, stoppers), stainless steel derived for instance from positive displacement piston pumps and fibers, typically cellulose or polyester based.

In all instances, one is expected to identify the source of these material defects or particles as a preventive action. Merely removing impacted units as a correction is

insufficient as an action item. Failure to perform a thorough investigation is a major reason for regulatory observations. The investigational path guides the root cause analysis upstream from the observation, to the process all the way to the incoming materials (Guidance for Industry 1999).

Typically the defects are described by size, occurrence, or number in multiple batches including retention batches or QA (Quality Assurance) reserves using a mix of validated assays and qualified orthogonal methods to achieve a full understanding. This investigation is further expanded to include the entire manufacturing infrastructure including inspection of all upstream equipment including that used to execute component preparation such as washing, sorting packing, depyrogenation, and sterilization. Finally all equipment and facility maintenance schedules are mapped with respect to timing the NC events. A holistic and comprehensive analysis by multidisciplinary functions is ideal to complete the CAPA and close out the NC.

A robust quality system requires a visual defect class risk assessment followed by mitigation plans to address the different categories of visual defects. This entire program must be rigorous, science based, and is referred to as risk management.

The combination of risk management (assessment and mitigation), root cause analysis (aided by forensics), defect libraries, and continuous process verification using validated inspection processes with SOP-based procedures for outliers, provide a solid foundation for the control strategy and the ability to justify NC closures to regulatory agencies.

Appendix: Inspection Attributes

1. Cosmetic
   (a) Surface scratches
   (b) Minor defects of the closures or label appearance
   (c) Major to critical defects of same
2. Content
   (a) Liquid:
      - Fill volume or weight
      - Observable matter as insoluble particles
      - Color: e.g., slightly pink or yellow
      - Clarity or degree of turbidity

   (b) Solid or Lyo cake:
      - Cracks
      - Collapse, shrinkage, etc.
      - Powder deposits above the cake
3. Functional

(a) Attributes related to handling, proper dosing, sterility, and stability

- CCI: defects such as cracks, vial crimp, etc.
- Container/closure and component shape dimensions
- Bent needles, needle shields, needle tip/hook, etc.
- Graduation marks
- Medical devices—scope can include injection aids such as an injection pen
- Opacity of container material or overwrap used as sometimes the contents are light or oxygen sensitive

4. Labeling, bar codes, etc. for accuracy, format, color, readability, etc.

(a) Text, artwork, 1 and 2 dimensional bar or QR codes
(b) Package inserts or leaflets
(c) Brand security anti-tampering and anticounterfeiting
(d) Braille format embossing for products sold in the EU member states

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USP Pharmacopeial Forum Vol. 38(6), Nov–Dec 2012

Chapter 16
Sterile Filtration: Principles, Best Practices and New Developments

Herb Lutz, Randy Wilkins, and Christina Carbrello

Abstract This chapter starts with a historical overview, description of sterile filtration applications, and performance requirements. Special attention is paid to the sterilizing final fill application for biotherapeutics with associated regulatory requirements. Sterile filters, their properties, manufacture, retention mechanisms, and economics are described. The chapter then covers how to develop, implement, and validate a sterile filtration process. Methods for filter selection, testing with scaled-down devices, sizing, system design, and operation are included. The use of filter bacterial challenge studies, system sterilization, and integrity testing for validation of the filtration process are covered. The chapter finishes with case studies in several areas the authors felt warranted special consideration.

16.1 Historical Overview

Early references to water purification by filtration include sand filtration in the Sushita Samhita Sanskrit text from the third or fourth century CE, and cloth filtration using a sleeve designed by Hippocrates in 400 BCE (Baker 1949). Wine clarification or fining employed flocculants and cloth filters. These were designed to enhance fluid clarity and thereby improve their appearance, odor, and their healthful quality. Cellulose fibers were applied to beer in 1892 (Enzinger 1892) and shortly thereafter, the Seitz brothers added asbestos and diatomaceous earth (Purchas and Sutherland 2002).

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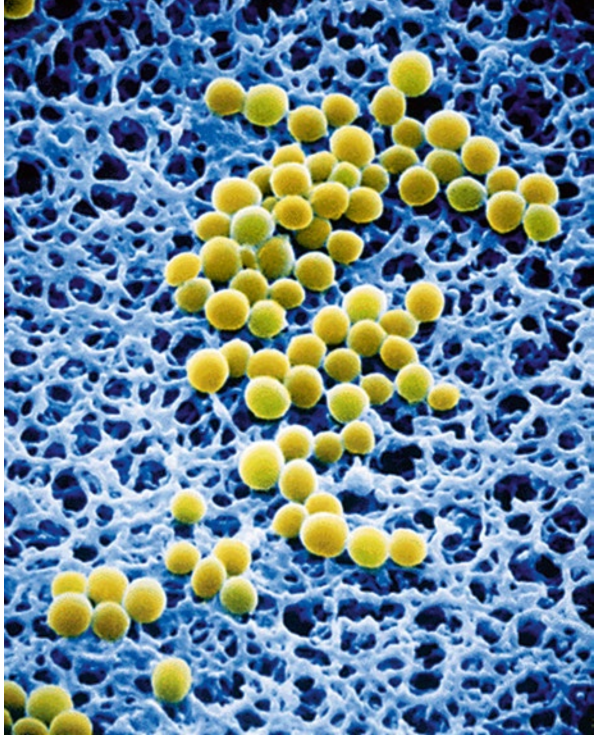

Fig. 16.1 *Staphyllococcus aureus* retained on the surface of a 0.45 µm cast Durapore™® membrane

The development and application of the microscope by Hooke and others revealed a new world of nonvisible microorganisms. Sintered glass and clay filters were used by Pasteur in 1884 for bacteria removal (Sykes 1965). John Snow in London and Robert Koch in Munich correlated sand filtration with reduced cholera bacteria and reduced cholera disease cases (Baker 1949). Zsigmondy and Bachmann (Zsigmondy and Bachmann 1922) introduced a cast cellulose ester membrane in 1922 that became widely used to measure the microbial populations and safety of drinking water throughout Germany (Fig. 16.1).

Along with the appreciation of the role of microorganisms in affecting health has come interest in controlling these microorganisms to ensure the safety of foods, medicines, and breathing gases. Sterilizing technologies such as heat, chemical treatment, and radiation have also been widely applied but can significantly degrade some products. Sterilization by filtration with minimal effects on the liquid and gas feed material is enabling for these labile products. This is especially important for expensive therapeutics with high purity specifications to maintain safety and efficacy. Filtration using membrane filters operating by size exclusion has

performed in a robust manner—insensitive to feed solution properties and operating conditions. It is estimated that billions of doses of sterile filtered pharmaceuticals have been delivered.

Sterile filters have evolved considerably over the years to increase their reliability in large-scale manufacturing, retention of smaller microorganisms, and degree of sterility assurance. New materials, formats, and multilayering strategies have enabled higher flows and reduced flow decay by plugging. Filter operation and use has also been facilitated by the evolution of integrity testing, validation services, and extractables and compatibility services. The field of sterile filtration is still evolving and more developments are expected in the future.

16.2 Applications

Sterile filtration delivers a very high level of purification to ensure a high probability of product sterility in liquids or gases. This makes it suitable for treating a variety of therapeutic liquids and gases used to treat sick patients such as parenteral fluids (Water for Injection or WFI, IV saline), injectables (protein therapeutics, vaccines), and breathing gases. Fermentation processes that produce products (biotherapeutics, beverages, fuels, and chemical raw materials) using a monoculture of microorganisms must also be protected from contamination by using sterilized liquid and gas feeds. Other applications can include sanitization of beverages (e.g., drinking water) or breathing gases for underwater diving.

A typical biotech manufacturing process involves product expression from microbial cells in a fermentor or mammalian cells in a bioreactor, followed by multiple purification steps, and filling to vials or syringes for administration. Sterile filters are used to filter both the liquids entering the bioreactor (e.g., growth media, antifoam, and bicarbonate additions for pH adjustment) and the gases (e.g., sparge air, vents). Sterile filters are also used to manage bioburden levels throughout the downstream purification process. This limits microorganism byproducts such as endotoxin from contaminating the therapeutic product and manages the load of microorganisms that must be removed in the final fill to ensure a high level of product sterility (Fig. 16.2).

The process requirements and validation needs differ between 1) sterile filters used to produce final product with a sterility claim, and 2) sterile filters without a sterility claim used to manage the level of bioburden in a process or improve the economics of manufacturing by lowering the risks of contamination and product loss.

The sterile filtration process contains the sterile filter, typically with inline pre-filters to prevent plugging, and with valves and vents to permit filter flushing, venting, sterilization by autoclaving or steaming, processing, recovery, and integrity testing (Fig. 16.3). The recent development of plastic assemblies that are pre-sterilized (e.g., by gamma irradiation) has simplified the assembly and its operation.

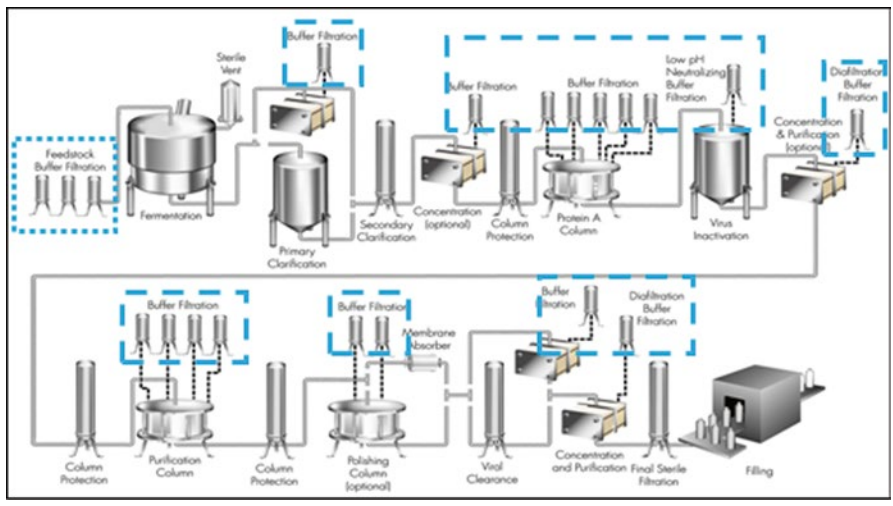

Fig. 16.2 Example biotech manufacturing process using sterile filtration

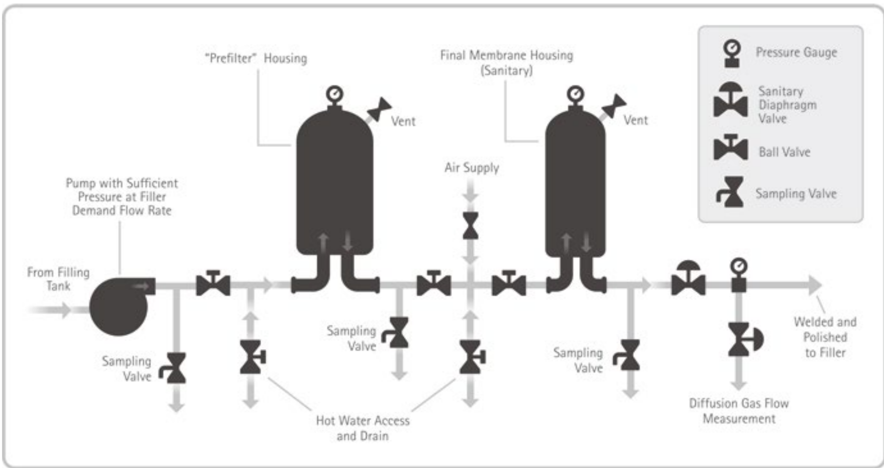

Fig. 16.3 Example sterile filtration process (Goel et al. 1992)

16.3 Sterile Filter Performance Requirements

16.3.1 High Microorganism Retention

Each application has a population (number and type) of microorganisms in the feed solution, referred to as the bioburden (Table 16.1).

Microorganisms are enumerated as colony forming units (CFU) per ml of solution (American Public Health Association 2005). The total CFU microbial count is the

Table 16.1 Small microorganisms found in sterile filter applications (Carter and Levy 1998; Chen et al. 2012)

Microorganism	Length × height (μm)
Burkholderia cepacia	1.0–1.15 × 0.43–0.46
Pseudomonas fluorescens	0.9–1.17 × 0.22–0.53
Ralstonia pickettii	1.37 × 0.48
Pseudomonas pseudocaligenes	1.06 × 0.32
Pseudomonas luteola	0.72–0.86 × 0.33–0.39
Stenotrophomonas maltophilia	0.88–1.40 × 0.44–0.52
Pseudomonas stutzeri	1.22–1.28 × 0.37–0.50
Pseudomonas testoseroni	0.99 × 0.38
Bacillus cereus	1.19 × 0.36
Rickettsia	0.3–0.5 × 0.8–2.0
Carnobacterium maltaromaticum	0.5–0.7 × 3.0
Leptospira licerasiae	0.1 × 6–12
Acholeplasma laidlawii	0.3 dia
Brevundimonas diminuta	0.68 × 0.31

CFU/ml concentration C times the ml volume of the feed V. Sterilizing-grade filters significantly reduce the microbial count in the feed challenge to a tiny or zero microbial count in the filtrate. This performance is quantified using a log reduction value (LRV):

$$\mathrm{LRV} = \log_{10}\left[\frac{C_{\mathrm{feed}}V_{\mathrm{feed}}}{C_{\mathrm{filtrate}}V_{\mathrm{filtrate}}}\right] \tag{16.1}$$

Higher LRVs mean larger reductions in bioburden and higher filtrate sterility assurance. One can use this to determine the risk of product contamination. Typical process stream can contain 0.1–50 CFU/ml with WFI having a USP spec of <0.1 CFU/ml (American Public Health Association 2005). Sterilizing-grade filters are qualified with a 10^7 CFU/cm^2 challenge (ASTM, Standard F838-05 2003) so a 10″ cartridge shows >10.8 LRV of bioburden removal. For equal feed and filtrate volumes, (16.1) requires WFI filtrate to have a concentration of $<10^{-11.8}$ CFU/ml. For common processes, 1 ml = 1 dose, so this becomes $<10^{-11.8}$ CFU/dose. For 1 dose, a $10^{-11.8}$ CFU is not physically meaningful. However, interpreting this value in the context of probability implies that less than 1 out of $10^{11.8}$ doses will be contaminated. The industry accepted value for sterilization processes is less than 1 out of 10^6 doses will be contaminated so this example shows a filtration process that meets industry criteria for sterility. Aseptic process media fills show contamination rates of <1 out of 10^3 doses (US FDA 2004), attributed to system contamination and false positives.

16.3.2 Filter Strength

Sterile filters require strength to maintain LRV under physical and chemical stress during processing. This includes being subjected to high pressure differentials and pressure shocks from valves opening and closing during filtration system

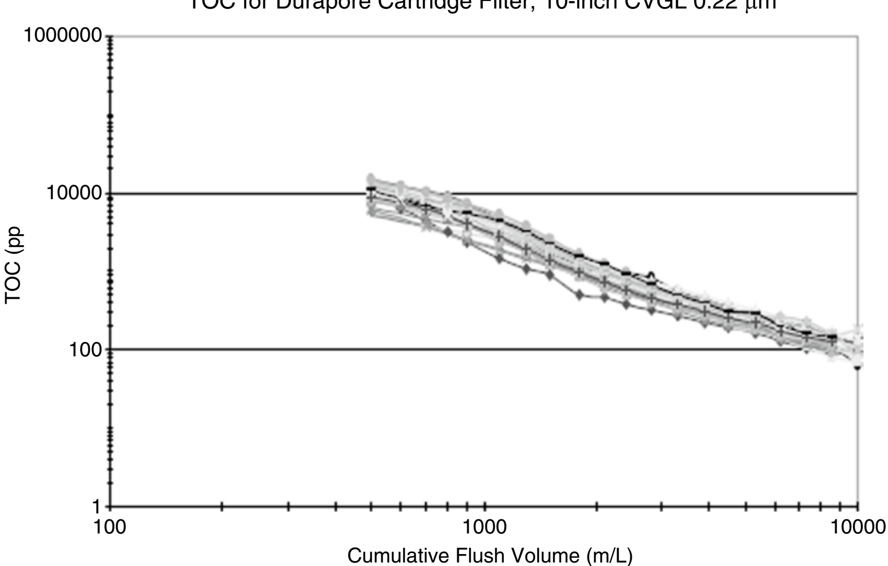

Fig. 16.4 Flush-out curve (Durapore Cartridge Filter Validation Guide 2012)

operation. It also includes maintaining strength under high temperature operations, both static (i.e., autoclaving) and dynamic (i.e., steaming). Polymer glass transition temperature and heat deflection temperature provide a guide. However, extended use in an application may also incur polymer creep (Goel et al. 1992).

Chemical attack can come from process fluids such as solvents but also from sanitizing agents using oxidation or extremes of pH. The increased adoption of single-use systems also requires insensitivity to sterilizing doses of gamma irradiation. All components in the sterile filter system must show minimal swelling, corrosion, and crazing. Filters with large surface areas and surface coatings may not strictly comply with the solvent compatibilities listed in polymer handbooks or manufacturer manuals.

16.3.3 Clean and Inert

Not only must microorganisms be removed but also significant amount of contaminants must not be released into the product (International Conference on Harmonization 2011). Pre-use flushing reduces various contaminants (ions, organics, particles, and endotoxin) in the filtrate. Some components in the filter system could potentially leach out during processing and be found in the filtered product. Extractables are components in the system that can be released into a liquid after extended exposure (International Conference on Harmonization 2011; Stone et al. 1994, 1996).

These give one an idea of the leachables released into the product during processing (Skidmore 2012; Yao 2001). These leachables need to be significantly below any toxicity levels in the drug product (Stone et al. 1994, 1996; USP 1990). USP Class VI testing employs rabbit implants and cell viability to assess toxicity levels (USP Section <85> and <88>). Vendor validation guides typically provide flush-out curves (Fig. 16.4) showing conductivity or nonvolatile residues (NVR) and total organic carbon (TOC) vs. L/m^2 flush (Durapore Cartridge Filter Validation Guide 2012). One convenient endpoint for flushing is to meet WFI levels which are generally recognized as safe. Higher post-flush levels are sometimes acceptable but require some additional data to demonstrate contaminant safety in the product and/or further removal in subsequent steps.

Particle shedding can be measured by light scattering or collecting on a downstream filter with microscopic examination. Relatively large particles >5 μm have a USP spec for injectables (USP Section <788>). Smaller metallic particles shed from pumps or mixers have been shown to act as a nucleation site for the formation of aggregates (Tyagi et al. 2009).

Filters must also not contaminate the filtrate with biological material. Most filter components do not facilitate bacterial growth. To demonstrate bioburden control, vendors lot release filters based on endotoxin levels in an extract (USP 85).

Inertness refers to the filtration system not having any significant effect on the product. Most filters in common use today show minimal (<2 g/m^2 frontal area) protein binding (Pitt 1987; Brose and Waibel 1996) (including support layers) and no significant product denaturing by shear or adsorption-desorption processes (Trusky et al. 1987). One must also check for binding of hydrophobic or negatively charged low molecular weight excipients (Bin et al. 1999, 2000; Nobuo et al. 1992; Guilfoyle et al. 1990; Kakemi et al. 1971; Zhou et al. 2008; Mahler et al. 2010). These can penetrate through the hydrophilic coating and bind to the hydrophobic base membrane polymer. Air interfaces associated with pumping or trapped air bubbles, especially in combination with shear, can also denature proteins (Thomas and Dunnill 1971).

16.3.4 Validateable

Validation involves developing scientific evidence demonstrating that a process can consistently and reliably meet performance claims associated with its intended function (US FDA 1990). Typical sterile filtration process claims include a high probability of filtrate sterility and no significant alteration of the purity of the filtrate. This requires a retention study where LRV is measured using a microorganism spiked into the feed solution processed through a scale-down model of the filtration process (US FDA 2004). A sterility risk assessment identifies the process parameters that may require further operating constraints to reliably meet the sterility claim. This includes: (1) the use of an integrity test to ensure the filtration system has no leaks and the filter is undamaged and properly installed in the mfg. process,

(2) mfg. process bioburden monitoring to ensure the microorganism used in the retention study continues to be relevant, (3) replicate mfg. scale runs to demonstrate the process is under control (i.e., performs consistently), and (4) validation of system sterility downstream of the filter.

Vendors generally provide confidential information to regulators using Drug Master Files containing details of manufacture, release specification and testing, and product qualification.

16.3.5 Economy

A sterile filtration process incurs costs of roughly $0.01–5.00/L associated with the filter, product losses, buffers, labor, capital equipment, and validation. Any risk to product sterility would impose the highest cost. For high value biotherapeutics, yield loss due to holdup in the system is the next biggest cost. In a large facility with multiple filters, flushing water could be a manufacturing bottleneck. Filter costs are generally small compared to product value except for some applications (e.g., beverage, animal vaccines).

Yield loss, flushing volumes, and filter costs scale with filter size. For nonplugging applications, high permeability filters are selected to deliver high flows at moderate pressures with modest filter areas. For plugging applications (i.e., permeability decays with filter throughput), multilayer sterile filters and inline prefiltration are used to handle the higher load of plugging particles.

16.4 Filter Media

16.4.1 Morphology

A wide variety of filter media exist but cast polymer membranes are the filters of choice for sterile filtration (Fig. 16.5). Membrane filters are rigid with a relatively narrow pore size distribution and can provide consistent high retention based on size exclusion. Thin granular or large fiber beds can be nonrigid, have a relatively wide pore size distribution, and provide low retention based on size and adsorption. They are generally referred to as depth filters. Hollow fiber membranes are not generally used for sterilizing applications due to their lack of strength and variability compared to flat sheet membranes.

Cast polymer membranes have a high porosity foam structure that imparts a relatively high permeability. Track etched filters use radiation exposure and etching to create a uniform pore structure with low permeability. Symmetric membranes have uniform structure throughout their depth while asymmetric membranes vary (Fig. 16.6).

Asymmetric membranes show high retention with improved permeabilities and capacities compared to symmetric membranes. It takes a relatively thin layer of small

Fig. 16.5 Filter surface view pictures using optical and scanning electron microscopy (SEM). Clockwise from *top left*—cast polyvinylidene difluoride membrane, track etched polycarbonate, glass fibers, and cellulose fibers impregnated with diatomaceous earth

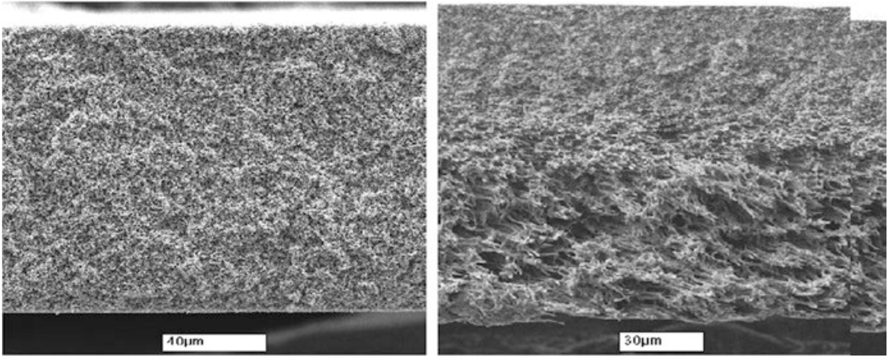

Fig. 16.6 Cast 0.2 **µ**m membrane filter side view pictures using scanning electron microscopy (SEM). (*Left*) Symmetric polyvinylidene difluoride Durapore™® membrane, (*right*) asymmetric polyether sulfone Millipore Express® SHF membrane

Table 16.2 Cast membrane microfilters

Nominal rating (μm)	Use	Water bubble point (psid)
0.1 Durapore®	Mycoplasma removal	70
0.1 Millipore Express®		105
0.22 Durapore®	Bacteria removal	50
0.22 Millipore Express®		58
0.45 Durapore®	Bacteria assay	28
0.65 Durapore®	Cell removal	14

diameter pores to be retentive (Leahy 1983) but a thicker membrane is required for mechanical strength. One can open up the pores in the support to increase permeability while maintaining strength. If the open support layer faces the feed solution, it can also serve as a pre-filtration layer to protect the retentive layer from plugging.

Membrane filters require a hydrophilic (water liking) surface for liquid applications and a hydrophobic (water hating) surface for gas applications. A surface chemistry can be applied to the base polymer to impart these characteristics. The surface chemistry must remain bound and stable during temperature cycles and process conditions.

16.4.2 Pore Size and Bubble Point Ratings

Different pore size estimates can be obtained on the same membrane using retention of model particles, optical processing software on an SEM picture, or porometry (bubble point) (Table 16.2). For sterile filters, microorganism retention is the key performance property. Pore size is a useful nominal value that is a rough indicator of relative performance.

As suggested by A. Einstein (Leahy 1983), at the bubble point upstream gas pressure, the gas–liquid meniscus is forced through the filter so convective gas flow or downstream bubbling is observed. This pressure is given by the modified Young–Laplace equation (Einstein and Muehsam 1923; Young 1885):

$$\mathrm{BP} = \frac{4k\gamma\cos\theta}{d} \tag{16.2}$$

where k = shape correction factor, γ = gas–liquid surface tension (72 dyn/cm for air–water at 25 °C), θ = contact angle (0° fully wet, 90° beaded up non-wetting), d = pore diameter.

16.4.3 Membrane Manufacture

Sterile filter membranes are most commonly made by casting polymers (e.g., polyethersulfone, polyamide, or various fluoropolymers) into porous films by

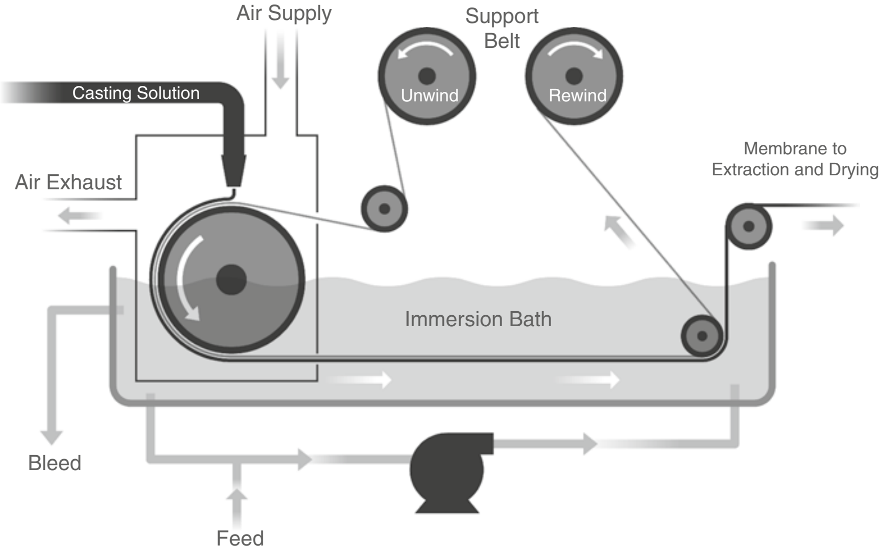

Fig. 16.7 Polymer membrane casting

immersion casting on a web support (Zeman 1996). A multicomponent, high polymer concentration (5–25 %) viscous solution (lacquer) is well mixed, degassed, and filtered to remove particles or trapped air. The lacquer phase separates upon contact with a nonsolvent bath into a foam-like structure and the polymer-lean phase becomes the pores of the MF membrane.

Flat sheet membrane is commonly formed by metering the lacquer through a slit to coat a support web lying on a casting drum. This web coat is fed into an immersion liquid bath that causes the lacquer solvent to diffuse out of the lacquer coat. The solid polymer phase then nucleates, grows, and coalesces into its final structure. Co-casting involves feeding two lacquers through two parallel slots to form two interconnected structures. Key control parameters can include the composition of the lacquer, composition of the immersion bath, and coating or extrusion thickness (Fig. 16.7).

Residual solvent and anti-solvent are washed out of the polymeric membrane structure in separate extraction baths. Hydrophobic polymers are surface modified or blended with hydrophilic polymers to render them hydrophilic and thereby reduce fouling, reduce product losses, and increase flux. Surface modification can include chemical grafting of hydrophilic groups to the base material surface or entangling hydrophilic polymers within the base material to coat the entire surface.

16.4.4 Particle Capture Mechanisms

Particle capture in depth filters has been successfully modeled using Newton's law of motion to predict particle trajectories (Tien 1989) under long-range forces (lift, drag, diffusion, gravity settling) and short-range molecular forces (electrostatic, van

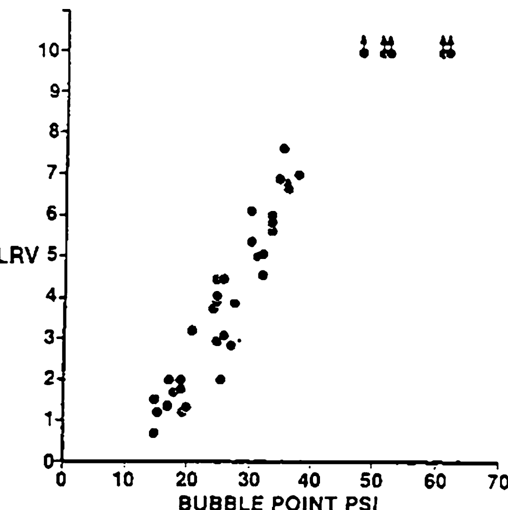

Fig. 16.8 *B. diminuta* LRV vs. Bubble Point Correlation (USP Section <85>)

der Waals, hydrogen bonding) that affect particles sticking to the filter surface. Modeling cast membranes as a fibrous structure, particles are carried through the porous medium by fluid flow where aerosols (gas borne particles) stick to the filter when they touch. Particle adhesion to the surface can occur through molecular adsorption sites and steric capture sites where particles are trapped by the filter structure. Particle migration to the filter surface by diffusion decreases with particle size while convective transport increases. This leads to a maximum penetrating particle size (0.045 μm in air for a 0.2 μm filter at 5 cm/s face velocity) (Rubow 1981; Accomazzo and Grant 1986; Grant et al. 1988; Leahy and Gabler 1984). Particle retention from gases by 0.2 μm filters is essentially complete regardless of size. The trajectory model shows the importance of pore size to adsorptive retention.

Hydrosols (liquid borne particles) may or may not stick as much to a filter surface since some solutions can attenuate adsorptive forces. However, as the pore size shrinks relative to the particle size, particles are increasingly held by steric or size exclusion, irrespective of the solution and particle surfaces.

The size exclusion retention mechanism is very robust (i.e., insensitive to feed composition or operating conditions) compared to adsorption. Sterile filters are designed to retain microorganisms by size exclusion to make sure they work reliably in a variety of applications (Leahy and Gabler 1984; Levy 1987a, b, 1998; Reti et al. 1979; Williams and Meltzer 1983; PDA 1998; Leahy and Sullivan 1978; Robertson 1995): (1) microscopic examination of the filter morphology shows a rigid regular structure with limitations on the particle size that could pass through it (Fig. 16.5), (2) coating the membrane to make it appear chemically similar to water minimizes retention by adsorption, (3) a strong correlation of LRV with bubble point demonstrates retention is primarily dependent on an independent physical measurement of pore diameter (Fig. 16.8), and (4) LRV is generally insensitive to feed composition or operating conditions. Particle retention on the membrane surface is referred to as sieving while retention within the depth of the filter by steric capture sites is referred to as entrapment.

16.5 Filter Devices

Sterile filter devices or modules containing flat sheet cast membrane comes in a variety of forms. Small-scale testing and validation can use die cut disks encapsulated in plastic or installed in stainless holders with a 13, 25, 47, or 90 mm diameter. Sealing these disks in stainless holders can be challenging and use of an extra prefiltration support layer is often needed to ensure good flow distribution (Giglia and Yavorsky 2007).

Membrane can be bonded to support disks with an underdrain and the disks stacked on top of each other. For small volume application, these stacked disk devices with low holdup are recommended. For larger volumes, pleated cartridges of 5″, 10″, 20″, or 30″ in length are used. Pleated cartridges are made by folding membrane into a zigzag or accordion shape with upper and lower support layers, cutting and then bonding the seam to create a pleat pack, then bonding end caps, and a cage support. These cartridges can be installed in stainless holders or used as capsules encapsulated in plastic. Multi-round stainless holders are used for large volume applications where a large number of cartridges can be installed. Devices can also incorporate multiple filter layers in series where the first layer acts as a prefilter to protect the cast sterilizing membrane (Fig. 16.9; Table 16.3).

Vendors use sensitive 100 % integrity tests and lot sampled retention testing to assure consistent retention performance.

Fig. 16.9 Sterile filter devices. Clockwise—encapsulated disk, pleated cartridges and capsules, encapsulated disk, cartridge housing, stacked disks, and encapsulated stacked disks

Table 16.3 Filter device properties

Device	Disks	Stacked disks	Pleated cartridge	Capsules
Use	Scale-down sizing and Validation	<50 L	>1,000 L	50–1,000 L
Filter area	1–100 cm^2	0.1–1.0 m^2	0.2–100 m^2	0.5–5 m^2
Unrecoverable holdup	1.5–3.7 L/m^2	1.5–3.7 L/m^2	2.5–3.7 L/m^2	2.5–3.7 L/m^2
Sterilization	Autoclave in holder	Gamma or autoclave	Steam-in-place	Gamma or autoclave
Operating limits	–	No SIP, 60 psi forward, 10 psi reverse	SIP, 80 psi forward, 50 psi reverse	No SIP, 80 psi forward, 50 psi reverse

100 m^2 includes multi-round housing assemblies

16.6 Process Development and Implementation

Final sterilizing filtration processes are a critical manufacturing step that affects product critical quality attribute of sterility. Follow this step-by-step approach and consult with vendors to ensure a reliable filtration process. Vendor's vast sterile filtration experience can greatly decrease time and resources required to complete key validation activities as well as assure the validation package is fully ready for regulatory scrutiny.

