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# Characterization of Therapeutic Proteins

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**Abstract** Therapeutic proteins are large biological molecules with complex structures and functions produced through complex manufacturing processes, which include multiple unit operations with finely tuned control parameters. The characterization of therapeutic protein products during development, manufacturing and at product release requires the development and qualification of appropriate analytical methods that measure physicochemical properties and biological activities. Analytical testing during product development forms the basis for identifying the critical quality attributes for the protein therapeutic product, establishing release and stability specifications, and developing an analytical comparability program that ensures safety and efficacy throughout the product life cycle. In this chapter we discuss analytical characterization in the context of the regulation of therapeutic proteins. We focus on polyclonal immune globulins, proteins for hemostasis, monoclonal antibodies, and other therapeutic proteins and emphasize the

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commonalities and also highlight differences in the application of laws, regulations, and guidance.

**Keywords** Analytical characterization, Biologics, Therapeutic proteins

**Contents**



# 1 Introduction

Therapeutic proteins are large molecules with complex structure and function. They comprise a sequence of amino acids folded into complex secondary, tertiary, and, sometimes, quaternary structures. They may be further modified either naturally by attachment of other molecular entities such as glycans or by designing biological or nonbiological modifiers such as polyethylene glycol molecules to extend product in vivo half-life or reduce immunogenicity. Furthermore, therapeutic proteins are often composed of a series of structural domains, which may be linked to separate functions directing interactions with any number of other molecules in the environment in order to confer their therapeutic benefit, including the duration of the activity in vivo. It follows, therefore, that molecular characterization of a therapeutic protein requires the development and application of a larger number of suitable analytical methods that measure an extended catalog of physicochemical properties as well as the protein's biological and, in certain instances, immunochemical activities. By comparison, small-molecule drugs often lack this structural and functional complexity (Fig. 1). Their small size and somewhat simpler three-dimensional structures make small-molecule drugs amenable to complete characterization by physicochemical methods, including absolute structural determination by X-ray diffraction.

The manufacture of therapeutic proteins is also complex. Therapeutic proteins may be purified from complex biological starting materials or may be produced in living cells through biotechnology. Starting materials contain the target protein

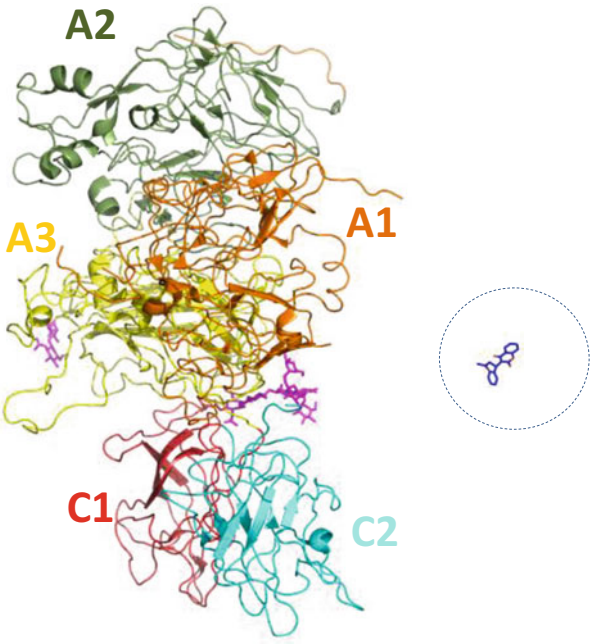


**Fig. 1** Three-dimensional structures of the active ingredients in two different drugs that affect coagulation, drawn to scale. Warfarin (Coumadin) is shown in stick representation in *dark blue* and is *circled* for emphasis. Coagulation Factor VIII is shown in cartoon representation with different domains *colored* and *marked*; glycans are shown in stick representation in *magenta*. Created with PyMOL using coordinates from DOI: 10.2210/pdb2bxd/pdb and DOI: 10.2210/pdb2r7e/pdb for Warfarin and Factor VIII, respectively

among a heterogeneous mixture of endogenous proteins, added chemicals or reagents, and potential microbial contaminants. Purification of therapeutic proteins, thus, requires multiple unit operations with finely tuned control parameters. Certain processes such as elution from an affinity resin or viral inactivation expose the protein to harsh conditions. Therefore, analytical testing schemes must be designed to ensure retention of the protein's molecular integrity and native conformation as well as demonstrate the removal of critical impurities throughout the manufacturing process. Finally, since modifications to a commercial manufacturing process are likely to occur throughout a product's lifetime, analytical testing plans must implement methods suitably sensitive to detect any consequent negative impact on product quality or safety.

The regulation of therapeutic proteins as biological products aims to ensure that new protein therapeutics entering the market are proven to be safe and efficacious and consistently manufactured to a high-quality standard and that they remain so for the entire life cycle of the product.

## 2 Regulatory Framework for Ensuring Quality Throughout the Product Life Cycle

Therapeutic proteins are regulated in the USA as biological products. The regulatory requirement for biological product quality is defined in statute and expanded in the regulations under Title 21 of the Code of Federal Regulations (21 CFR). The Public Health Service Act of 1944 (42 U.S.C.262) requires that biological products introduced into the market be licensed based on a demonstration that the product is safe, pure, and potent. Biological products are also subject to applicable laws in the Federal Food, Drug, and Cosmetic Act, which prohibits the marketing of adulterated drugs, including those not manufactured under current Good Manufacturing Practice (cGMP). Regulations governing cGMP for finished pharmaceuticals are further codified under 21 CFR part 211 (§211). To ensure drug product conformance to a consistent quality standard, cGMP regulations under §211.165 and General Biological Products Standards under §610 require release testing of every lot for specified quality attributes, which must include tests for identity, purity, potency, and sterility. In-process testing is further required to ensure batch uniformity and integrity of drug products (§211.110). Finally, to ensure that product quality attributes remain within appropriate limits throughout the product shelf life, §211.166 requires stability testing, identifying the appropriate storage conditions and establishing product expiration. Regulations under §211.165 also require that analytical methods used for release testing be validated for accuracy, sensitivity, specificity, and reproducibility.

Robust biopharmaceutical development programs that incorporate modern principles of Quality by Design (QbD) promote product quality throughout the product life cycle. QbD is the systematic approach to pharmaceutical development that begins with predefined objectives, emphasizes product and process understanding and control, and is based on sound science and quality risk management. International Conference on Harmonisation (ICH) Q8, Q9, Q10, and Q11 guidelines [1–4] describe enhanced approaches to product and process development based on QbD principles. The definition of a quality target product profile (QTPP) at product inception focuses product characterization and process design on meeting patient needs and clinical performance requirements at each phase of the product life cycle. Integrating quality risk management with comprehensive analytical characterization using state-of-the-art methods allows a confident definition of a product's critical quality attributes upon which the design of the manufacturing process can be based. Knowledge management of analytical data from product and process development is leveraged to define an appropriate control strategy, support process validation at the licensing stage, and enable continuous process verification post-approval.

In this chapter, we will discuss analytical characterization and testing paradigms in the context of the regulation of therapeutic proteins, focusing on recombinant therapeutics, monoclonal antibodies, and plasma-derived proteins. The experience of several regulating divisions will be discussed, emphasizing the commonalities but also highlighting differences in the application of laws, regulations, and guidance based on product class characteristics and regulatory experience. Monoclonal

antibodies and related products, most Fc-fusion proteins, and recombinant protein therapeutics are regulated by the CDER/OPQ/OBP,[1] while plasma-derived proteins and their recombinant analogues are regulated by CBER/OBRR.[2]

## 3 Analytical Characterization to Support Biopharmaceutical Development and Life Cycle Management

Analytical characterization serves three main purposes in biopharmaceutical development and regulatory approval: (1) definition of critical quality attributes, (2) establishment of release and stability specifications, and (3) development of an analytical comparability program. Analytical testing should be included in any regulatory application, and depending on the phase of development can be extensive. At the time of approval, there should be a history of all lots tested including purpose and summary results. Ideally, analytical characterization will also include results from stress stability studies. General strategies for generating adequate analytical data are described in ICH guidelines such as Q6B (specifications) [5], Q5E (comparability) [6], Q1A-Q1E [7–11], and Q5C (stability) [12]. Characterization includes determination of physicochemical, biological, and immunological properties and the impurity profiles. To support the advancements in the analytical field and selection of the best methods for particular purpose, guidelines do not recommend specific analytical technologies, but focus on the type of quality information to be collected. To ensure sufficient characterization and due to method limitations (e.g., limited resolution and sensitivity), the use of orthogonal techniques based on different physicochemical or biological principles may be recommended. Analytical methods used for final container testing should be validated, while methods used for characterization should be qualified (ICH Q6B [5], ICH Q2 [13]).

### *3.1 Critical Quality Attributes*

According to the ICH Q8(R2) [2] guideline, a critical quality attribute is "a physical, chemical, biological or microbiological property or characteristic that should be within an appropriate limit, range or distribution to ensure the desired product quality." Each unique product development program directs the

[1] Center for Drug Evaluation and Research/Office of Pharmaceutical Quality/Office of Biotechnology Products

[2] Center for Biologics Evaluation and Research/Office of Blood Research and Review

characterization of product-specific attributes within the regulatory framework of ensuring identity, quality, purity, potency, and safety.

### 3.1.1 Identity

As required by 21 CFR §610.14, "The identity test shall be specific for each product in a manner that will adequately identify it as the product designated on final container and package labels and circulars, and distinguish it from any other product being processed in the same laboratory."

### 3.1.2 Quality

The quality of a product is a complex property that includes characteristics of the biotherapeutic molecule, excipients, and solution properties. Molecular integrity of the active ingredient is ensured through extensive physicochemical characterization of primary structure, posttranslational modifications, higher-order structure, and biological tests, which provide evidence of the active conformation of the protein. General product quality tests may include pH, osmolality, appearance, color, or clarity of the reconstituted powder or liquid drug product. Robust quality systems and manufacture under cGMP provide continuous assurance that specified product quality is maintained throughout a product's shelf life.