16.6.1 Filter Selection

One can efficiently select an appropriate polymer, structure, and filter device by considering the performance attributes of compatibility, retention, flow rate, and capacity in turn.

As described in Sect. 16.3.3, chemical compatibility refers to filters swelling, dissolving, or losing strength when in contact with flushing, buffers, or process solutions. Compatibility issues with typical dilute aqueous streams are rare but can occur for extreme pH or solvents. Ask vendors for recommendations. Compatibility testing services may be available.

Compatibility also includes adsorption. As described in Sect. 16.3.3, protein, preservatives and surfactants can bind to the filter. This lowers initial filtrate concentration until adsorption sites are saturated and breakthrough occurs. Filter adsorption kinetics are fast so binding is generally an equilibrium phenomenon. Equilibration binding depends on component charge, hydrophobicity, and concentration, with low concentrations requiring the highest volume to achieve target concentration in the filtrate. Membrane adsorption is scalable with membrane area so breakthrough can be determined experimentally early in the filter selection process

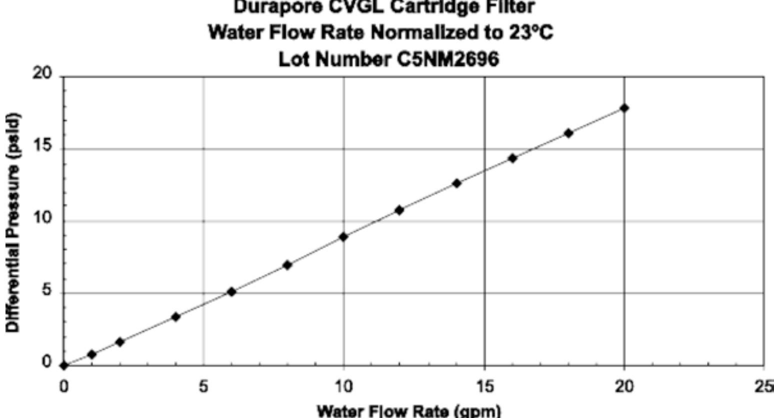

Fig. 16.10 Flow/differential pressure chart (Durapore Cartridge Filter Validation Guide 2012)

using a scaled-down system. Adsorption may require discarding the first few vials of product in a filling line.

Filter pore size or retention rating is selected for bioburden retention. One must monitor the manufacturing plant bioburden, size the smallest microorganisms, and select a sterile filter to retain these microorganisms. Sterilizing-grade filters typically have "nominal" pore size ratings of 0.2, 0.22, or 0.1 μm (Table 16.2). Depending on how these numerical ratings are assigned (microscopic examination, bubble point, particle retention, permeability,…), they alone do not assure sterilizing performance. Vendors must demonstrate bacterial retention capability in model systems to qualify a filter as sterilizing grade (ASTM, Standard F838-05 2003). See Blanchard (2007) for setting a bubble point specification.

16.6.2 Sizing

The amount of membrane required depends on process volume, time, and plugging. Filtration can also be performed at a specified constant pressure with a minimum flow after plugging, at a constant flow with a maximum pressure drop after plugging, or a combination of these. The process may have goals for volume, time, maximum differential pressure, and minimum flow.

In the absence of plugging, the flow resistance (psi/LMH) or permeability (LMH/psi) remains constant. Flow vs. differential pressure charts provided by the filter vendors (Fig. 16.10 for a 10″ cartridge with 0.7 m^2 area) represent the filter hydraulics with 1 cps viscosity fluid representative of dilute aqueous solutions at 23 °C. Sizing is readily obtained from flow, pressure, and viscosity specifications. This is matched with available sizes, and allowances for scaling and safety factors to select appropriate filter devices (Mok et al. 2012).

For fouling streams, the resistance (psi/LMH) increases with L/m^2 throughput. Some users might be concerned that filter plugging indicates a problem of reliability and that only filters that do not plug are suitable. While plugging does indicate that more filter area may be needed, plugging occurs in a consistent and predictable manner so that plugging filters can be sized to perform effectively in the application. Plugging particles can deposit on the surface or within the internal pore structure with different implications for how resistance changes with throughput. Capacity trials are needed to develop predictive plugging models for sizing. There are a number of published plugging models but the most generally applicable model is commonly referred to as V_{max} (Badmington et al. 1995; Bolton et al. 2006; Hermia 1982; Ho and Zydney 2000; Felo et al. 2010; Giglia and Straeffer 2012; Rajniak et al. 2008). V_{max} corresponds to a gradual pore plugging mechanism. Equation 16.3 shows how pressure drop or flow varies with throughput. Equation 16.4 shows the integrated form of how filtrate volume varies with time at constant pressure. These forms can be fit to short time, low throughput data using scale-down devices, and representative feed. The models are extrapolated to larger times and throughputs for sizing (Badmington et al. 1995). Models can also give insight into plugging mechanisms.

$$\Delta P / J = \left[\Delta P_0 / J_0\right]\left[1 - \frac{V}{AV_{max}}\right]^{-2} \tag{16.3}$$

$$A = \frac{V}{V_{max}} + \frac{V}{J_0 t} \tag{16.4}$$

Scaling may require factors to account for support layer and capsule fitting flow resistances (Rajniak et al. 2008; Giglia and Yavorsky 2007; Brown et al. 2009). Safety factors may also be applied to account for process variability (Lutz 2009). For a well-controlled process, cast membrane device permeabilities can be controlled to ±4 % CV consistency.

16.6.3 Single-Use Systems (Capsules) and Stainless Systems (Cartridges)

Sterility assurance requires minimizing connections post system sterilization, and demonstrating downstream components are free of defects that would allow bacterial ingress. Sterilizing an assembled system using gamma radiation for single-use or steaming-in-place (SIP) for stainless, minimizes the need for sterile connections. When sterile connections are needed, using robust sterile-to-sterile connections can eliminate ingress potential.

System integrity tests (Sect. 16.8) demonstrate the absence of leaks that would allow bacterial ingress. Air pressure hold tests are the easiest to implement option. Pressure hold test sensitivity increases as the test pressure increases and so these tests are common and robust for stainless systems. Often single-use systems have

Table 16.4 Systems comparison

Cost	Stainless systems	Single-use systems
Capital	Yes	No
Sterilization validation	Yes	No
Cleaning and setup	14 h	<1 h
Cleaning water	100 L/filter	0
Expendables (e.g., filters, tube sets)	1	1.5 to 5×

limited pressure capabilities, especially those using bags on the sterile side. Qualified connections and vendor conducted integrity tests can minimize risk of downstream defects.

Capsule systems tend to be simpler and more flexible than cartridge in stainless housing systems. For example, a capsule connected with flexible tubing can be easily moved to facilitate venting or draining. Conversely, stainless systems are more rugged and more resistant to impact, although impact would be unexpected and should be avoided in all cases. Cartridges in stainless systems minimize filter cost, but have higher capital and operating costs (Table 16.4).

16.6.4 Operation

Installation—The objective of installation is to assure integrity of the critical seals between upstream and downstream of the membrane. The filter element seal in a capsule is typically a thermoplastic bond created by the filter vendor at time of manufacture. These bonds are highly robust and require little concern on the part of the end user. In the unlikely event this bond is damaged in shipping or handling, the damage will be clearly evident during the device integrity test.

Cartridge filters seal to the housing using a double o-ring. It is critical that o-rings are seated properly, without kinks or cracks. To facilitate smooth installation and assure proper o-ring seating, it is helpful to wet the o-rings with purified water prior to installation. If the o-rings are damaged or installed incorrectly, the assembly will fail the integrity test.

Sterilization—The objective is to assure with a very high degree of certainty that the membrane and system downstream of the membrane are sterile before processing the product solution. There are three typical sterilization methods used for filters. Advantages and disadvantages of each follow:

- Gamma irradiation—Is highly effective on assembled systems using qualified components. This minimizes the potential for ingress from a post-sterilization assembly. While the sterilization process is qualified and conducted by the filter vendor, the end user needs to audit vendor's process and maintain vendor supplied documentation.
- Autoclave—Must be conducted and qualified in a way to assure removal of condensate and air that can cause cold spots. It can be conducted on individual

components on an assembled system. Sterilization an assembled system minimizes risk of ingress during assembly but increases risk of cold spots, especially when the system contains lengths of flexible tubing. Typical materials used in sterilizing-grade filters are thermally stable at autoclave temperatures and there is little risk of membrane or device damage from a properly operated autoclave process.

- Steam-in-place (SIP)—Must be conducted and qualified in a way to assure removal of condensate and air that can cause cold spots. For SIP system design, validation, and operation see Cole 2006; Agallaco 1990. SIP is very effective for sterilization of assembled stainless steel systems. Because SIP requires steam under pressure, use with plastic filter housings (capsules) must be avoided. Steam flowing through filter devices creates a differential pressure. To avoid filter damage, differential pressure must be controlled to relatively low levels (<5 psid).

Flushing—with purified water, WFI, or buffer is needed to remove extractables and wet the filter prior to performing the integrity test. Wetting requires more volume than extractables flushing.

The need for extractables flushing should be determined based on data relevant to the filtration system. An initial assessment can be made using filter manufacturer's qualification and support data, often found in Validation Guides (EMD Millipore 2012). More comprehensive data is obtained by performing extractables testing, whereby the filter device is statically extracted using model solvents that simulate actual product chemical attributes. Typical model solvents are pure water, ethanol, and acid or base solutions designed to simulate process fluid pH (Stone et al. 1994, 1996). Quantitative (NVR, TOC) and qualitative (HPLC, FTIR, etc.) analyses provide an assessment of amount and chemical composition of what might be extracted under worst-case conditions. A calculation of extractables concentration per dose and toxicity assessment will provide guidance on the need for additional testing. In most cases the filter extractables are considered low toxicity and safe.

Processing conditions also impact extractables concerns and the decision to flush. When the filtered material is going directly into final dosage form, the initial vials or syringes will contain the highest level of filter extractables, and this is the worst-case condition that should be assessed. When there is a hold tank between the filter and final filling, filter extractables are diluted, potentially to below detection levels. A comprehensive extractables assessment and justification for flushing procedures should be included in all cases. But a higher level of regulatory scrutiny might be expected for direct filling operations as compared to cases where an intermediate hold tank is used.

Wetting is necessary to assure accurate integrity test results. Smaller pore sizes are harder to wet and some hydrophilic coatings are harder to wet. Some coatings are impacted by steam exposure, making them less hydrophobic and more difficult to flush post-heat sterilization.

Table 16.5 Integrity testing failure rates (pre- and post-SIP are both pre-use)

Defect rates	Pre-SIP (%)	Post-SIP (%)	Post-use (%)
Catridge	0.0049	0.0073	0.0404
Stacked disk	0.0289	0.0294	0.0231

Integrity testing post-use is a regulatory requirement for filters used in critical sterilization applications (US FDA 2004; European Commission 2008). It provides assurance that the device is intact and free from defects that could compromise retention. EMEA Annex 1 also requires testing, post-sterilization, and pre-use (European Commission 2008) although other geographies leave this to the discretion of manufacturers.

Pre-use testing before sterilization will demonstrate that the filter was not damaged during shipping, handling, or installation. Pre-use testing post-sterilization adds assurance the filter was not damaged by the sterilization process. Either test reduces the chances of processing with a damaged filter and avoids the significant costs of rework or product scrap. A risk assessment to determine whether to pre-use integrity test should consider: Product value and the potential for rework, Integrity test history/likelihood of failure, the use of redundant filters, the cost of running the test, and contamination risks introduced by testing (sterility, dilution, false positives, etc.). Table 16.5 shows industry average failure rates calculated from a Millipore data base compiling product quality concerns. Poorly trained operators have higher failure rates than experienced well-trained ones.

Processing sterilizes the complete batch within validated process limits for time, volume/area, and differential pressure. Reasonable worst-case expectations for these process parameters should be considered during custom retention test design and execution. During processing the filter differential pressure and flow rate should be monitored and controlled to validated limits.

Recovery steps capture product held up in the filter system to improve yield. Fluid upstream of the filter can easily be forced through the filter using compressed gas. Table 16.3 shows the volumes held up within the membrane and downstream filtrate volume for a 20 psid applied gas pressure. Further recovery requires either pressure in excess of the filter bubble point (blowdown), a separate inlet on the filtrate side for sterile air, or the use of a liquid buffer displacement. If blowdown is to be employed the pressure required should be considered during retention validation.

16.7 Validation Retention Studies

As described in Sect. 16.3.4, validation of a sterile filtrate claim requires a retention test. ASTM F838-05 is a standard test protocol used by vendors to qualify sterile filters during filter development and as a manufacturing lot release criteria (ASTM, 2003).

The objective of the standard test is to challenge the entire filter area with bacterial cells ($\geq 10^7$ CFU/cm^2 of filter area) and assay the filtrate for presence of CFU of the challenge organism (0 CFU spec.). Scaled-down devices are challenged with ~10^7 CFU/ml to achieve this loading. The results are extrapolated to lower feed concentrations more representative of an actual manufacturing process. *B. diminuta* has been selected as the model challenge microorganism due to (1) its small size (0.68 μm long and 0.31 μm wide), (2) its model representation of a water borne, gram negative organism, and (3) because culturing and handling characteristics are well understood.

ASTM F838-05 is sensitive and effective as a development and QC tool but does not simulate end user processes. FDA recommends that microbial retention testing be conducted using the candidate pharmaceutical preparation under simulated processing conditions. Custom retention testing is a required component of sterilizing-grade filter validation when a there is a process claim of filtrate sterility (US FDA 2004).

Custom retention testing includes the following components:

- Test organism selection—based on assessment of the actual process bioburden, and comparison to *B. diminuta* relative to potential for membrane passage. *B. diminuta* has proven to be the effective challenge organism in almost all cases.
- Test parameter selection—test time, volume/area, and differential pressure are established based on expected worst-case process parameters (i.e., highest values).
- Viability—the ability of the test organism to survive in the process fluid is determined. If the test organism does not survive the test procedure or the test fluid will need to be modified. For example, if the drug product formulation includes a preservative, the test may be run with the drug product without the preservative.
- Test system—scaled down to 47 mm disk.
- Acceptance criteria—$\geq 10^7$ CFU/cm^2 filter area, 0 CFU in the filtrate, all worst-case operating parameters achieved, 3 filter lots tested with at least one lot having a pretest bubble point at or near the minimum manufacturers specification.
- A positive control 0.45 mm filter to show passage.

Regulatory requirements are subject to individual reviewer judgments that vary with geography and experience. It is useful to cite written references, provide supporting data or analysis. Vendors can be helpful. For nonstandard applications, it is recommended to meet with the agency to plan the validation strategy before implementation.

Custom retention testing has proved a valuable validation tool, successful in thousands of feed streams and process parameters. However there are a small number of feed types known to result in low level passage, including liposomes and some surfactant containing solutions. In these circumstances alternatives may include tighter pore size membranes, or redundant 0.22 um filters Sect. 16.10. Filter re-validation to accomodate process changes is outlined in (Table 16.6).

Table 16.6 Revalidation

Changes to Consider		Bacterial Retention	Product Bubble Point	Product Diffusion	Compatibility	Extractables
Filter	**Change in filter membrane** (type / materials of construction)	√	√	√	√	√
	Same membrane - Change in device type (from a capsule to a cartridge for instance)				√* *If original test is a full-device test	√
	Same membrane - Change in pore size	√	√	√	√ May need a letter with data to update the original report	√
	Same membrane - Change in filtration surface area	√* *If filtered volume / surface area is increased				May need a letter with data to update the original report
Process	**Increase in flux** ie. increased flow rate per surface area	√				
	Increase in total filter/product contact time	√			√* *If it exceeds contact time achieved during testing	√* *If it exceeds the time provided in original report
	Increase in control pressure	√				
	Increase in batch volume without a scaled increase in filter surface area	√* *If batch volume was a criterion met in the original study				
	Change in sterilization conditions generally increase in time, temperature, or number of cycles					√* *If the new sterilization conditions exceed those provided
	Change in filtration temperature	√* *Match test to process temp. when organisms can survive in that temp.	√	√	√* *If temperature is increased	√* If temperature is increased
	Change in sterilization method					√
Product	**Change in product formulation** (concentration, pH,...)	√	√	√	√	√* *If it would affect the choice of model solvents

16.8 Validation Integrity Testing

A manufacturer post-use integrity test demonstrates that a sterilizing-grade filter is free from defects. This allows retention study data to be applied to manufacturing batches. Integrity testing of liquid filters involves applying pressurized gas to the upstream side of a wetted filter and measuring the resulting gas flow through the filter. Gas flow below a specification indicates that the gas–liquid meniscus interface was held back by small pore capillary forces. Only flow associated with gas dissolution and diffusion is then observed. Gas filters use pressurized liquid on the upstream side of the unwetted hydrophobic filter with measurement of the liquid flow through the filter (water-intrusion). For liquid filters coated to make their surface hydrophilic, the surface coating must be stable to thermal and chemical stress so that integrity test values do not significantly change and create false negatives.

Regulatory guidance states “bubble point,” “diffusion” and “water-intrusion” tests may all be used, all based on the modified Young–Laplace equation (Young 1885; De Laplace 1806). The bubble point test involves ramping up the feed pressure until bubbles or a sharply higher gas flow is observed downstream, then making sure this breakpoint pressure is above or equal to the bubble point specification. Bubble point is more suited to filters with surface area less than 0.2 m^2. The diffusion test involves measuring downstream gas flow at a fixed feed pressure, then making sure the flow is below the specification. Diffusion tests are suited to large filter areas and filters with a high degree of asymmetry. Consult with the filter vendor to determine the best test for any specific filter and application (Emory 1989a, b; Meltzer 1989a, b).

All tests can be influenced by degree of membrane wetting, environmental temperature, instrumentation, and operator inputs:

- Consistent Test Fluids—Filter suppliers provide test specifications for standard fluids (e.g., water and air for hydrophilic filters, alcohol and nitrogen for hydrophobic filters). Test results will vary with the surface tension of the wetting liquid, the contact angle, and the test gas solubility in the wetting liquid. Trace amounts of surfactants can influence the contact angle and surface tension. In some cases (e.g., post-use testing), fluids other than the filter supplier’s specification may be used. In these cases comparative testing should be conducted to determine the expected test results with the nonstandard fluids (PDA 2008).
- Robust Wetting/Flushing Procedure—Thorough wetting of the filter membrane is essential for accurate bubble point or diffusion tests. A general water wetting procedure is 1 lpm/0.1 m^2 filter area for 5 min. Specific wetting conditions for a given filter type should be available from the filter supplier. Wetting effectiveness can be enhanced by wetting under pressure.
- Validated Test Method—Measurement or detection of gas flow through the filter can be done using either manual or automated methods. If manual methods are used, operator training is essential. Automated equipment removes operator subjectivity and assures consistent measurement. Automated instrumentation must be validated (e.g., IQ/OQ/PQ) with PQ demonstrating that the instrument is capable of accurate results for filters with known bubble point and/or diffusion test properties.
- Stable Test Environment—Vendors specifications are given for stable ambient temperature. Corrections are needed for a cold room or hot WFI to account for surface tension and gas solution-diffusion. Automated instruments determine gas flow by measuring changes in upstream gas pressure, and assume that all pressure changes are the result of gas flowing through the filter. Since changes in gas temperature also impact pressure, one must limit upstream gas temperature changes to ±1 °C during the test.
- Operator Training—For manual tests, operator training and certification should provide documented evidence that the operator is capable of identifying the filter bubble point, or measuring the diffusion rate. Using filters with known bubble point and/or diffusion in blind tests is an effective certification method. For automated tests, it is essential that the operator is capable of interpreting the instrument output.

Instruments will determine and report PASS/FAIL, but also should show pressure/flow vs. time trends either on screen during the test, or on the printout. Operator capability to interpret these trends is essential for assuring accuracy of PASS results. It is also very helpful for troubleshooting FAIL results, as pressure/flow vs. time trends vary for different types of failure modes.
- Standard Operating Procedures—SOPs should include concise instructions for performing routine tests and for troubleshooting. False failures due to poor wetting, upstream leaks, or temperature changes are not uncommon. A well designed troubleshooting process will define a series of retests that will result in definitive understanding of filter integrity, as well as insight into the root cause of false failure.

16.9 Redundant or Serial Filtration

FDA Aseptic Process Guidelines of 2004 state—"Use of redundant sterilizing filters should be considered in many cases" (US FDA 2004). EMEA Annex 1 states "Due to the potential additional risks of the filtration method as compared with other sterilization processes, a second filtration via a further sterilized microorganism retaining filter, immediately prior to filling, may be advisable" (European Commission 2008).

Use of two sterilizing-grade membranes in series may be considered "bioburden reduction/final filtration," "redundant filtration" or "serial filtration". For processes using "bioburden reduction/sterile filtration," the bioburden reduction filter may or may not be integrity tested. Only the second filter inline is considered the critical sterilizing filter and must pass post-use integrity testing. It is considered "redundant filtration" when either filter is qualified to provide sterile filtrate and the second is used as a back up in the event the primary filter fails integrity testing. "Serial filtration" may be used in the rare cases when a single stage sterilizing filter provides insufficient sterility assurance. Examples of feed streams known to be challenging for single stage sterilizing filters include fluids containing liposomes, adjuvants, and some surfactants. The potential need and effectiveness for serial filtration would result from the custom retention validation study. At one time it was suggested that two 0.45 μm filters in series could be considered in cases where the product is retained by 0.22 μm filters (e.g., vaccines). Successful retention validation with serial 0.45 μm filters may be difficult.

Redundant filtration may result in saving a batch of product in cases where one filter fails. The decision to use redundant filtration may consider the following risk factors:

- Product value and potential for rework should the final sterilizing filter fail.
- Filter cost as related to history of failure.
- Operational complexity—note that if either of two filters are to be relied on for sterility, the interstitial space between the two must be properly sterilized and kept sterile throughout the process.
- Product holdup volume.

16.10 Challenging Applications

For the vast majority of applications, sterile filtration processes are straightforward to develop, easy to validate and perform effectively. However, a small group of applications can be challenging due to sizing or retention issues. High viscosity applications associated with high concentration proteins or the addition of thickeners in ophthalmics can lead to large sizing. This can require the use of high pressure and elevated temperature operation. Filtering a non-Newtonian fluid (where the viscosity changes with flow rate) requires modification of the standard V_{max} model (Hermia 1982).

Plugging solutions are typically addressed using inline pre-filtration. This requires screening tests to select a pre-filter that both protects the final sterilizing filter from plugging while not showing significant plugging itself. More complex models are needed for sizing (Felo et al. 2010; Giglia and Straeffer 2012). Pre-filter costs are generally lower than sterile filter costs so it is worthwhile to prevent sterile filter plugging.

Retention challenges can occur when solutions have high salinity ($\gg$150 mM) that shrinks the challenge organism by osmotic pressure (Koch 1984). Some solution components such as surfactants and antibiotics can also reduce the surface tension of a bacteria cell wall and effectively serve to decrease its size (Lebleu et al. 2009). Some feedstocks present a validation challenge because the feed solution is lethal to the retention study model organism. This is accommodated by first, filtering the solution, then, performing a retention study in a separate buffer. The intent is to demonstrate that the feed solution did not alter the filter to prevent organism retention.

Some operating conditions can be challenging. For example, extended use can allow multiple generations of microorganism growth. This can result in the progressive migration of the small infant microorganisms through the filter in what is called "grow-through" (Reti et al. 1979; Christian and Meltzer 1986; Acucena and Wilkins 2012; Kaushal et al. 2013). Grow-through has been demonstrated in static "membrane on agar" experiments (Acucena and Wilkins 2012). Time is therefore a critical consideration for sterile process validation, and regulatory scrutiny is likely to increase for extended time processes. No known cases of grow-through have been verified in biopharmaceutical processing.

In noncritical applications such as the beverage industry, filters may be reused. It can be challenging to accurately mimic these applications in a scale-down validation lab. Cleaning and batch to batch contamination are also major concerns. Generally the resources required to develop, validate, and conduct sterile filter cleaning operations far outweigh any cost savings obtained from filter reuse (Acucena and Wilkins 2012).

16.10.1 Emulsions and Liposomes

Oil-in-water emulsions and liposomes are growing in use as vaccine adjuvants and drug delivery vehicles. They can plug sterile filters and reduce microorganism

retention (Carbrello and Rogers 2010; Onraedt et al. 2010; Folmsbee and Moussourakis 2012). Adjuvant process filtration typically occurs before aseptic combination with the antigen since the antigen/adjuvant combination is often too big for sterile filtration. As drug delivery vehicles, the drug is often incorporated during liposome formation and the conjugate is sterile filtered. Because there are typically no additional downstream steps to remove bioburden, achieving a sterile effluent is the primary goal of this step.

Factors suspected in compromising sterile filtration and filter capacity include product particle size close to the size of the bacteria that need to be removed, low surface tension, and high viscosity of the process fluids. In one study, elevating the filtration temperature to 23 °C and decreasing the processing time provided a sterile product, regardless of operating pressure. Higher filter plugging also decreased microbial retention so higher capacity filters and lower throughputs improve performance. These effects limit the window of operating conditions and show the importance of screening operating conditions in process development. Validation must employ a scale-down model of the manufacturing process without introducing additional pre-filtration steps.

For emulsions and liposomes, not every combination of filter and operating conditions may give you robust retention and high capacity. However, in all the cases that we have studied to date, a combination of filter choice and operating conditions has been found to provide robust retention and acceptable filter capacity.

16.10.2 Spirochetes

Spirochetes are long, helically shaped bacteria. They provide particular challenges to sterile filters because of their shape. The elongated shape and flexibility of these organisms causes them to orient with the flow in a fluid shear field and slip through the filter, much like spaghetti through a strainer. These organisms are not part of the relevant bioburden for most processes and are therefore not part of routine filter validation studies. However, there have been instances of these organisms in bioprocesses. Therefore, is it important for manufacturers to understand the bioburden risks of their process and facility and to design studies for particularly difficult organisms, like spirochetes, when appropriate.

Processing conditions are very important to consider when designing sterile filter processes to remove spirochetes. Both temperature and flux can impact the microorganism morphology and its ability to penetrate the filter. In addition, worst-case conditions for retention may be different for organisms with different shapes. In most cases, using a tighter filter, such as a 0.1 μm filter, will provide complete retention. However, in some cases, processing conditions may also need to be optimized.

As with other challenging retention situations, it may be necessary to choose process conditions that are not optimized for high capacity in order to achieve robust retention of spirochetes. However, in most cases the right combination of filter choice and process conditions can be found to provide robust retention performance.

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Chapter 17
Intravenous Admixture Compatibility for Sterile Products: Challenges and Regulatory Guidance

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Abstract Intravenous (IV) administration of many sterile drug products requires admixture preparation using a diluent, brief storage in an IV container, and dosing through an infusion device. To ensure patient safety and drug efficacy, regulatory agencies require that the sterile drug product is compatible with the diluents and the infusion devices. Therefore, admixture compatibility and stability studies are key components of the pharmaceutical development process. On the surface these studies may seem straightforward, but in practice they require detailed planning, meticulous execution, and appropriate data analysis. The purpose of this chapter is to discuss various requirements and challenges associated with conducting IV admixture studies and the related regulatory guidance.

17.1 Introduction

Intravenous (IV) administration of many sterile drug products requires admixture preparation using a diluent prior to administration. A pharmaceutical admixture consists of a drug product mixed with an appropriate diluent in a suitable dosing/delivery device for the purpose of parenteral infusion to the patient. Regulatory agencies, as a part of registration requirements, have listed specific requirements for the demonstration of the compatibility of the drug product with the diluents and with the infusion devices. For example, as per ICH Q8 guideline, the compatibility of the drug product with reconstitution diluents should cover the recommended in-use shelf life, at the recommended storage temperature and at the likely extremes of concentration (ICH 2009). Similarly, guidance for industry from the U.S.

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Food and Drug Administration states that for parenteral dosage forms, the appropriate containers, filters, and tubing materials should be identified (US Department of Health and Human Services and Drug Administration 2010). Therefore, admixture compatibility and stability studies, which may include evaluation of the compatibility and in-use stability of the drug product with the diluents as well as the dosing/delivery devices, are key components of the pharmaceutical development process. The importance of admixture compatibility and stability studies is further magnified by the fact that in most cases the patient is exposed to the admixture and not the undiluted drug product.

In spite of the high relevance, there is limited literature available for scientists and the drug development community to provide guidance on the design and execution of these admixture compatibility and stability studies (Hawe et al. 2012). Although each product may have specific requirements, the general aspects of admixture compatibility and stability studies remain unchanged and may be applicable to all sterile products intended for IV administration. The purpose of this chapter is to discuss various challenges associated with conducting admixture compatibility and stability studies and the related regulatory guidance.

17.2 Background

17.2.1 Diluents

The most commonly used diluents for IV administration are 0.9 % sodium chloride injection, USP (normal saline), 5 % dextrose injection, USP (D5W), Ringer's Injection, USP and Lactated Ringer's Injection, USP. In most cases, the diluent solution is iso-osmotic. Therefore, the drug upon significant dilution with the diluent is expected to be iso-osmotic. In some cases the diluent may be supplied as concentrated solution and is expected to be diluted with appropriate amounts of drug to obtain iso-osmotic solution for IV administration. As an example, 10 % Dextrose Injection, USP, hypertonic solution, is supplied with the expectation that it will be diluted with a compatible IV fluid to provide a 5 % final dextrose concentration for intravenous infusion.

17.2.2 Components

During clinical administration, the drug admixture may come into contact with the following components:

- IV container—In many instances, the drug admixture may be prepared up to 24 h prior to dosing in the IV container.

- Infusion line—The admixture comes in contact with the infusion line during infusion, which may last between a few minutes to a few hours.
- IV catheter—The catheter usually has a much smaller contact area. However, a peripherally inserted catheter line which is considerably longer may have significant contact with the drug admixture.
- Filters—Filters of 5 μm or smaller pore size (either in-line or add on) are commonly used during infusion to remove any adventitious particles from the admixture.
- Syringes—In cases where the infusion volume may be small the dose may be administered through a syringe pump (e.g., pediatric patients).

Another component that may need to be evaluated is the infusion pump. The use of peristaltic infusion pump instead of gravity assisted drip does not involve contact with the drug. However, the use of pump in the case of biologics may potentially cause drug degradation due to high sheer-related stresses (Wang 1999).

17.2.3 Materials of Construction

Historically, IV containers and infusion lines were made from polyvinyl chloride (PVC) (Sacha et al. 2010). The main reasons for using PVC-based materials are their high strength and flexibility, transparency, ease of sealing, good resistance to sterilization procedures, and relatively low cost (Sacha et al. 2010; Smith et al. 1989). PVC-based infusion devices are made flexible by addition of bis(2-ethylhexyl) phthalate (DEHP) as a plasticizer. However, since the DEHP is not chemically bonded to PVC, it can leach into the drug solutions, especially those containing nonaqueous components such as fats or surfactants (Pearson and Trissel 1993). There have been increasing concerns of adverse health effects of DEHP (US Food and Drug Administration 2010).

An alternative to DEHP plasticized PVC is Tri-2-ethylhexyl trimellitate (TOTM) plasticized PVC, which is believed to have lower toxicity (ExxonMobil Biomedical 2001; Czuba et al. 1996), and has been shown to have lower migration rates (Czuba et al. 1996; Ito et al. 2008).

Another alternative is to use a PVC container that is lined with polyethylene (PE) on the fluid contact surface. PE is believed to act as a barrier and minimize the migration of plasticizer DEHP into the drug solution (Sacha et al. 2010; Czuba et al. 1996). Moreover, other non-PVC materials have also emerged as alternatives, e.g., ethyl vinyl acetate (EVA) and polyolefins (non-PVC, non-EVA). Table 17.1 lists the representative materials of construction of IV containers and infusion lines that are currently available.

Table 17.1 Representative materials of construction[a] of IV containers and infusion lines

IV containers	Infusion lines
• PVC + DEHP • PVC + TOTM • EVA • Polyolefin (non-PVC, non-EVA) • Polyolefin/polyamide co-extruded plastic with PE lined fluid contact surface	• PVC + DEHP • PVC + TOTM • PE lined PVC • Polyolefin

[a]In order to determine the material composition, one may need to contact specific vendor

17.3 Challenges of Admixture Studies

17.3.1 How to Design an Efficient Study to Meet all Requirements of Clinical Dosing?

It is critical to define the scope of the admixture study based on the clinical dosing strategy. In most cases the information about clinical dosing plan is limited to the dose range defined in terms of mg of dose per kg of patient weight (mg/kg, mpk) or mg of dose per unit patient body surface area (mg/m^2). The scope of the admixture study may include the definition of admixture concentration levels to be tested, identification of suitable IV container/infusion line type, and identification of the size of IV container and fill volumes from the perspective of worst case scenarios. The goal is to provide maximum flexibility to the clinicians while minimizing the number of experiments and required study materials.

17.3.2 Admixture Concentration Levels

The goal of the admixture compatibility and stability studies should be to evaluate suitable concentration range that would allow administration of all the desired dose levels in the clinic for a range of patient weights. Therefore, testing the lowest and highest concentrations required in the clinic can bracket the entire concentration range. The first estimation of the lowest and the highest concentration levels can be made considering the lowest dose level/lowest patient weight and highest dose level/highest patient weight combinations for representative IV container sizes (Table 17.2). However, in some cases there could be analytical challenges associated with the low concentration limit, which would require increasing the concentration. This is discussed more in Sect. 17.3.4.

17.3.3 Exposure Temperature and Time

Admixture compatibility and stability studies should be designed to support the clinical requirements such as hold times and conditions. In the case of a lyophilized

Table 17.2 Example dose concentration calculations for fixed volume in the IV container

	Required concentration (mg/mL)			
	Representative scenario 1: 100 mL container is desired		Representative scenario 2: 250 mL container is desired	
Dose level (mg/kg)	50 kg patient	100 kg patient	50 kg patient	100 kg patient
0.1	0.05	0.1	0.02	0.04
0.3	0.15	0.3	0.06	0.12
1	0.5	1	0.2	0.4
3	1.5	3	0.6	1.2
5	2.5	5	1	2
10	5	10	2	4
20	10	20	4	8

Table 17.3 Beyond-use dating guidelines from USP <797>

	Risk level		
	Low	Medium	High
Room temperature (°C)	≤48 h	≤30 h	≤24 h
2–8	≤14 days	≤7 days	≤3 days
≤–20	≤45 days	≤45 days	≤45 days

drug product, the stability of the reconstituted drug product prior to admixture preparation may also need to be evaluated. USP <797> (USP 797) provides guidance for beyond-use dating of compounded sterile preparations in the absence of sterility testing (Table 17.3).

Although USP <797> provides guidelines to identify the risk level of the sterile preparation, the most conservative approach would be to assume that the product is at the high risk level and minimize probability of microbiological contamination. This approach is supported by the finding that the compounding procedures followed in the pharmacies are not highly standardized and may not always ensure the highest level of aseptic handling (Kastango and Bradshaw 2004). Therefore, based on the storage requirements, the physical and chemical stability of the admixture can be evaluated for the maximum length of time allowed by Table 17.3 (e.g., up to 24 h at room temperature). If desired, a 25 ± 2 °C or equivalent incubator may be used to provide a more controlled simulation of the USP controlled room temperature.

If there is acceptable physical and chemical stability for the duration of the study, it may be acceptable to recommend the maximum allowable beyond-use dating as dictated by USP <797> (USP 797). However, in the absence of microbiological testing to minimize risk one may consider restricting the storage period to fall short of the maximum allowable period listed in Table 17.3 (e.g., up to 8 h at room temperature when up to 24 h is allowed as listed).

For lyophilized drug product, the reconstitution precedes the preparation of admixture solution. In that case, the physical and chemical stability of the

reconstituted drug product in the original container-closure system may also need to be evaluated for a representative length of time (ICH 2009; US Department of Health and Human Services and Drug Administration 2010). The discussion presented above would still apply to this case. In addition, the recommended exposure time limit at required storage temperature should be cumulative of the reconstituted drug product storage in the original container-closure and the admixture solution storage in the IV containers.

17.3.4 Analytical Challenges

Concentration level below levels of quantification. As discussed in Sect. 17.3.2, the admixture studies could be bracketed by using two concentration levels calculated from the lowest and the highest dose to be given in the clinic. While the upper concentration limit may be in the same order of magnitude as the concentration of the undiluted drug product and therefore usually not challenging from an analytical perspective, the lower concentration limit could occasionally be much lower than the analytical level of quantification. As an example for biological products such as monoclonal antibodies, the lower concentration limit of 0.05 mg/mL in Table 17.2 may be challenging from analytical perspective. In such scenarios, suitable adjustments need to be made either to the dosing plan or analysis. For low dose levels, the total admixture volume in the IV container may need to be lowered to ensure analytically feasible concentration.

Sample replicates. It is best to have samples tested in triplicates for optimization of precision of measurement. In practice, though, this may lead to a significant increase in the number of samples and drug product requirements. Therefore, duplicate samples may be a viable option. In the cases where the duplicate sample loads are still too high, average sample composites may be considered. In such a situation, the actual analytical sample will be a composite of two or more individual samples prepared in the same way. Even though a single sample will be tested, it will reflect an averaging of the conditions of individual samples.

Admixture sample storage prior to testing. In certain cases where the samples cannot be tested immediately, or if the samples are required to be shipped to different testing sites, they may be frozen to minimize changes. However, a probe study or prior experience should be used to evaluate the effect of freezing on sample stability.

Limited sample volume. Composite samples may also be used if sample volume is limited. As an example, particulate matter testing by USP light obscuration requires approximately 25–30 mL of sample. However, an infusion line may only contain a total line volume 10 mL. In this case, multiple infusion lines will need to be combined to generate sufficient volume for the Light Obscuration characterization (see Sect. 17.3.9 for more discussions on the approach of using multiple infusion lines).

Impurities in the diluent. Certain diluents may contain impurities which can interfere with the analytical techniques employed to test admixture samples. For example, 5-hydroxymethylfurfural or related substances in Dextrose Injection, USP solution are degradation products of dextrose and absorb UV light (~280 nm), thus interfering with the UV A280 concentration measurements (USP 2004).

Need to test diluent. Certain tests may require measuring the diluent samples for assessing any background signal. In most cases it may be sufficient to collect diluent sample at the initial time point since no changes are expected during the course of the admixture study. For example, particulate matter testing by HIAC may need background particle count of the diluent solution. Diluent measurement may also be required if it contains an impurity interfering with UV assay measurement. In certain cases the diluent may need to be run through assay to identify any interactions with the drug over time. Based on individual study needs, appropriate diluent sampling scheme should be used.

17.3.5 Low Infusion Volumes (Requirement of Syringe Pumps)

As discussed in Sects. 17.3.2 and 17.3.4, analytical limitations may require lowering the admixture volume in the IV container to maintain a certain concentration level. However, certain low dose levels may require infusion of very small admixture volumes. For example, only 5 mL would be needed for a 50 kg patient receiving 0.1 mpk dose at 1 mg/mL concentration.. This scenario may be even more likely in pediatric patients with much lower body weights. The option of using a constant volume IV container (e.g., 50 mL) may not be feasible due to potential of significant drug losses from adsorption (i.e., small amount of drug in contact with entire container surface) and also due to the drug concentration falling below the levels tested in the admixture study.

In such cases, the use of syringe pump may be a suitable alternative where the admixture of a particular concentration may be prepared in an IV container and the desired volume is filled in the syringe. Therefore, representative syringe materials (e.g., polypropylene, latex-free) should also be evaluated for any compatibility/stability issues with the representative admixture solutions. Since the syringe may be used only for the lowest dose, it may be sufficient to test the lowest admixture concentration level in the syringe. In addition, since the syringe can be filled in a short period of time, it may not be necessary to study extended hold times. Therefore, admixture stability in the syringe may only be required to be studied for shorter duration (e.g., 2–4 h at room temperature). Note that even though a syringe may be used to infuse the low volumes, it is required to be filled from an IV container containing the admixture solution of a suitable concentration. In addition, if a syringe is used, the recommended exposure time limit at required storage temperature should be inclusive of the storage period in the syringe.

17.3.6 IV Container Overfill Volume

IV containers filled with diluents usually contain an overfill volume to ensure that the label claim volume is met. A published study with 162 D5W IV containers from different vendors found that the mean volume was 110.20 mL for 100 mL containers (Blad et al. 2000). We have also found that 250 mL normal saline IV containers from one vendor had overfill volumes ranging from approximately 12 to 30 mL.

If the excess fill volume is not considered, the concentration of the study drug in the admixture solution can be off target and may lead to undesirable consequences related to under-medication (Blad et al. 2000). Therefore, the concentration range covered in the admixture study may be made slightly broader than that required in the clinic to accommodate for concentration variations due to container overfill. To precisely achieve target concentrations during admixture studies, the dilutions can be prepared by weight. However, assuming that the pharmacist in the clinic would require a simpler process, it is recommended to test a statistically significant number of IV containers (e.g., 10 containers) to determine the average overfill volume and provide this information to the clinic. It is important to note here that even though failure to consider overfill will results in an off-target concentrations (over dilution), the patient would still receive the total intended amount of drug as long as the entire container is dosed followed by infusion line flush, assuming there are no incompatibilities associated with over dilution.

Overfill volume would not be an issue if an empty IV container is being used to prepare the admixture since the required amount of diluent can be accurately added to the container. However, this approach introduces additional manipulation steps of removing the diluent from another source and adding it to the empty container.

17.3.7 Leachables

Generally, many of the stability indicating assays for the admixture solution are product-related. For example, acceptance criteria for stability include monomer concentration (for biologics), product purity, and subvisible particulate matter levels in solution (see Sect. 17.3.8 for more details). However, the presence of leachables from the infusion device contact material is often overlooked.

Excipients, which are used to stabilize the drug product formulation, may also facilitate leaching within the admixture solution (Bee et al. 2011). For example, excipients used to solubilize insoluble compounds in the drug product may also solubilize less soluble compounds from contact material that ordinarily would not leach (Zimmerman et al. 2003).

For biologic products, surfactants such as polysorbate 80 (PS80) and polysorbate 20 (PS20) are commonly used to minimize the interaction of the drug with various interfaces encountered during manufacturing, storage, and handling (Kerwin 2008). Their amphipathic nature gives them the unique ability to preferentially interact

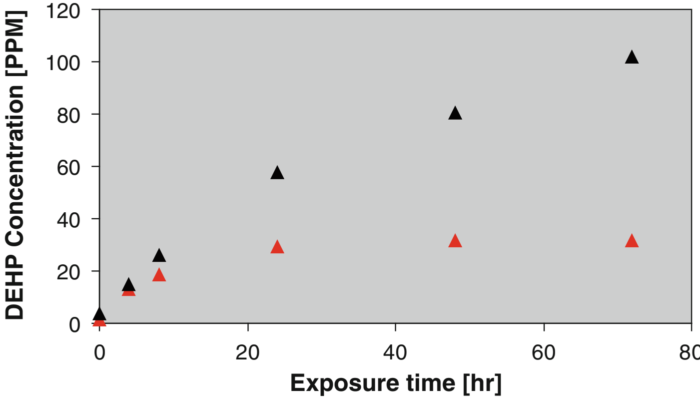

Fig. 17.1 Leaching of DEHP as a function of exposure time under ambient conditions for 3 mg/mL of PS80 (*black*) and 3 mg/mL of PS20 (*red*)

with many surfaces and compete with the drug molecule thus imparting formulation stability. However, during infusion of the sterile drug, the same amphipathic nature gives these excipients the ability to solubilize hydrophobic molecules like DEHP used to plasticize PVC containers into the admixture solution (see Sect. 17.2.3). In Fig. 17.1, we show the level of DEHP leached from PVC containers resulting from the presence of 3 mg/mL PS80 and PS20 in solution.

17.3.8 Acceptance Criteria

The analytical tests employed in the study should be able to detect any physical and chemical degradation of the drug molecule during storage in the admixture solution. The typical tests that can be conducted for therapeutic proteins may include assay by UV A280, purity by HP-SEC, charge profile by HP-IEX, biological potency, pH, physical appearance, osmolality, and subvisible particulate matter count. Small molecules may require assay and degradation products by HPLC, pH, physical appearance, osmolality, and subvisible particulate matter count.

In most cases, the analytical tests being used in the admixture study will be the same as those used for testing and release of the drug product. Therefore, drug product release specifications can be used as a guide while setting acceptance criteria for the analytical tests of admixture study.

For some tests, the same acceptance criteria as that of the drug product may be applicable, e.g., physical appearance, HPLC assay, HP-SEC purity, and biological potency. However, for other assays the acceptance criteria may need to be modified (e.g., UV A280 test) due to factors such as additional variability associated with

container overfill volume, and interference from any potential impurity in the diluent (e.g., 5-hydroxymethylfurfural present in dextrose) (USP 2004). As an example, if the release specifications for UV assay are 85–115 % of target, and the drug product lot used in the study was close to 85 % of target, any further decrease in the concentration due to container overfill variability may result in an out-of-specification result. One way to address this issue would be to set the acceptance criteria with respect to the results from the initial sample of the admixture study (e.g., 85–115 % of initial). In this regard, the experience obtained from the probe studies can be of great help in understanding the variability associated with admixture preparation and also making suitable adjustments to the dosing strategy. For example, if adsorption losses on the infusion line are observed, recommendation may be made to prime the line with admixture and discard certain volume prior to infusion. In the admixture study it would ensure meeting the target concentration specification, whereas in the clinic it would ensure that the target dose is delivered to the patient.

Another test where release specifications may not apply is pH, since the diluent solutions are usually not buffered and therefore can have a large range of pH values. Therefore, dilution of the drug product with these diluents may push the pH out of the release specification range, especially in the case of low concentration admixtures. To circumvent this issue, the pH test may have “report results” as the acceptance criteria.

Before applying any of the validated analytical methods for testing admixture samples, it is important to make sure that the validated method covers the range of concentration suitable for the admixture samples.

Particulate matter test is another example where it is quite difficult to set acceptance criterion. Although USP <788> (USP 788) is a compendial test, there are unresolved questions about how to adapt it to testing admixture solution. For example, (a) should there be an acceptance criterion associated with the IV containers at all since the patient is only exposed to the infusion line drip? (b) If yes, which criterion is appropriate to apply to the IV container (Test 1.A, small volume, or Test 1.B, large volume; what if the clinic will use containers below and above 100 mL size?) (c) Which criterion (Test 1.A, small volume, or Test 1.B, large volume) to apply to the samples from infusion line drip? Due to all these unresolved issues, an appropriate strategy may be to list the particle count numbers on a per mL basis in comparison with the results obtained from undiluted drug product testing. If there are any incompatibilities related to storage of admixture solutions in the infusion devices, the admixture particle counts may increase. It is to be noted that the particle count of the diluent solution should also be measured to assess the background count of the diluent.

In the case of a lyophilized drug product, the stability of reconstituted solution may also be monitored for a certain period of time, e.g., 24 h at room temperature. The release specifications of the drug product may be used as the acceptance criteria for this purpose.

Based on the discussion above, sample acceptance criteria for a sterile drug product are presented (Table 17.4).

Table 17.4 Sample acceptance criteria for admixture of sterile drug product

Test	Representative acceptance criteria
Description	
Clarity	Clear to opalescent; may contain particles
Color	Report results by "Y" ref solution
pH	Report results
UV A280 (biologics only)	Initial samples: 85–115 % of target All other samples: 85–115 % of initial
Potency by competitive ELISA or other suitable bioassay (biologics only)	50–150 % of reference
HP-SEC (biologics only)	High mol. wt species: ≤5.00 % Monomer: ≥90.0 % Late eluting peaks: report results
Contents by HPLC (small molecule)	Initial samples: 85–115 % of target All other samples: 85–115 % of initial
Degradation products/impurities (small molecule)	Total degradation/impurities must not be more than 0.3 % absolute change from the initial sample
Subvisible particulate matter	Comparable to reconstituted DP vials (on a per mL basis)
Osmolality	300 ± 50 mOsm/kg

17.3.9 Other Challenges

Surface incompatibilities. Another challenge associated with admixture solutions which is especially critical at low concentration levels is the potential for drug losses due to adsorption (small amount of drug in contact with entire container surface). At high dilution ratios the concentration of surfactant, if present in the drug product may also fall below critical micelle concentration, making the drug loss at surfaces even more likely. This could seriously compromise the dose accuracy in the clinic. In addition to surface adsorption, there could be instabilities caused by surface-induced denaturation in the case of proteins (Hawe et al. 2012 and references therein)

Choice of diluent(s). The choice of diluent(s) for the admixture compatibility and stability studies should be made according to the requirements of the clinical program. However, any gross incompatibilities observed in probe admixture studies may help eliminate those choices. In certain cases, the preformulation experience may also help eliminate some of the choices. For example, in the case of biologics, incompatibilities with sodium chloride may have been observed in the preformulation studies in the form of one or more of the following: increase in opalescence, particle formation, shaking stress instabilities, and/or soluble aggregate formation. In those cases, normal saline may not be the best choice for diluent. Similarly, certain biologics may be prone to destabilization due to interaction with reducing sugars, rendering them incompatible with diluents containing dextrose or glucose (Fischer et al. 2008). In certain cases the diluent may contain trace amounts of impurities,

which may interfere with the analytical tests. In that case, appropriate adaptations may be required in the analytical methodology, which are discussed in details in the analytical section.

Most appropriate IV container size. Since the objective of the admixture compatibility and stability studies is to determine any incompatibilities with the material of construction of the IV container, it may be ideal to maximize the contact surface area to fill volume ratio to simulate the worst case scenario. Therefore, the IV container should be of the largest size that may be used in the clinic and should be filled with the least amount of admixture that may provide enough volume for all the planned analytical tests.

Worst case exposure simulation for the infusion line. In the clinic the infusion will be performed over a certain period of time (few minutes to a few hours). The admixture study could simulate the infusion by dripping the representative amount to cover the dripping time required and collecting the drip in a suitable container. It is always a good practice to evaluate an extended duration of the drip time to give clinics some flexibility. However, another viable alternative to dripping is to fill the infusion lines with the admixture solution, incubate for a predetermined length of time, and then drain in a suitable container. The latter option allows for maximum contact time of the admixture with the infusion devices and therefore simulates worse case scenario. Moreover, this option is more convenient since it is very difficult to control the drip rate without using a pump. However, more care needs to be exercised while collecting the samples since the lines may need to be cut to collect the liquid trapped below the airtight in-line filter.

Requirement of transportation of admixtures. In most cases the admixtures are prepared at the dosing site but there may be a requirement of transportation of the admixture containers from one site to another (Kupfer et al. 2009; Sreedhara et al. 2011). Sreedhara et al. (2011) showed that severe instabilities were caused by subjecting an IgG1 monoclonal antibody admixture in normal saline. Even though each protein is unique, however, in general the agitation of admixtures should be avoided as much as possible.

17.4 Regulatory Requirements

Regulatory agencies, as a part of registration requirements, have listed specific requirements for the demonstration of the compatibility of the drug product with reconstitution diluents and with the infusion containers. As an example the “ICH Harmonized Tripartite Guideline—Pharmaceutical Development Q8” states the following: “The compatibility of the drug product with reconstitution diluents (e.g., precipitation, stability) should be addressed to provide appropriate and supportive information for the labeling. This information should cover the recommended in-use shelf life, at the recommended storage temperature and at the likely extremes of concentration.

Similarly, admixture or dilution of products prior to administration (e.g., product added to large volume infusion containers) might need to be addressed" (ICH 2009). In addition, there are specific requirements listed in the Dosage and Administration section of labeling required by 21 CFR 201.57(c)(3) as described in the Guidance for Industry document titled "Dosage and Administration Section of Labeling for Human Prescription Drug and Biological Products—Content and Format" (US Department of Health and Human Services and Drug Administration 2010). These include:

- Procedure to reconstitute the drug product (if applicable)
- Dilution procedure to form admixture solution
- Specific handling instructions (shaking, shear) to maintain stability of the biological drug product
- Allowable concentrations of the admixtures
- Storage conditions and durations
- Compatible IV container and infusion line materials and allowable IV container sizes
- Compatible diluents
- Specific dosing instructions

Based on the discussions provided in the previous section (Sect. 17.3), it is clear that appropriate design of admixture studies plays a critical role in fulfilling these regulatory requirements.

17.5 Conclusions

IV admixture studies are an integral part of developing a safe and efficacious sterile drug product intended for IV administration. In this chapter we have listed various challenges and regulatory expectations associated with the IV admixture studies. Although each product may have unique requirements, the general aspects of IV admixture studies remain similar and may be applicable to all sterile products intended for IV administration. We hope that the discussions presented here will help researchers identify critical admixture issues for their products and also gain some insights into addressing those issues.

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Chapter 18
Basics of Sterilization Methods

Gregory W. Hunter

Abstract The manufacturing of parenteral formulations should include a sterilization step in order to maintain product quality. How the most common sterilization techniques are performed and some of the advantages and disadvantages of each type will be summarized. Sterilization methods that will be covered include the fundamentals of steam, radiation, and ethylene oxide terminal sterilizations, as well as aseptic processing. Once a sterilization cycle or procedure has been chosen and developed, its effectiveness should be validated according to the guidelines of the respective authorities. Some of the documentation that regulators may review to decide whether they believe a method is acceptable is summarized in this chapter. While regional regulatory authorities may differ on what types of information should be submitted to their agencies and what should be reviewed during field inspections of a manufacturing facility, the focus for this chapter will be validation information and Good Manufacturing Practices aspects of sterilization techniques.

18.1 Introduction

It is generally understood in the pharmaceutical industry that parenteral formulations must include some sort of sterility assurance for the product in order to maintain product quality. This chapter will briefly summarize how the most common sterilization techniques are performed and some of the advantages and

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disadvantages of each type. Once a sterilization cycle or procedure has been chosen and developed, its effectiveness should be demonstrated or validated according to the guidelines of the respective authorities. This chapter summarizes the general factors that regulators may review to decide whether they believe a method is acceptable. Regional regulatory authorities may differ on what types of information should be submitted to their agencies and what should be reviewed during field inspections of a manufacturing facility, but validation information and Good Manufacturing Practices (GMP) aspects of sterilization techniques are common aspects that should be applied for successful product registration outcomes. Much of the documentation that regulatory authorities may review is described in guidance and publications written by the U.S. Food and Drug Administration or international regulatory agencies, and as part of international standards documents. Note that this chapter only details a portion of the documentation that regulatory agencies may review for registration of sterile products and cannot be considered an exhaustive compilation. Although sterility testing after sterilization and control of bacterial endotoxin may be key components of a successful product release, the performance of these aspects of finished product manufacturing will not be discussed here.

Unfortunately, a simple, practical method of sterility assurance that can quickly verify the absolute absence of viable microorganisms in every individual unit of a batch that may be composed of tens or even hundreds of thousands of units is not yet practical. Therefore more indirect means are used to demonstrate effectiveness of sterilization cycles. Throughout the chapter, *sterility assurance level* (SAL) will be a metric for sterilization goals. SAL is the probability of a single unit being non-sterile after exposure to the sterilization process (ANSI/AAMI/ISO TIR11139:2006). SALs are typically expressed as exponents. For example, if the probability of non-sterility of a batch after application of a sterilization technique is one in a thousand, the SAL for this process would be written as "10^{-3}." International authorities generally agree that a SAL of 10^{-6} or better is sufficient for pharmaceutical products and devices (ANSI/AAMI/ISO TIR11139:2006; FDA's Guidance for Industry 2004; The Japanese Pharmacopoeia Fifteenth Edition 2006; EudraLex 2008). While there does not seem to be a modern scientific basis for the choice of the 10^{-6} value (Agalloco 2011), it does represent a reasonable and achievable endpoint for sterilizations.

Another key concept involves the timing of the sterilization. A process that renders an article sterile *after* it has been put into its final packaging is known as a *terminal sterilization* process. Processing that renders a drug product sterile and maintains that sterility *before* it has been put into (sterilized) final packaging is known as *aseptic processing*. As noted in Sect. 4.1, aseptic processing entails rather exhaustive procedures and engineering to bring about the sterility assurance desired. In addition, available guidance suggests that injectable products should be sterilized in their final containers whenever possible (EMEA 2000) although aseptic processing may be considered. Therefore, terminal sterilization procedures are often the first processes explored for pharmaceuticals to be sterilized, and these procedures will be treated first in this chapter.

18.1.1 Heat Sterilizations

Terminal sterilizations using heat are common techniques for solutions and suspensions. Autoclaves are readily available at most manufacturing facilities and are useful for providing moist heat (steam) sterilizations. Autoclaves can vary in capacity from small "desktop" units to room-sized and therefore can be designed with just about any batch size of product desired. To proceed, the autoclave chamber is loaded with the objects to be sterilized in a defined pattern determined beforehand. Once loaded, the chamber is sealed, and then air is removed and replaced by steam (Coon and Sadowski 2011). The heat from the steam kills microorganisms, and the effectiveness of the sterilization depends both on the amount of time the items in the load are exposed to the steam and the efficiency of steam penetration into all product surfaces and spaces. The time a product, component, or equipment will need to be exposed to the steam at a defined temperature will likely be short—on the order of 12–20 min. In addition to terminal sterilization of pharmaceutical products, moist heat may be used to sterilize the manufacturing equipment used for aseptic processing either as part of an autoclave load or already assembled in a steam-in-place operation.

Dry heat sterilization may be accomplished using ovens specially designed to handle components of pharmaceuticals. If only sterilization (and not depyrogenation) is desired for the items in the load, cycles consisting of temperatures of 160 °C for 2 h may be needed (EudraLex 2008). Dry heat is most desirable if powders or other items that cannot tolerate water are to be sterilized.

18.1.2 Heat Sterilization Validation

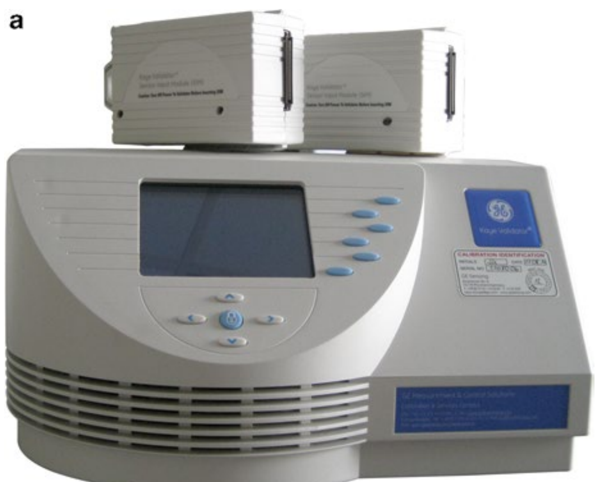
Once identified as an optimal choice for use with a particular product, the effectiveness of the sterilization method should be demonstrated. There are several tools available for the validation of heat sterilization processes. One tool is the measurement of thermal exposure using thermocouples at defined positions in the load. Another is deployment of biological indicators (BIs) adjacent to or at other sites in the load. These tools should be used simultaneously to validate heat sterilization process.

BIs are preparations of a specific microorganism that may be placed within a sterilization load during validations. To validate that the process provides a 10^{-6} SAL under worst-case conditions, the microorganism chosen should be more resistant to the process than microorganisms present in the manufacturing facility. Spore-forming microorganisms become resistant to inactivation by heat and chemical treatments by assuming a dormant state under adverse conditions. Upon encountering optimal growth conditions the spore-forming organisms can assume a vegetative state in which the reproductive capability of the organism can be resumed (Matthews et al. 1994). *Geobacillus stearothermophilus*, a spore-former, is an organism found in many different soil and aqueous environments that can grow at temperatures

Fig. 18.1 An example of a biological indicator used for a heat sterilization validation. Photo courtesy of Mesa Laboratories, Inc

greater than 70 °C (Jurado et al. 1991). Since its optimal growth temperature range is much higher than organisms that are likely to contaminate pharmaceutical products, *G. stearothermophilus* represents an excellent BI choice for steam sterilizations, and is widely available commercially in preparations that are validation-ready (Lemieux 2006). These preparations include spores affixed to paper or other materials, BI suspensions, and so-called self-contained BIs in which the organism and a suitable growth medium are contained within glass ampoules. Other BI organisms that may be used in some types of dry heat validations are *Bacillus subtilis* and *B. atrophaeus*. Figure 18.1 shows a preparation of BI that may be used in specialized validation procedures.

BIs may be deployed in the load in positions found to be the most difficult to heat. In order to meet the necessary 10^{-6} SAL acceptance criterion, each location monitored by a BI generally should contain at least 10^{6} spores of the organism. Each BI preparation should also have an associated *D-value*, or the time required in minutes for a tenfold or one log cycle reduction in population under the relevant sterilization conditions. *D*-values for a *G. stearothermophilus* BI preparation for a terminal sterilization validation often range from 1 to 3 min. The magnitude of the *D*-value for the BI may be used to roughly estimate the dwell times for a sterilization load during cycle development and validation. For example, since the SAL desired represents at least 12 log cycles of population reduction of the BI (six logs reduction to get to a population of 1, or 10^{0}, then six more logs to reduce the



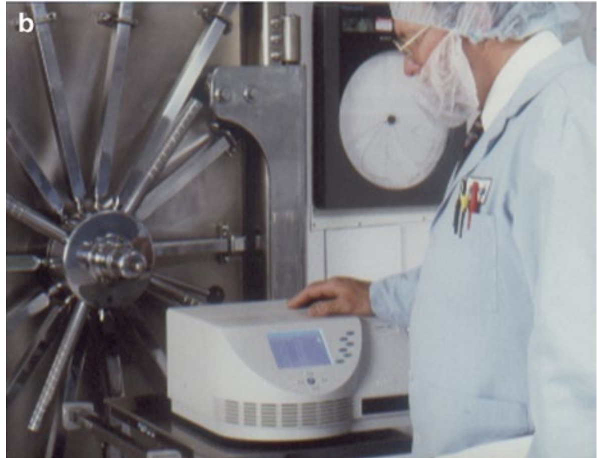


Fig. 18.2 (**a**) Example of a data logger for a sterilization validation. (**b**) In preparation for a validation. Photographs obtained with the permission of GE Sensing & Inspection Technologies GmbH

organisms to a 10^{-6} probability of survival), then in general the time that the product is exposed to the steam in a validated moist heat process should be at least 12 times the *D*-value listed with the BI. Once placed into and sterilized with the load, it is expected that all the spores in the BI preparations should be killed to achieve a successful validation. This may be verified following a validation run by incubating the BI under appropriate temperatures and conditions as directed by the BI manufacturer.

Thermocouples, in combination with a data logger (such as that in Fig. 18.2), provide a real-time readout of the temperature at critical points within the load. The use of multiple thermocouples deployed at various positions within the load therefore can provide a "map" of the killing power of the steam. Calibration of the thermocouples should be traceable to national standards. A parameter called F_0 may be used as a measure of cumulative thermal exposure of the load at a monitored location. The parameter may be calculated from the following equation:

$$F_0 = \sum 10^{(121.1-T)/z} \Delta t,$$

where Δt is the time interval between measurements (usually in minutes), T is the temperature in degrees Celsius, and z is the number of degrees Celsius required to change the BI's D-value by one factor of 10. The 121.1 figure that appears in the exponent of the equation refers to a common steam sterilization temperature in degrees Celsius. Values around 12–15 represent a rough figure for an acceptable F_0 depending on the nature of the validation it is used with; for example, European Pharmacopoeia requirements are for a minimum of 15 min under saturated steam at 121 °C, implying an F_0 of at least 15 for successful validation (European Pharmacopoeia 2011). F_0 and complete BI spore kill should both act as primary acceptance criteria for a steam sterilization validation to ensure that a 10^{-6} SAL can be achieved.

To validate the moist heat sterilization of an autoclave load, a study of the *heat distribution* using a collection of thermocouples should be performed in order to determine the hardest to heat points (lowest F_0 values) in the load during a sterilization run. The results of the heat distribution study should be used to determine worst-case positions for monitoring in the validation. *Heat penetration* determinations monitor the worst-case and other prominent positions from heat distribution experiments with thermocouples and BIs to determine whether F_0 and BI kill acceptance criteria have been met. Three autoclave runs that meet acceptance criteria should be performed (EudraLex 2008; http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM072171.pdf). Once a load is validated, future routine runs should be performed using the same load patterns (although not necessarily with BIs included in the load). Annual requalification with one run using a similar procedure as the validation is suggested. If loads or operating parameters change, complete revalidation may be required.

Dry heat sterilizations follow somewhat similar outlines as steam sterilizations, although the identity of the BI differs as noted above. More specific details concerning validation of moist and dry heat will be included in later chapters of this book.

18.1.3 Heat Sterilization Trade-Offs

Advantages of heat terminal sterilization include the ease, speed, and convenience of having most of the elements of a successful sterilization program on site. There are disadvantages to this approach however, and they include possible heat sensitivity of the product and the fact that products such as powders in glass or plastic containers may not allow for acceptable amounts of heat penetration into their interiors. Dry heat sterilizations usually require higher temperatures and times; therefore dry heat cycles may be even less desirable for heat-sensitive products.

18.1.4 Heat Sterilization Regulatory Aspects

Though the regulatory approaches in various countries may differ, most of the regulation of heat sterilizations will be through review of documentation and not observation of the processes. For any sort of sterilization, agencies will as a matter of course want to be aware of the types of products to be sterilized and their characteristics such as volume or size of container and the nature of the dosage form in order to understand the limitations and goals of the validation. In addition, evidence that the autoclave and related equipment is working properly should be made available. At a minimum, the following descriptions and documentation could be reviewed if the regulatory agency requires it:

- Instrument manufacturer and model
- Volume or capacity of the instrument
- Performance specifications of the instrument such as temperatures and vacuum pressures if applicable
- Verification that you schedule preventive maintenance and make repairs as necessary
- Thermocouple calibrations both before and after validation runs
- Traceability of calibration standards to appropriate regional standards

Be prepared to present a description of the cycle and the following documentation:

- A validation master plan or protocol describing the facility's general strategy for the relevant validation(s)
- Product loading pattern(s)
- Treatment times for the load articles
- Validation acceptance criteria such as F_0, spore kill, positive/negative control requirements, and any additional dwell time criteria
- Requalification schedule and procedure

Next, you should justify to the authorities through documentation that your placement of monitoring devices (BIs and thermocouples) for the final validation is appropriate:

- Evidence that appropriate heat distribution studies have been performed
- Demonstration that the most difficult to heat spot in the cycle has been identified and has been monitored in the validation

Documentation and description of the BIs should include

- Vendor name and certificates of analysis for a representative lot of BI
- D and z-values as well as the spore population for each lot
- Procedures describing storage and handling of the BIs

You should have a compilation of the raw data and summaries of the validation activities that includes

- Individual thermocouple readouts for each validation run

- Procedures describing the incubation conditions for the BIs as well as positive/negative controls
- Outcome of the BI incubation and calculations of F_0 from the thermocouple data
- Verification that appropriate facility personnel have reviewed and approved the outcome of the validation

Finally, for routine sterilizations, you should be prepared to present:

- Procedures for how the sterilization will be monitored
- Procedures describing what the facility will do if the sterilization fails.