### 3.1.3 Purity

According to 21 CFR §600.3(r), "*Purity* means relative freedom from extraneous matter in the finished product, whether or not harmful to the recipient or deleterious to the product." Impurities in a therapeutic protein starting material should be removed to the extent possible, and the remaining impurities should be defined and controlled to specified, justified limits. Impurities can be defined as product related or process related. Product-related impurities include molecular variants and degradants, such as aggregates, glycoform variants, or other degradation products of the active ingredient. Examples of process-related impurities include host-cell impurities or culture additives from cell cultivation processes, reaction by-products, leachates from resins used for chromatography purification, solvent and detergent used for viral inactivation, or components from buffers added to reach the desired pH or ionic strength during purification. Characterizing impurities to the greatest possible extent enables a manufacturer to design, validate, and monitor a purification process to ensure continued product purity and safety. Specific recommendations for mitigation immunogenicity risk related to the presence of impurities can be found in FDA Guidance for Industry: Immunogenicity Assessment for Therapeutic Protein Products (August 2014 [14]).

### 3.1.4 Potency

A suitable potency assay is crucial to manufacturing control and clinical use of protein therapeutics and is required by statute and regulation. According to 21 CFR §600.3(s), "The word *potency* is interpreted to mean the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result." Potency assays may be in vitro or in vivo tests, which provide a *quantitative* measure of the product attribute linked to its primary mechanism of action.

The ICH Q6B guideline [5] defines three categories of potency assays: (1) in vivo animal-based, (2) in vitro cell-based, or (3) biochemical assays using purified systems. The potency, which is defined in units of activity that are product specific, is determined for each product by comparing it to a suitably characterized, product-specific reference standard, which can be international, national, or in-house.

The use of a voluntary consensus standard such as an international standard or a regional standard as a primary standard is recommended whenever such standards are available. These standards are established in collaborative studies based on the need, e.g., when several products in a class are present on the market. Various CBER, WHO, and NIBSC standards have been established and are available for use by biologics manufacturers. It is recommended that in-house potency reference standards (secondary standards) be established using well-characterized product lots, whose potency assignments are traceable to the consensus standard. Reference standards should be established early in development and updated to reflect changes in manufacturing process. The potency assay should be validated as suitable by the time of pivotal trials.

At present, no international reference standards are available for monoclonal antibodies and most other therapeutic proteins. In such cases, ICH Q6B [5] recommends that an in-house primary reference standard be prepared from materials representative of production and clinical materials. The potency of the product (measured in potency assays) should be compared to data from the in-house reference standard and reported in "in-house units." As product evolves during process development, so do the in-house reference standards, so care should be taken to qualify the new standard against the previous iteration. It is crucial that criteria for potency have a narrow acceptable range to ensure control over product drift. By the time of a BLA submission, a two-tier system should be implemented where the primary reference standard should be representative of the materials used in the pivotal clinical studies and that a working reference standard be appropriately qualified against the primary reference standard.

## *3.2 Safety*

### 3.2.1 Adventitious Agent Safety

For all biologics, orthogonal procedures aimed at drastically reducing the risk of pathogen contamination of the product are in place. Final products intended for parenteral administration are required to be sterile (21 CFR §610.12). Endotoxin levels should also meet USP limits specific for the route of administration (USP < 85>). For parenteral products, the limit is no more than 5 EU/kg/h. For emerging pathogenic agents, such as prions associated with TSE, guidelines have been developed that address the novel concerns with these agents [15].

To ensure that products are free of adventitious viruses, manufacturers of licensed protein therapeutics implement the viral safety tripod recommended in the ICH Q5A guideline: (1) careful selection and extensive characterization of starting materials, other raw materials, and excipients, (2) viral clearance validation of targeted steps in the manufacturing process, and (3) in-process testing for adventitious viruses, as applicable.

For plasma protein therapeutics, there is a real potential for viral contamination of plasma material. Therefore, a multilayered approach to blood donation safety is implemented at each blood establishment to enhance viral safety. Overlapping safeguards include donor screening, donor deferral, infectious disease testing, quarantine of donated plasma until tested and demonstrated to be free of infectious agents, and compliance with cGMP for deviation investigation and corrective and preventive action. Various guidelines have been published by the agency to ensure implementation of this system.[3] For products that are made through biotechnology, viral safety is enhanced by establishment of a highly controlled, tiered cell banking system and extensive testing of each cell bank for relevant endogenous or adventitious viruses.

For mAbs and other products that are amenable to platform manufacturing processes, generic and modular virus clearance studies may be performed. A "generic" clearance study is one in which virus removal and inactivation is demonstrated for several steps in the purification process of a model antibody. These data may then be extrapolated to other antibodies following the same purification and virus removal or inactivation scheme as the model antibody. A modular clearance study is one that demonstrates virus removal or inactivation of individual steps during the purification process (column chromatography, filtration, pasteurization, solvent/detergent, low pH, etc.). Each module in the purification scheme may be studied independently of the other modules. Different model mAbs may be used to demonstrate viral clearance in different modules, if necessary. If the purification process of a product mAb differs at any of the virus removal or

[3] For FDA guidance documents for blood and plasma products, refer to http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/default.htm

inactivation modules from the model mAb, this module must be studied independently from the model. The other, identical modules in the procedure may be extrapolated to the product mAb.

For therapeutic proteins not manufactured using a platform technology, a clearance study demonstrating the effectiveness of viral removal or inactivation for select unit operations in the purification process is necessary to validate the viral inactivation capability of the process. The conditions and operating parameters, including column dimensions, buffer, temperature, time, etc., in the study should closely mimic the worst-case scenario of the manufacturing scale production condition [16].

### 3.2.2 Immunogenicity

While biologics provide remarkable therapeutic benefits, they can also trigger the development of anti-drug antibodies (ADA) that could result in loss of efficacy or adverse events. The immune responses may vary in clinical relevance, ranging from antibody responses with no apparent clinical manifestations to life-threatening and catastrophic reactions [14, 17]. The immunogenicity risk of a therapeutic protein should therefore be considered at the earliest stage and reevaluated at each stage of product development. The evaluation should consider factors such as product origin and characteristics, the immune responses of concern, the target disease indication, and the proposed patient population. Although certain quality attributes of biologics have been related to immunogenicity [17], the ability of a molecule to elicit an immune response cannot be reliably predicted by physicochemical characterization or by studies using animal models. Immunogenicity of a protein product is therefore assessed during clinical trials using binding and neutralizing ADA assays to evaluate the production of ADAs. In most cases, binding and neutralizing ADA results obtained with validated assays are required to support licensure. For high-risk products, such as protein products of nonredundant endogenous cytokines, critical enzymes, and growth factors, implementation of preliminary validated assays in early trials may be necessary to obtain real-time data and to minimize the risk of neutralization of the endogenous protein counterpart and subsequent loss of its physiological function [18].

## *3.3 Specifications*

The ICH Q6B [5] guideline defines a specification as, “a list of tests, references to analytical procedures and appropriate acceptance criteria, which are numerical limits, ranges or other criteria for the tests described.” Specifications are a subset of product quality attributes chosen to confirm product quality as it relates to safety and efficacy. Tests and acceptance criteria should be based on levels shown to be safe and effective in clinical trials supporting licensure, in the context of

manufacturing process capabilities. Analytical methods used to measure specifications should be validated. The approach to setting specifications may be different for each product class and should be justified in the BLA.

## *3.4 Stability*

Manufacturers should demonstrate acceptable product quality not only at the time of product release but also throughout its shelf life (21 CFR §211.137). The quality of the product changes with time and is affected by multiple factors including temperature, humidity, and light. Interactions of the product with the container closure system and resulting from stress occurring, for example, on the liquid/air interphase may affect the molecular integrity of the product. Stability testing is, thus, performed to establish adequate storage conditions and shelf life and is performed in the same container closure system in which the product is marketed (21 CFR §211.166). Changes to the primary, secondary, and higher-order structure during degradation processes may not only reduce the amount of active product but also affect product safety by increasing the risk of the immune response.

Relevant tests demonstrating stability should be selected, and they usually include characterizing appearance, protein concentration, activity, molecular integrity (fragmentation, aggregation), quantification of particulate matter, pH, water content for lyophilized products, pyrogenicity and/or bacterial endotoxin testing, and sterility. Stability testing starts during product development and upon approval of a BLA is performed annually to ensure that product characteristics remain within specifications. Enhanced stability testing is performed when manufacturing changes are introduced. Product stability profile is characterized under the normal storage conditions to establish the product shelf life and also at a higher temperature (s). Even with extensive characterization, changes to the complex product characteristics, which include the composition of the product and its physicochemical properties, may go unnoticed. Degradation profiles observed under accelerated or forced degradation conditions help early identification of changes that may potentially impact safety and efficacy during the product shelf life. Performing stability studies at elevated temperature(s) may help identify changes that affect product stability and to establish comparability of the product before and after manufacturing change.

## *3.5 Comparability*

Extensive characterization that is performed during product development helps ensure that products maintain their quality, safety, and efficacy throughout life cycle. The properties of the product used in clinical trials serve as a critical comparator, i.e., the quality of the product after change has to be comparable or

better than of the product tested in pivotal clinical trials. Product comparability pre- and post-manufacturing change is established based on the analysis of data collected from product characterization, routine batch analyses, in-process control, process validation and evaluation, and product stability (ICH Q5E, [6]). Data collected after change are compared to predefined criteria established based on quality information accumulated before the change is made. For certain applications and when prospectively established, successful completion of such comparability protocols can impact regulatory pathway (and review timelines), for example, enabling a sponsor to file what would be a prior approval supplement (PAS) as a "changes being effected in 30 days" (CBE30) supplement.