Recommendations from the International Organization of Standardization (ISO) document published as ISO 17665, although developed for medical devices, may also be useful for additional understanding of the documentation necessary for steam terminal sterilization of pharmaceuticals (ANSI/AAMI/ISO 17665-1:2006).

18.1.5 Radiation Sterilizations

Another way to terminally sterilize a parenteral product is to use ionizing radiation to kill or inactivate the existing *bioburden* or microbial load of the product. Radiation sterilizes by inducing the formation of free radicals that damage DNA (Hammad 2008) within the bioburden of the product rendering the affected microorganisms inactive. Sterilizing a product using radiation is relatively simple conceptually; a load of product is conveyed past or around a stationary radiation source until the load absorbs the required radiation dose. Since radiation penetration past the surface may be shielded by the mass of the load, the denser the load, the greater the exposure time necessary to allow the inside portions of the load to absorb a sterilizing dose (Mehta 2008). For the vast majority of loads, the identity of the material to be sterilized is not as important as its mass on the magnitude of the dose absorbed in the interior of the load. Other influences on absorbed dose include distance from the load to the source and activity of the source. Radiation exposures or doses are typically expressed in kilograys (kGy).

18.1.5.1 Gamma/E-Beam

One common radiation sterilization technique employs gamma irradiation to kill microorganisms. Gamma rays are energetic enough to penetrate past the surface of the load into its interior. To sterilize them, loads are placed on a carrier and conveyed into a shielded room past a gamma emitter, typically ^{60}Co pencils arranged on racks (Mehta 2008). The carrier containing the product is conveyed so that both sides of the load are exposed to the source for approximately the same amount of time. This ensures that the surfaces of the load absorb roughly the same dose all the way around. Since the source activity decays with time, exposure time for a load is adjusted and the ^{60}Co source replaced periodically.

Electron beam or “e-beam” processes is another radiation technique used to sterilize articles. In this form of radiation sterilization, an accelerator generates a

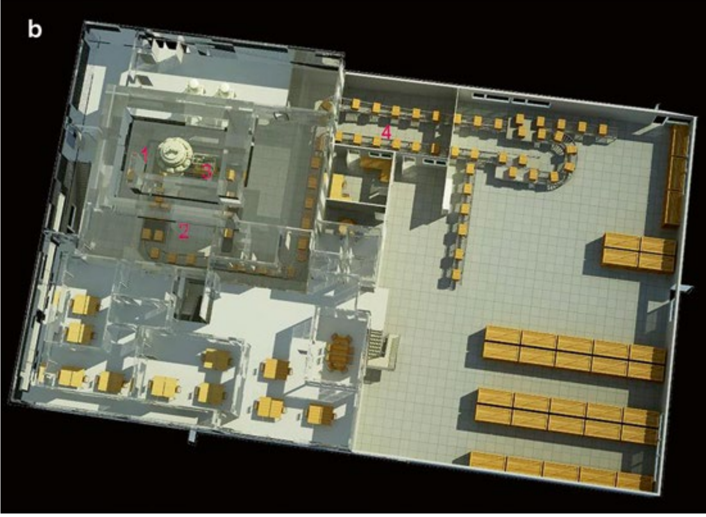


Fig. 18.3 (**a**) A Rhodotron® electron beam accelerator. The diameter of the unit is 3 m. (**b**) Layout of a representative e-beam facility, 1-Rhodotron®, 2-Load staging area, 3-Scanning horn, 4-Conveyor line. Photographs courtesy IBA Group

concentrated stream of electrons and directs the stream through a scanning horn onto the items to be sterilized. Loads are conveyed into range of the electron stream. The accelerator can then scan the electron stream across the length and width of the load. Unlike the cobalt gamma sources described above, e-beam accelerators may be turned off when not needed. An example of an e-beam sterilization apparatus and conveyor scheme is in Fig. 18.3.

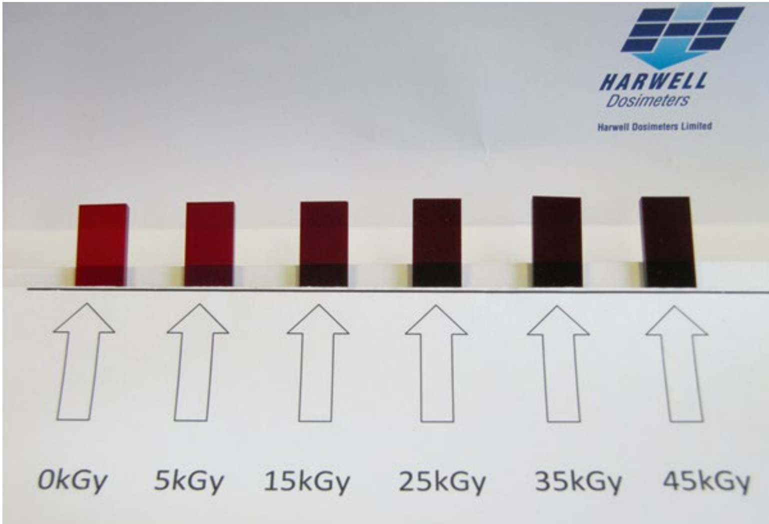


Fig. 18.4 Radiation dosimeters following selected doses. Photo obtained with the permission of Harwell Dosimeters Ltd

18.1.5.2 Cycle Development

Radiation dosimeters placed within the load to be sterilized measure the dose absorbed at that point. An example of a radiation dosimeter that may be used for sterilization procedures and how it responds to irradiation is shown in Fig. 18.4. Dosimeters commonly used in sterilization cycle development as well as routine sterilizations are thin acrylic films that undergo a color change upon exposure to ionizing radiation. This color change may be read in a spectrophotometer at a suitable wavelength and compared to standards. Absorbance values are sensitive to path length and therefore thicknesses of the dosimeters must be determined precisely. Since the relationship between color presented and radiation dose recorded may differ based on many factors, calibrations must be performed for each new batch of dosimeters (Sharpe and Miller 2009).

Development of a radiation sterilization process may employ a different strategy than that of a moist heat terminal sterilization, but the desired SAL of 10^{-6} can still be employed as a goal and a useful monitoring metric. Another way to establish a radiation process involves a bioburden approach that employs the ISO 11137 methods for both determining a suitable radiation dose and verification that the conditions in which the method was developed are still valid with time (ANSI/AAMI/ISO 11137-2:2006). These procedures were developed for medical devices, but also may be used for terminal sterilization of pharmaceuticals. The ISO methods allow the development of an irradiation cycle with at least a 10^{-6} SAL by establishing the average (naturally occurring) bioburden of product units, performing dose verification experiments to an SAL that is measurable using a conventional sterility test then extrapolating the results to a find a final routine radiation dose.

ISO 11137–2:2006 describes several different methods to establish a radiation dose for routine use. If the goal is to reduce the dose to the minimum level to achieve a 10^{-6} SAL (Method 1), ten units at random are selected from three representative batches and tested to determine the average number of microorganisms per unit. This number may be used to calculate or read from a table a verification dose that should provide a 10^{-2} SAL. Then, 100 additional units are irradiated at the verification dose and tested for sterility. If no more than two units of irradiated product are positive for growth, the verification dose may be used to scale up to a 10^{-6} SAL for routine use. [1]Once established, the appropriateness of the radiation dose is determined by periodic audits of the sterilization dose; ISO 11137–1 recommends once quarterly for most processes. In addition to Method 1, the ISO document allows for substantiation of two preset doses, 15 and 25 kGy, assuming appropriately low microbial counts per unit of product. A verification dose study and periodic audits are still performed for these options.

18.1.6 Radiation Sterilization Validation

Although biological indicators such as *Bacillus pumilis* spore strips traditionally have been used for validating radiation processes in a somewhat similar manner to biological indicators for steam sterilization processes, this practice has declined in recent years because of the difficulty in identifying a standard microorganism that represents a worst-case challenge to the sterilization process (United States Pharmacopeia 2011). Nevertheless, validations of radiation sterilization processes that involve biological indicators may sometimes be used in situations where other validation methods may not be practical. Before beginning a validation involving BIs, a dose-mapping study to determine the minimum and maximum positions of radiation dose absorption should be performed. Some guidance on how a dose-mapping study could be carried out may be found in ISO 11137–3. For the validation, radiation dosimeters are deployed at the minimum and maximum positions determined in the dose-mapping studies, as well as other positions within the load. Biological indicators with counts of at least 10^6 colony forming units (CFU) per unit are also deployed in a manner analogous to that of heat sterilization validation mentioned in Sect. 1.2. After subjecting the load to a production radiation cycle, the dosimeters are checked to assure that an acceptable range of doses has been absorbed at all the positions within the load. The biological indicator strips are incubated in a suitable growth media and checked for growth. If none of the indicator strips is positive for growth, the process can be deemed to have achieved a SAL of 10^{-6}.

[1] ISO 11137–2 bases its rationale for allowing two positives upon the assumption that the probabilities of occurrence of numbers of positives around an average of one positive are distributed according to the Poisson distribution. With this distribution, there is a probability of 92 % that zero, one or two positives will occur.

Radiation process validations need not necessarily involve BIs however, and may take the form of demonstrating that the source and related equipment function as intended. Part of this process involves demonstration that installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ) of the irradiating equipment or source and facility are appropriate. ISO 11137–1 describes general requirements for IQ, OQ, and PQ for the common types of radiation sterilization. Both gamma and e-beam validations may follow the ISO 11137 guidelines. The periodic verification dose audit may be considered part of the requalification since it verifies that conditions have not changed since the sterilization procedure was developed and shown to be effective. European requirements for high dose e-beam sterilizations may also include a demonstration that the product does not develop radionuclides (European Union 1992).

18.1.7 Radiation Sterilization Trade-Offs

The use of gamma radiation to sterilize products comes with a number of advantages and disadvantages. The advantages include the fact that the products need not be heated to extreme temperatures to absorb sterilizing doses of radiation, nor is there a need for a "wash-out" period or check for sterilant breakdown products after sterilization as there would be for gas sterilization techniques (see Sect. 3.1). Gamma radiation has been shown to kill most viruses (Nims et al. 2011), although validation of viral clearance potential of radiation cycles is not normally required. Disadvantages include the fact that many pharmaceutical companies do not keep a gamma radiation source within their facility; any such sterilization often requires a contracting facility to perform this function. In addition, gamma rays may degrade both product and packaging (e.g., (Loo et al. 2010; PDA Technical Report 1992)). The amount of degradation varies considerably with product and some process development studies may be required to determine a radiation dose that will attain the requisite SAL but keep the extent of the damage tolerable.

Advantages of e-beam sterilization include higher dose rates and therefore the possibility of higher throughputs than with gamma radiation (Chmielewski et al. 2008). The higher dose rate of e-beam processing also may allow reduced oxidative degradation of the product components in some cases (Woo and Purohit 2002). In addition, the accelerator may be set up to deliver electron beams at various intensities, allowing for many possibilities for different processing procedures. Disadvantages involve the fact that electron beams are not as inherently energetic as gamma rays and thus may not penetrate as far into a load or be as useful for higher density materials as gamma irradiation. As with gamma sterilizations, e-beam is not available in most pharmaceutical manufacturing facilities, and therefore may necessitate the use of a contract facility. There is also potential for degradation of some types of materials with e-beam sterilizations (Lucas et al. 2004; Singh et al. 2011).

18.1.8 Radiation Sterilization Regulatory Aspects

Of course, information about the radiation dose ultimately chosen and load diagram for routine sterilizations should be available for review. For calibration of radiation dosimeters, users may turn to ISO/ASTM standards (ISO/ASTM 51261-2002).

To demonstrate that radiation processes are under control, documentation and procedures as described in ISO 11137–1 should be available. For example, some of the documentation that might be needed for IQ is as follows:

- Operating procedures for source and conveyor
- For gamma sources, activity of the source and location of individual components of the source
- For e-beam, descriptions of the electron energy, beam current, scan width, and scan uniformity
- Documentation of methods and results of testing to verify operation to design specifications

For OQ:

- Documentation of methods and results of testing to verify operation to design specifications
- Confirmation of the calibration of instrumentation
- Dose-mapping study methods, raw data, and results
- Evidence that the relationship between timer, conveyor speed, and radiation dose is known

For PQ:

- Dose-mapping study methods, raw data, and results
- Definition of the load(s) to be tested during PQ including its (their) density if necessary
- Definition of containers for the product(s)

From the qualification information, a process specification should be developed to describe the operating parameters for routine sterilization. To demonstrate control of the routine process for the product a facility should be prepared to present documentation describing:

- Loading pattern(s) developed for the product(s)
- Conveyor path to be used
- Maximum and minimum acceptable dose
- Target sterilization dose
- Routine dosimeter positions for monitoring the dose
- Maximum time allowed between completion of packaging and the radiation treatment.

For verification dose determination and auditing, at a minimum you should have documented:

- Raw data showing the results from verification dose testing
- Frequency and timing of auditing
- Procedures for determining product units to be sampled
- Procedures for performing the sterility and bioburden tests on the product and interpretation of the results
- Acceptance criteria for verification dose testing
- Procedures to be followed when an audit fails

Note that ISO 11137 guidelines describe additional documentation that may be needed for sterilizations of some types of products. Additional material to show that the product can withstand a routine radiation treatment may also be reviewed. If biological indicators are used, studies characterizing the resistance of the BI relative to the bioburden may be necessary (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM072171.pdf).

18.1.9 Ethylene Oxide Sterilizations

Sterilization may also be accomplished without either heat or radiation by the introduction of gaseous chemical sterilants into specially designed chambers containing the loads to be sterilized. The most commonly used chemical sterilant, ethylene oxide, works as an alkylating agent (https://www.osha.gov/dts/sltc/methods/organic/org030/org030.html). Ethylene oxide can covalently bind to nucleophilic sites of purine DNA bases (Huang et al. 2011), making the gas effective for breaking down nucleic acids in viruses and bacterial cells. Ethylene oxide sterilizations are sensitive to humidity and temperature (Oxborrow et al. 1983). The sterilization process using ethylene oxide involves four stages: a preconditioning phase to stabilize moisture content of the load, transfer of the product to the sterilization chamber, ethylene oxide introduction and dwell time, and a degassing phase. In the preconditioning phase, the load is held at a temperature of about 40–50 °C for a day or two. For routine ethylene oxide sterilizations, *B. atrophaeus* BIs at spore concentrations of at least 10^6 per unit are commonly used for monitoring. After this phase, the load is transferred to a sterilization chamber; air is removed from the chamber, and replaced with ethylene oxide. After a defined dwell time, ethylene oxide is pumped out of the chamber and the load is degassed to remove excess ethylene oxide and ethylene oxide breakdown products. Air may be used for degassing, and the load may be required to dwell within this “wash” for about a day for some loads. The product may be released following verification that ethylene oxide is sufficiently removed and BIs spores have been killed. Gaseous sterilants are by nature able to sterilize surfaces only, making them most useful in the pharmaceutical industry for sterilizing certain

types of containers and not usually as a means of terminal sterilization for finished parenteral products. However, certain packaging materials such as Tyvek are permeable to ethylene oxide and allow sterilization of the package contents (Mankel 2008).

Other chemical sterilizations may involve agents such as hydrogen peroxide, chlorine dioxide, and glutaraldehyde. These agents are not often used for sterilization of finished pharmaceutical products, but may be used for sterilization of some types of manufacturing equipment. These types of sterilizations will not be dealt with extensively here.

18.1.10 Ethylene Oxide Sterilization Validation

Validation of ethylene oxide processes most commonly follow a half-cycle approach suggested by ISO 11135–1 (ANSI/AAMI/ISO 11135-1:2007) and ISO 11135–2 (ISO 11135-2:2008(E)). BIs are located in difficult to sterilize locations as described previously for heat sterilization methods. The reader should be cautioned that while suppliers of *B. atrophaeus* may provide a *D*-value for their spore preparations, this value may have been calculated with a dry heat process in mind and this should not be confused with a *D*-value determined for an ethylene oxide process. Validation dwell times for the loads in the presence of the gas are typically half of what is used for production purposes, but other sterilization parameters such as temperature, humidity, pressure, and ethylene oxide concentration should be the same as in routine production. Incubation of BIs and establishment of controls positive for growth after the runs should be performed according to manufacturer's instructions. In order to determine the most difficult to sterilize portion of the load for monitoring purposes, some development work for the cycle should be performed beforehand so that optimal positions can be documented. Three successful half-cycle validation runs should be performed to verify that the full cycle used for routine sterilization is acceptable. ISO 11135 suggests that requalification needs be reassessed in an annual review of changes to chamber engineering or performance (ANSI/AAMI/ISO 11135-1:2007; ISO 11135-2:2008(E)). Depending on the outcome of the assessment and nature of the product release procedure, a requalification may be performed once every 1 or 2 years.

To validate that ethylene oxide and its breakdown products can be removed by the degassing process, testing should be performed to verify that ethylene oxide and its breakdown products do not remain in the sterilized material (EudraLex 2008; Adler 1965). FDA and ISO have published guidance regarding maximum residue limits in medical devices (http://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/UCM078413.pdf; AAMI/ANSI/ISO 10993-7 1995).

18.1.11 Ethylene Oxide Trade-Offs

There are trade-offs when using ethylene oxide. Advantages include the relatively modest temperatures required for sterilization as well as freedom from some of the degradative effects of ionizing radiation treatments. Disadvantages include the amount of time required for treatments—the preconditioning, degassing, and breakdown product detection are all fairly time consuming relative to other terminal sterilization techniques. In addition, ethylene oxide reacts with some materials (ISO 11135-2:2008(E)) and therefore careful screening of any materials that may come into contact with the gas is necessary. The gas is flammable (Use of Ethylene Oxide as a Sterilant in Medical Facilities 1977) and an irritant of the eyes and skin as well as potentially carcinogenic (https://www.osha.gov/dts/sltc/methods/organic/org030/org030.html) and may present safety issues if handled improperly or by untrained personnel.

18.1.12 Ethylene Oxide Sterilization Regulatory Aspects

For a validation of an ethylene oxide process, regulatory agencies may request descriptions of the sterilizer and the cycle control parameters mentioned in Sect. 3.2 such as temperature, ethylene oxide gas concentrations, pressure, humidity, half-cycle exposure time, etc. ISO 11135–1 emphasizes IQ, OQ, and PQ documentation (ANSI/AAMI/ISO 11135-1:2007) and review of this material by regulatory agencies is possible. Documentation concerning the routine control of the cycle such as load pretreatment, exposure time and temperature, total amount of ethylene oxide gas used, and degassing after the cycle may also be reviewed (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM072171.pdf). Other possible documentation that could be reviewed is noted in ISO 11135. Keep in mind that calibration documentation for any equipment such as that used to record temperature, pressure, and humidity may be subject to review. BI parameters such as *D*-value and spore concentration may also be subject to review.

18.1.13 Aseptic Processing

Aseptic processing is when a bulk product and its container are sterilized separately and packaging and sealing are performed in such a manner that the sterility is maintained throughout the procedure. While the sterilization of containers used for the product conceivably may employ any of the sterilization methods previously discussed, under most circumstances sterilization of bulk liquid product before filling into final containers requires filtration using sterilizing-grade filters. Once the bulk liquid product is filtered, the goal of aseptic processing is to keep the liquid microbe-free until it is safely sealed in sterilized containers—and this is not a trivial proposition. Microbial contamination may come from either the environment itself or be

shed from humans in that environment; care must be taken to avoid transfer of the bioburden from these sources to the sterilized product. The exclusion of microorganisms from a sterilized bulk liquid during the processing steps before filling and sealing a product into sterilized containers requires careful design of the facility where an aseptic processing takes place. Although most aseptic processing necessitates some personnel contact with materials and equipment during manufacturing, minimization of human intervention is one of the most important factors in assuring success. Unfortunately, a quick review of all the aspects of aseptic processing will necessarily be incomplete. Discussion of issues such as cleaning and sterilization of manufacturing equipment or design of vial filling areas cannot be adequately managed in a short review. However, sterile filtration, clean room design, and personnel flow are three common aspects that should be integrated into any successful plan and each requires a large amount of documentation and validation to demonstrate the effectiveness of the whole process.

18.1.13.1 Filters

A successful sterilizing filtration works by retaining all the bacteria in a fluid on the filter, but there is more to it than that. Filters used for aseptic processing are rated at 0.2 μm porosity or smaller (FDA's Guidance for Industry 2004; EudraLex 2008). Beyond this, philosophies around the world differ. In the United States, filters should be designed to retain at least 10^7 CFU per cm^2 of filter area of *Brevundimonas diminuta* (chosen because of its small size—about 0.3 μm in diameter) (FDA's Guidance for Industry 2004) whereas to meet European Union requirements, the maximum suggested bioburden in front of a final sterilizing filter is 10 CFU per 100 mL of fluid (European Agency for the Evaluation of Medicinal Products Committee for Proprietary Medicinal Products 1996). This suggests that in some cases it would be prudent to employ two sterilizing-grade filters in series for production purposes.

For routine production, a filter integrity test will suffice to show that the filtration process is under control. Most filter vendors define the pressures their products can tolerate with common liquids such as water or isopropyl alcohol. Vendors cannot however completely predict pressure ratings for their filters when the vast array of conditions and products to which the filters may be subjected is considered. Therefore, during the development of the filtration process, a suitable filtration pressure should be determined. Then during the production of a sterile product, the filter should be integrity tested before (as recommended by European guidance) and/or after (European and the United States) use. For example, a bubble point test of the filter may be performed in order to verify integrity. Bubble point testing is based on the fact that liquid is held in the pores of the filter by surface tension and capillary forces. The minimum pressure required to force liquid out is a measure of the pore diameter. When this pressure is reached, a stream of bubbles that can be detected visually should appear downstream of the filter. Other integrity tests such as forward flow testing (PDA Technical Report 1998) may be used instead of the bubble point test.

Table 18.1 US and ISO 14644–1 classifications of cleanrooms. ISO 5, ISO 7, and ISO 8 are the most commonly used classifications for aseptic processing

US and ISO air classifications							
	Maximum number of particles per m³ of air						US standard
ISO class	≥0.1 μm	≥0.2 μm	≥0.3 μm	≥0.5 μm	≥1 μm	≥5 μm	209E
ISO 1	10	2	–	–	–	–	–
ISO 2	100	24	10	4	–	–	–
ISO 3	1,000	237	102	35	8	–	–
ISO 4	10,000	2,370	1,020	352	83	–	–
ISO 5	100,000	23,700	10,200	*3,520*	832	29	*100*
ISO 6	1,000,000	237,000	102,000	35,200	8,320	293	–
ISO 7	–	–	–	*352,000*	83,200	2,930	*10,000*
ISO 8	–	–	–	*3,520,000*	832,000	29,300	*100,000*
ISO 9	–	–	–	35,200,000	8,320,000	293,000	–

Production pressures should not exceed the maximum pressure determined in the integrity tests. Regulatory agencies recommend sampling the bioburden of the bulk liquid to be filtered before filtration (FDA's Guidance for Industry 2004; EudraLex 2008).

18.1.13.2 Cleanrooms

The areas where pharmaceutical products are manufactured need not be sterile, nor is it practical to make them so. However, the avoidance of contamination from microorganisms does dictate that these rooms have potential contaminants controlled. Airborne particulates are the indicator of choice for control of potential contaminants. Although it is difficult to assign concrete numbers to the correlation, in general the greater the level of airborne particulates, the greater the assumed bioburden in the area (United States Pharmacopeia 2011). Among the international standards documents, ISO 14644–1 (ISO 14644-1:1999(E) 1999) deals with the classification of clean rooms including those used for, among other possible operations, manufacturing pharmaceuticals. Table 18.1 shows the ISO cleanliness classifications that one may encounter in pharmaceutical manufacturing and the equivalent US nomenclature for these standards. The figures in italics in the table represent the classifications that are typically used for aseptic processing. The U.S. Federal Standard 209E shown in the table header has been discontinued, but the nomenclature for air classes based on this standard is still used in the United States. To further confuse the matter, facilities in the United States and Europe have traditionally adopted different nomenclature and philosophies for assignment of clean room designations. Table 18.2 shows the European Union classifications. While limits on other particle sizes may be defined for the various room classifications, ≥0.5 μm particles are the most frequently measured and monitored and represent the most common reference point for aseptic processing. Annex 1 to the European

Table 18.2 European classification of cleanrooms (EudraLex 2008)

European Union air classifications				
	Maximum number of particles per m^3 of air			
	At rest		In operation	
Grade	≥0.5 μm	≥5.0 μm	≥0.5 μm	≥5.0 μm
A	3,520	20	3,520	20
B	3,520	29	352,000	2,900
C	352,000	2,900	3,520,000	29,000
D	3,520,000	29,000	Not defined	Not defined

Union Guidelines to Good Manufacturing Practice for Medicinal Products for Human and Veterinary Use also singles out ≥5.0 μm particles for emphasis since even low levels may be an indicative of contamination in the clean room. Note also the distinction in the European guidelines between "at rest" and "in operation"—a distinction that does not exist in US or ISO standards. At-rest classifications for grade B and C refers to the status of the areas when all processing equipment installed but no operating personnel are present whereas in-operation status is under working conditions. Japanese requirements for classified areas are similar to those of the European Union although ≥5.0 μm particles do not appear to be as emphasized and US classifications are recognized (The Japanese Pharmacopoeia Fifteenth Edition 2006). Annex 1 suggests that grade A areas be used for operations with high risk of contamination such as those with open vials or ampoules; grade B is for aseptic preparation and as a background for the grade A areas; grade C is for operations with less risk of contamination such as preparation of solutions to be filtered. US aseptic processing guidelines (FDA's Guidance for Industry 2004) suggest similar uses for their equivalent cleanroom classifications—class 100 (ISO 5) areas are suggested for high risk operations, and areas adjacent to aseptic processing lines should meet class 10,000 (ISO 7) at minimum. US FDA guidance (FDA's Guidance for Industry 2004) suggests that particulate levels in ISO 5 be measured using particle counters at locations no more than one foot from "work areas" and that probes intended to measure particles in other areas be located in areas where product is at potential risk.

Regardless of which system is used for classification, High Efficiency Particulate Air (HEPA) filters are typically used to reduce the particulates measureable in the air in the areas hosting aseptic processing. In grade A/class 100/ISO 5 areas, unidirectional air flow systems direct filtered air usually from overhead downward toward intakes containing filters located near the cleanroom floor and care should be taken not to disrupt this flow during operations whenever possible. In order to verify that the classified areas remain under control, particulate levels may be measured in each area using procedures from ISO 14644–2 (ANSI/IEST/ISO 14644-2:2000). Positive pressures should be maintained between adjacent rooms of differing classifications to minimize ingress of contamination. US and European guidelines suggest for rooms of higher air cleanliness to have at least 10–15 Pa maintained between adjacent rooms of differing classification (with doors closed). In addition to monitoring

of particulates, cleanrooms for the aseptic processing of pharmaceuticals require monitoring of the cleanroom environment according to the U.S. Code of Federal Regulations (21 CFR 211.42(c)(10)(iv)) and European Union GMP requirements (EudraLex 2008). Such monitoring should include

- Active air monitoring—microbes in the air in the clean areas that may be sampled using devices such as slit-to-agar or surface air system samplers.
- Surface monitoring—contact or touch plates or swabs that can sample surfaces such as walls, floors, or product contact areas.
- Passive air monitoring—bacterial culture plates ("settle plates") exposed to the environment allowing any airborne bacteria above the plate to fall onto it.

Microbial limits for each room class should be established. European, Japanese and US pharmacopeias all have recommendations for each type of environmental monitoring in each type of classified area as well as suggested frequency of sampling. The regional compendia describe two sets of microbial limits for each classified area: *alert* limits (requiring investigation) or *action* limits (requiring corrective action). Locations of monitoring plates and devices should be documented in an environmental monitoring plan including diagrams where appropriate. Note that due to the need to process each of the types of plates above, traditional monitoring methods for microorganisms do not usually provide results in real-time. US guidance recommends trending of the historical environmental monitoring data to identify potential adverse tendencies (FDA's Guidance for Industry 2004).

18.1.13.3 Personnel

The goal of managing the flow of personnel and materials into and around the clean rooms in an aseptic processing area should be to ensure that the possibility of introducing human-borne microorganisms onto or into the sterilized components of the product is negligible. Of course, humans must still intervene occasionally in even the most automated processes to introduce materials and containers, establish (aseptic) connections between pieces of equipment, troubleshoot problems, and perform in-process testing. Although some variation in movement around cleanrooms is inevitable, a prearranged plan for how the personnel flow should proceed should be available. When in classified areas, personnel should wear sterilized protective apparel such as gowns and gloves as well as face, hair, and shoe coverings. The apparel should be low-shedding material to reduce the risk of introducing additional particles into the aseptic processing areas. Flow of personnel should minimize the possibility of interrupting any unidirectional flow of air in the room. Common-sense approaches such as moving personnel away from contamination-sensitive areas when not performing interventions should be encouraged.

Good manufacturing practice for aseptic processing requires that persons assigned to intervene in clean rooms have adequate training. This training should include instructions on gowning, procedures for and frequency of personnel

microbial monitoring and adequate instruction on the tasks to be performed to complete the manufacture. Personnel engaged in manufacturing in aseptic areas should practice good sanitation habits and if ill should not participate in cleanroom activities.

18.1.14 Aseptic Processing Validations

A calculation of an approximate SAL for a single aseptic processing run is more mathematically elusive than with the other techniques discussed in this chapter. A BI cannot monitor an aseptic process adequately, so for validation a simulation of the process, or *media fill*, using a medium suitable to support growth of microorganisms likely to contaminate the product during manufacturing should be performed. This media fill should simulate all the steps and use all the equipment required to manufacture the product. In practice, this means that the growth medium should contact all equipment and container surfaces that the bulk product may encounter during the processing. In addition, any instances of possible interventions by personnel during the process should be included in the simulation, such as aseptic connections of equipment, addition of product components to the controlled areas, troubleshooting, and routine sampling. The media fill should include an environmental monitoring plan mimicking the plan used for routine production. The medium should be held at each step of the process for durations at least as long as the longest hold time for that piece of equipment. Once all the hold times have been simulated, the medium is filled into sterile containers, the containers incubated under suitable conditions to support bacterial growth, and all the containers inspected visually for turbidity to determine whether growth has occurred. To show that the simulation has not altered the medium such that microbial growth can no longer be supported, a test of the growth promotion potential of the medium using standardized bacterial stocks and microorganisms isolated directly from the facility should be performed on vials that have already been utilized in the simulation and found negative for growth.

For operations that involve filling into vials, while the total worst-case filling time should be simulated, it may not be necessary to duplicate a representative batch size to have a successful media fill. FDA recommends that a typical run size should be in the range of 5,000–10,000 units. For operations with production sizes under 5,000, the number of filled units should at least equal the maximum batch size made on the processing line (FDA's Guidance for Industry 2004). In some cases where very large numbers of units are filled, run sizes greater than 10,000 should be considered. Line speed for a media fill should be chosen and justified based on number and time of interventions, choice of simulation run size and intended time of vial exposure. The total time for the simulation should include all the necessary work shifts that may occur during routine manufacturing.

US regulatory guidance recommends at least three consecutive successful simulations for validation purposes followed by twice annual requalification after

validation (FDA's Guidance for Industry 2004). Recommendations for acceptance criteria for a simulation vary according to regional authority. For example, for individual fills, US authorities recommend the following criteria for simulations:

1. When filling fewer than 5,000 units, no contaminated units should be detected.
   - One (1) contaminated unit is considered cause for revalidation, following an investigation.
2. When filling from 5,000 to 10,000 units:
   - One (1) contaminated unit should result in an investigation, including consideration of a repeat media fill.
   - Two (2) contaminated units are considered cause for revalidation, following investigation.
3. When filling more than 10,000 units:
   - One (1) contaminated unit should result in an investigation.
   - Two (2) contaminated units are considered cause for revalidation, following investigation.

European Union recommendations are similar to those of the United States (EudraLex 2008). If an investigation of contaminated units is needed, it should include identifying the contaminating organism.

Filters also should be validated for their ability to retain bacteria and produce a sterile effluent. The filter validation need not take place within the manufacturing facility where the operation will take place; it may be carried out in a contract facility, for example. Validation conditions should at least simulate conditions of production. For US validations, after conditioning with the product, filters should be challenged with about 10^7 CFU of *B. diminuta* per cm^2 of filter surface (see Sect. 4.1.1) suspended in a reasonable simulation of the product (ASTM, Committee F 838-05 2005). Effluent should then be checked for sterility using appropriate media and incubation conditions.

Validation of HEPA filters in cleanrooms involves testing the filtration efficiency, leaks and uniformity of velocity of the filters. Aerosols with defined particle diameter are used to challenge the filters for filtration efficiency and leaks. The procedures for these types of validations are detailed in the ISO 14644 series of guidances (ANSI/IEST/ISO 14644-2:2000; ISO 14644-3:2005). In areas where unidirectional flow is intended, directionality and turbulence may be tested with aerosols that are visible (FDA's Guidance for Industry 2004).

18.1.15 Trade-Offs with Aseptic Processing

The main advantage of aseptic processing of course is that it does not require high temperatures or harsh chemicals to be effective. One disadvantage is that viruses may not be retained by 0.2 μm sterilizing-grade filters; much smaller porosity (and

correspondingly lower throughput) filters are needed to significantly retain virus particles. In some cases, even some types of bacteria may fail to be retained on sterilizing-grade filters (Sundaram et al. 2001). Another disadvantage of aseptic processing procedures using filtration is that in the process of filtering, particles and filter components may be shed into the liquid on the downstream side and the risk of these contaminants getting into finished product should be mitigated.

18.1.16 Aseptic Processing Regulatory Aspects

Documentation from the validations previously described may of course be reviewed by regulators. Regulatory review of aseptic processing involves observation of the sterilization and filling areas (Dosage Form Drug Manufacturers cGMPs 1993; EMA (European Medicines Agency) 2010) and perhaps even the process as it takes place. Documentation alone may not satisfy a regulatory agency whose job necessitates understanding how the aseptic process is intended to work and judging the location and adequateness of the monitoring. Upon inspection of your facility be prepared to answer questions about the kinds of activities that occur in your cleanroom.

Media fill documentation should include a general protocol describing each step in the process and summaries of results from each run. To gauge the appropriateness of the simulation, supporting documentation that may be reviewed includes individual records of media fills and related product manufacturing records. Regulators may pay particular attention to timing and impact of interventions on the media fill. Since a failure of a media fill run may indicate that integrity of the aseptic processing system is compromised, requalification runs of the media fill may be evaluated to gauge when the failure occurred with respect to any product lots that may have been impacted around the time of the failure.

Filter validation documentation such as the bacterial retention study may be reviewed as well as verification that filter pressures used for production are equivalent to those tested in validation. A diagram of the plan for personnel flow in the cleanrooms should also be available for review.

Environmental monitoring locations and frequencies may be evaluated for appropriateness based on risk assessments provided by your facility. Monitoring results for relevant periods should be available for review, and any investigations or corrective actions executed as a consequence of alert or action levels being exceeded should be included with these reports. Results from the identification of organisms found in environmental monitoring may also be used to judge the adequacy or impact of any investigations and corrective actions.

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Chapter 19
Avoiding Common Errors During Viable Microbial Contamination Investigations

Kenneth H. Muhvich

Abstract Pharmaceutical companies are required by regulatory authorities to conduct thorough investigations into microbial contamination events related to aseptic manufacturing of sterile products. Often a variety of errors are made during the course of such investigations. In many cases, those are errors in judgment which preclude finding the Root Cause of the problem and prevent identification of a long-term solution. The major goals of such an investigation are to locate the source of the contaminating microorganism and then to determine the Root Cause, i.e., how that microbial contaminant got into the "sterile" product or aseptic processing area. Often wrong assumptions are made based upon previous experience or lack of sufficient technical knowledge. Extraordinary Environmental Monitoring (EM) is typically required to locate the source(s) of the microbial contamination. Examples of errors in judgment (Wrong Thinking) and case studies are provided to aid the reader in conducting the best possible sterility assurance failure investigations.

19.1 Background

The author is frequently asked to assist pharmaceutical companies that are conducting investigations into microbial contamination events related to aseptic manufacturing of sterile products. Over the past decade he has observed a variety of errors that were made during such investigations. In many cases, those errors in judgment precluded finding the source (Root Cause) of the problem and prevented identification of a long-term solution. Therefore, the purpose of this chapter is to help the reader avoid common mistakes that others have already made.

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19.2 Introduction

As described in Section XI “STERILITY TESTING” Part C of the FDA’s Guidance for Industry entitled *Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice* a thorough investigation of sterility test positive should be performed (U.S. Department of Health and Human Services et al. 2004). Such an investigation is also required by Section IX, Part A.9 *Interpretation of Test Results* (U.S. Department of Health and Human Services et al. 2004) when test units are contaminated with viable microbes during process simulation runs (media fills). Chapter <1116> of the current US Pharmacopeia provides some additional guidance on how to conduct a sterility assurance failure investigation. Yet, in the author’s opinion, neither document provides enough meaningful information to aid one in performing an in-depth investigation. The goal of this chapter is to provide practical advice regarding the performance of a sterility assurance failure investigation.

19.3 Typical First Steps

How do people typically begin an investigation into a sterility assurance issue? What do they do first? Most people aren’t very experienced in performing failure investigations. The reason for this is that most sterile product manufacturing facilities are, for the most part, under an acceptable state of contamination control. So, people haven’t seen many Environmental Monitoring (EM) excursions, Sterility Test, or Process Simulation Test (Media Fill) failures. In many pharmaceutical companies there is no formal plan or internal guidance other than what is written in an Out-of-Specification (OOS) Standard Operating Procedure (SOP). The typical scenario described in an OOS SOP is to form a cross-functional team (Operations, QC, Validation, etc.) that is tasked with conducting the investigation. There may also be a checklist containing items that must be investigated. Use of a checklist to begin an investigation is acceptable, but should not be viewed as all inclusive for the balance of the investigation. Checklists should be open-ended so that one can utilize outside-of-the-box thinking and so that one can explore areas that have not already been identified as possible causes for the contamination event(s). Some OOS SOPs may include a decision tree to help get the investigation off on a solid footing. In author’s opinion, these are typically very helpful if constructed properly.

19.4 Goals for the Investigation

The first and most important goal for the investigation is to determine the source(s) of the microbial contamination. In author’s opinion, it is not possible to consistently identify a real Root Cause for a microbial contamination event without knowing the

source or sources of the contaminating microbe(s). Often inexperienced individuals think that the source of the contaminating microbe and the Root Cause are the same thing, but that is not the case. Simply put, the source of the contaminating microbe is where it resides in the manufacturing facility. The Root Cause is how the microbe got into the product or found its way into a highly classified aseptic processing environment.

Finding the Root Cause for the contamination event is a regulatory authority, e.g., FDA and EMA, expectation. However, finding the definitive Root Cause, i.e., smoking gun, for a microbial contamination event is a difficult task. Furthermore, a definitive Root Cause is not identified in a large number of cases despite exhaustive due diligence efforts. In author's experience, if one can find the definitive Root Cause 15–20 % of the time then the proper level of due diligence has been expended.

In the author's experience, finding a Probable Cause for a microbial contamination event is the most common outcome of a sterility assurance failure investigation. One should be able to identify one or more Probable Causes in at least 60 % of the cases. If an investigation results in several Possible Causes, then (in author's opinion) that means that sufficient efforts have not been expended in investigating the contamination event to date and more work still needs to be done. Possible Root Causes result when a pharmaceutical manufacturing facility doesn't look long or hard enough for the source of the microbial contamination. One must locate the source(s) of the microbial contamination to have any real chance of finding the Root Cause.

19.5 Wrong Thinking: Errors in Judgment

"Wrong Thinking" is defined for the purpose of this chapter as using thought patterns that will prevent finding the source(s) of the particular microbial isolates and the Root Cause for the contamination event. The most common type of Wrong Thinking is making assumptions based upon a little bit of information or knowledge. For example, information about the isolate from a contaminated media fill test unit is obtained from an "online" source or from a Microbiology textbook. Then the commonly listed source(s) in the reference(s) are automatically assumed to be the same for the microbial isolate from their pharmaceutical product manufacturing facility. But, that may not be the case at all. For example, a particular bacterium may have been described as "human borne" in a scientific reference. However, that doesn't mean that an operator in a clean room was actually the source of the particular bacterium which contaminated a product lot, resulting in non-sterility. In the author's experience, such narrow-minded thinking is not helpful. Unfortunately, Regulatory Authority inspectors sometimes make the same mistake and require unnecessary tasks (Corrective and Preventative Actions, CAPAs) to be performed without proof of the contamination source.

Another type of Wrong Thinking takes place when a microorganism is isolated from a contamination event and the investigation team automatically assumes that they know "where it came from." Someone remembers seeing that microbe in a

particular area of the facility before. Then they assume that this area is the contamination source. That may indeed be the case. However, the microbe may have been eradicated from that area, but has remained undetected in a totally different area of the sterile product manufacturing facility. Often secondary contamination sources are not detected because the personnel conducting the investigation are not open-minded and ignore that possibility. Such secondary contamination sources are analogous to metastases from a primary cancer tumor.

Yet another type of Wrong Thinking occurs when the investigation is focused on a really tight time frame (window of time) around the date that the product or media fill was actually filled. The author has seen searches for the contaminant only concentrate on a few days or weeks on both sides of the contamination event. Oftentimes it takes a much wider data search, e.g., as much as a year in the past, to locate the microbial contamination source. The particular microbe may have been in the classified areas, e.g., Grade C or D, of a sterile product manufacturing facility for quite a long time, but it wasn't detected because only microbes from Alert or Action Level excursions were identified. One may have to analyze a year's worth of EM data to figure out what is going on.

Another type of Wrong Thinking is failure to conduct a thorough review of EM data for all classified areas. Instead the focus of the investigation is kept only on the affected aseptic filling room and/or line. Yet, many aseptic manufacturing facilities do not have a true "Aseptic Core," i.e., cleanest areas (Grade A, ISO 5) totally surrounded by less clean areas from a particulate and viable microbial contamination perspective. So, microbes may exist in areas that are close to the filling room and line that is undesirable and may contribute to product contamination.

Another type of Wrong Thinking is failure to consider the possibility that the filling line itself was contaminated. The following statement reflects that sort of attitude: "There isn't anything wrong with my filling line. It must be the Microbiology Lab's fault!" Some sterile product manufacturers assume that the filling line and the clean room surrounding it are in an adequate state of contamination control. Those sort of statements are usually based upon the assumption that EM Trend Reports are acceptable and the fact that the most recent media fill(s) "PASSED." The author actually heard the following statement made during a recent sterile product failure investigation:

"The FDA was happy with our data and said that we had a model filling operation." This statement represents a failure of facility management to admit that there was any possibility that the manufacturing area could be responsible for the contamination seen in the true sterility test positive (for bacterial growth).

Another type of Wrong Thinking is assuming that the Environmental Monitoring data that was used to make decisions during the failure investigation came from "worst case" sample sites. That may not have been the case at all. A proper Environmental Monitoring Performance Qualification (EMPQ) must be performed to ensure that sample sites used routinely represent the areas with the highest risk for product contamination.

Another type of Wrong Thinking is coming or jumping to conclusions about the contamination event without substantial (sufficient) information and data to support

them. For example, when an anaerobic *Propionibacterium acnes* strain was isolated from the Fluid Thioglycollate canister of a sterility test, the conclusion was drawn that this bacterium "Must have come from the operators" that were present during the aseptic filling operation. Some of the team members investigating the sterility test failure recognized that *P. acnes* are normal flora of the human skin. Yet, in this particular case, the *P. acnes* actually existed in areas of the filling room that were not sanitized very well or at all and were directly adjacent to the aseptic filling line. The presence of the *P. acnes* was detected when anaerobic EM was performed.

Another type of Wrong Thinking is when one assumes that the sterility test samples won't be contaminated externally during selection, handling, and transport to the microbiology lab. Or, another possibility is that one might understand that the sterility test samples need to be decontaminated, but fails to use a sporicidal disinfectant. Instead, a disinfectant like 70 % Isopropyl alcohol, which only kills vegetative cells, is used for "decontamination" of sterility test samples.

Another type of Wrong Thinking and perhaps the "**Biggest Trap**" that one can fall into is what I call the "**Similar Circumstance.**" You have seen a particular type of contamination before at another facility with the same microorganism. So, you automatically assume that the source and/or Root Cause for the current contamination event is also the same as the one that you had seen before. This is an easy trap to fall into and the author has been guilty of this type of Wrong Thinking himself. When this happens, there is a failure to perform a thorough investigation of the current contamination event. CAPAs are performed based upon the previous knowledge not an in-depth investigation of current events. This scenario often leads to additional contamination events, because the source of the microbe and/or the Root Cause were not identified and properly mitigated.

Another example of Wrong Thinking is assuming that triple-bagged gamma-irradiated RODAC plates were properly decontaminated as they were transferred into the aseptic processing area. The author is aware of one instance when mold (*Penicillium* species) was transferred from a laboratory refrigerator to the aseptic core of a facility because of failure to properly decontaminate the outer bag containing those plates with a sporicidal disinfectant. In another similar instance an inexperienced lab technician was trying to be efficient and labeled plates in the lab on the bench top. During the labeling the outside of the plates was contaminated with a filamentous mold, which was then transferred into the aseptic manufacturing areas. One would ordinarily assume that there was "no chance" that something like this could happen, but in reality it did.

Another example of Wrong Thinking was assuming that gamma-irradiated "bunny suits" in plastic bags were properly decontaminated when they were transferred into the Grade B gowning room. But, in reality, the outside of the bags containing the sterile gowning materials contained a huge bioload (hundreds of CFUs per 25 cm^2), which was not eliminated by the decontamination procedure in place at the time. The firm had assumed that 70 % Isopropyl alcohol was suitable for material transfer decontamination at the interface of controlled and classified areas. However, 70 % IPA is not sporicidal, so the outside of the bags containing the gowning materials was not completely decontaminated. Filamentous molds and *Bacillus*

species present on the plastic bags containing the gowning materials survived and contaminated the classified aseptic processing areas.

Another example of Wrong Thinking was assuming that all of the materials used in a particular Sterility Test were in fact sterile. Dimethyl sulfoxide (DMSO) was used to solubilize a drug product so that it could be tested for sterility using a membrane filtration method. DMSO is bactericidal and readily destroys vegetative cells, but it may not destroy bacterial endospores. In one particular case that the author is aware of, a *Bacillus* species was isolated from the Tryptic Soy Broth Sterility Test canister. When this sterility test positive was investigated, it was discovered that the DMSO used was *non-sterile* and contaminated with the identical *Bacillus* seen in the false-positive sterility test. The sterility test for that drug product had been conducted for more than 2 years using DMSO and many product lots were tested. No problems were detected until the DMSO was contaminated with a spore-forming microorganism which was resistant to its antimicrobial effects. In this particular case the manufacturing area was assumed to be at fault, because the sterility test was performed in an isolator and the product was considered to be truly contaminated. Four rooms of manufacturing equipment (sterile closed system) were dismantled and swab samples were taken for viable microbes. More than one thousand surface (swab) samples were taken, but no *Bacillus* species of any sort were recovered within the sterile product manufacturing equipment or the filling room.

Another example of Wrong Thinking was assuming that the "qualified vaporized hydrogen peroxide (VHP) cycle" would decontaminate 100 % of the microbes existing on the outside of sterility test samples and testing materials. This assumption ignored points-of-contact of materials on racks and mating of surfaces which prevents flow of VHP. The assumption was also made that the VHP was able to penetrate the Sterility Test isolator load of testing materials and sterility samples. Another error in judgment was made because the personnel assumed that the loading pattern was the same as that originally qualified. That wasn't the case and the amount of material per load had almost doubled over time. There was also the assumption made that as long as "you can see space" between the various items in the load that VHP will penetrate/flow everywhere that it needs to go in order to be able to effect surface decontamination. It was also assumed that wiping of sterility test samples with a sporicide would be effective. However, the wiping procedure did not achieve the necessary contact time required to destroy all of the bacterial endospores present on the outside of the sterility test samples. All of the assumptions described above prolonged discovery of the definitive root and caused multiple sterility test failures.

Another example of Wrong Thinking was evident when the decision was made by "Management" *not* to interview the operators who were present during the aseptic filling of a sterile product. That particular product lot had failed sterility testing performed in an isolator. The assumption was made that interviews conducted more than 2 weeks after the batch was filled would fail to yield any meaningful information. However, in the author's experience, operators often remember "Oh Dear Events" that could have contributed to the microbial contamination seen, but were not recorded in the batch record. If one asks the operators, they are just as likely as not to remember something important that could have caused the batch contamination.

This is important to prevent repeat microbial contamination events. The author has personally seen operator interviews that gleaned extremely important information which lead to the discovery of the Root Cause for the contamination.

Another example of Wrong Thinking is when one assumes that the disinfectant efficacy results for compendial and "in-house" isolates can be extrapolated to any adventitious (foreign) microbes that are brought into the manufacturing facility. That may not be the case at all. Furthermore, increased frequency of isolation of any particular microorganism may be indicative of inadequate contact time or use of the wrong type of disinfectant. The possibility exists that any "new" microorganism seen in the facility could be innately more resistant to the currently used disinfectants. The author has seen several examples of this for filamentous molds and *Bacillus* species.

19.6 Extraordinary Environmental Monitoring

Extraordinary Environmental Monitoring is defined by the following:

- Monitoring of numerous nonroutine clean room sites
- Using swabs to sample/get into "nooks and crannies," which are areas that are difficult to clean and sanitize
- Increasing sampling frequency

Why does Extraordinary EM work? The author will readily admit that by the time that a sterility assurance failure has occurred (failed media fill or sterility test) clean rooms, hoods, etc., have already been cleaned and sanitized several times. So, how is it possible to detect any viable microbes? If poor cleaning technique(s) and sanitization practices are used, one can still find the source of the microbial contamination if enough effort is exerted. This is particularly true if one samples in hard-to-reach areas, which are cleaned and sanitized poorly, if at all. The following statements were made to me when I suggested performing extraordinary EM when a product sterility failure occurred:

"The facility has already been cleaned and sanitized fourteen (14) times since the product batch was filled. You'll never find anything! It's a waste of time, effort and resources." The individual who made those statements probably made the following assumptions:

- That cleaning and sanitization had been performed properly
- That the disinfectants used were sterile
- That the disinfectants were used at the proper dilution (concentration)
- That the disinfectants used in his facility had been properly qualified for the current use dilution
- That the contact time qualified in the Microbiology Laboratory is achievable in the aseptic manufacturing and areas as well
- The filling equipment was cleaned from inside to outside

- That the filling equipment was cleaned from top to bottom
- That the filling room was cleaned and sanitized from the cleanest areas to the least clean areas

Disinfectants have to be "wet" to be effective against viable microbes. Air flows in clean rooms can be so substantial that a disinfectant will "dry off" before the necessary contact time is achieved. So, as a result, it may take more than one application to kill certain spore-forming microorganisms. For example, a *Paenibacillus* species isolated from a sterility test required two applications of SporKlenz® to destroy its spores in an aseptic manufacturing facility.

Properly performed trend analysis of routine EM data is typically sufficient to prevent product contamination events with viable microbes. However, Extraordinary EM is a valuable investigative tool for discovering the location of microbial contamination sources in facilities where sterility assurance failures have actually occurred.

19.7 Case Studies

Case Study #1—*Ralstonia pickettii* was isolated from multiple sterility test failures at a sterile product manufacturing facility. This particular bacterium had never been seen in the sterile manufacturing areas, i.e., aseptic core, before. The facility management assumed that because no Alert or Action levels were exceeded that the aseptic filling room was not the source of the problem. In fact the following statement was written in the initial draft of the sterility test failure investigation:

> …based on EM data it appears that the facility was in control at the time of use and that it was unlikely that the clean room environment was the source of the bioburden (*Ralstonia pickettii*)

However, when extraordinary Environmental Monitoring was performed, *R. pickettii* was found in areas that had been wet by purified water, as well as in sinks and drains. The particular *R. pickettii* strain was resistant to the preservative system (Parabens) used in the sterile multiple-dose products manufactured at the facility. The *R. pickettii* present in the pre-filtration drug solutions passed through integral 0.2 μm filters and several batch failures for sterility occurred.

The assumptions made in this particular case allowed bacterial contamination of the "sterile" product solutions and delayed finding the Root Cause.

Case Study #2—Several sterility test failures were seen at a contract testing laboratory. The sterility tests were performed in an isolator. A dematiaceous (black) mold was found in multiple Steritest Canisters containing Tryptic Soy Broth. The mold colonies excreted a water soluble black pigment in and on the agar plates, which is very unusual and allowed easy presumptive identification of those colonies. The following factors contributed to the false–positive sterility test failures:

- No Environmental Monitoring was ever performed in the room containing the sterility test isolators

- No preventive maintenance was performed on the isolators
- No cleaning program in place for the isolators or the room that they resided in
- Viable microbial contamination was observed from several EM samples taken inside the isolator after testing sessions, but there was no recognition that contamination inside the isolator was a problem
- No sporicidal agent had ever been used for sanitization in the sterility testing area
- No sporicidal agent was used for decontamination of sterility test samples or testing materials prior to introducing them into the isolators

The testing lab failed to recognize that EM "hits" inside the isolator was a risk to product testing. They did not investigate these unusual results, because they assumed that the VHP cycle would take care of any contamination present. In addition, they had no experience with or knowledge about how to conduct an investigation.

Fortunately, almost all of the contract lab's clients had robust EM programs and none of them had ever seen that dematiaceous (or any other) mold in the filling rooms of their sterile product manufacturing facilities. Some of these manufacturers were confident enough in their EM program to retest their products at another laboratory and release product. Yet other firms rejected their sterile product lots.

This particular case illustrates what happens when assumptions (Wrong Thinking) are made both in the laboratory testing environment and during the sterility test failure investigation itself.

Case Study #3—Pre-filtration bioburden viable counts were detected at a level of 100 CFU/mL of product solution. A coryneform, Gram-positive bacterium was isolated, which turned out to be a *Corynebacterium striatum*. In this particular case, the sterile product manufacturing firm relied on the raw material vendor Certificate of Analysis alone for bioburden load information; they did no in-house confirmatory testing. The product formulation did not include a preservative. In addition, many formulation components were growth promoting. Raw material suppliers had never been audited by the firm as required by cGMPs and the raw materials had never been tested by the firm itself for bioburden load. Also, the bulk product solution was held for >96 h at ambient temperature, which presumably allowed the *C. striatum* to grow.

The investigation performed relied on a checklist type of form and many areas that needed to be explored/ investigated were *not* on that list. The list was limited to a few areas of concern and did not allow "thinking outside the box." The documentation was minimal and not informative, i.e., not enough details were provided to be helpful. Furthermore, the investigation team assumed that contamination came from manufacturing operators. They came to this conclusion without any data to indicate that operations personnel were a problem. Yet, the personnel contamination rates for that facility were very good; less than 2 % of EM samples taken from manufacturing operators demonstrated microbial growth. No visual monitoring of aseptic technique by the Quality Unit was performed to determine if the operators were conducting themselves properly during aseptic filling of product lots. Equipment sanitization was only performed using 70 % IPA. Furthermore, many pieces of product manufacturing equipment were sanitized manually that could have been steam sterilized-in-place.

When Extraordinary EM was performed as an investigative tool, it was apparent that the operators had excellent aseptic technique and clean room conduct. No further problems with high product bioburden were seen after audits of raw material suppliers were instituted and in-house raw material test method was validated and testing performed on a routine basis. So, this case typifies what can happen when the investigation is fraught with assumptions and is too narrow in scope.

Case Study #4—Media fills were performed to requalify filling lines after the annual shut down and the results for several aseptic filling lines failed to meet acceptance criteria. *Paenibacillus* was isolated from all of the turbid vials. Significant new construction and refurbishment had taken place during the facility shutdown period. Routine cleaning procedures were probably inadequate to remove all of the debris present. During the investigation it was discovered that no disinfectant efficacy studies had been performed for currently used disinfectants. So, we then recommended that a disinfectant efficacy study be performed as part of the investigation. Interestingly, the disinfectant efficacy studies showed that a 20 min contact time with SporKlenz® was required to kill this spore-forming *Paenibacillus*. SporKlenz® would have to be applied twice to be effective in production areas. So, acidified bleach (500 ppm, neutral pH 7.0) was used instead to eradicate *Paenibacillus*, because a much shorter contact time was required. In this case, the facility management assumed that routine cleaning and sanitization procedures would be adequate to decontaminate the aseptic processing areas after the facility shutdown activities. This assumption initially precluded them from discerning the Root Cause of the investigation.

Case Study #5—Very low level yeast contamination (*Rhodotorula rubrum*) seen on a particular filling line, but only when media fills performed. Only one or two vials contaminated in each and every media fill over 3 years. Only 15 EM samples were positive for *R. rubrum* out of several thousand taken throughout the facility. The investigation showed that the stopper placement machine used on that filling line was corroded beneath the stopper bowl connection area and that was colonized with *R. rubrum*. Only swab samples taken during Extraordinary EM were positive for the yeast. The Root Cause of this chronic contamination problem was that operators had ignored the SOP which required dismantling and proper cleaning and sanitization of the stopper placement machine because significant effort was required. The assumption made by facility management was that all SOPs were being adhered to was wrong.

Only through extraordinary EM sampling was the source of the yeast contamination found. Wrong assumptions contributed to both the contamination problem itself and the discovery of the Root Cause.

Recommendation: During annual shut down of sterile manufacturing facilities, filling equipment can be dismantled, inspected for product and media residues, cultured and sanitized (if necessary). In the author's experience performing this sort of a preventative maintenance with an eye towards contamination control is extremely valuable for any sterile product manufacturing firm.

19.8 Conclusions

When performing a Sterility Assurance Failure Investigation one should make no assumptions and keep an open mind. Aggressive Extraordinary Environmental Monitoring should be performed to locate the source of the microbial contamination seen in product (sterility test failure) or process simulation runs (media fills). Failure to do so will preclude determination of the definitive Root Cause for the microbial contamination.

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Chapter 20
Validation of Rapid Microbiological Methods (RMMs)

Jeanne Moldenhauer

Abstract Microbiological testing is foundational to the operation of pharmaceutical facilities. This testing is used to assess the microbiological quality and attributes of the product, components, ingredients, environment, and the utilities. Unfortunately conventional microbiological methods are limited by the time it takes to grow the microorganism under the specified test conditions. These methods have traditionally required days until the results are obtained. Sterility testing and mycoplasma testing are often cited as the most serious offenders, with a minimum 14- or 28-day release time respectively. Significant costs are associated with holding the product during this time. As such, there has been an increasing interest in the use of rapid microbiological methods (RMMs), which are also known as alternative microbiological methods.

These new methods offer many advantages, from the ability to gain results in real time to those where the results are obtained in a much shorter time. Depending upon the method they may also significantly improve the accuracy of the method, the limit of detection, or other key attributes associated with the method. One of the hindrances associated with these methods has been defining what is necessary to validate these methods. This chapter provides guidance on the validation of RMMs.

20.1 Introduction to Rapid Microbiological Methods

Introduction to rapid microbiological methods (RMMs) were introduced to the pharmaceutical industry about 20 years ago. Vendors of these methods believed that the benefits of the new technologies would be immediately adopted in the

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pharmaceutical industry. This was not the case. Pharmaceutical companies were fearful of the methods, and the regulatory acceptance of these methods. While many companies proceeded to evaluate the technologies in research or development settings, the anticipated widespread implementation of the systems did not occur. Approximately 10 years ago, the first submission of an RMM for regulatory approval occurred in the United States when Glaxo submitted the PallChek® technology using adenosine-triphosphate bioluminescence for use as a product release test for the microbial limits test. This was significant as it was replacing a compendial product release test for nonsterile product.

Following this initial regulatory submission, the same company submitted a proposal to perform bioburden monitoring of water using the Scan*RDI* system. (Note: In some countries this same system is denoted the ChemScan.) This technology utilizes solid-phase laser-scanning cytometry to detect viable microorganisms in a few hours. For this method there is no need for the organisms to be cultured or grown as part of the test method. This methodology provided replacement of a compendial product release test for a sterile product.

The interest in the implementation of RMMs increased significantly after the publication by Gressett et al. (2008) indicating that a rapid method for sterility testing using the Scan*RDI* had been approved by FDA. Since that time, Jennifer Gray of Novartis gave presentations that a rapid sterility test method was approved by FDA using the Milliflex Rapid (Gray et al. 2008).

Another major advancement during this time period was the introduction of the IMD-A, by BioVigilant. This instrument introduced companies to the concept of the potential for real time results for viable microorganism monitoring in their environmental monitoring program. Since this technology did not require the use of culture media in addition to real time results, companies saw the potential for cost savings associated with the method. As such, there was an increased interest in these new methods.

20.1.1 Types of Conventional Test Methods

There are three basic types of microbiological evaluations conducted: determination of whether an organism is present (presence-absence tests), if organisms are present determination of how much is present (enumeration tests), and if an organism is present what organism is it (identification tests) (PDA TR33 2000).

20.1.1.1 Presence-Absence Tests

This type of technology simply determines whether a microorganism is present in a sample. Sterility testing is a typical test using this type of assessment (Moldenhauer 2003).

20.1.1.2 Enumeration Tests

These tests are used to determine the number of microorganisms present. The results obtained are affected by many characteristics including: the test conditions, culture medium, incubation conditions, whether the organism is or is not stressed or shocked, and whether it is a stock culture or an environmental isolate (Moldenhauer 2003).

The variability with this type of test is high when using conventional methods. It is common to assume that values within 30 % are equivalent (PDA Technical Report Number 21 1990). This type of test is used for determining counts in environmental monitoring and other tests where specific counts are necessary.

20.1.1.3 Identification Methods

Numerous systems exist for the characterization and identification of microorganisms. Many of them have their origins in the clinical setting. Some technologies are manual and use classical methods of identification while others are automated and utilize other technologies.

20.1.2 Types of New Technologies

Many of the new technologies available to replace microbiological methods have their foundation in other sciences like chemistry, molecular biology, optics, and so forth. These technologies have been categorized in the Parenteral Drug Associations (PDA's) Technical Report Number 33 (2000) as growth-based methods, viability-based methods, cellular component or artifact-based technologies, and nucleic acid-based technologies. Since the publication of the PDA document, systems have been released using various types of spectroscopy. As more methods have been developed, there are also methods that incorporate more than one type of technology into the system, e.g., may be able to detect, enumerate and identify microorganisms or addition of a viability assessment to a system that uses another technology like spectroscopy to detect microorganisms. Some publications refer to the systems that use more than one type of technology as combination systems.

20.1.2.1 Growth-Based Technologies

Systems in this category assume that premise for organism detection is dependent upon measurement of attributes that require growth of the microorganisms. Some of the types of systems that are included in this category are ATP Bioluminescence, Colorimetric Detection of Carbon Dioxide Production, Measurement of Changes in Headspace Pressure, Impedance, and Biochemical Assays (Moldenhauer 2003).

20.1.2.2 Viability-Based Technologies

This category of technologies allows the user to detect and/or enumerate viable microorganisms present without requiring growth of the microorganism. As such, it is possible to have different results using a viability-based technology for enumeration vs. a growth-based technology. The viability-based technology is likely to include those organisms that are not able to be cultured under the conditions used by the growth-based technology. Some of the systems included in this category are Solid-Phase Laser-Scanning Cytometry and Flow Fluorescence Cytometry (Moldenhauer 2003).

20.1.2.3 Artifact-Based or Component-Based Technologies

Technologies based upon artifacts or components utilize the presence of specific cellular components. For example, the bacterial endotoxin test method tests for the presence of endotoxin. Some of the systems using this technology are fatty acid profiles, enzyme-linked immunosorbent assays (ELISA), and fluorescent probe detection (Moldenhauer 2003).

20.1.2.4 Nucleic Acid-Based Technologies

Technologies that are based upon some type of nucleic acid, e.g., RNA or DNA, fall into this category. Some of the examples of systems using this technology are DNA probes, ribotyping or molecular typing, and polymerase chain reaction (PCR) (Moldenhauer 2003).

20.1.2.5 Spectroscopy Systems

Many of the instruments from the chemistry laboratory are now being used for microbiological applications. Some of these instruments include Maldi-TOF (Shelep 2011), Fourier transform infrared spectroscopy (FTIR) (Wenning et al. 2010) and Raman spectroscopy (Ronningen and Bartko 2009). The Maldi-TOF is used for microbial identifications, while Raman is used for detection, enumeration, and identification.

Optical spectroscopy measures the interactions between light and the subject of interest. The scattering of light that occurs when light is disturbed by its interactions with particles. This scattering can be used to determine whether particles are present in the air. It can be combined with laser technologies to detect fluorescence. This type of technology has been used for real time environmental monitoring of air. Coupled with an appropriate viability marker both viable and nonviable particles can be detected (Anonymous 2011a).

20.2 Guidance for the Validation of Rapid Microbiological Methods

The first information provided as guidance for the validation of RMMs came from the PDA's Technical Report Number 33 (2000). This document provided useful information, however it was written before most companies had completed a successful validation of these systems. At the time of this writing, the document is currently being revised.

The *United States Pharmacopeia* (*USP*) published an informational chapter <1223> on the Validation of Alternative Microbiological Methods. It was formally completed in 2006 (USP 2006). This chapter modified the criteria for Validation of Compendial Methods in USP <1225>, which describes the requirements for validation (usually chemistry methods), and made them applicable to microbiological methods. While this chapter addresses alternative methods, it does not have specific details relative to the validation of identification methods (Anonymous 2011b).

In recent years there has been a trend to separate "rapid" identification methods from other rapid or alternative methods. This is not a function of implying that they are not rapid methods, but rather that the validation is quite different from the other methods for qualitative and quantitative analysis. The USP has published a draft monograph <1113>, Microbial Identification. It is currently being retitled to Microbial Characterization, Identification, and Strain Typing. Included in this monograph is a description of how to verify these methods (Anonymous 2011c).

The European compendia, *Pharm Europa*, also published a chapter 5.1.6, Alternative Methods for Control of Microbiological Quality, which provides an overview of alternative microbiological methods (qualitative, quantitative, and identification methods) as well as guidance for the validation of these methods (Pharm Europa 2006).

Both of these compendia are considering revisions to these chapters. In recent years there have been numerous presentations and publications enhancing the statistics in these chapters and newer technologies that have been released (for USP, Sutton and Tirumalai 2011; for EP, Verdonk 2011).

The International Conference for Harmonization (ICH) has published information on the validation of analytical methods in their document Q2 (R1), Validation of Analytical Procedures: Text and Methodology (ICH 1995).

20.3 The Validation Process

Validation of RMMs has mimicked the validation processes traditionally used for equipment and method validation. Agalloco (1993) defines validation as, "Validation is a defined program which in combination with routine production methods and quality control techniques provides documented assurance that a system is performing as intended and/or that a product conforms to its predetermined specifications.

When practiced in a 'life cycle' model it incorporates design, development, evaluation, operational and maintenance considerations to provide both operational benefits and regulatory compliance." Method validation has been described as the program to confirm that an analytical procedure used for a specific test is reliable, reproducible, and suitable for its intended purpose. Most RMMs include equipment, software, consumables, reagents, and human interactions which all must be considered in the validation process (Anonymous 2011b).