## 4 The Development and Characterization of Different Classes of Protein Biotherapeutics

Below we describe approaches to analytical characterization for four different classes of proteins: (1) polyclonal immune globulins, (2) coagulation and hemostasis proteins, (3) monoclonal antibodies, and (4) other therapeutic proteins. Chronologically, these products span the entire history of the FDA ranging from the first human protein (an immune globulin preparation used for the prevention of measles) approved in 1934 [19] to the most recent recombinant proteins made in large quantities in bioreactors. As scientific knowledge and clinical experience with these products have progressed, so have the analytical methods used to characterize and monitor their quality attributes during development, manufacturing, and at release. cGMP manufacturing practices and regulatory expectations are that all products, even those first approved decades ago, be characterized to the extent possible using current analytical methods. Appropriate studies are used to bridge existing product information, including clinical safety and efficacy information with current analytical methodology and manufacturing practices. Thus, it follows that analytical methods in general are common for all product classes discussed here. What is different, as it will become apparent in the following sections, are the experiences in the product-specific regulatory divisions of FDA. These experiences, their relation to product mode of action, its manufacturing process, and the history of clinical use safety and efficacy have flavored the analytical emphasis in each division and provide the basis for the fit-for-purpose approach to characterizing therapeutic proteins described here.

## *4.1 Plasma-Derived Immunoglobulins*

Polyclonal immune globulin products are biological therapies comprised of human or animal plasma or serum-derived products.[4] At present, there are 38 such products approved by the US FDA, manufactured by 15 different firms. Of these, DigiFab® and CroFab® contain the Fab part of the immunoglobulin G (IgG) molecule, whereas the active ingredient in Anascorp® and Botulism Antitoxin Heptavalent is the $(Fab')_2$. The remaining approved products are intact antibodies (Table 1).

### 4.1.1 Quality Polyclonal IgG Products Start with Quality Plasma

Plasma used for making human polyclonal IgG products is collected in licensed and inspected plasma or blood donation centers. The quality of the starting material is essential for the ultimate safety and quality of the end products. Thus, to ensure that donated plasma is safe from hazards to human health and to minimize the likelihood of transmitting infectious disease, multiple measures are in place. These include screening of donors by questionnaire, instituting good collection practices in the donation centers, and testing of donated plasma for blood-transmitted pathogens. Many of these mandatory practices are codified in appropriate subparts of 21 CFR, Part 640, and more specific details are set out in FDA and international guidelines [20–23]. Other safeguards aimed at lowering potential risk for contamination of US products with transmissible spongiform encephalopathies (TSEs), such as variant Creutzfeldt-Jakob disease (vCJD), are specified in a recent FDA guidance document [15]. Additional processes are incorporated at the IgG product manufacturing site to ensure that plasma pools collected in the US licensed donation centers (including all manufacturing intermediates and final products) are segregated from those not originating from centers not licensed by the FDA.

Polyclonal IgG biologics are often categorized as either normal or specific. The only difference between the two product categories lies in the specificity of the polyclonal antibodies they contain. Specific products have high titers of antibodies directed against particular pathogenic agents, such as viruses (e.g., hepatitis B, rabies, varicella, and vaccinia) or bacterial toxins (e.g., tetanus toxoid, botulinum toxin). Antilymphocyte and antithymocyte polyclonals are used to suppress the adaptive immune system in transplant settings. For specific IgG products sourced from human plasma, donors often receive vaccinations and are tested to ensure that their serum contains sufficient amounts of the specific antibodies of interest. Animal plasma is obtained from healthy animals that have high titers of desired antibodies achieved through immunization and boosting with the specific antigen(s). Animals are almost always used to make products directed against toxins, venoms,

[4] Regulatory oversight for these products is the responsibility of the Laboratory of Plasma Derivatives, Division of Hematology Research and Review in the Office of Blood Research and Review.

**Table 1** IgG products currently marketed in the USA

| | Product name | Manufacturer/sponsor | Proprietary name |
|---|---|---|---|
| *Normal IgG* | Immune globulin intravenous (human), 10% liquid | Biotest Pharmaceuticals Corporation | Bivigam® |
| | Immune globulin intravenous (human) | CSL Behring AG | Carimune® NF, Nanofiltered |
| | Immune globulin intravenous (human) | Instituto Grifols, S.A. | Flebogamma® 5% DIF |
| | Immune globulin intravenous (human) | Instituto Grifols, S.A. | Flebogamma® 10% DIF |
| | Immune globulin (human) | Grifols Therapeutics Inc. | GamaSTAN® S/D |
| | Immune globulin infusion (human), 10% | Baxalta (formerly Baxter Healthcare Corporation) | Gammagard Liquid® |
| | Immune globulin intravenous (human) | Baxalta (formerly Baxter Healthcare Corporation) | Gammagard® S/D |
| | Immune globulin intravenous (human), 5% liquid | Bio Products Laboratory Limited | Gammaplex® |
| | Immune globulin injection (human), 10% caprylate/chromatography purified | Grifols Therapeutics Inc. | Gamunex® C |
| | Immune globulin injection (human), 10% | Grifols Therapeutics Inc./Kedrion | Gamaked® |
| | Immune globulin subcutaneous (human), 20% liquid | CSL Behring AG | Hizentra® |
| | Immune globulin infusion 10% (human) with recombinant human hyaluronidase | Baxalta (formerly Baxter Healthcare Corporation) | HyQvia® |
| | Immune globulin intravenous (Human) 5% liquid | Octapharma Pharmazeutika Produktionsgesellschaft m.b.H. (Vienna) and Octapharma AB (Sweden) | Octagam® 5% liquid preparation |
| | Immune globulin intravenous (human) 10% liquid | Octapharma Pharmazeutika Produktionsgesellschaft m.b.H. (Vienna) and Octapharma AB (Sweden) | Octagam® 10% liquid preparation |
| | Immune globulin intravenous (human), 10% liquid | CSL Behring AG | Privigen® |
| *Specific IgG* | Centruroides (scorpion) immune $F(ab')_2$ (equine) injection | Rare Disease Therapeutics Inc. (RDT) | Anascorp® |
| | Anthrax IGIV | Emergent BioSolutions | Anthrasil® |
| | Antivenin (*Latrodectus mactans*) (black widow spider), equine origin | Merck | Antivenin |
| | Botulism immune globulin intravenous (human) | California Department of Public Health (CDPH) | BabyBIG® |

(continued)

**Table 1** (continued)

| | Product name | Manufacturer/sponsor | Proprietary name |
|---|---|---|---|
| | Botulism Antitoxin Heptavalent (A, B, C, D, E, F, G) (equine) | Emergent BioSolutions | BAT® |
| | Antivenin (*Micrurus fulvius*) (North American coral snake), equine origin | Pfizer | Wyeth® Antivenin |
| | Crotalidae [pit viper] polyvalent immune fab (ovine) | BTG International Inc. | CroFab® |
| | Cytomegalovirus immune globulin intravenous (human) (CMV-IGIV) | CSL Behring AG | Cytogam® |
| | Digoxin immune fab (ovine) | BTG International Inc. | DigiFab® |
| | Hepatitis B immune globulin (human) | Biotest Pharmaceuticals Corporation | Nabi-HB® |
| | Hepatitis B immune globulin (human) | Emergent BioSolutions | HepaGam B® |
| | Hepatitis B immune globulin (Human) | Grifols Therapeutics Inc. | HyperHEP® B S/D |
| | Rabies immune globulin (human) | Grifols Therapeutics Inc. | HyperRAB® S/D |
| | $Rh_o$(D) immune globulin (human) | Grifols Therapeutics Inc. | HyperRHO® S/D (Full Dose) |
| | | | HyperRHO® S/D (Mini-Dose) |
| | Tetanus immune globulin (human) | Grifols Therapeutics Inc. | HyperTET® S/D |
| | Rabies immune globulin (human) USP, heat treated | Sanofi Pasteur SA | Imogam® Rabies HT |
| | Rho(D) immune globulin (human) | Kedrion Biopharma Inc. | RhoGam® Ultra-Filtered PLUS |
| | | | MICRhoGAM® Ultra-Filtered PLUS |
| | $Rh_o$(D) immune globulin intravenous (human) | CSL Behring AG | Rhophylac® |
| | Varicella zoster immune globulin (human) | Emergent BioSolutions | VARIZIG® |
| | Vaccinia immune globulin intravenous (human) | Emergent BioSolutions | VIGIV |
| | $Rh_o$ (D) immune globulin intravenous (human) | Emergent BioSolutions | WinRho® SDF |
| *Antithymocyte IgG* | Equine thymocyte immune globulin injection, solution | Pfizer | Atgam® |
| | Antithymocyte globulin (rabbit) | Genzyme Polyclonals S.A.S. | Thymoglobulin® |

or other highly potent antigens which may not be safe for immunization of human donors. By contrast, normal immunoglobulins represent a diverse antibody repertoire and are made from pooled human plasma from donors not receiving any specifically targeted vaccinations. As specified in 21 CFR, §640.102(d), each lot of normal human IgG represents a pool of material obtained from not less than 1,000 donors. In manufacturing practice, many more individual donations are frequently pooled to manufacture large batches of IgG. It is recommended that the size of plasma pool be limited to no more than 60,000 donations [24–26].

### 4.1.2 Application of Good Practices in Polyclonal IgG Product Manufacture

Manufacturing processes and analytical techniques used for isolating and evaluating pure and potent immune products obtained from human and animal plasma have evolved considerably in recent years. While the impetus on the manufacturers' side has been to increase yield to satisfy rising demand, the focus on the regulatory side has remained on ensuring safety while fulfilling the public health need for these products. Presently there are several general purification schemes used for licensed products; details on these can be found in recent reviews [27–30]. Many of them are derived from the original methods discovered and refined by Cohn and his collaborators [31–33] representing a series of finely tuned and closely controlled manufacturing steps to ensure the quality attributes of marketed products. The need for strict controls of the manufacturing process parameters cannot be overemphasized, given that seemingly minor changes or deviations can alter product characteristics, sometimes resulting in unanticipated and severe adverse outcomes [34–36].

As for all protein biologics, a series of analytical tests designed to monitor product quality and quantity are performed during the manufacturing process of IgG products. As for other protein therapeutics, these in-process tests, implemented at different key manufacturing steps, provide the data for the historical database that will be accumulated during the lifetime of the IgG product. This database is used to ensure the process is performing according to expectations and to establish comparability, discussed in detail earlier in this chapter. Many of the in-process testing methods used in manufacturing IgG products are validated. Those that are not should meet the bar of being scientifically sound and reflect current laboratory standards and methodology.

### 4.1.3 Parameters Measured to Ensure Polyclonal IgG Product Quality

The analytical measurements used to characterize polyclonal IgG preparations at release can roughly be divided into the following categories: those that measure (1) active ingredient indicative parameters, (2) impurities, (3) final product solution properties, (4) excipients, and (5) parameters related to viral safety.