Since using RMMs in a pharmaceutical environment requires that they be validated, it is important to consider the needs and requirements for validation even before purchasing the system. It is useful to develop a validation strategy, a validation master plan (VMP), a User's Requirement Specification (URS), a test plan and the appropriate validation protocols.

20.3.1 Concerns for Purchasing a Rapid Microbiological System

A significant investment is required for many of the new rapid microbiological technologies on the market. As such, it is important to understand what requirements must be met prior to purchase of the system. There are several different areas from which requirements may originate, for example validation needs and expectations, regulatory expectations, scientific expectations, and so forth.

20.3.1.1 Assessing the Technical Capabilities of the System

Prior to purchase, one should understand exactly what the equipment or system is capable of doing. For example, what is the method sensitivity? What categories of products can be tested using this technology? What types of testing can be performed using this technology? How many samples can be processed in what time period? What is the level of automation in the system? One should also assess whether the system will be able to meet the various requirements that must be met.

It is also important to perform feasibility or proof-of-concept testing. This may be performed at the end user site, a contract testing laboratory, or at the vendor's site. During this evaluation, it is important to assess whether the rapid technology being considered will work with the items to be tested with the system. For example, if the system requires filtration through a specified filter, it is important to determine if your test articles can be filtered through the specific filter. For systems using fluorescence, you must assess whether your test articles will interfere with the test methods.

20.3.1.2 Assessing the Regulatory Requirements to Be Met

Prior to purchase of the system, it is useful to determine what requirements must be met for implementation of the system. Additionally, one should take into consideration what regulatory requirements must be met to implement the system. Some

systems may easily be shown to be equivalent to existing methods and no additional regulatory concerns need to be met. Others may require prior approval of a regulatory body before they can be implemented. For those requiring regulatory submission, it may be necessary to have substantial support from the vendor to be able to gain regulatory approval. In the United States, this may be aided by the vendor having a drug master file (DMF) on file with FDA. In Europe, one may need the vendor to provide data confidentially to the regulator for review as part of the product application. It is beneficial to work with the vendor to assess their willingness to support these activities prior to purchase. Some companies actually tie the necessary supporting requirements into their purchase agreement.

20.3.1.3 Assessing the Cost

It is important to understand the cost associated with the rapid technology selected. In some cases the bulk of the expense is in the initial purchase of the equipment. For some systems, the bulk of the cost is associated with the on-going costs of operating the system. Many companies require formal documented reviews of both the costs and how the money spent for the system will generate a return on investment (ROI). It is important to understand the impact of the spending on the company's bottom line. Additional costs are associated with the validation, and in some cases additional equipment that must be purchased in support of the new technology. For example a new method for sterility testing may require that one purchase specialized isolators in which the new technology will be used.

20.3.1.4 Assessing the Capabilities and Sustainability of the Vendor

For most of the rapid technologies currently available, there is a single source of the system. This limits the end users ability to obtain supplies for the system or to gain technical information on the system. As such, the vendor selected can have a significant impact on your ability to successfully validate the system and continue to use the system on an on-going basis. In most cases, you will require a significant amount of support from the vendor during the validation process and to support any issues that arise after implementation.

There are several considerations for selection of a vendor of a rapid technology including (Anonymous 2011b):

- The systems used by the vendor to manufacture the system
- The documentation associated with the manufacture of the system
- The systems established for change control and the associated notifications for users of significant changes
- The regulatory status of the company, e.g., regulatory inspections, deficiency letters
- Whether there are other users of the system that may be used as references?

- Do other companies offer the supplies, parts, or equipment for commercial sale?
- What type of training is provided?
- What support services are provided, e.g., calibration, preventative maintenance, troubleshooting, regulatory services?
- Are support services available locally, or if not, what are the associated costs with obtaining support services?
- Has the vendor validated the system and is it in a reference document like a DMF?
- Are validation protocols or guides available to support validation activities?
- How often is software updated and how is this handled with the end user?

20.3.2 Developing a Validation Strategy

Rapid technologies vary in their complexity and their level of automation. Additionally, the scientific basis for how the system works varies. It is important to develop a validation strategy that includes validation of the hardware, software, and method. Depending upon the system selected, this may be performed concurrently or separately. It is important to determine the steps that will be incorporated into the validation plan for the technology selected. There are significant differences between the technologies available. The level of complexity of the system and the intended use of the system may impact the considerations that are applicable in the validation strategy.

Some of the considerations in developing a strategy include (Anonymous 2011b):

- User's requirement specification (URS)
- Functional design specification (FDS)
- Supplier assessment
- Risk assessment
- Validation master plan (VMP)
- Design qualification
- Factory acceptance testing (FAT)
- Site acceptance testing (SAT)
- Installation qualification (IQ)
- Operational qualification (OQ)
- Performance qualification (PQ)
- Method validation (MV)
- Personnel training
- Documentation requirements, e.g., standard operating procedures (SOPs), Protocols
- Requirements traceability matrix (RTM)
- Summary report

20.3.3 User's Requirement Specification

In order to purchase the right rapid technology for your site, it is important to understand what your expectations for the system are. Some expectations will be specific to the applications of the system you want to buy, while others may be general requirements to meet your company's requirements. You should have a clear understanding of what you want from the system before you purchase it. These expectations are incorporated into a formal document called the URS. The URS serves as the foundation for several other documents including the RTM and the various tests and acceptance criteria incorporated in the validation documents.

Some of the considerations in developing a URS are (Anonymous 2011b):

- What is the intended purpose of the system, e.g., this system is intended to replace the conventional product release sterility test
- What are the specific performance requirements that must be in the system
- A description of how the system will be used, e.g., in the laboratory, online, in a manufacturing environment
- Characteristics of the hardware that must be met, e.g., size requirements, power requirements
- Characteristics of the software that must be met, e.g., stored on a chip, PC compatible software
- Expectations for reports
- Compatibility and/or communication with other systems, e.g., networks, testing equipment, utilities
- Descriptions of how data will or should be managed
- Necessary requirements for safety
- Requirements for support services or needs, e.g., preventative maintenance, calibration
- Concerns for engineering or physical requirements, e.g., must fit in a specified space or area, must be cleanroom compatible
- Training that should be provided
- Minimum requirements for an acceptable supplier, e.g., audit support, regulatory expectations
- Financial considerations that must be met, e.g., cannot exceed a price of X

The complexity of the technology and its intended use will determine the complexity of this document and whether all of the above listed considerations are necessary for the document.

20.3.4 Functional Design Specification

All of the functional requirements for the system are described in the FDS. Some companies choose to include these requirements in the URS and skip the development of this document. The FDS requirements are intended to ensure that the URS

requirements for performance will be met. This document is very specific and detailed, which leads to development of a lengthy document.

The FDA should describe the system functionality, configuration, the necessary inputs and outputs, the environment in which the system must operate, the utilities required and/or used, system architecture, system interfaces, the types of data, and the system security. Some FDS documents also identify where and how the expectations will be evaluated to ensure compliance to the criteria for the requirement. The testing is typically conducted within the qualification protocols, or depending upon the requirement in the FAT (Anonymous 2011b).

Some examples of the typical information included in the FDS are (Anonymous 2011b):

- System description
- Purpose
- Scope of the FDS requirements for system documentation, e.g., user manuals, procedures, technical documentation
- Physical specifications for the system, e.g., size, power, operating environment, utilities
- Specifications for the computer system, e.g., type of computer, system operating system (including version numbers in some cases), supporting software, requirements for the computer hardware and accessories, networking requirements, printer requirements, databases, and so forth.
- Requirements for system security, e.g., multilevel password system, methods for record retention, necessity of an audit trail, compatibility with requirements for 21CFR Part 11, and so forth
- Validation attributes that must be met, e.g., the compendial validation expectations like accuracy
- Customization required for the system
- How the system responds to deviations and errors, e.g., alarms
- Whether there are parts of the system that will not be utilized or tested

The complexity of the system and the intended use will determine the level of detail required in this type of document.

20.3.5 Supplier Assessment

Verifying that the supplier is able to provide the services and systems that are necessary for your operation is critical when you are dealing with a single source supplier. This includes assessment of the quality systems utilized by the supplier, the testing used to release the products manufactured by the supplier, where the products are manufactured, and the financial stability of the company. Additional considerations should be assessed if any or all of the validation is conducted by the supplier, e.g., whether the supplier understands and follows the good manufacturing practices applicable to your industry.

These assessments may be accomplished in a variety of ways like use of questionnaires, soliciting written information from the supplier, and/or conducting an assessment at a supplier's site.

20.3.6 Risk Assessment

The concept of risk management, also known as quality risk management (QRM), has been endorsed by numerous regulators for the pharmaceutical industry. As such, there is an expectation that one understand the risks associated with a RMM to be utilized at a pharmaceutical site. The specific risks will vary depending upon the technology selected and the type of testing to be conducted using the technology. For example, the risks associated with a final product release test may be very different from the risks associated with a technology used to monitor process control in the facility.

One should also consider how the risks of the new technology are different from the risks associated with the conventional methods currently being used. There are many tools available for conducting a risk assessment.

20.3.7 Validation Master Plan

A VMP may be established for the company site, microbiology, and/or the specific process or equipment to be validated. Regardless of the scope of the master plan, it provides guidance on all of the required validation activities that must be conducted for the acceptance of the system. Some of the common considerations in a VMP include description of the system(s) covered, scope of the document, the types of documentation that must be generated, required supporting systems, testing expectations and in some cases the acceptance criteria that must be met, training, and the departments or individuals responsible for the approval of protocols, execution of the studies, and final reports. Typically, there is also guidance on how one should address issues (deviations or exceptions) that occur during execution of testing and how the testing documents can be amended to make changes.

20.3.8 Design Qualification

Design qualification (DQ) is conducted by many facilities, though not all, to ensure that the proposed design of the system or equipment is appropriate for its intended purpose. There are a variety of methods available for how to conduct this qualification. One way to accomplish this is to determine if the vendor's design is appropriate to meet the various requirements you have established in the URS.

20.3.9 Factory Acceptance Testing

In some cases, testing is performed at the vendor site prior to shipping the equipment to the end user. This testing may include functions which require special equipment that the end user does not have. Some choose to perform testing at the factory site which would cause the system to be returned if found at the end user site. Depending on the company, this testing may be formally documented in protocols, or performed in laboratory notebooks. In some cases, this testing is referenced in subsequent validation protocols without repeating the testing.

20.3.10 Site Acceptance Testing

Some companies perform testing once the system is obtained at the end user site prior to the official validation studies. These studies may be a repeat of the some of the tests conducted in the FAT in order to ensure that the system received operates the same way it did when at the vendor site.

The level of documentation generated and required varies.

20.3.11 Installation Qualification

The installation qualification (IQ) is a formal protocol that is designed to ensure that the system as received and installed at the end user site meets the specified requirements. In some cases, companies contract with the vendor of the equipment to perform this testing. It is good practice for end user personnel participate in this activity, even when the vendor conducts the studies to gain knowledge of the system. The documentation generated becomes a technical handbook for the system as supplied to your site. This information can be invaluable when determining at a later date whether replacement parts are or are not identical to those installed. It is at the end user's discretion on whether the hardware and software are tested concurrently or as separate documents (Moldenhauer 2003).

Some of the considerations included in this evaluation include (Moldenhauer 2003):

- Verification that the items received and installed agree with those specified in the purchase order
- The items received were not damaged in shipping or during the installation process
- All of the required supporting documentation for the system was received
- The required documentation is complete
- Specified utilities are available and properly connected
- Verification that the system was installed correctly

- Wiring of the system is as specified, if applicable
- Version numbers for software or EPROMs used are documented
- Models, serial numbers, and operating ranges are documented for key components
- Applicable hardware and software are documented to be present
- Peripheral equipment and accessories are present and properly connected and configured for use, if applicable
- Drawings are available for the system and/or installation, as applicable
- Back-up and/or recovery copies of all software have been made and are available
- Dip switch settings are documented
- Cabling connections are documented and as specified
- Configurations are documented
- Verification that log books are established and maintained for the system
- Verification that the system has been incorporated into a change control system

20.3.12 Operational Qualification

The operational qualification (OQ) is designed to document that the system operates as expected. Depending upon the complexity of the system, the hardware and software may be tested concurrently or separately. When developing these testing requirements it is useful to establish testing requirements that will be repeated or reevaluated for system updates and revisions, for example one might establish a standard test set of data. There are many different ways to accomplish these expectations (Moldenhauer 2003).

Some approaches to conducting this testing involve including actual product testing during the evaluation of the system (PDA 2000). This is not the only way to conduct testing, however. For example some companies have successfully used testing plans that conduct testing with a product or solution that serves as a standard, and then product testing is evaluated as part of the method validation. This type of model is similar to the approach used with equipment used for testing for bacterial endotoxins. One of the reasons this type of model is attractive is that when a failure occurs one can attribute it to the system, rather than wondering whether the product or the system is at fault (Moldenhauer 2003).

The amount of testing performed varies based upon the complexity of the system, the type of testing performed, and how the data is used, e.g., process control test or product release test.

Typical considerations in an operational qualification protocol include (Moldenhauer 2003):

- Verification that the installation qualification protocol has been completed
- All critical equipment requiring calibration have been calibrated
- The required system SOPs have been issued and implemented

- Certifications have been completed for any items requiring certification
- Alarms are functional and operate as designed
- Error messaging is functional and operates as designed. For some systems it is not possible to identify and/or test all error messages in the qualification. One may be able to use a risk assessment procedure to determine the number and type of error messages that should be tested in the qualification process
- Instrument operation, e.g., generation of expected results, standard curves, assays, expected results with specified organisms, and so forth
- Inputs and outputs operate correctly
- Interfaces and connections operate correctly
- Software structure and documentation is complete
- The system sequences in the specified order, and timing, if applicable
- Potential sources of interference have been evaluated and mitigated, if necessary. For example: radio frequency interference, electromechanical interference, shielding of wiring, light interference, and so forth
- Ancillary equipment is functional and interfaces with the system correctly
- Back-up and recovery procedures are effective
- Security procedures are effective
- Stress testing, e.g., the system operates correctly when the maximum number of systems are operating in the laboratory or under stresses of environmental conditions
- Data management capabilities are operational as specified
- Operator training or qualification is completed
- System suitability or standard curves are completed and acceptable
- Preventative maintenance programs have been established and implemented for the system
- Safety programs have been established and implemented for the system
- Data archiving occurs as specified
- Audit trails are accurate
- Reports are accurate
- Electronic signatures comply with 21CFR Part 11, if applicable
- Verification that validation attributes or criteria are met

20.3.13 Establishing Validation Attributes or Criteria

Recommendations for validation criteria have been established in several different documents including the compendia and industry guidance. There are some minor differences in the criteria across documents. As such, it is important to clearly identify the requirements that must be met you're your site. Within these documents, there are differences in the criteria to be met depending upon whether it is a qualitative, quantitative or identification test method (PDA 2000; USP 2006; EP 2006).

The validation criteria may be described as follows however as all of the guidance documents are currently under revision one should check the most recent guidance when writing validation protocols (Anonymous 2011b):

- Accuracy is used to describe the closeness of the test results obtained using the alternative method to those obtained using the conventional or reference method. It should be evaluated across the operating range for the test method. It is frequently expressed as the percentage of recovery of microorganisms using the method.
- Limit of detection describes the smallest number of microorganisms that can be detected using the specified test conditions. This is the level at which the method can detect the presence or absence of microbes present.
- Limit of quantification describes the smallest number of microorganisms that can be accurately enumerated in a test sample using the method.
- Linearity describes the ability of the system to produce results proportional to the organism concentration in a test sample within a specified range of microorganisms.
- Precision describes the level of agreement among the individual test results when the method is used repeatedly to multiple samplings of test organisms across the range of the test. It is frequently expressed as the variance, standard deviation, or the coefficient of variation.
- Range describes the interval between the upper and lower concentrations of microorganisms that have been shown to be determined with accuracy, linearity, and precision.
- Robustness describes the ability of the method capacity to be unaffected by small but deliberate variations in the parameters used for the method. Usually this testing is performed by the supplier of the equipment and either included in the DMF, or provided in a report to the end user.
- Ruggedness describes the degree of precision of test results that are obtained when the test samples are processed using a variety of normal test conditions. This might include different analysts conducting the tests, testing the samples on different pieces of equipment, using different lots of reagents, testing in different laboratories, and so forth.
- Specificity describes ability to detect a range of microorganisms for the method that are appropriate with the intended use of the system. One should also evaluate the potential for false positives and false negatives with the system. False results may be a result of interference from other items used in the testing, inability to distinguish between viable and nonviable cells, background noise, and so forth.
- Equivalence or comparative testing describes the testing and the results obtained from the alternative method compared with the same testing performed with the compendial or reference method. This assumes that the test samples utilize equivalent standardized microbial cultures. This is usually tested as part of the performance qualification.

Table 20.1 describes the expected tests for different guidance documents and test types.

Table 20.1 Validation criteria expected for different test types

Validation criteria	Type of test	USP <1223>	Pharm Europa 5.1.6	PDA Technical Report Number 33
Accuracy	Qualitative		✓	
	Quantitative	✓	✓	✓
	Identification		✓	
Limit of detection	Qualitative	✓	✓	✓
	Quantitative	✓		✓
	Identification			
Limit of quantification	Qualitative			
	Quantitative	✓	✓	✓
	Identification			
Linearity	Qualitative			
	Quantitative	✓	✓	✓
	Identification			
Precision	Qualitative		✓	
	Quantitative	✓	✓	✓
	Identification		✓	
Range	Qualitative			
	Quantitative	✓	✓	✓
	Identification			
Robustness	Qualitative	✓	✓	✓
	Quantitative	✓	✓	✓
	Identification		✓	
Ruggedness	Qualitative	✓		✓
	Quantitative	✓		✓
	Identification			
Specificity	Qualitative	✓	✓	✓
	Quantitative	✓	✓	✓
	Identification			
Equivalence or comparative testing	Qualitative		✓	✓
	Quantitative		✓	✓
	Identification		✓	

20.3.14 Statistical Evaluations

Statistical evaluations are expected in the compendial validation requirements for RMMs. Additionally, they are discussed in the PDA's Technical Report Number 33. However, many have struggled with the statistical methods described in these chapters. Numerous publications and presentations have been given that provide alternative methods for conducting these evaluations. Van den Heuval et al. (2011) and Schwedock (2011) provide alternative methods and explanations for the statistics frequently used.

It is useful to involve your own statisticians early in the development of validation requirements and testing.

20.3.15 Performance Qualification

The performance qualification should be conducted as an integrated unit, i.e., hardware and software. Additionally, items like printers or ancillary equipment required in the process should be concurrently evaluated during the qualification. This testing provides documented evidence that the system performs as expected operating in the user environment and utilizing the user's systems and procedures. Some companies validate the equipment and the method concurrently, while others separate the method validation out (Moldenhauer 2003). It is useful to separate the method validation into a separate evaluation especially when the item being tested has a potential to interfere with the test.

There are different ways to perform this testing. Some compare the conventional method to the alternative method for a period of time or across a specified number of batches to show equivalence. During the testing, a minimum of three separate evaluations should be performed. This may be three different lots of material, or three unique study evaluations (Anonymous 2011b).

The data generated in these studies should be evaluated for equivalence of the alternative method to the compendial or reference method. Depending upon the rapid technology selected, many are superior to the existing method (Anonymous 2011b). It is important to select a statistical method that is appropriate for the type of results that should be obtained, the number of samples being tested, and the expected level of microorganisms that should be detected using the method.

Some of the typical considerations in this type of protocol include (Moldenhauer 2003):

- Testing to show that all of the compendial validation criteria are met under the conditions of testing, e.g., with the standard products tested and the test samples. (Some companies only evaluate these parameters during the OQ.)
- Documentation of the system reliability, accuracy, reproducibility, and consistency over time.
- Verification that the system operates and performs as expected in the user's environment, using user procedures, and operated by users.
- Reports generated should be accurate.
- Changes to information that should be incorporated into audit trails are accurate.

Other examples of testing plans are included in the PDA's Technical Report Number 33 (PDA 2000).

For some systems, like those used for air monitoring, one may want to run the conventional test method side by side with the alternative method for a period of time to show equivalence.

20.3.16 Method Validation

There are a variety of different ways to conduct method validations. For example, alternative sterility test methods may be validated using the sterility test validation

procedures specified in the compendia. Other test methods may repeat evaluation of the validation criteria when the test is conducted using specific products.

The testing should be conducted as part of a formal protocol.

20.3.17 Personnel Training

Different rapid technologies have different levels of complexity of operation. In some cases, minimal operator experience or training are required while other systems require a specialized education and prolonged training before the system can be operated correctly. One should identify who is responsible for the training, both initially and on an on-going basis. Many vendors are quick to provide training at the time of sale. The problem can arise in handling operator turnover. Frequently, only a few personnel are trained to use these new technologies and the site may not have other individuals that can train new employees that may need to use the system.

There are some contract laboratories and/or consultants that may be able to provide the training required on an on-going basis.

Systems should be established to verify the competency of the personnel that will be operating these systems. Additionally, requirements should be established describing at what phase in the project operator training should be conducted.

20.3.18 Documentation Requirements, e.g., SOPs, Protocols

The minimum expected documentation associated with the system should be defined. Additionally, one should define at what stage in the project the documentation should be issued.

Some of the typical types of procedures that should be generated include (depending upon the complexity of the system, these may be individual procedures or the requirements incorporated into a small number of procedures):

- Operation of the system
- Exceptional operation of the system, i.e., what to do when something goes wrong
- Preventative maintenance for the system
- Calibration of the system
- Cleaning of the system
- Method validation requirements, if applicable
- Methods for assessing whether products are compatible for use with the system, if applicable
- Receipt, testing and acceptance or rejection of supplies for the system
- Qualification of analysts that operate the system
- Change control for the system (hardware, software, methods, and procedures)
- Security procedures, e.g., assignment of system administrator's, levels of passwords, control of passwords, and so forth

- Control of the system software
- How to handle system updates
- Procedures for data management and evaluation
- Back-up, recovery, and archival procedures
- Disaster recovery and contingency plans, e.g., what to so if the single source system fails
- Specific procedures for different methods used

20.3.19 Requirements Traceability Matrix

Once the various requirements for the system have been established in the URS and/or FDS, it is important to assess whether all of the requirements have been successfully met by the system purchased. The RTM is a document that provides a linkage between the requirements established and the location of the testing that has been established to ensure that the requirement is met. This document is very useful in assessing whether the validation documents generated are appropriate to ensure that the system meets the established requirements.

This document is considered a living document that is updated and revised throughout the validation process (Anonymous 2011b).

20.3.20 Summary Report

Final reports should be generated and approved at the completion of each phase of the validation testing. This report should be accurate, complete, and approved by those organizations that approved the protocol.

20.4 Other Types of Testing, e.g., Air Monitoring

Some of the alternative methods available today are used for testing samples that are not liquid based. Most of the original requirements specified in the compendia, and the PDA's Technical Report Number 33 were written for liquid-based testing. As such, some of the companies for air monitoring equipment have struggled with application of these methods to air samplers.

In other cases, vendors out of necessity have performed much of the validation for validation criteria and included the testing in a DMF because many pharmaceutical companies do not have the necessary equipment to perform the testing, e.g., challenging an air sample with a single microorganism.

20.5 Implementation of the System

Following completion of the initial validation, the system is released for implementation and use at the site. This release for site use may be dependent upon other factors like whether the method must be submitted to a regulatory agency for approval prior to release.

20.6 Qualification of Additional Sites or Additional Equipment

Depending upon the rapid technology selected and the intended use, more than one system may be needed at your site. In some cases, the methods are initially validated at a corporate site and then transferred to another site where routine testing is performed. With the substantial amount of work required to conduct the validation, there are frequently concerns on whether validation of subsequent systems can be reduced.

When the new technology is submitted to a regulatory agency as a comparability protocol or a request for scientific advice, it may be useful to describe the reduced testing plan proposed for reduced testing of subsequent systems. If no submission is made, one can also discuss these plans with the local regulatory inspectors for the site. The following is a description of how some companies have chosen to deal with testing of additional systems.

When considering a reduced testing plan is being considered, it is important to understand whether the new system is "identical" vs. a system that is similar and uses the same basic technology. Identical systems may provide more opportunities for reduced testing.

20.6.1 Equipment Evaluations

There are many possibilities to create differences between systems based upon how they are installed. As such, it is common to perform a complete IQ on each system purchased. The contents of the IQ may be an exact duplicate of the initial IQ for the first system, other than data entries made.

It is appropriate to repeat sufficient testing from the OQ to ensure that the system is operating as intended. The total amount of testing conducted will be dependent upon the system, and how it will be used.

If the repeated IQ tests and OQ tests show that the system functions the same way as the original piece of equipment, the PQ may be omitted or significantly reduced. A risk assessment should be conducted to assess the tests that need to be repeated.

20.6.2 Software Evaluations

When the same software is installed on more than one unit, the requirements for testing may vary. Some companies treat it the same way as equipment with an IQ and OQ for each system. Other companies have chosen to verify the software identification, including the version number and any other updates to show it is equivalent and only perform some minimal tests to ensure that all of the applicable sections of the software have been installed. The rationale for the test plan should be described along with assessments of the associated risks.

20.6.3 Method Evaluations

Typically, it is not necessary to repeat the method validations for additional pieces of equipment added. Rather, this type of situation is addressed by the method transfer programs within the facility.

20.7 On-Going Activities

Following the initial validation of the equipment, one should take care to maintain the equipment and methods in a validated status. As such, it is important to define the requirements and methods to be used for subsequent revalidation of the equipment. Additionally, one should ensure compliance with procedures for change control, preventative maintenance, and so forth.

The performance of the system over time should also be assessed to ensure that it is operating as shown in the validation. If there are a number of deviations or exceptions, one should assess whether there are critical parameters that should be added to the validation criteria for the system. If the deviations or changes necessitate changes or modifications to the system this should trigger an assessment of whether validation is necessary for the system

20.8 Conclusion

While the validation of alternative methods can be daunting, the benefits of completing these activities can offer improvements in the time to results, the quality of data recovered, or cost savings that make it worth the work to validate and implement the systems.

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Chapter 21
Validation of Moist and Dry Heat Sterilization

Jeanne Moldenhauer

Abstract In recent years the Parenteral Drug Association has issued several technical reports that had a significant impact on the validation of steam sterilization. They provide more formalized guidance on each different step of the validation process. Many of these documents have required significant upgrade of the methods used for validation. In addition, increased regulatory scrutiny on sterilization has utilized some of these documents as references for the "state-of-the-art" sterilization. Currently new guidances are also being issued on dry heat sterilization. This chapter describes the current expectations for validation of dry and moist heat sterilization cycles.

21.1 Introduction

Many different sterilization processes are used within the pharmaceutical industry to sterilize components, equipment, and final products. Sterilization is utilized at various phases throughout the manufacturing process. Some of the commonly used types of sterilization processes include aseptic filling, moist heat sterilization, dry heat sterilization, ethylene oxide sterilization, radiation sterilization, e-beam sterilization, and chemical sterilization. This chapter discusses the methods utilized for the validation of both moist heat sterilization processes and dry heat sterilization processes. There are many commonalities in the methods used for validation of these processes. Where differences exist, they will be described.

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21.2 What Is Sterilization?

Sterilization processes are used to destroy or eliminate the microorganisms that are present on the surfaces or materials in order to aid in providing a safe pharmaceutical product for the patient. All sterilization processes are intended to result in materials or surfaces that have become sterile, or free from viable microorganisms. While this dictionary definition of sterility sounds good, in reality we do not have a way to test or evaluate for the absence of all viable microorganisms (Agalloco 2008).

When products are labeled sterile, most assume that the vendor is claiming to meet the dictionary definition of sterility. This is further complicated by the fact that most sterile products are released using a compendial sterility test methodology. Again, most assume that passing this test indicates that there are no viable organisms present. In 1956 Bryce identified two critical limitations of the compendial sterility test method. He cited that the viable organisms present in the test sample can only be cultivated if they are able to do so under the specific test conditions. Additionally, the number of samples tested is so small (it is a destructive test) that at best it can only be used to provide a gross estimate of the "sterility" of the test sample (Bryce 1956).

Knudsen (1949) added to Bryce's comments, to indicate that the sample size is not of a statistically significant population to accurately estimate sterility. As such, the compendial sterility test is a poor indicator of test sample sterility. The ability to accurately detect contamination with a 95 % confidence level is only 15 % when using this test method. The probability of microorganisms surviving is 10^{-1} or 10 %. While it is possible to improve these numbers by increasing the sample size, most have not considered this type of increase. Since this is a destructive test, the test samples used in a statistically valid sample plan would be costly and possibly wasteful (DeSantis 2008).

The sterility test method would be more reliable if one were able to detect single microorganisms, in-line, and without destroying the sample. When such a method is available for commercial use, the validity of the test may be greatly improved (Sutton and Moldenhauer 2004).

The sterilization processes utilized are intended to eliminate the microbial contamination that may be present. Scientists have carefully researched the destruction of microorganisms (Agalloco 2008). This topic can be more complicated than it appears. There is no single definition of when a cell is dead. Many use the definition that the cell is unable to continue to replicate. Microbial cells can have long dormant phases. Some cells can form spores which are resistant to adverse conditions and exist in the spore state for very long periods of time. Some have even reported spores that have existed for hundreds of years. But, when the cell is exposed to favorable growth conditions these spores can be revived and replicate (Setlow 2009). Some other microorganisms that are not able to form spores can form viable-but-non-culturable (VBNC) cells. Oliver, et al. (1995) states that most of the estimates of the number of microorganisms present are much lower than the real number of cells present. Part of the problem in estimating counts is that the ability to recover microorganisms is dependent upon the culture methods used.

We utilize a sterilization process to eliminate the viable microorganisms, to a specified level of sterility assurance (SAL), which is also called the probability of a non-sterile unit (PNSU). We perform studies to show that the sterilization process is effective. Generating the documentation to show effectiveness and control of the process is called validation. Good science and engineering practices are used to assess the functions and capabilities of the sterilization process.

Regulatory guidance from different agencies indicates that manufacturers of pharmaceutical products are responsible for performing validation studies to ensure that the critical aspects of the system or operation are controlled and operating to perform their specified functions (Annex 15 2001; FDA 2003).

There are many different definitions of validation, but all of them include requirements to provide documented evidence of the system's performance within specified parameters. Agalloco (1993) provides a definition that is comprehensive and descriptive. His definition is, "Validation is a defined program which in combination with routine production methods and quality control techniques provides documented assurance that a system is performing as intended and/or that a product conforms to its predetermined specifications. When practiced in a 'life cycle' model it incorporates design, development, evaluation, operational and maintenance considerations to provide both operational benefits and regulatory compliance."

21.3 Guiding Principles for Validation of Sterilization Processes

There are a variety of fundamental principles of validation that are applicable when validating sterilization processes in pharmaceutical applications. Good science and engineering practices should be used for the validation. The scientific method is used in designing and executing the studies, typically documented in a protocol or series of protocols. This involves assessment of the critical performance aspects for the sterilizer and development of methods to evaluate this performance. The experiments are designed based upon the hypothesis developed (e.g., sterilization will be accomplished to a specific sterility assurance level). The testing is conducted to assess whether the hypothesis developed is or is not true. The observations made and the results obtained lead to the development of a conclusion. The conclusion identifies whether the testing is acceptable (Agalloco 2008).

Another principle used is the expectation that testing will be conducted to verify that all critical parameters are met. This is accomplished using test equipment which is properly calibrated, or qualified as appropriate. For calibrated equipment, the accuracy should be traceable to established national standards (Agalloco 2008).

One of the items in many definitions is the requirement for reproducibility of the system's performance (consistency of performance). In the case of sterilization systems, it is expected that the performance be evaluated within a specific sterilization cycle as well as across a series of cycles (Agalloco 2008). For sterilization, this includes a minimum of three consecutive acceptable cycles as part of the initial

validation. An assessment should be conducted to determine the number of studies that should be performed to ensure appropriate testing has been conducted to demonstrate consistent and reliable performance of the system.

There is an expectation that the worst case conditions will be evaluated during the validation studies. Among the worst case conditions for sterilization, it is common to test at or below the minimum allowable conditions for exposure time and/or temperature.

A principle that has developed over the years for sterilization is "the bugs don't lie." While this statement has been attributed to several different individuals, it is widely accepted in the pharmaceutical industry. This statement is meant to indicate that the microorganisms used as part of the test system evaluate the performance of the sterilizer process as a whole. Whether they live or die is a reflection of all the conditions to which they were exposed during the sterilization cycle. For example, it is possible to obtain thermal data that indicates an acceptable cycle was delivered to a sterilizer load, and yet they have biological indicator results which show that an unacceptable condition occurred. Over the years many have thought "there is something wrong with the biological indicators," when in reality the expected sterilization process was not delivered to all of the areas where the biological indicators were placed within the load.

Another common phrase is "if it isn't documented, it didn't happen." As such, it isn't enough to execute the validation studies; there must be documented evidence that the studies were conducted. The data should be accurate, legible data generated in accordance with good manufacturing practices (GMPs) and the data must be maintained in a way that allows it to be found and reviewed whenever necessary. Most sterilizer validation studies are documented as part of protocols or standard operating procedures (SOPs).

It isn't enough to validate the sterilizer at one time. One must also maintain the equipment in a validated state. This includes maintaining the equipment, software, support systems, and such so that the validation state is maintained. Appropriate controls should be established to maintain the system in a state of control. Procedures should be established to monitor and evaluate changes made to the system, i.e., it should be part of a change control system. When changes are made, they should be reviewed to assess the impact on the validated state of the system. Preventative maintenance should be conducted to ensure that the system is maintained in "good" operating condition. Access to the system should be limited to appropriately qualified and trained individuals.