### Active Ingredient Indicative Parameters

For IgG products, key indicative parameters, i.e., those that measure active ingredient quantity, purity, and structural integrity in the final product, typically include total protein content, absolute and relative amount of IgG proteins, molecular size distribution, protein banding pattern, and other IgG biophysical properties. Unlike other protein biologics, there are a few product-specific release tests explicitly required for normal IgG products derived from human plasma. Potency tests for antibodies against measles, diphtheria, and poliomyelitis are codified in 21 CFR §640, Subpart J.[5] When performed by bioassay, these measurements provide information about biological function of the IgG lot at release and throughout its shelf life. Additionally, these parameters are an indicator of the manufacturing process consistency. For these reasons, binding only assays are not, as a general rule, acceptable methods for determining potency. Cell culture or in vivo neutralization assays are the methods accepted by the FDA for measuring these specificities.

The same potency assays are not generally required for specific IgG products, but potency tests measuring the neutralizing activity of the specific IgGs are. Due to methodological limitations, there have been instances when binding assays have been used in lieu of bioassays for some specific IgG products. This has been acceptable in cases, such as hepatitis B-specific IgG products, where the protective target levels of anti-Hbs were well known from epidemiologic or clinical studies [37].

Other antibody specificities can be measured, especially when characterizing a new product or as part of a comparability protocol. Examples include pneumococcal, B19, H. influenza B, or hepatitis A-specific antibodies.

### Impurities

As for all protein biologics, impurities in polyclonal IgG products are categorized as either product related or process related.

Immunoglobulin-related impurities usually characterized in IgG products include IgM, IgE, IgA, or undesirable types or forms of IgG molecules. The latter include IgG aggregates and IgG fragments, which in addition to being measured at product release, also serve as stability indicators. High aggregate levels can cause clinical adverse events [38], whereas fragments measure product integrity during storage. Their increase over time can provide an early indication of pending potency loss. Anticomplementary activity (ACA) is measured as a functional property of IgG aggregates, and high levels are potential indicator of infusion-related adverse events. Antibodies against blood-group antigens A, B, and Rho(D) are

[5] 21 CFR §601.104 specifies that the minimum levels for diphtheria antibodies should be 2 international units per mL, and those for measles and poliomyelitis type 1, type 2, or type 3 should be compared to the levels found in CBER reference.

also measured and controlled in IgG products since high levels of these antibodies are associated with clinically significant hemolysis in recipients of these blood groups [39].

A second category of impurities is other plasma proteins that may co-purify with IgG at clinically significant levels. Several of these entities, i.e., pre-kallikrein activator (PKA) and activated coagulation Factor XI (FXIa), have been implicated in clinical adverse reactions [38, 40] and as such are measured in final release tests and controlled by release specifications. Depending on the manufacturing process, certain products may have additional release specifications to control product-specific protein impurities. Additional product qualities are measured when characterizing a new product or as part of a comparability protocol. Examples include other coagulation factors (fibrinogen, Factor IX, FXII), other plasma proteins (i.e., albumin, fibronectin, apolipoprotein A1, antithrombin III, C1-esterase inhibitor, α2-macroglobulin, transferrin), and lipids.

Process-related impurities include process-specific residues such as solvent or detergent from the viral deactivation steps, leachates from chromatographic column resins, filters as well as their housing, and buffers or other chemicals used at different steps in the process. Examples include polysorbate 80 (PS80, Tween 80), tri(*n*-butyl)phosphate (TnBP), and heavy metals. This class of impurities is controlled by designing a robust purification process that can perform well under "worst-case" conditions. Their levels are monitored by in-process and release tests; action limits and release specifications that ensure these impurities remain within acceptable levels are instituted. If these (or any impurities) are not removed to achieve sufficiently low levels, toxicity assessments and, in some cases, toxicology studies are performed to assess potential risk to patients under "worst-case scenario" conditions. If warranted, stricter process controls or additional mitigation steps are incorporated during the manufacturing to ensure adequate safety margins in clinical use, including for susceptible populations.

### Final Product Solution Properties and Excipients

Final product solution properties often measured for polyclonal IgG products include pH, osmolarity, particulates (visible and subvisible), and appearance. The latter includes color and clarity for liquid products and, for lyophilized products, appearance and reconstitution time for the dried cake.

Polyclonal IgG products are formulated at a very high nominal protein concentration, ranging from 50–200 g/L. To ensure that native, biologically active conformation is preserved, excipients are added as stabilizers in the final formulation of these products. Most of the excipients fall under two classes of chemicals: sugars (i.e., maltose, sucrose, and glucose) or amino acids (i.e., glycine and proline). Both the choice and concentration of the excipient are critical parameters that help limit IgG aggregation and ensure product quality throughout the life cycle. Thus, these properties are measured and controlled at lot release.

Parameters Related to Microbial Growth and Pathogen Safety

Another group of analytical techniques are those used to measure parameters such as sterility and pathogen safety. Some of these concepts (i.e., sterility and viral clearance) are similar among all the different protein products discussed in this chapter and have already been mentioned. Others, such as bio-burden levels at upstream manufacturing steps, are monitored and controlled, and pyrogenic bacterial constituents are measured in final product using rabbit pyrogen test or LAL. Monocyte activation test has emerged as a possible method for measuring pyrogen activity in IgG products. Validation studies should be submitted to support any changes to existing release tests. Other parameters such as the level of antibodies directed against hepatitis B surface antigen (anti-HBsAg) that have been found to be important as markers for the stability of the preparation and provide an additional measure of viral safety are also measured.

### 4.1.4 Emerging Areas in Analytical Measurement Development

With the ever-increasing use of IgG products in auto-immune disorders, especially those with neurological manifestations [41], there is a lot of attention being given from the regulators, industry, and scientific community to defining the unique product attributes which underlie efficacy in these disease states and establishing methods to measure them. There is growing evidence that efficacy of polyclonal IgG products in auto-immune indications is Fc mediated [42]. Thus, discovering accurate and precise methods to measure Fc-receptor binding activity which correlates with effector function(s) has become a priority. An optimal such measure would be a bioassay connecting Fc-receptor binding of the IgG preparation with a biological readout such as activation or inhibition of a functional measurement, for example, cytokine release. The appropriate readout should also be related to the pathophysiology of the disease. Given that the underlying mechanisms of many auto-immune conditions are not only complex but also incompletely understood, picking the "ideal" assay represents a challenge and an area that would benefit from further research.

Other areas of regulatory interest include improving the methodology for measuring IgG aggregates in the nanometer range and developing neutralizing assays for specific IgG products where such assays are not available. For the latter, challenges remain in validating neutralizing assays for several viral agents, such as CMV, HBV, and HCV, and continued research would be beneficial.

## *4.2 Recombinant Therapeutic Proteins for Hemostasis*

Proteins of the hemostatic system span a broad range of sizes, shapes, structures, and quality characteristics, which direct multifaceted interactions. Complex interactions, however, among these widely varying hemostatic proteins drive the

common goal of hemostasis, the system for maintaining vessel patency in response to injury.

### 4.2.1 The Hemostatic System

Hemostasis in response to vessel injury is a complex process involving coordinated interplay among subendothelial matrix components, the endothelial lining, blood cells, and finally the components of the blood coagulation cascade leading to the formation of a fibrin clot (Fig. 2). The physiological balance of procoagulant, anticoagulant, and fibrinolytic activities among the players of the blood coagulation cascade is designed to tailor clot location, size, and stability in accordance with the need at the injury site. The clot-forming cascade is propagated through a series of multicomponent, proteolytic complexes operating on the surfaces of activated cells, such as platelets, white blood cells, and endothelial cells. These cell surface

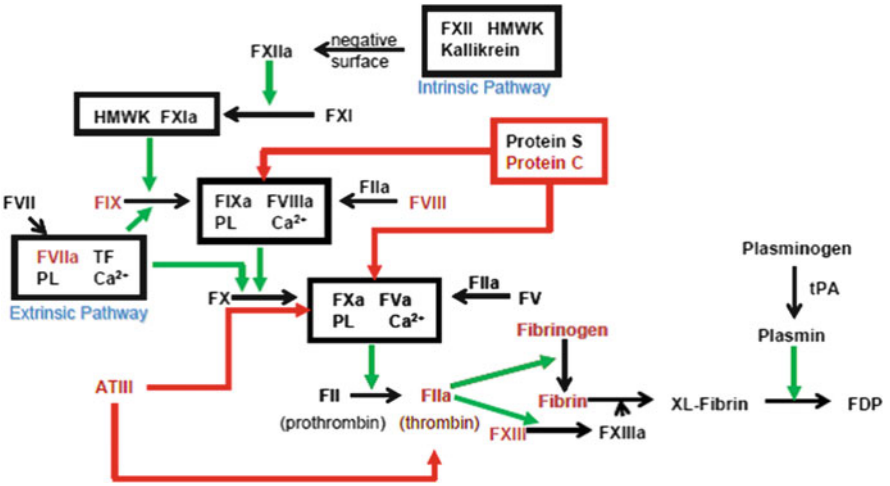


**Fig. 2** The hemostatic system: The ability to form and maintain a blood clot in the right place, at the right time, involves proper, physiological coordination of procoagulant, anticoagulant, and fibrinolytic pathways. As depicted, each pathway executes a tightly controlled cascade of sequential proteolytic activation reactions, operated by specific proteolytic complexes, which are designed to amplify each system while responding to feedback mechanisms. Blood coagulation proteins circulate as inactive zymogens or cofactors designated as "factors" with assigned roman numerals. Inactive zymogens and cofactors are sequentially activated at the site of injury, through the cascade depicted in the figure. As illustrated, extrinsic and intrinsic pathways operate through proteolytic complexes (in *black boxes*) comprising an activated proteolytic enzyme, an activated cofactor, calcium, and phospholipid which serve to activate each zymogen in succession. Progression of the blood coagulation cascade is subject to modulation (indicated through *red arrows*) by the protease inhibitor, antithrombin III, and the anticoagulant complex comprising activated forms of protein C and protein S. Disorders of hemostasis may be treated by replacement therapy with hemostasis protein concentrates purified from plasma or produced through recombinant DNA technology. Currently marketed therapeutic concentrates are indicated in *red*.

assembled complexes accomplish the sequential activation of their serine proteases so that thrombin can be generated locally to convert fibrinogen to an insoluble, cross-linked fibrin clot at the specific site of injury. The importance of physiologically functional hemostasis is evidenced by the occurrence of pathologic bleeding or thrombotic disease caused by its disruption. Inherited disorders of hemostasis may result from a missing or nonfunctional coagulation factor, anticoagulant, or fibrinolytic protein. In addition, acquired bleeding or thrombotic disease may be a consequence of environmental stimuli [43]. The current US market offers a number of licensed, safe, and efficacious plasma protein therapeutics indicated for disorders of hemostasis. Licensed products include purified coagulation factor or anticoagulant protein concentrates, covering many proteins required for proper hemostasis. Hemostasis protein therapeutics may be purified from human or animal plasma or expressed through recombinant DNA technology. Figure 2 indicates in "red" those proteins for which there are currently licensed concentrates. The majority of licensed products are indicated as replacement proteins to correct disorders of hemostasis through on-demand treatment, routine prophylaxis, or perioperative management of bleeding. Thrombin and fibrinogen are components of fibrin sealant products, which mimic the final step in blood coagulation, to stop minor surgical bleeding when standard surgical practices are ineffective or impractical.