21.4 What Is the Difference Between Moist Heat and Dry Heat Sterilization?

Moist heat sterilization is defined as a sterilization process that uses steam under pressure, as is conducted in an autoclave. The steam is considered saturated when the steam and the water are in equilibrium. A closed container of water, when heated to

temperatures in excess of 100 °C, will demonstrate an increase in the steam pressure. As the temperature rises, so does the pressure. There are steam tables that can be utilized to show the relationship between temperature and pressure. When the steam is saturated, raising the pressure using external means will result in the steam starting to condense back into water. Superheated steam is defined as steam whose temperature, at a given pressure, is higher than that indicated by the equilibration curve for the vaporization of water (PDA Technical Report Number 1 2007).

The thermal energy (heat) delivered by different heating media using different types of moist heat sterilization processes like saturated steam, air/steam mixtures, or superheated water, at the same temperature, is significantly different (PDA Technical Report Number 1 2007).

In a moist heat sterilization cycle, the microorganisms present are destroyed by coagulating and denaturing the cell's enzymes and structural proteins. Typical sterilization cycles require that the exposure time be at 121 °C in the range of 15–30 min for the spores most resistant to the sterilization process. These processes can be performed at other temperatures that yield equivalent lethality to those at a specified reference temperature.

Moist heat sterilization processes are used to sterilize equipment in support of the sterile processing of pharmaceutical products, cleaning supplies used in some clean room operations, primary packaging materials, terminal sterilization of liquid-filled containers, and so forth.

Dry heat sterilization utilizes hot air that is free of water vapor, or has very little water vapor. As such, the moisture present has little effect on the sterilization process. This type of sterilization is one of the earliest methods used. It is considered to be less complicated than moist heat sterilization. To achieve sterilization, much higher temperatures are used for much longer time periods. In pharmaceutical applications it is common to use temperatures in excess of 250 °C, although some guidance indicates that one may use temperatures greater than 160 °C. Dry heat kills microorganisms by destructive oxidation of essential cell constituents. Although there are spores of microorganisms that are resistant to dry heat sterilization, most frequently endotoxin challenges are conducted concurrently with the validation of these cycles. When appropriately controlled endotoxin challenges are used, biological indicators are not required since the endotoxin challenge is more severe than the resistance of the biological indicator. Dry heat sterilization processes are used for glassware, and some equipment that can withstand the higher temperatures for the prolonged periods of time required.

One might think that if you are using a steam sterilizer, you always have moist heat. That may not be true. It isn't enough to supply moist heat (steam) into the sterilizer. The moist heat has to come into contact with the items being sterilized. Items that make it impossible for all of the air to be removed from them and steam to penetrate may be achieving sterilization through dry heat only. It is important to ensure that the items being sterilized are coming into contact with the sterilizing media that you intend to use.

21.5 Moist Heat Sterilization

Moist heat sterilizers are available in both batch configurations and continuous sterilizers. Although continuous sterilizers are available, few companies choose to use this type of sterilizer. Most of the pharmaceutical sterilizers used are operated as a batch unit, i.e., material is moved to the sterilizer, processed in the sterilizer, and following sterilization is moved to the next production step. Batch sterilizers are available in a variety of sizes from laboratory-sized units to units that handle two or more sterilizer trucks or pallets of loads. There are some sterilizers the size of railroad cars.

The sterilizers may be specific for one type of sterilization process or may be able to perform more than one type of sterilization process (multifunction sterilizers). Some of the typical sterilization processes available are saturated steam, steam–air mixtures, air overpressure cycles, and those which use superheated water (e.g., water spray, raining water, water immersion, and rotary sterilization cycles) (PDA Technical Report Number 1 2007).

In the PDA's Technical Report Number 1 (2007) they have further divided moist heat sterilization cycles into those used for porous (and hard goods) loads and those used for liquid loads. In this document they have defined porous loads as "loads of materials for which the contaminant microbial populations are inactivated through direct contact with the steam supplied to the sterilizer. For porous loads heat transfer is through steam condensing directly on items being processed, unlike fluid loads where steam acts principally as an agent for heat transfer." Some examples of porous loads are those used to sterilize filter cartridges, garments, stoppers, tubing, and so forth. This same document defines liquid loads as "Liquid filled container loads within the production setting are usually homogeneous, comprised of containers of a single size, single fill volume, and derived from a single lot. Some examples of liquid loads are liquid filled vials and syringes. Liquid load cycles are developed and validated frequently using the Product Specific Approach though the overkill method may also be used" (PDA Technical Report Number 1 2007).

21.5.1 Determining Worst Case Conditions

Overkill cycles are used for sterilization of porous loads, hard goods, and some terminal sterilization activities, when the item is not sensitive to the heat delivered in this type of cycle. When performing overkill cycles, the worst case cycle is often determined by lowering the sterilizing temperature by 1 °C and reducing the exposure time by a few minutes from the set-point conditions. Alternatively, if one uses a load probe, this may be accomplished by executing the study with a lower F_o than the values routinely allowed. Another approach that is used in validation is the *half cycle* approach, where the sterilization time used in the performance qualification is doubled for the routine production cycle. This approach is only useful if the

excessive heat provided will not adversely affect the product. The cycles developed for an overkill process are expected to totally destroy the biological indicator challenge utilized (Agalloco 2008).

Product-specific cycles are used most often for terminal sterilization of products. For many companies using these types of cycles, the validation is performed at the minimum conditions allowed for acceptable release of the product. These requirements may require total destruction of the biological indicator or have a requirement for a specific biological indicator log reduction or $F_{\text{Biological}}$. Additionally, there are requirements established for the maximum heat delivered to the product, typically as specified upper time/temperature requirements and/or a provision for the maximum allowable F_0 (the maximum allowable lethality delivered to the product). Since the resistant bioburden is an integral part in determining the lethality required, these types of cycles require an ongoing, vigilant evaluation of bioburden recovered in the plant, including an assessment of its heat resistance.

21.5.2 Load Configurations

The load size and the load arrangement is also an important factor in the efficacy of the sterilization cycle. For porous loads, it is important that the items be placed on the sterilizer carts or pallets in such a way that they do not adversely affect the sterilization of other items in the load. It is common to have a fixed loading pattern, which identifies the specific locations where items are to be placed. Heavier items are placed on the bottom of the load. One should also be aware of how condensate may be formed in the cycle. Care should be taken to ensure condensate from one item is not "dripping" onto other items in the load. Specific loading patterns are also used for liquid loads. The containers may be placed into trays or on sterilizer carts in a specific arrangement. It is common to have detailed drawings or photographs which clearly delineate the loading arrangements to be used.

Since the items in the sterilizer may be removed from the sterilizer and transported to other areas for use or may not be used immediately, provisions are made to ensure that the item sterilized maintains its sterile state. This may include wrapping the item with sterilizer wrap or placing it into sterilizer bags or pouches. When this is done, the methods should be established so that the wrapping or pouching process does not adversely affect the air removal or steam penetration.

As part of the cycle development activities, one should determine which items heat up faster than others and which are the slowest to heat up. Within specific items studies are performed to evaluate the slowest-to-heat area of the item, which is where the thermocouples are placed.

Many companies validate both the minimum and maximum allowable loading configuration. However, the PDA's Technical Report Number 1 (2007) indicates that neither of these configurations may be the worst case.

21.5.3 Monitoring of Cycles

Some of the typical parameters monitored for moist heat sterilization cycles include come-up time, exposure time and temperature, cooling time or final temperature, water temperature (for superheated water cycles), fan speed (for fan cycles), pressure, time at which air is added to the cycle (air overpressure), and so forth.

21.6 Dry Heat Sterilization (and for Some, Depyrogenation)

Dry heat sterilization cycles utilize hot, dry air to accomplish sterilization. These processes have not been studied as extensively as their moist heat counterparts. There are differing opinions on the predictive nature of the microbial destruction or endotoxin reduction. Some choose to perform calculations to assess the lethality delivered and the theoretical endotoxin reduction while others challenge these calculations. The calculations used to determine the theoretical reduction of endotoxin are limited by the various *D*- and *z*-values used in the calculation. Since there is not a single acceptable *D*- and *z*-value, the calculations yield a variety of results depending upon the value utilized (Agalloco 2008).

When endotoxin challenges are utilized, biological indicators are not typically required as the endotoxin reduction provides more challenge to the system than the destruction of the biological indicator (PDA Technical Report Number 1 2007). For those studies which must show depyrogenation in addition to sterilization, the endotoxin challenge should show a minimum three-log reduction of endotoxin.

Dry heat sterilizers are available in both batch and continuous configurations. Continuous configurations typically have a tunnel.

21.6.1 Dry Heat Ovens

These units are validated in a similar fashion to moist heat sterilizers. Studies are performed measuring temperature distribution, heat penetration, and biological (or endotoxin) challenges. If sterilization is the only objective of the cycle, spores of *Bacillus subtilis* are typically used as biological indicators in the cycle.

21.6.1.1 Worst Case Conditions

The oven validation studies are conducted using worst case conditions. Typically, this is accomplished by reducing the exposure time and/or temperature.

21.6.1.2 Load Size and Configuration

Dry heat sterilization is not as effective as moist heat sterilization. For this reason, the loading in a dry heat oven is even more important. The loading configurations should be established and tightly controlled, as the hot air may not have the ability to compensate for differences in the loading configuration. It has been reported that in some cases minimum loads may receive lesser amounts of heat delivered than in the corresponding maximum load (Agalloco 2008). Condensation of saturated steam provides a greater amount of heat delivered to the product than is available in a dry heat oven.

Ideally it is recommended that every load configuration be validated, although some companies still try to use minimum and maximum loading.

21.6.1.3 Monitoring of Cycles

Some of the typical parameters monitored for dry heat sterilization cycles include exposure time and temperature, cooling time or final temperature, information on the blowers used, and so forth.

21.6.2 Dry Heat Depyrogenation (and Sterilization) Tunnels

Tunnels are validated in much the same way as a dry heat oven. The key validation parameters are the belt speed (which corresponds to the amount of time the item is subjected to the sterilization process) and the exposure set-point temperature. Biological indicators are not used in these cycles, since the intent is to perform depyrogenation. The endotoxin challenge units should be prepared directly inoculating the endotoxin onto the surface of the items to be processed in the tunnel. Data should be available to show the level of the challenge is sufficient to assess a minimum three-log reduction of endotoxin. Additionally, it is important to develop data on the recovery efficiency for the endotoxin method. For example, it might look like a total reduction of endotoxin occurred, when in reality the method was not appropriate to show the recovery of endotoxin from the surface of the item.

21.6.2.1 Loading

With a continuous sterilizer, there is not the same type of loading configuration or arrangement as is possible with a batch sterilizer. The items are "pushed" into the sterilizer on the conveyor belt. In most cases, those units at the beginning and trailing edges of the items typically represent the worst case conditions (Agalloco 2008).

Many companies choose to validate the largest and smallest size containers processed through the tunnel, without challenging each intermediate size container.

21.6.2.2 Worst Case

To challenge the worst case conditions a company may lower the conveyor belt speed (exposure time) and/or the exposure temperature.

21.7 Are Sterilization and Sanitization the Same Thing?

Sanitization is defined as the destruction of microorganisms that may or may not be pathogenic, on surfaces using chemicals or heat. While sanitizing destroys the microorganisms it may or may not achieve the same lethality offered by sterilization cycles. When using either of these terms, it is useful to clearly define the intended meaning within your documentation system.

Frequently, companies use the term sanitization when describing processes used to reduce bioburden, without necessitating all of the validation and documentation associated with a sterilization cycle. For example, the microorganisms may be destroyed, but the cycle is not designed nor expected to meet the same levels of sterility assurance as a validated sterilization cycle.

21.8 What Regulatory Requirements Must Be Met for Sterilization Validation?

The criticality of sterilization operations results in many regulatory expectations for the validation of these processes. Within these regulatory expectations there are differences in how the validation should be conducted and what parameters are most important in the validation (HTM-2010 1994; FDA 1994; BSEN285 2006, 2009). It is important to understand the regulatory requirements for sterilization that must be met for your affected product.

In 1976, the FDA published the *Proposed Current Good Manufacturing Practices for Large Volume Parent*, which is also called the GMPs for LVPs (FDA 1976). This document was drafted by the Agency in response to problems with sterility in LVPs, that resulted in the deaths. While this document was withdrawn by FDA and never officially issued, many of the requirements within this document have become the small "c" in cGMPs relative to sterilization validation used for terminal sterilization. Some examples include requirements for temperature distribution evaluations and heat penetration evaluations, calibration of instruments, use of biological indicators as part of the validation process, and worst case evaluations (Agalloco 2008).

Additionally, organizations like the Parenteral Drug Association (PDA) have issued technical reports that reflect industry standards for validation. Among the PDA documents governing sterilization are:

- PDA Technical Report 1, Revised 2007 (TR 1) validation of moist heat sterilization processes cycle design, development, qualification and ongoing control (PDA Technical Report Number 1 2007)

- PDA Technical Report 2 (TR2) validation of dry heat sterilization and depyrogenation cycles (PDA 1980)
- PDA Technical Report 48 (TR48) moist heat sterilizer systems: design, commissioning, operation, qualification and maintenance (PDA Technical Report Number 48 2010)
- PDA Technical Report 7 (TR7) depyrogenation (PDA Technical Report Number 7 1981)

The International Organization for Standardisation (ISO) also has published a variety of documents for sterilization processes. In addition to sterilization, they have documents for calibration and biological indicators. Some of the ISO documents include:

- ISO 14160:2011 Sterilization of health care products—Liquid chemical sterilizing agents for single use medical devices utilizing animal tissues and their derivatives—Requirements for characterization, development, validation and routine control of sterilization processes for medical devices
- ISO 20857:2010 Sterilization of health care products—Dry heat—Requirements for the development, validation and routine control of a sterilization process for medical devices
- ISO 25424:2009 Sterilization of medical devices—Low temperature steam and formaldehyde—Requirements for development, validation and routine control of a sterilization process for medical devices
- ISO 11137—Sterilization of health care products package

All of the documents from regulatory agencies and industry are living documents. They change over time to reflect current expectations for sterilization validation.

Compendial expectations for sterilization and sterility assurance also exist. The appropriate documents for your region should be consulted when establishing the minimum validation requirements that should be met.

21.9 The Basis of Sterilization Processes: Understanding How Microorganisms Die

Over the years a significant amount of research has been conducted studying the kinetics of microbial death. They found that the process of destroying or sterilizing the cells occurs at a defined and consistent rate that is dependent upon the variables that affect the reaction rate, as shown in Fig. 21.1. The *D*-value is determined by plotting the number of viable cells, those that survived, and determining the time it takes to reduce the microbial population by one-log at a specified set of sterilization conditions (Agalloco 2008).

Understanding this relationship has led to the development of a number of calculations that can be used to model the microbial destruction and the probability of sterility associated with a sterilization cycle.

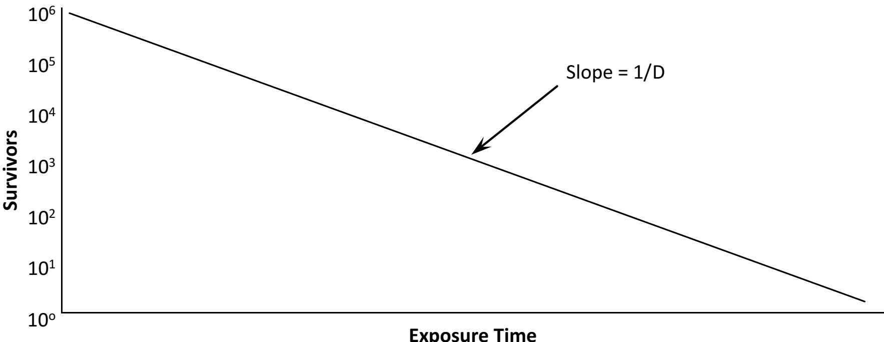

Fig. 21.1 Microorganisms die in a logarithmic fashion at a defined set of parameters

One of the most effective types of sterilization is moist heat (steam) under pressure. To use this methodology, the item to be sterilized must be able to withstand heat and moisture without damage. It is reported that most microbes grow in the range of −50 to 80 °C, although some form spores at temperatures outside of these ranges (DeSantis 2008).

For this reason, sterilization applications utilize the understanding of how microorganisms die, to predict the probability of a survivor in a sterilization process. This is expressed as PNSU or the probability of a non-sterile unit. For moist heat sterilization the expected PNSU is less than 10^{-6}, which means that there is no more than a one in a million PNSU. Not all sterilization processes predict the probability of non-sterility. For example, an aseptic process validation measures the rate of contamination present at the time the process simulation test was performed but does not provide a probabilistic measure of the sterility assurance.

Copious information is available that indicates that microbial death can be described as a first-order chemical reaction. This implies that the death is a single molecule reaction, probably as a result of a denaturation of a specific critical molecule in the cell (DeSantis 2008).

21.9.1 Biological Indicators

Some types of bacteria are able to form structures that are resistant to adverse conditions called spores as a defensive survival method. The genus and species of bacteria resistant to a specific sterilization process may be different across sterilization processes. Additionally, the same bacterial spore may have different levels of resistance to varying sterilization processes. The level of resistance to a sterilization process is expressed as the *D*-value. *D*-values are specific for a sterilization medium and a specific temperature. By definition the *D*-value is the amount of time in minutes that are required at a specific reference temperature to reduce the microbial population by one-log. The higher the *D*-value at a specific set of sterilizing conditions, the more resistant the organism is to the sterilization process.

Mold spores are formed by differing species of mold as a reproductive tool. They allow the mold to reproduce. As such, they do not have the same type of moist heat sterilization resistance as bacterial spores.

21.9.1.1 *D*-Values

Geobacillus stearothermophilus spores (D_T ~ 1.5–6.0 min in various parenteral solutions) are considered one of the most resistant spores for moist heat sterilization. As such, many regulatory documents specify the use of these spores in the validation of moist heat sterilization cycles. Some other organisms that have sufficient resistance to be appropriate challenges for validation of moist heat sterilization cycles include *Bacillus subtilis* 5230 (D_T ~ 0.5 min in 0.9 % saline), *Bacillus smithii* (formerly *Bacillus coagulans*, FRR B666) (D_T ~ 1.5 min in sterile water), and *Clostridium sporogenes* ATCC 7955 (D_T ~ 0.3–7.0 min in various parenteral solutions) (Sadowski 2009; Moldenhauer 2011). It is important to know the regulatory expectations in your country when selecting the appropriate biological indicator challenge to use.

For dry heat sterilization, several types of spores may be used. Typically, spores of *Bacillus subtilis* have been used. Some have successfully used *Geobacilllus stearothermophilus*. Typically the most resistant of spores will have a *D*-value of 6–10 min at 170 °C. If the cycle is used to depyrogenate and is run at a much higher temperature, the microbial *D*-values may be only a few seconds (Anonymous 2011a).

Selection of the biological indicator to use includes consideration of many things including an organism that is not pathogenic; an organism that is easy to cultivate; an organism that can be cleaned, harvested, and grown with relative ease; an organism more resistant to the sterilization process than the items being sterilized; an organism which is stable; an organism which is not inhibited by the items it will be used to test; and an organism which provides reproducible results when tested at a specific temperature (Sadowski 2009).

For moist heat sterilization cycles, the biological indicator to be used in the process is based upon the sterilization cycle–design model (approach) used. Most companies using an overkill model choose to use *Geobacillus stearothermophilus.* When product-based models are used, the other acceptable biological indicators are frequently used (Sadowski 2009).

21.9.1.2 *z*-Values

Another important term when using biological indicators is the *z*-value. This term refers to the temperature dependence on the *D*-value. It represents the number of degrees of temperature that are required to achieve a tenfold change in the *D*-value. For example, if the *z*-value is 10 °C, changing the sterilizing temperature from 120 to 130 °C will yield a tenfold change in the *D*-value. As such, a much shorter sterilization time will be required at the higher temperature. The *z*-value tends to be constant across a broader range of temperatures. *Geobacillus stearothermophilus* spores

have a typical z-value of 10 °C for temperatures between 100 and 135 °C. *Bacillus subtilis* spores used for dry heat have a typical z-value of 20 °C in the range of 150–190 °C. For dry heat cycles used to concurrently depyrogenate using temperatures of 225–300 °C, the z-values used are between 45 and 53 °C (Agalloco 2008).

21.9.1.3 *F*-Value

The F-value or thermal death time is used along with the temperature data and z-value to estimate the lethality for moist and dry heat sterilization cycles (Agalloco 2008). It is the time in minutes required to deliver a sterilization cycle equivalent to that of the reference temperature T. For the majority of moist heat sterilization cycles the reference temperature is 121 °C and for dry heat sterilization cycles it may be 160 or 250 °C.

The reference temperature benchmarks the cycle in terms of the heat delivered at the specified temperature. The F-value is specific for a defined reference temperature. Equation (21.1) provides the equation for the F-value (PDA 2007; DeSantis 2008).

$$F = \int 10^{(T_0 - T_{\mathrm{ref}})/z}\, \mathrm{d}t$$

This formula approximates to:

$$F = \Sigma 10^{(T_0 - T_{\mathrm{ref}})/z}\, \Delta t \tag{21.1}$$

where

T_0 = temperature within the item being heated
T_{ref} = reference temperature
z = z-value of the challenge organism
$\mathrm{d}t$ = the change in time (minutes)
Δt = the chosen time interval (minutes)

For moist heat sterilization, when the reference temperature is 121.1 °C and the z-value is 10 °C, the F-value is called F_0. For dry heat sterilization, many use the term F_{H} where the reference temperature is 170 °C and the z-value is 20 °C. Some companies use the term F_{P} for dry heat that is used also to depyrogenate, using a reference temperature of 250 °C and a z-value between 40 and 50 °C (Agalloco 2008).

Figure 21.2 provides an example of a microbial survivor curve for moist heat sterilization. Although this example is for moist heat, similar curves can be performed for dry heat. In this figure, the starting population of resistant microorganisms N_0 is 10^6. The D-value at the specified reference temperature is 2.5 min. The desired level for the PNSU is 10^{-6}. The desired probability of survivors in this graph is designated as N_{F}, or the final population (PDA Technical Report Number 1 2007).

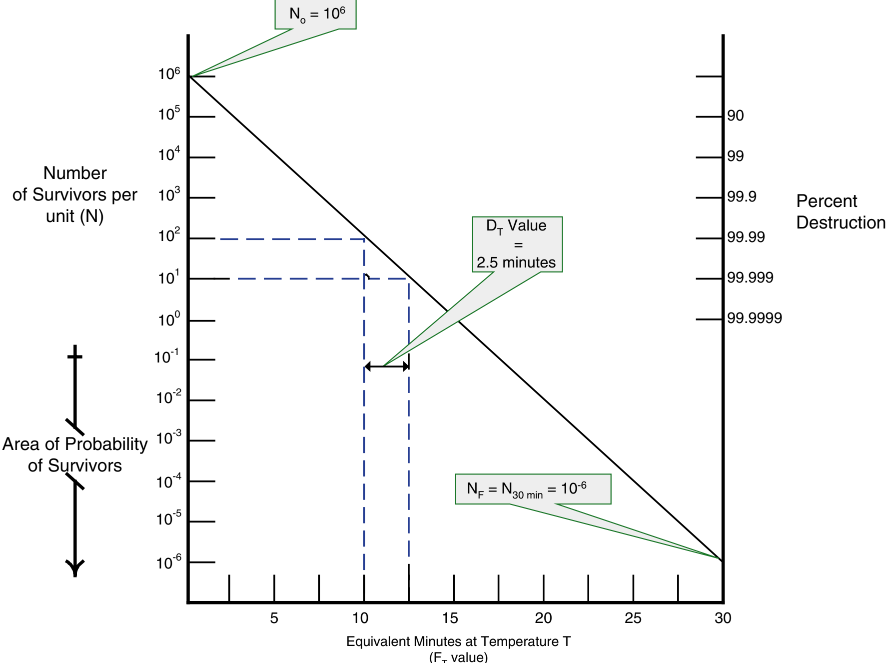

Fig. 21.2 Microbial survivor curve

The microbial survival curve model is representative of first-order kinetics, i.e., the death kinetics of single-celled spores. While this model may not be representative of all of the different types of challenge systems, this is still the best model available (PDA 2007).

To properly use this model in microbial destruction calculations it is important that the challenge organism is in a homogeneous culture and that the constant lethal stress or the equivalent lethal stress is applied to the challenge (PDA 2007).

21.9.1.4 Probability of a Non-sterile Unit

The PNSU calculation is used to determine the probability (risk) of obtaining a non-sterile unit at the end of the sterilization cycle. Most regulators have accepted that an acceptable probability is one in a million, or 10^{-6} for sterilization. Some individuals assume this is a synonymous term with sterility assurance level (SAL). In reality the exponent for the SAL should be a positive number rather than the negative exponent in a PNSU value. Many prefer the term PNSU since the values of the exponents are not ambiguous. The PNSU can be calculated by solving for *B*, using (21.2) (Agalloco 2008).

$$\mathrm{Log}_{10} B = \mathrm{Log}_{10} A0(F_0 / D_{121}) \tag{21.2}$$

where

B = probability of a non-sterile unit
A = number of presterilization bioburden microorganisms
F_0 = the minimum F_0 observed in the cycle
D_{121} = the D-value of the bioburden microorganism at the reference temperature of 121 °C

Note: For cycles using other F-values or D-values, the values would be substituted for F_0 and D_{121}.

21.10 The Physical Characteristics of a Sterilization Cycle

In addition to the assessment of the biological performance of a sterilization cycle, one must also assess the physical performance of the sterilizer, including assessment of the temperature and pressure in the sterilizer chamber and the load.

21.10.1 Temperature

Both moist heat and dry heat sterilization cycles are dependent upon the heat (thermal energy) from the sterilizer being delivered to the items being sterilized. The temperature delivered determines the rate of the chemical reactions that take place resulting in destruction of the microorganisms. Temperature evaluations are conducted in two different ways, temperature distribution studies and heat penetration studies. The temperature distribution studies assess the temperatures throughout the sterilizer chamber. The heat penetration studies are used to assess the heat delivered to the items being sterilized.

Temperature distribution studies and heat penetration studies are conducted using temperature monitoring devices, usually thermocouples. The thermocouples selected for use should be appropriate for the temperature range being monitored. Some companies choose to use resistance temperature detectors (RTDs), because they have a higher level of accuracy than thermocouples. However, thermocouples are less expensive, have a faster response time, and they are made to withstand the "abuse" they get during validation studies. The thermocouples selected for use must be calibrated and accurate to a specified level for the temperature range in which they will be used (Agalloco 2008).

Care should be taken when placing the thermocouples in the sterilizer load for validation. When placing temperature distribution probes, it is important that the

probes are not coming into contact with other surfaces that may produce results that are not representative of the temperature in the sterilizer chamber. Placement of heat penetration probes also requires care. The probes should be placed within items in such a way that they do not block off the path of steam penetration to the item. Additionally, the placement of the probes must not create artificial openings where steam could penetrate the item in a way that is not possible in routine sterilization. For thermocouples that have wires which attach to the data recorder, one must also be sure to position the wires so that they do not become damaged by the handling of the load being placed into or out of the sterilizer.

The operation of some sterilizers makes it difficult to utilize wired probes as part of the validation process, e.g., probes that operate through a tunnel or probes in sterilizers that rotate or agitate. There are probes that have been designed to wirelessly provide the temperature data to the data logger.

Some sterilizers also utilize load probes. These probes are permanently installed in the sterilizer and monitor temperature in both validation and routine production cycles. When installed, the temperature values for these probes are also monitored during the validation cycle.

For sterilizers that utilize recirculating water for cooling, thermocouples are also used to assess the temperatures attained in the water prior to initiation of the cooling process. There is an expectation that the water be sterilized during the cycle so that the water used for cooling of the product is sterile.

21.10.2 Pressure

Pressure is monitored in moist heat sterilization cycles. The pressure has lesser importance than temperature; however, it is used to ensure that saturated steam is used during the exposure dwell period. There are some regulators who routinely check the temperatures observed on recording charts to the expected pressure readings from steam tables.

Pressure can play a significant role in ensuring functional product at the completion of the cycle. For example, inadequate overpressure in a plastic cycle may result in deformed containers.

21.10.3 Calibration Expectations

The key instrumentation for monitoring or recording temperature and pressure data for the sterilizer should be calibrated in such a way that the calibration can be traced to a recognized national standard. Additionally, the thermocouples used in the validation should be calibrated before and after each study.

21.10.4 Other Monitoring Devices

One may choose to utilize a physical or chemical indicator and/or a physical/chemical integrator to monitor the sterilization cycle. There are a variety of indicators available for different types of sterilization. One of the simplest types of indicators is the use of autoclave tape, which changes color if the tape has been processed in the sterilizer. It does not provide information on the quality of the cycle, and in many cases does not indicate whether a complete cycle was performed. Physical/chemical integrators assess the process conditions in the cycle, typically integrating the effects of multiple process parameters, and provide some guidance on the cycle delivered. One such integrator provides a line showing whether a minimum F_0 value was achieved in the cycle.

Neither indicators nor integrators are to be utilized in place of biological indicators. They do provide useful information, like indicating that a load has been processed through the sterilizer.

21.11 Selecting an Approach for Sterilization Validation

There are established models or approaches that can be used to determine the sterilizer validation criteria like minimum lethality that must be met. In the past, there were three models based upon the thermal death time curve, an overkill model, a combined biological indicator–bioburden-based model, and an absolute bioburden model. The key differences in these models are the amount of heat delivered to the product, the effect on stability of the product, and the cost of the sterilization cycle (e.g., the utilities used). The PDA reissued their technical report on moist heat sterilization and converted the combined biological indicator–bioburden-based model and the absolute bioburden model into a product-specific approach to cycle design (PDA 2007). The intent of each model or approach is to determine the lethality required to deliver the desired PNSU.

The semilogarithmic survivor curve model can be used to determine the appropriate PNSU, which in most cases is 10^{-6}. This can be expressed as shown in (21.3).

$$\mathrm{Log}\,N_{\mathrm{F}} = -F/D + \mathrm{Log}\,N_0 \tag{21.3}$$

In order to determine the lethality that is required for a specific sterilization cycle, this equation can be rearranged to solve for the F-value, as shown in (21.4) (PDA 2007).

$$F = (\mathrm{Log}\,N_0 - \mathrm{Log}\,N_{\mathrm{F}}) \times D_{\mathrm{T}} \tag{21.4}$$

where N_0 represents the starting population of the biological challenge organism, e.g., 10^6. The D-value is specific for the microorganism chosen as the biological indicator. N_{F} represents the final population of microorganisms, which is usually 10^{-6}, or the desired PNSU.

21.11.1 Overkill Cycle Models

An overkill cycle provides the most heat to the items being sterilized. It has the highest cost associated with use of the sterilizer. This added heat has an impact on the stability of the item following sterilization. This type of cycle provides a high level of sterility assurance regardless of the level of bioburden present and regardless of the heat resistance of that bioburden. We commonly assume that the starting population of bioburden is 10^6, with a $D_{121\,°C}$ of at least 1 min, and a z-value of 10 °C. To have a PNSU of 10^{-6}, that would indicate a final biological population or N_F that is 10^{-6}. These values can be entered into the equation to determine the F-value, i.e., the expected lethality. This calculation is shown below using (21.5) (PDA 2007).

$$F_0 = D_{121^\circ C} \times (\mathrm{Log}\,N_0 - \mathrm{Log}\,N_F) \tag{21.5}$$

$$F_0 = 1.0\,\mathrm{min} \times (\mathrm{Log}10^6 0\mathrm{Log}10^{-6}) = 12\,\mathrm{min}$$

Within the definition of overkill in the PDA's Technical Report (PDA 2007), an overkill cycle must deliver a lethality of at least 12 min under the specified conditions. The PDA's Technical Report (PDA 2007) added to this definition stating: "a cycle designed with the overkill design approach can be defined as a sterilization cycle that is demonstrated to deliver an $F_{\mathrm{Biological}}$ and F_{Physical} of at least 12 min to the items being sterilized."

F_{Physical} is defined as the term used to describe the delivered lethality calculated based on the physical parameters of the cycle. The F_{Physical}-value is calculated as the integration of the lethal rate (L) over time. The lethal rate is calculated per a reference temperature (T_{ref}) and z-value using the equation: $L = 10^{(T - T_{\mathrm{ref}}/z)}$ (PDA 2007). This typically refers to the data obtained from the temperature measurement devices, e.g., heat penetration probes.

$F_{\mathrm{Biological}}$ is defined as a term used to describe the delivered lethality measured in terms of actual kill of microbiological organisms on or in a BI challenge system. The $F_{\mathrm{Biological}}$-value is calculated as the $D_T \times \mathrm{LR}$, where D_T is the D-value of the BI system at the reference temperature, T, and LR is the actual log reduction of the BI population achieved during the cycle (PDA 2007).

Addition of the requirements for $F_{\mathrm{Biological}}$ to the definition of an overkill cycle was a change over previous definitions. Few companies prior to this time routinely monitored or assessed $F_{\mathrm{Biological}}$ as part of their routine qualification requirements. For many companies who had cycles in place for many years, converting to this definition of an overkill cycle necessitated repeating or revising the cycle development studies previously conducted.

There is a separate definition of overkill cycles within the European regulations. In the European regulations for terminally sterilized dosage forms, an overkill cycle is specified as sterilization by moist heat at 121 °C for 15 min (PDA 2007).

Most regulators expect that an overkill cycle model be used for the sterilization of equipment and components utilized in aseptic manufacturing operations.

It is possible to use this type of sterilization model for some terminal sterilization activities, providing that the items being sterilized can withstand the cycle conditions.