### 4.2.2 Analytical Characterization of Recombinant Factor VIII and Factor IX

Successful analytical programs provide conclusive evidence that demonstrates preservation of native protein structure, retention of all functional properties, and control of impurities. Advances in manufacturing and analytical techniques have allowed comparable approaches for complete characterization of the vast array of hemostasis proteins whether purified from plasma or recombinant DNA (rDNA)-derived cell culture. This section will focus on analytical paradigms for two recombinant coagulation factors: Factor VIII and Factor IX. Factor VIII is the missing protein in hemophilia A, likely the first documented bleeding disorder (Talmud, circa 200 CE) [44]. Factor IX is the missing protein in hemophilia B, a bleeding disorder prevalent in European royal families in the nineteenth and twentieth centuries [45]. Although highly divergent in structure and quality characteristics, Factor VIII and Factor IX are connected through their interdependence as components of the proteolytic complex responsible for activating Factor X. Factor VIII and Factor IX products currently occupy the majority of the commercial market for hemostasis protein concentrates. Furthermore, both proteins have been the targets of significant engineering efforts designed to enhance their clinical performance.

## Primary Structure

Coagulation Factor VIII is a large, glycosylated protein, which undergoes multiple, directed proteolytic cleavages in order to create the biologically active molecule. The ~330 kDa protein contains 2,332 amino acids arranged in domain structure: A1–A2–B–A3–C1–C2. For the full-length molecule, several posttranslational, proteolytic cleavages in the B domain produce a series of divalent cation-linked, two-chain molecules. The N-terminal, heavy chain, comprising A1, A2, and cleaved B domains, displays heterogeneity upon analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), exhibiting several bands possessing molecular weights (MW) between 210 kDa and 90 kDa. The light chain comprising A3, C1, and C2 domains has an MW of ~80 kDa [46]. There are four commercially available recombinant full-length Factor VIII concentrates. Thrombin cleavage of Factor VIII followed by subunit analysis using SDS-PAGE or HPLC methods is particularly useful for confirming Factor VIII domain structure. During development of expression systems for recombinant Factor VIII, it was discovered that removing the B domain increased expression and decreased molecular heterogeneity through removal of the majority of glycosylation sites and heavy chain size heterogeneity while retaining in vivo procoagulant function. SDS-PAGE analysis of B domain-deleted Factor VIII yields a simplified banding pattern comprising a single-chain molecule with MW ~170 kDa, a ~90 kDa heavy chain, and an ~80 kDa light chain [47]. Four commercially available Factor VIII concentrates are based on rDNA constructs that delete the B domain.

Factor IX circulates as a single chain, ~56 kDa molecule possessing five structural domains in the following order from amino to carboxyl terminus: Gla-EGF1-EGF2-activation peptide-protease domain. Similar to Factor VIII, directed proteolytic processing and unique posttranslational modifications are required in order to generate the biologically active molecule [48]. Four recombinant Factor IX concentrates are commercially available.

## Posttranslational Modifications Important for Pharmacologic Action

The design of expression systems to produce biologically active recombinant Factor VIII or Factor IX must consider the fidelity of posttranslational proteolytic processing, glycosylation, sulfation, and γ-glutamyl carboxylation, all of which have reported impact on pharmacokinetic or pharmacodynamic properties. Recombinant expression of mature, fully processed Factor IX requires co-expression of a signal peptide-processing enzyme. Critical to the function of Factor IX and other vitamin K-dependent hemostasis proteins is the posttranslational addition of carboxyl groups to specific glutamyl residues contained within the N-terminal (Gla) domain. Factor IX contains 12 sites for γ-carboxylation. A high level of γ-carboxylation must be achieved in order for recombinant Factor IX to properly interact with activated cell surfaces as part of its procoagulant mechanism of action. Anion exchange chromatographic separation followed by peptide mapping

strategies enhanced by sensitive mass spectrometric techniques may be used to accurately quantitate the degree of γ-carboxylation. Tyrosine sulfation has been described for Factor VIII and Factor IX. Particularly important for Factor VIII function are sites on acidic peptides, which direct thrombin activation of Factor VIII. A critical site at Tyrosine1680 mediates Factor VIII binding to von Willebrand factor, a large multimeric protein that stabilizes Factor VIII in circulation [43, 46, 48]. Since heterogeneity in glycosylation gives rise to structural diversity and impacts clinical pharmacology, complete characterization and tight control of protein glycoforms are critical to product quality and manufacturing consistency. Factor VIII and Factor IX proteins produced in nonhuman cell lines have been characterized to contain minimal quantities of *N*-glycolylneuraminic acid (NGNA), a sialic acid substituted for the more common *N*-acetylneuraminic acid (NANA) and galactose-α-1,3-galactose (α-Gal), with recent advances in mass spectrometric techniques allowing more sensitive α-Gal detection [49]. Although no direct adverse clinical consequences have been reported, NGNA and α-Gal levels are tightly controlled in all Factor VIII and Factor IX concentrates.

## Purity and Impurities

Purity is defined as the relative freedom from extraneous matter whether or not harmful to the recipient or deleterious to the product. It is an expectation that impurities in Factor VIII or Factor IX therapeutic concentrates be removed to the extent possible and that the remaining impurities be defined and controlled to specified, justified limits.

### *Product-Related Impurities*

As for other recombinant proteins, Factor VIII and Factor IX concentrates are controlled for aggregates, polymeric forms, truncated forms, fragments, and oxidized molecular variants, as applicable, using peptide mapping, HPLC, and SDS-PAGE methods. Control of aggregates in the final product, most commonly by size-exclusion chromatography (SEC), is particularly critical due to their potential for inducing an immunogenic response. Nonspecific adsorption of proteins on the matrix of SEC columns can confound the analysis and give results that do not accurately represent the aggregate content present in the product. Nonspecific adsorption can be minimized by preconditioning the column through multiple injections of the protein prior to sample analysis, allowing a layer of the protein to coat binding sites on the column matrix. However, such preconditioning may also reduce the pore size of the matrix and change the performance characteristics of the column by reducing peak resolution and separation range. In addition, because of the dynamic equilibrium between the adsorbed protein and the protein in solution, the adsorbed protein may slowly leach into the eluate giving aberrant results. Therefore, orthogonal methods to analyze aggregates, such as dynamic light scattering or analytical ultracentrifugation, are essential in validating an SEC

method for its intended purpose. Factor VIII and Factor IX circulate as inactive precursors until their activation is initiated in vivo, in response to injury. Since premature activation is undesirable, purification procedures and quality control tests must ensure retention of the protein in its unactivated state. Therefore, product-related impurities in Factor VIII or Factor IX products with the greatest potential negative impact to product quality and safety constitute the activated forms. Of particular concern, activated Factor IX (FIXa) impurity has been associated with reports of thromboembolic complications and is routinely characterized and controlled in all Factor IX products [50].

*Process-Related Impurities*

Strategies described in the ICH Q6B guideline [5] for characterization and control of process-related impurities associated with recombinant cell culture systems and downstream purification steps are also routinely applied to characterization and control of recombinant Factor VIII or Factor IX concentrates. It is an expectation that host-cell protein (HCP) be characterized and controlled using an in-house assay specific to the product cell line and validated for adequate detection of all relevant proteins. Two-dimensional SDS-PAGE and immunoblotting techniques have been applied to the development and validation of suitable HCP enzyme-linked immunosorbent assays (ELISA). Affinity chromatography strategies are often employed during Factor VIII or Factor IX purification schemes. Sensitive in-house assays specific for affinity ligands should be developed and validated to control for affinity column leaching. Unique to Factor IX concentrates is the control of the signal peptide processing enzyme impurity.

## Viral Safety

Viral safety is of utmost concern for a class of products with a history of virus transmission. Plasma-derived fibrinogen concentrate, first marketed in 1947, was removed from commercial distribution in 1977 due to transmission of hepatitis [51]. Then came the AIDS crisis in the early 1980s, which deeply impacted the hemophilia community. By the mid-1980s, methods for inactivation of blood-borne viruses had been implemented in manufacturing processes followed by the first recombinant Factor VIII and Factor IX products in the 1990s. Manufacturers of all Factor VIII and Factor IX concentrates are required to validate two orthogonal virus reduction steps, of which one step must be an inactivation method, such as solvent/detergent or heat treatment.

## Biological Activity

ICH Q6B [5] advises that in vitro assessment of biological properties is an essential addition to physicochemical analyses in establishing a therapeutic protein's higher-order structure. For Factor VIII and Factor IX, biological activity is defined by the

potency unit and further characterized to demonstrate conformational fidelity through in vitro functional assays, which measure the full complement of intermolecular interactions responsible for procoagulant activity.

*Potency*

Potency is the quantitative measure of the product attribute linked to its primary mechanism of action. Assaying functional activities of hemostasis proteins in the complex mixture of patient plasma originally led scientists to define an activity unit for each plasma protein, including Factor VIII and Factor IX, as that amount contained in 1 mL of normal human plasma. Wide variations in the normal human population and the lack of agreement among functional assays performed in different laboratories necessitated the development of an international standardization program, which has been in place for over 40 years, is sponsored by the World Health Organization (WHO), and is facilitated by the National Institute for Biological Standards and Control (NIBSC) in the UK. WHO international standards (IS) for Factor VIII and Factor IX concentrates are prepared by NIBSC, are calibrated through international collaborative studies involving recognized experts from industry and regulatory authorities, and are established by the Expert Committee on Biological Standardization of the WHO [52]. WHO IS are intended as primary reference standards against which each manufacturer should calibrate in-house potency reference standards from well-characterized product lots.

Potency of Factor VIII and Factor IX concentrates may be determined by either of two methods: a one-stage clotting assay or a two-stage chromogenic substrate assay. Both methods were designed to represent the in vivo mechanism of procoagulant function by measuring the outcome following assembly and activation of the complex responsible for activating Factor X, termed the "Tenase" complex. As illustrated in Fig. 2 above, the Tenase complex comprises activated Factor VIII (FVIIIa) as the cofactor, activated Factor IX (FIXa) as the proteolytic enzyme, and phospholipid and calcium for proper assembly on an activated cell surface. The clotting assay was designed to mimic the in vivo mechanism of action by using a plasma substrate deficient in Factor VIII or Factor IX (as applicable) and measuring the correction of a prolonged clotting time as a function of added factor concentration. Variability in the clotting method led to the development of a more purified system designed to directly measure enzymatic activation of Factor X as reflected in the cleavage of a chromogenic substrate. Because reported discrepancies between the two assays have ranged from ~10–50%, with the chromogenic substrate assay generally giving higher values for recombinant products, the most challenging aspect of developing a recombinant Factor VIII product has been the decision on which assay, clotting or chromogenic, is most appropriate for product potency labeling. Clinical practice in the USA supports the use of the clotting assay, while the chromogenic assay is mandated for potency labeling of products in Europe [53]. Current development programs should include both assays for product characterization and clinical development with a decision on which assay will ultimately be used for commercial release and stability dependent upon analysis of all development data [54]. Although the clotting assay is currently used for

potency assignment of all licensed Factor IX concentrates, comparative assessment of chromogenic and clotting activities is recommended for complete characterization of recombinant Factor IX products since assay discrepancies have been reported [55].

### *In Vitro Functional Tests*

Many of the in vitro functional tests currently used to assess Factor VIII or Factor IX quality are based on reconstitution of the Tenase complex and monitoring the generation of Factor Xa detected by a chromogenic substrate. By varying component concentrations and experimental conditions and performing kinetic analyses, the affinity of molecular interactions and degree of in vitro procoagulant functionality can be characterized [56]. Factor VIII and Factor IX function also depend on binding to phospholipid, which can be measured by surface plasmon resonance or Factor Xa generation assays. Factor VIII binding to von Willebrand factor (critical for Factor VIII stability in circulation) can also be measured using surface plasmon resonance assays. The activation of Factor VIII or Factor IX is intrinsic to in vivo function. In vitro assays have been developed for evaluating kinetics of activation and subsequent inactivation of Factor VIII by thrombin or Factor Xa. Similar assays have been developed to characterize the kinetics of Factor IX activation by Factor XIa. Activated protein C confers its anticoagulant activity through direct cleavage of Factor VIII; therefore, in vitro assays designed to specifically characterize activated protein C inactivation kinetics for Factor VIII are included in complete in vitro functional characterization strategies [57]. Thrombin generation assays, which measure kinetics of thrombin generation in complex systems from whole blood to defined combinations of purified proteins, are gaining wide use as global assays for evaluating product quality and in vivo performance [58]. In general, the ability of in vitro functional tests to assess product quality relies on comparative testing of the product under development to a licensed comparator. In vitro functional tests are highly valuable in supporting manufacturing changes to licensed products by comparative testing of pre-change to post-change product.

## Immunogenicity

Preservation of native protein conformation is key to maintaining product safety and efficacy. Failure to implement analytical programs to guarantee retention of protein conformation for the duration of a product's shelf life may result in loss of therapeutic benefit or increased risk of immunogenicity. The development of Factor VIII or Factor IX "inhibitors," antibodies against the Factor VIII or Factor IX molecule, remains the major negative clinical consequence of hemophilia treatment. Factor VIII inhibitors develop in ~30% of patients with severe hemophilia A, and Factor IX inhibitors, some with anaphylactoid consequences, develop in ~5% of patients with severe hemophilia B. Despite suggestions that recombinant products may confer higher immunogenic risk, there is no conclusive evidence, to date,

showing a difference between plasma-derived and recombinant product immunogenicity [59]. Reports in the early 1990s of increased Factor VIII inhibitor incidence resulting from Factor VIII products denatured through certain viral inactivation processes highlighted the importance of robust analytical programs designed to demonstrate retention of protein conformation and clinical programs suitably designed to monitor for inhibitor development [60].

#### 4.2.3 New Generation Products

New generation Factor VIII and Factor IX proteins bioengineered for better clinical performance have been licensed, and others are in development. PEGylation, Fc fusion, and albumin fusion strategies have been successfully applied to create Factor VIII or Factor IX analogues with increased circulating half-lives [61]. Development and life cycle management of these novel proteins have warranted the implementation of equally novel and sensitive analytical methods to ensure retention of full functionality without increased risk of immunogenicity. Particularly challenging can be the development and validation of suitable functional assays for quality control of the non-coagulation fusion moieties. The agency encourages manufacturers to qualify novel, product-specific, and sensitive new technologies to enhance product knowledge and understanding, facilitating Quality by Design approaches to life cycle maintenance of product quality.

### *4.3 Monoclonal Antibodies*

#### 4.3.1 Introduction

The first therapeutic mAb, OKT3, was licensed in 1986 for the treatment of acute renal allograft rejection. Although it was an effective treatment, a majority of patients made anti-drug antibodies (ADA) against this murine mAb, which blocked its therapeutic effectiveness [62]. In general, murine mAbs are immunogenic, have a short half-life, and are inefficient at eliciting effector functions in humans [63]. The therapeutic potential of mAbs began to be realized in the late 1990s after the introduction of chimeric and humanized mAbs, which contain human Fc regions. Subsequently, mice engineered to express human antibodies and phage display technology introduced human mAbs as clinical candidates. Since the early 2000s most mAbs entering clinical development are humanized or human. Of the 23 mAbs approved since 2009, 14 are human, 5 are humanized, 3 are chimeric, and 1 is murine.

### 4.3.2 Selecting and Engineering MAbs for Specific Applications

The majority of mAbs in development continue to be intact mAbs, mostly IgG1, but IgG2 and IgG4 mAb are used when the mAb is designed to have little to no effector function. In addition to intact mAbs, the diversity of mAb and related products includes antibody-drug conjugates (small-molecule drugs and radioimmunoconjugates), other types of antibody conjugates (bacterial toxins, enzymes, cytokines), antibody fragments (Fab, sFv, single V domain constructs), bispecific antibodies (full length or based on sFv or single V domain fragments), antibody cocktails, and Fc-fusion proteins. To date, the agency has approved ten Fc-fusion proteins, three antibody-drug conjugates, two therapeutic radioimmunoconjugates, and one bispecific mAb.

Intact antibodies have two functional domains, the Fab region for binding to antigen and the Fc region, which imparts effector function. Knowledge of Fc-glycan structures and amino acid residues in the Fc region that play a role in effector functions, such as complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (ADCP), led to engineering approaches that can enhance or reduce specific effector functions [64–72]. Engineering either the Fab or Fc regions can enhance the pharmacokinetic properties of a mAb [73–76].

Although the IgG3 isotype elicits high levels of effector function, especially CDC, it is not commonly used for therapeutic mAb, because it is susceptible to proteolytic cleavage and has a shorter half-life compared with the other IgG isotypes. However, specific mutations of amino acid residues in the IgG3 hinge region are being used to take advantage of IgG3 effector function properties [77].

### 4.3.3 Common Manufacturing Considerations to Ensure MAb Quality and Consistency

Posttranslational Modifications (PTMs)

Each mAb is subject to posttranslational modifications (PTMs) and degradation over time. One advantage of intact mAbs is that many PTMs in conserved regions and degradation products are well understood and apply to all products of this class. Many of these PTMs and degradation products occur naturally in endogenous IgG [78]. The risks to patient safety of these common PTMs are generally understood. Therefore, the challenge for sponsors is to evaluate the risks of PTMs and degradation products that are unique to each product.

### N-Terminal Pyroglutamic Acid and C-Terminal Lysine

Many mAb PTMs result in charge variants that have the potential to impact stability or biological activity. Two of the most common PTMs resulting in basic charge variants are due to N-terminal glutamine and C-terminal lysine. These amino acid residues are often processed from N-terminal glutamine to pyroglutamic acid (PE) or the C-terminal lysine is cleaved, resulting in more acidic species. These PTMs have no known impact on mAb function or pharmacokinetics (PK) [79, 80].

The conversion of N-terminal glutamine to PE occurs spontaneously and mostly in the bioreactor; however, buffer composition and temperature can also lead to this conversion [81]. N-terminal glutamate can also be converted to pyroglutamic acid but does not result in a charge difference. Therefore, other methods such as LC/MS/MS are needed to characterize this variant.

The conversion to PE by either pathway, as well as C-terminal lysine cleavage, occurs in vivo and reflects normal modifications of both therapeutic mAbs and endogenous IgG [78, 79, 82–84].

C-terminal lysine cleavage occurs rapidly upon administration to patients [85]. As a quality attribute, it can be a predominant source of charge heterogeneity, with 0, 1, or 2 C-terminal lysine residues per molecule. To assess the contribution of C-terminal lysine, as well as other PTMs, to charge heterogeneity, samples can be treated with carboxy peptidase B to remove the C-terminal lysine [84]. A comparison of the charge profile of untreated and treated samples reveals the charge heterogeneity due to the presence of C-terminal lysine or other PTMs.