21.11.2 Product-Specific Models

Overkill terminal sterilization cycles are too harsh for many products in the pharmaceutical environment. Terminal sterilization is desired for pharmaceutical products as a method to provide for patient safety. Since many overkill cycles are too harsh, alternative models have been developed for those products with some thermal sensitivity, which are called product-specific models or approaches. With this type of approach, one must sufficiently reduce the microbial population to an acceptable level of sterility assurance while maintaining the product's attributes. In order to accomplish this, the models take into account the starting population of the microorganisms present (bioburden) and the heat resistance of those organisms, along with provision of a safety factor. These values are utilized in the calculation to determine the minimum lethality. Use of this type of model requires the manufacturer to commit to ongoing monitoring of the product bioburden and its heat resistance. An example of the product-specific design model calculations is illustrated using (21.5) (PDA 2007).

Manufacturing site data for bioburden indicated:

$$N_0 < 10 \text{ resistant organisms per unit of product}$$

$$D_{121^\circ\mathrm{C}} < 0.25 \text{ min}$$

In order to add a safety factor, the following values were used:

$$N_0 = 10^2$$

$$D_{121^\circ\mathrm{C}} = 0.4 \text{ min}$$

The desired PNSU was determined to be 10^{-6}

$$F_{121^\circ\mathrm{C}} = (\mathrm{Log}10^2 - \mathrm{Log}10^{-6}) \times D_{121^\circ\mathrm{C}} = 3.2 \text{ min}$$

The above example indicates that the model would deliver the desired PNSU using an F_0 of 3.2 min. However, many regulators would expect that additional precautions be established in support of cycles with an F_0 less than 6 min, e.g., aseptic filling prior to terminal sterilization or other methods designed to ensure microbiological control.

21.12 Developing a Validation Strategy

Moist and dry heat sterilizers vary in their complexity and their level of automation. There are also differences in the types of cycles available to achieve sterilization. It is important to develop a validation strategy that includes validation of the hardware, software, and specific cycle. It is important to determine the steps that will be incorporated into the validation plan for the selected sterilizer. The level of complexity of the system and the intended use of the system may impact the considerations that are applicable in the validation strategy.

Some of the considerations in developing a strategy include (Anonymous 2011b):

- User's requirement specification (URS)
- Functional design specification (FDS)
- Supplier assessment
- Risk assessment
- Validation master plan (VMP)
- Design qualification (DQ)
- Factory acceptance testing (FAT)
- Site acceptance testing (SAT)
- Commissioning or engineering studies
- Installation qualification (IQ)
- Operational qualification (OQ)
- Performance qualification (PQ)
- Personnel training
- Documentation requirements, e.g., SOPs, protocols
- Requirements traceability matrix (RTM)
- Summary report

21.13 User's Requirement Specification

Sterilizers are expensive capital investments. It can be disastrous to find out after purchase that the sterilizer will not perform the tasks you need. In order to purchase the right sterilizer for your site, it is important to understand what your expectations for the system are. Some expectations will be specific to the sterilizer you want to buy, while others may be general requirements to meet your company's requirements. You should have a clear understanding of what you want from the system before you purchase it. These expectations are incorporated into a formal document called the URSs. The URS serves as the foundation for several other documents including the RTM and the various tests and acceptance criteria incorporated in the validation documents.

Some of the considerations in developing a URS are:

- What is the intended purpose of the system, e.g., to moist heat sterilize glass vials and prefilled syringes; to provide a continuous process of sterilization and depyrogenation of glass vials
- What are the specific performance requirements that must be in the system, e.g., temperature or pressure ranges you need in a system, type of cooling
- A description of how the system will be used, e.g., a batch process to accommodate multiple filling lines, a continuous process tied to a specific vial washer and aseptic filling line
- Characteristics of the hardware that must be met, e.g., size requirements, power requirements, welding expectations, standards that must be met, provisions to use data loggers and thermocouples
- Characteristics of the software that must be met, e.g., stored on a chip, PC compatible software, certified to be compatible with 21CFR Part 11 requirements
- Expectations for reports, e.g., what types of information must be documented, how often should data be documented, how are alarms handled
- Compatibility and/or communication with other systems, e.g., networks, testing equipment, utilities
- Descriptions of how data will or should be managed, e.g., is it stored after the cycle is complete, how long is data archived, who can amend the data
- Necessary requirements for safety, e.g., interlocks on doors preventing them to open before product has cooled to a specific temperature, tests for pressure, valve releases, manual overrides
- Requirements for support services or needs, e.g., preventative maintenance, calibration
- Concerns for engineering or physical requirements, e.g., must fit in a specified space or area, must have a separate area for the engineers to access the system separate from the clean room
- Training that should be provided
- Financial considerations that must be met, e.g., cannot exceed a price of X

21.14 Functional Design Specification

The functional requirements for the sterilizer are described in the FDS. Some companies choose to include these requirements in the URS and skip the development of this document. The FDS requirements are intended to ensure that the URS requirements for performance will be met. This document is very specific and detailed, which leads to development of a lengthy document. It is more common to generate this type of document when the sterilizer is being custom-manufactured as opposed to a system that is commercially available with minimal customization.

This document adds to the information in the URS, describing how the sterilizer should operate or function to meet all of the requirements in the URS. For example, if the URS requires that a recorder be provided to document the cycle the FDS

might specify the exact report content and format that must be met, along with the time intervals for reporting data.

Some examples of the typical information included in the FDS are:

- System description
- Purpose
- Requirements for system documentation, e.g., user manuals, procedures, technical documentation
- Physical specifications for the system, e.g., size, power, operating environment, utilities
- Specifications for the control system, e.g., type of computer, system operating system (including version numbers in some cases), supporting software, requirements for the computer hardware and accessories, networking requirements, printer requirements, databases, and so forth
- Requirements for system security, e.g., multilevel password system, methods for record retention, necessity of an audit trail, compatibility with requirements for 21CFR Part 11, and so forth
- Cycle parameters that must be included in the system and corresponding information on where parameter tables must be available and configurable
- Supporting equipment required, e.g., requirements for sterilizer trucks, special handling equipment, interfaces to other production equipment
- Error handling, e.g., what must be alarmed, what types of alarms are required, how the alarms silenced, how are the alarms documented
- Other requirements

The complexity of the system and the intended use will determine the level of detail required in this type of document.

21.15 Supplier Assessment

For sterilization systems, one may want to verify the knowledge of the supplier with the regulatory requirements for your locale. You may wish to contact other users to obtain references. If this is a supplier you have not previously used, it may be prudent to evaluate the technical support available for the system. For example, are there local service representatives or does one need to have representatives come in from other areas.

21.16 Risk Assessment

The concept of risk management, also known as quality risk management (QRM), has been endorsed by numerous regulators for the pharmaceutical industry. As such, there is an expectation that one understands the risks associated with a sterilizer to be utilized at a pharmaceutical site. The specific risks will vary depending upon the system selected.

21.17 Validation Master Plan

The requirements for the validation of sterilization processes should be included either in the site master validation plan or in a specific validation plan. Some companies identify these requirements in corporate policies. This type of plan is useful in ensuring the desired expectations. This type of master plan might include general requirements like overkill cycle models are used for in-process sterilizers used to sterilize equipment and components for aseptic processing and product-specific models are used to terminally sterilize products. This document might also include information like, validation must include temperature distribution, heat penetration, and biological indicator or endotoxin challenges concurrently. Depending upon the level of detail included for your facility, this document might describe the minimum number of studies required, type of product loading, and so forth.

21.18 Design Qualification

Design qualification is conducted by many facilities, though not all, to ensure that the proposed design of the sterilizer is appropriate for its intended purpose. There are a variety of methods available for how to conduct this qualification. One way to accomplish this is to determine if the vendor's design is appropriate to meet the various requirements you have established in the URS. Some companies accomplish this by having a multidisciplinary group meeting to discuss the design proposals and the URS.

21.19 Factory Acceptance Testing

Sterilizers have a substantial size and weight that makes them expensive to ship. This would make them difficult to return to the vendor. In order to mediate this concern some companies conduct testing at the vendor site prior to shipping the equipment to the end-user. This testing may include functions which require special equipment that the end-user does not have. Some choose to perform testing at the factory site which would cause the system to be returned if found at the end-user site. Depending on the company, this testing may be formally documented in protocols, or performed in laboratory notebooks. In some cases, this testing is referenced in subsequent validation protocols without repeating the testing.

21.20 Site Acceptance Testing

Some companies perform some initial testing once the system is obtained at the end-user site prior to the official validation studies. These studies may be a repeat of some of the tests conducted in the FAT in order to ensure that the system received operates the same way it did when at the vendor site.

The level of documentation generated and required varies.

21.21 Commissioning or Engineering Studies

Once the sterilizer has been installed at the intended location, there are some activities conducted prior to initiating the formal validation of the system. Some of the activities that may be conducted include cleaning, addition of lubricants, and so forth. It is also beneficial to execute some runs of the sterilizer to take a “look-see” on how the system is operating. The engineers frequently use this time to ensure that the system is sequencing as expected, no major alarms occur, and a general assessment if the systems appears to be operating reliably so that validation can commence. This is performed to reduce the risk of failures during the actual validation process. Typically these activities fall under the responsibility of the engineering/maintenance department.

This type of evaluation may also include commissioning of the equipment, which includes verifying that it is performing sufficiently to proceed with validation. Additional information on this topic can be found in the Parenteral Drug Association’s Technical Report No. 48 Moist Heat Sterilizer Systems: Design, Commissioning, Operation, Qualification and Maintenance (PDA 2010).

21.22 Qualification of the System

The equipment qualification refers to an assessment of the hardware/software used for the sterilization process. It does not address the actual sterilization cycle or the items being processed in the sterilizer. The equipment qualification includes verifying the proper installation of the equipment as well as verifying that it is operating correctly, prior to assessing its capability to sterilize the desired components, equipment, or product. These activities are conducted in the Installation Qualification (IQ), the Operational Qualification (OQ), or in a combined document that covers the Installation and Operation Qualification (I/OQ). Whether the protocols are combined or separate documents frequently is a function of how complex the equipment qualification process is. The same requirements apply in either case.

21.22.1 Installation Qualification

The installation qualification (IQ) is a formal protocol that is designed to ensure that the system as received and installed at the end-user site meets the specified requirements. In some cases, companies contract with the vendor of the equipment to perform this testing. It is good practice for end-user personnel to participate in this activity, even when the vendor conducts the studies to gain knowledge of the system. The documentation generated becomes a technical handbook for the system as supplied to your site. This information can be invaluable when determining at a later date whether replacement parts are or are not identical to those installed. It is at the end-user’s discretion on whether the hardware and software are tested concurrently or as separate documents (Moldenhauer 2003).

Some of the considerations included in this evaluation include:

- Verification that the items received and installed agree with those specified in the purchase order and design specifications
- The items received were not damaged in shipping or during the installation process
- All of the required supporting documentation for the system was received
- The required documentation is complete, e.g., User Manuals, Technical Manuals
- Specified utilities are available and properly connected to the sterilizer
- Verification that the system was installed correctly
- A listing has been compiled with all of the key devices in the system along with a description of information like the model numbers, serial numbers, operating ranges, voltages, and so forth
- Wiring of the system is as specified, if applicable
- Version numbers for software or EPROMs used are documented
- Models, serial numbers, and operating ranges are documented for key components
- Applicable hardware and software are documented to be present
- Peripheral equipment and accessories are present and properly connected and configured for use, if applicable
- Drawings are available for the system and/or installation, as applicable
- Back-up and/or recovery copies of all software have been made and are available
- Dip switch settings are documented
- Calibration records are available for all instrumentation
- Cabling connections are documented and as specified
- Configurations are documented
- Verification that log books are established and maintained for the system
- Verification that the system has been incorporated into a change control system

This document should include all of the needs for operation of the unit as well as the needs to ensure compliance with the applicable regulatory agencies, health and safety requirements, and so forth. When obtaining proposals from vendors, it is useful to actually track where each requirement from the URS is reflected in the item to be purchased. Key information to include is expected capacity of the unit, types of loads to be sterilized, any safety requirements, and the expectations for documentation from the system. Additional information on this topic can be found in the Parenteral Drug Association's Technical Report No. 48 Moist Heat Sterilizer Systems: Design, Commissioning, Operation, Qualification and Maintenance (PDA 2010).

It is beneficial to make this document as comprehensive and detailed as possible. This document becomes the technical handbook for the sterilizer over time. It is especially useful when trying to assess the appropriateness of replacement parts, and whether they are equivalent to the original installation.

21.22.2 Operational Qualification

The operational qualification is important as it allows you to assess whether the system it operating at the specified conditions, prior to adding the influence of the item to be sterilized into the mix. It may also be called the Dynamic Equipment Qualification. Once you initiate sterilization of items within the unit, if a problem occurs it is not clear whether it is the sterilizer performance at fault or effects of the item in the sterilizer. The OQ of the hardware and the software or control system may be performed within a single document or in separate documents. The utilities used to support the sterilization system, e.g., clean steam, oil-free compressed air, and the like, should be qualified either concurrent with the OQ or prior to the OQ being conducted.

The OQ should identify all of the operating variables along with the normal ranges of operation. Studies for dry heat sterilizers typically have a wider temperature range than the corresponding moist heat sterilizers. This is due to the lower heat capacity delivered by dry heat vs. that delivered by steam.

21.22.2.1 Empty Chamber Temperature Distribution

Studies are conducted using temperature distribution probes, usually arranged in a fixed or geometric pattern, to evaluate the uniformity of heat delivered within the sterilizer chamber. Unless heat is delivered to all areas of the sterilizer chamber, one cannot ensure that all of the items in the sterilizer will be appropriately sterilized in the cycle. During these studies it is common to find that some of the sterilizer parameters may need to be modified, for example, the bleeder valves, water spray nozzle directions, directional air flow, and so forth. The data obtained from these studies is also used as baseline data for evaluating the sterilizer's performance characteristics over the life of the sterilizer.

Penetration probes are not typically used in these evaluations. It is important that the temperature distribution probes are properly located and that they do not come into contact with either the sterilizer trays or the sterilizer itself.

21.22.2.2 Software Evaluations

During the OQ, it is also common to evaluate several features of the control system including, for example:

- Verification that it sequences properly through the various stages of the sterilization cycle
- Verification that the alarms trigger at the specified parameters (limits)
- Verification that security features are enabled as designed, like passwords, security levels, and administrative functions

- Verification that printouts provide all of the documentation required in the URS
- Verification that all stored records are accurate and retrievable, as specified in the URS
- Verification that the control system interacts with specified valves and other hardware as specified

21.23 Sterilization Cycle Development

After the OQ is complete, verifying that the sterilizer is operating correctly, cycle development studies are initiated. The requirements may differ somewhat for sterilization of porous/hard good loads and those for terminal sterilization of liquids, or for dry heat sterilization in ovens or tunnels. The PDA's Technical Report Number 1 (PDA 2007) provides a decision tree that walks through the various evaluations that should be considered in assessing the process type to be used for moist heat sterilization.

Cycle development studies should be used to determine the physical parameters required to appropriately sterilize the component, equipment, or final product. This includes placing the item into a specified loading pattern, except for dry heat tunnels. Part of this process is to determine a range of acceptable parameters that may be met in performing the studies. The end result of this process should be an item that is sterile and functional post-sterilization. Studies for cycle development should be formally documented (PDA 2007).

21.23.1 Development Support Activities

In order to conduct cycle development studies, it is important to conduct some evaluations prior to the actual cycle development.

21.23.1.1 Determination of the Slowest-to-Heat Location

This evaluation is important for both moist and dry heat sterilization cycles. A key consideration when performing validation studies using these items is where to place the penetration probes to measure the item's temperature accurately. Additionally, one wants to monitor at the "cold spot" of the item, to ensure that the entire item has seen the recorded temperature (at minimum). As such, it is necessary to perform studies that assess the heating characteristics of the item. These studies may also be called component-mapping studies.

Many components, especially within equipment loads, are complex and it would take numerous probes to determine all of the "cold spots." In order to minimize the number of probes and studies required, some assessment is conducted to evaluate

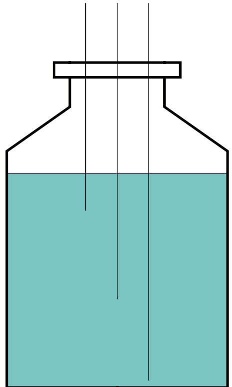

Fig. 21.3 Example of mapping in a liquid-filled vial

those spots expected to be more difficult to heat, like those with the greatest mass, potential for entrapped air, long hoses, and those items that encompass combinations of these types of concerns. There are differences in evaluating "cold spots" for those items which present issues in removing air vs. those that have a high mass. If air removal is the concern, then the probes would be placed within the wrapped item so that the tip of the probe is adjacent to the item, so that one can determine the equilibration time. The equilibration time represents the effectiveness of the air removal. Items with a significant mass tend to have a temperature lag to the sterilizer chamber temperature, due to the energy transfer not the temperature in the sterilizer chamber. As such, the probes should be placed to measure the heat penetration in contact with the item. This information aids in the determination of the load come-up time (PDA 2007).

Component mapping studies may be performed in the production sterilizer, laboratory sterilizer, or BIER vessels, providing that the sterilizer used has the same type of sterilizing conditions. It is possible to place several different probes in an item concurrently to collect additional data. It is important that the items being sterilized are prepared and wrapped in the same way they will be treated in production (PDA 2007).

Mapping studies are also performed for liquid-filled containers; however, frequently it is assumed that these studies are not required for containers of 50 mL size or smaller, due to the small size of the container. The studies usually look at different levels of solution within the container as illustrated in Fig. 21.3.

21.23.1.2 *D*-Value Studies

Biological indicators used as the challenge to a sterilization process have a measured *D*-value, thermal resistance to the sterilization process, at a set of specific conditions. There are a variety of parameters which affect the heat resistance of a

biological indicator (Moldenhauer (1999). These types of evaluations are conducted for biological indicators used for both moist and dry heat sterilization.

- Size of the inoculum
- Type of organism
- Sporulation and growth media composition
- pH
- Phase of spore maturity
- Incubation temperature for recovery of organisms

The surface on which the biological indicator is placed can have an affect on the heat resistance of the biological indicator. For example, placing a biological indicator on the surface of a porous rubber stopper can have increased heat resistance, since the biological indicator is protected by the porosity of the stopper and the poor transfer of heat from the rubber. In general, we assume that placement on a glass or stainless steel surface does not affect the *D*-value sufficiently to be of concern. Biological indicators placed on paper carriers can show different resistance based upon the quality of the paper used as a carrier. For porous/hard good loads, one must evaluate the biological indicator's heat resistance when directly inoculated on the rubber stoppers being sterilized. Rubber inherently is a poor conductor of heat. Data has been published to show that the heat resistance is affected by the rubber composition. This effect varies based upon the specific biological indicator used, the formulation of the stopper, and the types of treatments to which the stopper is subjected (Rubio and Moldenhauer 1995). As knowledge in this area has increased, concerns have arisen with other items that may change the biological indicator resistance. This has led to companies starting to evaluate the heat resistance of tubing and other materials processed in the load.

For liquid loads, the biological indicator should be placed in direct contact with the liquid to determine the *D*-value. The *D*-value can change based upon the strain of biological indicator used as well as the formulation of the liquid product.

If a carrier is used for the biological indicator, the sterilization cycle design should take into account the *D*-value on the carrier as well as the *D*-value determined when in direct contact with the liquid or component being evaluated.

21.23.1.3 Determination of the Selected Loading Configuration (Load Patterns)

The configuration of the load is important in both moist and dry heat sterilization cycles. These arrangements should take into account the necessary capacity required to support production activities as well as ensure that effectiveness of the sterilization process can be achieved. For example, if a water spray cycle is used, the loading should allow for adequate drainage of the water that comes into contact with the items being sterilized. When sterilizing hard goods, many of the items need to be wrapped in sterilization wrap to protect the sterile surfaces until they are used. If sterilization wrap is used, it should be non-shedding and provide a microbial

barrier (keeping the item sterile). The amount of wrapping should not be excessive, as this can reduce the effectiveness of air removal, steam penetration, and temperature penetration.

Larger mass items should be placed on the lower level of sterilizer trucks, in order to reduce the risk of wetting by condensate. Care should be taken to ensure that condensate from items minimizes drip onto other items (PDA 2007).

It is common to either photograph or generate drawings that clearly identify the loads selected. Additionally, only the loads that are successfully qualified should be used for production activities.

The appropriateness of a loading configuration can be assessed by reviewing the physical data generated and additionally it is useful to calculate the capability analysis of the penetration data for the load, e.g., C_{pk}. When the value is lower than expected, which for most sterilization processes is a minimum of two, the loading can be reduced or rearranged to increase the C_{pk} value.

21.23.1.4 Sterilization of Porous/Hard Goods

Validation of sterilization for porous items in a sterilizer load can be difficult. It is important to ensure that the air normally present in the load items is removed during the process to ensure that the desired heat is delivered to the items being sterilized. As such, the data generated must show that sufficient air is removed from the chamber and load prior to the exposure conditions of the cycle. Additionally the air and noncondensable gases need to be removed from the steam supply provided to the sterilizer. It is also critical for saturated steam (must have an appropriate dryness level) be provided to the sterilizer (PDA 2007).

The number and type of studies required during this process may vary, depending upon the site's knowledge of the items to be sterilized. Some of the typical parameters evaluated as part of this process include the following (PDA 2007):

- Jacket temperature
- Number and depth of pre-vacuum conditions
- Steam pulse levels
- Time and conditions for chamber heat-up
- Exposure time and temperature
- Allowable time for cooling (exhaust phase) and drying
- Air break

When possible, it is useful to standardize cycles to aid in reducing the ongoing validation and revalidation costs.

21.23.1.5 Sterilization of Liquid Loads

In a liquid load, the liquid inside of the container is heated by the transfer of heat from the outside of the container. The water contents steam and provides the sterilant inside the container. Nonaqueous solutions, like suspensions and emulsions,

require use of a rotation or agitation cycle to keep the load in motion. Several types of sterilization processes may be used including saturated steam, overpressure cycles, steam–air mixtures, and superheated water. For many liquid cycles air removal is not performed; however, it is important to ensure that a system is used to ensure uniform heat distribution within the chamber (PDA 2007).

The containers in a load heat do not necessarily heat at the same rate. Items closer to the outside may heat more quickly than items in the center of the load. The concern is to be sure that the items receiving the least heat are sterile and the items which receive the most heat are not adversely affected from a functional and stability point of view. As such there are several concerns to address including (PDA 2007):

- Ensuring that the load configuration in production matches the load configuration qualified
- Uniform delivery of heat to the load, in order to prevent under- or overheating
- Product bioburden in the load is within preestablished limits (cycle development and validation)
- For overpressure cycles, the overpressure is sufficient to maintain product functionality

There are some additional concerns for liquid filled containers, including (PDA 2007):

- The heating of the container surfaces is efficient to ensure that sterilizing conditions are maintained across the entire load
- There is a provision for efficient cooling of the load after sterilization so that the product quality attributes are maintained
- The product is stable
- Minimization of container breakage or deformation to maintain integrity
- Product formulation is maintained and understood to keep the solution resistance to the biological indicator within established parameters

It is also important to evaluate the temperatures of containers within the load in various positions in the sterilizer. If both minimum and maximum loads are used, both configurations should be evaluated.

21.23.1.6 Determining the Cycle Parameters to Use

The parameters to measure in a sterilization cycle are dependent upon the type of items to be sterilized, e.g., porous/hard goods loads, terminal sterilization loads, dry heat sterilization, or dry heat depyrogenation. In addition, one must also determine the criticality of these parameters. Some parameters are so important, frequently deemed critical parameters that when they are not met the sterilization cycle should be deemed unacceptable. Other parameters, while important, may be mitigated; for example, a pressure failure at some parts of the cycle might be mitigated by 100 % inspection of the load for a type of physical defect.

The PDA's Technical Report Number 1 provides a listing of process parameters to be considered for moist heat sterilization of porous loads including (PDA 2007):

- Jacket temperature and/or pressure at all phases of the cycle
- The number and depth of vacuum pulses prior to exposure
- The level of the steam charge prior to exposure
- The number and requirements for positive pressure pulses prior to exposure
- The chamber come-up time
- The exposure time
- The exposure temperature set-point
- The temperature of the chamber drain during exposure
- The chamber temperature during exposure
- The allowable temperature range for the load probe during exposure
- The chamber pressure during exposure
- The minimum allowable F_0 during exposure
- The allowable time for the load to cool down after exposure
- The drying time after exposure
- The rate for vacuum breaks

Depending upon the sterilizer configuration used, not all of the stated parameters are applicable.

The PDA's Technical Report Number 1 provides a listing of process parameters to be considered for liquid-filled moist heat sterilization loads including (PDA 2007):

- The jacket temperature and/or pressure prior during the cycle
- The fan rotations per minute during the cycle
- The agitation rate during the cycle
- The water flow rate during the cycle
- The chamber water level prior to exposure
- The heat-up time prior to exposure
- The temperature heat-up rate prior to exposure
- The rate of temperature heat-up prior to exposure
- The pressure ramp-up rate prior to exposure
- The temperature set-point during exposure
- The exposure time
- The chamber pressure during exposure
- The redundant heating media temperature during exposure
- The load probe temperature(s) during exposure
- The minimum allowable F_0 for the load probe during exposure
- The minimum allowable F_0 for the load probe postexposure
- The temperature cool-down rate postexposure
- The pressure ramp-down rate postexposure
- The load cool-down rate following exposure
- The maximum allowable load probe accumulated F_0 postexposure. Note: while PDA expresses this term postexposure, some companies actually calculate the value accumulated only in the exposure period.

For dry heat sterilization loads some of the items to be documented include:

- Blower parameters for the hot air
- Positions of the baffles in cycle development
- Load configuration
- Exposure temperature
- Exposure time
- Cooling parameters, which may include a safe temperature for handling the load after sterilization

For dry heat depyrogenation, one typically monitors the same types of parameters as in an oven, except for load configuration. Belt speed is monitored to reflect the requirements for exposure time.

The applicability of these parameters is determined by the type of sterilization process selected.

21.24 Performance Qualification

The purpose of the Performance qualification (PQ) studies is to demonstrate that the sterilization process consistently meets its predefined requirements. A minimum of three acceptable studies is required. If minimum and maximum loads are performed, a minimum of three studies for each load configuration are performed. There are some provisions for bracketing of load configurations, e.g., qualification of the maximum and minimum container size may justify the elimination of testing for intermediate container sizes. The IQ and OQ should be successfully completed prior to initiating the PQ studies. Supporting systems like utilities necessary for the process should also have been completed by this time.

The PQ includes both physical and biological qualification of the sterilizer load. Today, it is common practice to perform the studies concurrently. For moist heat sterilization, the physical qualification data yields an F_{Physical}-value while the biological data yields an $F_{\text{Biological}}$-value. These values should be correlated to ensure that both the physical and biological parameters for the cycle design have been met.

These studies should be performed at "worst case" conditions. This may be achieved in a variety of ways including:

- Reducing the exposure set-point temperature
- Reducing the exposure set-point time
- Reducing the F_{o} for load probe-controlled cycles
- Reducing both the exposure time and temperature

For dry heat studies, the heat penetration data generates an F_{H} or F_{P}-value.

21.24.1 Physical Qualification Studies

The term physical qualification studies refers to the studies which include temperature distribution and heat penetration data. This data confirms the uniform

distribution of heat within the sterilizer chamber and that sufficient heat is delivered to the item being sterilized, as evidenced by the F_0, F_H, or F_P-value obtained. In the PDA's Technical Report Number 1, this value is designated $F_{Physical}$ for moist heat sterilization (PDA 2007). Usually these studies are run concurrently, although there are still some companies that choose to run them separately.

It is important that the probes used in these studies are reliable for the temperature range in which they are used and that they meet the requirements for calibration and accuracy. Additionally, the probes should be properly placed in the load. Companies used either random patterns or fixed patterns for probe placement. The distribution probes should not come into contact with other surfaces in the chamber. Penetration probes should be positioned within the item being sterilized at the slowest to heat area of the item. For dry heat studies, the heat penetration probe should be in direct contact with the item being sterilized.

These studies should be performed in the production sterilizers where the load will be sterilized.

Cycle parameters and load configurations could be determined in the cycle development studies and verified in the qualification studies. The minimum lethality established for the cycle design should be met in these studies also. Studies should be performed for both minimum and maximum loading configurations for moist heat sterilizers and for the load configurations used in dry heat sterilizers, unless minimum and maximum loading configurations are used.

21.24.2 Biological Qualification Studies for Moist Heat Sterilization Cycles

Biological qualification of the load is used to refer to the requirements for microbiological challenges which confirm that the lethality in the cycle delivers the microbiological kill designed for the cycle. These studies are typically performed concurrently with the physical qualification studies. They are performed using the "worst case" sterilization cycle, in terms of a minimum sterilization challenge. The studies should be run in the production sterilizers where the product will be routinely sterilized. Defined loading patterns should be used. The cycles should deliver the appropriate $F_{Biological}$ as required in the cycle process design.

21.24.2.1 Challenges for Porous Load Cycles

Traditionally *Geobacillus stearothermophilus* is used as the challenge organism in this type of cycle. The typical cycle design utilizes an overkill approach. Data should be generated using the biological indicators directly inoculated onto the items or carriers may be used. Carriers are available in a variety of formats including paper, wire, stainless steel, aluminum, or other appropriate materials. The biological indicators should be placed in the most difficult-to-sterilize areas of the item. Some examples include within long tubing, areas where it is difficult to achieve air

removal and/or steam penetration, within the pleats of filter cartridges, and so forth. When these studies are performed concurrently with physical qualification, care must be taken to ensure that placement of thermocouples does not impact either the air removal or the steam penetration such that the biological challenge is seeing different conditions than if the probes were not in position (PDA 2007).

Biological challenge studies should be performed for both minimum and maximum load configurations.

21.24.3 Biological Challenges for Dry Heat Sterilization

Historically, spores of *Bacillus subtilis* are used as the biological challenge in dry heat sterilization studies. The *D*-values for dry heat sterilization are important, as they are with moist heat sterilization. Studies are typically performed concurrently with heat penetration and temperature distribution studies.

21.24.4 Biological Challenges for Dry Heat Depyrogenation (and Sterilization)

For this type of study the biological indicator is replaced with an endotoxin challenge unit. The endotoxin used should be obtained from a recognized source, who is licensed (if required in your region). A sufficient challenge level should be used to be able to demonstrate a minimum three-log reduction of endotoxin.

21.24.5 Maintenance of Sterility

Once an item is sterilized it should maintain its “sterile” condition until it is used. For example, a stoppered vial should stay sterile throughout the shelf-life of the product. Data should be generated to show that the item sterilized maintains its sterility through the sterile use-life. Methods for assessing the integrity of pharmaceutical packaging are included in PDA’s Technical Report Number 27: Pharmaceutical Packaging Integrity (PDA 1998).

21.25 Evaluations of Equivalence

Validation studies are performed in large numbers at most facilities. It is valuable to minimize the number of additional studies that must be performed. Each study requires both resources and financial cost. The bigger concern in most cases is the delay while waiting for the study to be performed. Thus, there is a desire to determine

if the times can be reduced by determining equivalence to existing processes eliminating or reducing the time required for another validation study.

21.25.1 Sterilizer Equivalence

Equivalence can be addressed with different types of evaluations. One consideration is the ability to use sterilization data from one sterilizer in the qualification of another sterilizer. While this has been accepted by some regulatory bodies, there is an expectation that the sterilizers use the same sterilization process, have the same physical size, construction, and basic operation. It is expected that these sterilizers should be in the same general location, e.g., the same plant although maybe not in direct proximity of each other. In this scenario, each sterilizer would be tested to ensure that the operation of the sterilizer is within the expected norms prior to utilizing data from another sterilizer for qualification (Agalloco 2008).

This evaluation can be made for both moist and dry heat sterilization applications.

21.25.2 Equivalence of Different Containers/Fill Volumes

Evaluating minor differences in container dimensions and/or in the fill volumes within a container can be accomplished by performing side-by-side statistical evaluation of the heat penetration in both containers (or fill volumes). A test like a Student *T*-test may be used for this evaluation.

This evaluation can be made for both moist and dry heat sterilization applications.

21.25.3 Equivalence of Different Load Configurations

When evaluating changes to the load configurations for moist heat sterilization of porous loads or hard good loads, the heat penetration data can be compared between the two load configurations in both the minimum and maximum loading arrangement. This evaluation may include a statistical evaluation of the data also.

Care should be taken in evaluating equivalence of load configurations in dry heat sterilizers due to concerns with the heat penetration in the load.

21.26 Personnel Training

Sterilizers are expensive and complex pieces of equipment. It is important that the individuals operating this equipment be thoroughly trained. Some companies have established certification programs for sterilizer operators.

21.27 Documentation Requirements, e.g., SOPs, Protocols

All of the activities surrounding the validation of sterilizers should be thoroughly documented. This may be in the form of protocols or SOPs that govern the process.

21.28 Requirements Traceability Matrix

Once the various requirements for the system have been established in the URS and/or FDS, it is important to assess whether all of the requirements have been successfully met by the system during the validation process. A RTM is a document that provides traceability between the requirements established and the location of the testing that has been established to ensure that the requirement is met. This document is very useful in assessing whether the validation documents generated are appropriate to ensure that the system meets the established requirements.

This document is considered a living document that is updated and revised throughout the validation process.

21.29 Summary Report

Final reports should be generated and approved at the completion of each phase of the validation testing. This report should be accurate, complete, and approved by those organizations that approved the protocol.

21.30 Ongoing Activities

Once validated, it is important to maintain the sterilizer within its qualified parameters. As such, it is important to monitor the sterilizer's performance. Some of the evaluations should include review of the cycle parameters during routine use, evaluation of deviations that occur, review of maintenance activities, and change control. Should a significant number of deviations occur, one must consider whether there are additional parameters that should be added for evaluation in routine qualification to better control the sterilizer's performance. Re-qualification should be conducted on a periodic basis, as well as when deemed necessary by maintenance or other activities.

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