### Proline Amidation

A recently identified PTM resulting in basic species is the amidation of the penultimate proline residue after C-terminal lysine cleavage [86]. This modification is fairly common among mAbs of different IgG isotypes and is catalyzed by peptidylglycine alpha-amidating monooxygenase in cell substrates after C-terminal lysine cleavage [87]. The reaction is sensitive to levels of copper in the bioreactor [88]. This PTM does not appear to impact biological activity or PK [79, 86].

### Deamidation

Asparagine deamidation leads to aspartic acid or isoaspartate, which are found in acidic species of mAbs [79, 89]. The rates of conversion vary for each asparagine and are influenced by the surrounding sequence. Some conserved asparagine residues in the Fc region of IgG1 and IgG1 can be deamidated, with Asp384, being the most susceptible [90]. Endogenous antibodies are also deamidated at this site [78, 90]. The levels of deamidation at these constant region sites are not known to have an impact on biological function. Deamidation in either heavy chain

or light chain CDRs has also been reported, and the impact on binding to antigen can vary from little to significant impact on biological activity [89–95].

### Oxidation

Oxidation occurs predominantly at methionine residues but can also occur at tryptophan, cysteine, histidine, and cysteine residues. Oxidation of methionine in the heavy chain constant region (Met256 and Met432) can reduce binding to protein A and G [96] and FcγRIIa 131H and FcRn [97, 98]. However, the levels of oxidation that reduce binding to these receptors are generally higher than typical levels due to manufacture and product shelf life and do not have an impact on PK of a mAb when compared with an unmodified IgG [99].

There are examples of oxidized residues in heavy chain or light chain CDRs that decrease binding to the antigen. Oxidized methionine and cysteine in the VH region of OKT3 impact binding to CD3 [91]. Oxidation of tryptophan, but not methionine, in an anti-RSV mAb resulted in loss of activity [100].

### Glycation

Glycation occurs on lysine residues during manufacture or storage when reducing sugars are present in culture media or formulations. Because a mAb generally contains many lysine residues, glycated forms, which are acidic species, can be extremely heterogeneous. Glycation also occurs in vivo on both endogenous antibodies, as well as therapeutic mAbs, and does not appear to affect PK or effector function [79, 101]. For example, mAbs with up to 10 or 17% glycation in a heavy chain CDR [79, 102] or near complete glycation through forced glycation studies [103] did not have reduced biological activity. Forced glycation studies also showed that high levels of glycation (~42%) did not decrease binding to FcγRIIIa, FcRn, or protein A [101].

### Glycosylation

Antibodies have a single N-linked glycosylation site at Asn297 in the Fc CH2 domain. The biantennary glycan structure contains the core heptasaccharide, and additional sugars attached to the core heptasaccharide result in heterogeneous structures. The predominant forms in unengineered cell lines are generally fucosylated, and galactose may or may not be present (G0, G1, and G2). Terminal sialic acid; afucosylated G0, G1, and G2; and high-mannose forms are generally present at low levels but can usually be quantitated by mass spectrometry, capillary gel electrophoresis, and HPLC with fluorescence detection. Other structures can be detected but usually are present at such low levels that they cannot be quantitated.

Although all species, including plants, synthesize the same core heptasaccharide with the same additional monosaccharides, there are species-specific differences [104, 105]. For example, there are species-specific differences in terminal sialylation and galactosylation [104], and relative to endogenous human mAbs, therapeutic mAbs produced in mammalian cell lines are hypogalactosylated [106]. Another difference is that human glycan structures can have a bisecting GlcNAc, which inhibits the addition of fucose. CHO cells do not express *N*-acetylglucosaminyltransferase-III, which is the enzyme that carries out this reaction [104].

Specific glycan structures play a role in antibody effector function. The complete removal of the glycan results in the loss of both CDC and ADCC activity [107]. Galactose is associated with CDC activity such that its removal results in a significant, but not complete reduction of activity [107, 108].

The presence of fucose inhibits ADCC activity [109, 110] specifically when mediated through FcγRIIIa. IgG and FcγRIIIa interact through a rare carbohydrate to carbohydrate interaction, with which fucose on the Ig glycan interferes [111, 112]. However, a mAb where the glycan is fucosylated on one constant region chain and is afucosylated on the other has enhanced ADCC activity relative to a fully fucosylated mAb but still has about 50% of the activity of a fully afucosylated mAb [113]. For mAbs where ADCC is a desired mechanism of action, CHO cell lines have been engineered to knock out the fucosyltransferase gene [114–116] or by adding genes that express *N*-acetylglucosaminyltransferase-III and α-mannosidase II, which block the addition of fucose by promoting structures containing a bisecting GlcNAc [117].

Heavy and light chain variable (V) regions can also contain N-linked glycan structures [118], which may enhance or interfere with antigen binding [119]. Some germ line gene VH and VL sequences contain the consensus glycan attachment Asn-X-Ser/Thr sequence, but others can be generated through the somatic mutation process [120]. V region glycosylation has been associated with various diseases, for example, 79% of VH sequences in follicular lymphoma patients had novel glycan attachment sites in CDRs [121], and V region glycosylation of autoantibodies can influence antigen binding and possibly the pathogenic nature of these autoantibodies [122]. For therapeutic mAbs that have potential V region glycosylation sites, it is important to determine if the Fab is glycosylated and, if present, the structures of the Fab glycan.

Cetuximab, first approved in 2004 for patients with metastatic colorectal cancer and subsequently for head and neck cancer, is glycosylated on the VH region. The glycan structure contains a galactose-α-1,3-galactose (α-gal) linkage, which is produced by the murine cell substrate. This structure was shown to induce anaphylaxis in patients who had preexisting IgE anti-α-gal antibodies prior to treatment [123].

### 4.3.4 Product-Related Impurities

Antibody fragments and aggregates are critical quality attributes of intact mAbs that can impact the potency of the product. V region-containing fragments generally maintain their ability to bind antigen. However, if effector function is part of the mechanism of action, both V region and Fc region fragments will have reduced potency. Aggregates often have reduced antigen-binding capacity but may demonstrate enhanced potency if presented as immune complexes to Fcγ receptors. Therefore, it is necessary to understand the causes of these degradation pathways in order to improve the manufacturing process to further remove fragments and aggregates from the drug substance and to optimize the formulation to reduce their formation over the shelf life of the product.

#### Fragments

The hinge region of IgG is susceptible to cleavage by proteases and nonenzymatic cleavage. Fragmentation of mAbs at the hinge region can occur in vivo by exposure to endogenous or bacterial proteases [124]. Residual proteases in host-cell proteins can also lead to fragmentation [125]. However, most of the fragmentation of mAbs is nonenzymatic and can occur during manufacturing or under normal storage conditions, although temperature and pH stress enhance the chemical reactions. Several studies investigated chemical reactions of nonenzymatic cleavage, such as peptide bond hydrolysis or β-elimination. These reactions typically have sequence specificity [126]; see [127] for a review). Different pH and temperature conditions can lead to fragmentation, but the presence or absence of the Fc-glycan structure does not [128, 129]. The rates of fragmentation were shown to be similar for five different IgG1 mAbs, suggesting that fragmentation is largely dependent on the primary sequence of the hinge region [129].

The presence of copper or iron in the presence of histidine can also result in nonenzymatic cleavage. Cleavage of alemtuzumab at 37°C was due to the presence of copper and was further elevated at high pH. This fragmentation was also temperature dependent; no fragmentation occurred below 20°C but increased at temperatures above 37°C [130]. Interestingly, in IgG mAbs containing a lambda light chains, cleavage was observed at a different site within the hinge region, and there was also cleavage of the lambda light chain. These reactions were due to the presence of iron in the drug substance. However, iron alone did not induce the cleavage but required the histidine in the formulation buffer for this reaction. This cleavage reaction was not seen in IgG mAbs with kappa light chains [131].

Aggregates

Antibody aggregates can form during upstream or downstream manufacturing, as well as during the shelf life of the product [132, 133]. Thermal, freeze-thawing, pH, light, and mechanical stress are known to induce aggregation of mAbs. Aggregation can also occur in the presence of human plasma, which is dependent on the diluent used for preparation of the infusion for intravenous infusion [133]. Formulation optimization studies are crucial for the long-term stability of mAbs.

Antibodies naturally contain aggregation-prone motifs, which may play a role in their ability to bind antigen, FcγR, or protein A [134]. However, these motifs play a role in aggregation of mAbs over their shelf life. Specific aggregation-prone amino acids in either the V region or C region can be engineered to maintain antigen binding and other functional properties of the mAb while reducing aggregation [134, 135].

Several studies applied different forms of stress to mAbs to understand the factors that contribute to aggregation. In a study by Joubert et al. [136], different forms of freeze-thaw, pH, mechanical, chemical, and thermal stress were applied to IgG2 mAbs, human and mouse IgG1 mAbs, and IVIG. Aggregates were characterized by orthogonal methods and divided into seven classes based on the biophysical properties of the aggregates, such as total aggregation, size, morphology, etc. Similar classes of aggregates were formed in each of the samples when treated by the same stress conditions. This suggests that specific types of aggregates are formed by specific types of stress, which is consistent with an earlier study [137] that showed the Fab fragment is more sensitive to heat stress, while the Fc region is more susceptible to low pH stress. On the other hand, in a study by Nicoud et al. [138], stressed IgG1 and IgG2 mAbs under identical conditions showed differences in the kinetic mechanisms of aggregate formation.

The simplest form of aggregates is non-covalent reversible dimers, which are seen under normal storage conditions. However, some dimers are covalent, and while some maintain activity, others lose activity. A study on epratuzumab showed that 70% of the dimers were covalent with three different forms; Fab/Fab dimers, Fab/Fc dimers. and Fc/Fc dimers. The Fab/Fc dimers were the predominant form [139], but all forms maintained binding activity. A different study [140] treated an IgG1 with three different forms of stress that all resulted in Fab/Fab dimers. Dimers generated by two of the stress conditions had reduced potency in a cell-based assay, but the dimers from all three stress conditions had enhanced binding to FcγRIIIa by surface plasmon resonance. A study by Luo et al [141] also showed enhanced binding of dimers to FcγRI, FcγRII, and FcγRIII.

### 4.3.5 Mechanism of Action

Understanding the mechanism(s) of action (MOA) of a mAb is a continuous process and may depend on the indication, new knowledge of the target biology, and antibody-Fc-receptor interactions. Antigen binding is a critical quality attribute of all mAbs. Whether antibody effector function plays a role in the mechanism of

action for a given mAb depends on many factors including whether the target is expressed on a cell surface or is soluble; the presence of an Fc region; the IgG isotype; or specific engineering of the Fc region to enhance or reduce effector function. Aspects of antigen binding and the potential to bind FcγR and complement should be part of a thorough characterization of a mAb. Potency assays that reflect the predicted mechanism of action should be developed for release and stability testing. When effector function is expected, a cell-based potency assay with appropriate target and effector cells demonstrated both antigen binding and effector function. Methods that bridge binding to the target as well as FcγR can also be developed as characterization and release methods [142].

### Antigen Binding

Binding to the antigen is an MOA for every mAb, which should be characterized for binding affinity and on and off rates. Binding to cell surface antigens on appropriate target cells should be demonstrated. Homology between the human target and primate or other species is important in order to determine the relevant animal models for preclinical studies.

Some mAbs against soluble ligands may work simply by blocking the binding of that ligand to its receptor, resulting in the inhibition of downstream signaling effects. The mechanism of the anti-CD25 mAb, daclizumab, first approved for the prophylaxis of kidney graft rejection, was believed to be due to blocking IL-2-mediated signaling of activated T cells. However, in multiple sclerosis, additional mechanisms resulting from IL-2 neutralization have become apparent, such as the activation and expansion of regulatory NK cells, blocking of dendritic cells from presenting IL-2 to primed T cells, and modulation of the development of innate lymphoid cells [143].

Other mAbs against soluble targets, such as the TNFα antagonists, are thought to work primarily through TNFα neutralization; however, these agents can also bind to membrane forms of TNFα which can lead to apoptosis and cytokine suppression through reverse signaling and may include antibody effector functions such as CDC and ADCC [144].

### Effector Function

There is a hierarchy among IgG isotypes regarding their ability to carry out effector function. IgG1 and IgG3 isotypes are chosen when effector function is desirable and IgG2 and IgG4 isotypes are generally chosen when effector function is not. Jiang et al. [145] published a reasonable approach describing the characterization of potential effector function and developed a control strategy for mAbs that includes effector function as part of their MOA, which is dependent on whether the mAbs are anticipated to have high, intermediate, or low potential for effector function.

In general, there is an expectation that the characterization of mAbs with a low potential for effector function should be performed early in development. No additional studies would be needed unless new information became available suggesting that effector function may contribute to the MOA. For mAbs where effector function is expected to contribute to the MOA, there is an expectation that characterization will include relevant cell-based bioassays as well as bind to complement and FcγRs. Analysis of glycoforms (galactose and fucose) that impact effector function should also be included in the characterization. The control strategy should ideally include a bioassay representing the most relevant proposed MOA.

Until recently, most potency assays for mAbs with effector function were CDC assays, because this is generally a robust method that can meet requirements for appropriate validation and quality control. While CDC potency assays represent a relevant biological activity, it is thought that for many mAbs, ADCC and ADCP may be more important MOAs in patients, especially oncology patients [146–148].

ADCC assays have been included in characterization studies, but not as potency assays, because the effector cells were peripheral blood mononuclear cells (PBMCs) derived from human donors. Because of donor-to-donor variability, these ADCC assays were not amenable for quality control purposes. However, over the last 5–10 years, NK cell lines have been developed that replace PBMCs as donor cells in the ADCC assay [149], and when available to a sponsor, these assays are now incorporated as part of the control strategy. Reporter gene assays have also been developed as a surrogate for an ADCC assay [150, 151], but these methods are not a direct measure of ADCC activity and should be demonstrated to be representative of the mAb's ADCC activity.

ADCP assays for characterization are just beginning to be developed. These methods use human PMBCs differentiated into macrophages or murine macrophages [148, 152–154] and are not amenable as quality control methods. However, ADCP methods using cell lines as effector cells are under development, and we expect they will be used first for characterizing mAb effector function and eventually as release and stability methods.

Along with developing relevant antibody effector function characterization and potency assays, understanding the most relevant effector function in any given patient population is a challenge. Different effector cell types have varying levels of activity in vitro [151] and this may also reflect in vivo efficacy. Different effector cells are present at different sites of disease, and other than NK cells, which express only FcγRIIIa and FcγRIIc, other effector cells, such as monocytes, macrophages, dendritic cells, and neutrophils, express all the FcγR [155, 156].

Other aspects of the antigen/mAb interaction influence effector function activity. Higher levels of EGFR expression correlate with Fc-mediated effector function in vitro [157]. Of particular note, in vitro ADCC studies with cetuximab using NK cells from healthy donors and squamous cell carcinoma of the head and neck (SCCHN) and colorectal cancer cell lines correlated with the high affinity FcγRIIIa V/V polymorphism [158]. However, a more recent study using NK cells derived

from SCCHN patients showed that the FcγRIIIa genotype was not associated with the clinical outcome but rather had an innate cytotoxicity capability [159].

The intracellular domain of the antigen also appears to play a role in effector function mechanisms. Using intact CD19 and HER2 and chimeric constructs swapping the intercellular domains of the molecules, Tiroch et al. [160] showed that mononuclear cells could trigger ADCC with the appropriate mAb for all the constructs, while polymorphonuclear granulocytes were only effective against wild-type HER2 or the CD19/HER2 intracellular domain chimeric protein.

Overall, while relevant bioassays may be available for characterization and release of mAbs, there may not be a complete understanding of the MOA for specific indications. Since there are many effector cell types with different expressions of FcγRs, methods that assess binding of the mAb to all the FcγRs are important for characterization and comparability exercises. Focusing on afucosylated glycans and NK cell ADCC activity may not always be a reflection of the in vivo MOA.

### 4.3.6 IgG Isotype-Specific Characterization

IgG2 MAbs

The IgG2 isotype is often chosen when effector function is not intended as a mechanism of action for a therapeutic mAb. Unlike the other IgG isotypes, human IgG2 contains four cysteine residues in the hinge region, which result in different IgG2 disulfide isoforms, termed IgG2-A, IgG2-A/B, and IgG2-B [161, 162]. These disulfide isomers occur in endogenous and myeloma-expressed IgG2, as well as in therapeutic mAbs. However, for some therapeutic mAbs, the isoforms may have different potency in cell-based assays [163].

The disulfide isomers were subjected to site-directed mutagenesis, and it was shown that specific cysteine to serine mutations would reduce the disulfide heterogeneity while maintaining in vitro activity [164]. However, disulfide isomers may also be exploited for enhanced activity. A recent study showed that IgG2 mAb, in particular the IgG2-B disulfide isoform, provides superior FcγR-independent activity relative to other isotypes to immunostimulatory mAbs [165].

In addition to the disulfide isoforms, IgG2 can also form covalent dimers, which are thought to increase the avidity of the IgG2 response against bacterial capsular polysaccharides [166]. Therefore, therapeutic IgG2 mAbs should be thoroughly characterized for disulfide isomers and covalent dimers, which should be controlled if they demonstrate different in vitro potency. Capillary gel electrophoresis [167] and capillary zone electrophoresis [168] methods can distinguish the disulfide isoforms.

IgG4 MAbs

The IgG4 isotype is also chosen when effector function is not an intended mechanism of action. Although IgG4 has two interchain disulfide bonds in the hinge region, the specific sequence surrounding the two cysteine residues on each H chain results in an unstable hinge region, which leads to the formation of IgG4 half antibodies (or monomers). This property is associated with the presence of the serine 228 residue immediately prior to the second cysteine residue ([169] and references therein). The result of half-antibody formation is that the IgG4 monomer can undergo Fab arm exchange to form bispecific IgG4 heterodimers with other IgG4 antibodies present in serum [170]. Although additional sequences in the CH3 domain have also been demonstrated to play a role in Fab arm exchange [171], mAbs containing a serine to proline mutation at position 228 do not undergo Fab arm exchange in vivo [172, 173].

It has been proposed that IgG4 half antibodies may have advantages over mAb fragments when it comes to their half-life. To this end, specific mutations in the CH3 domain were identified that result in monovalent IgG4 that may be useful for clinical development [174].

IgG4 half mAbs, with or without the serine to proline mutation at position 228, should be characterized and controlled throughout product development. Western blot [175], chip-based SDS-PAGE [176], and capillary SDS [177] methods have been developed to detect IgG4 half antibodies. Methods that provide quantitative results are preferred.

### 4.3.7 Future Trends in MAb Development

Therapeutic mAbs are currently the largest class of biologics in development. This is due in part to the success of antibody engineering the Fc region to reduce immunogenicity, the ability to use platform manufacturing processes, and a good understanding of general quality attributes. Better understanding of disease pathways and the identification of new targets, in combination with continued engineering of Fc regions, glycan structures, and development of novel constructs, such as antibody conjugates, bispecific products, mAb cocktails, and Fc-fusion proteins, provide a robust pipeline of mAbs and related products. Finally, we anticipate that in the next few years, there will be a better understanding of how different effector cell types respond to mAb-based immune complexes through different FcR.

## *4.4 Other Therapeutic Proteins*

Therapeutic proteins encompass a wide variety of proteins products including replacement enzymes, cytokines, hormones, and toxins. Many expression systems such as bacteria, yeast, plant and mammalian cells, transgenic animals, and even natural sources are used to manufacture these proteins. The purification processes

are generally tailored to characteristics of each product and are aimed at maximizing the capture of the target and removal of process- and product-related impurities. Due to the lack of a single robust affinity purification step such as the protein A column for monoclonal antibody purification, the purification process for the majority of therapeutic proteins varies depending on the property of each protein. In addition, structural modifications or variations for each protein are specific to the protein, and the impact by these modifications on product quality can vary from product to product. There is no "platform" approach toward characterization of all therapeutic protein products. Here, we discuss some general guidelines for characterizing these products and how appropriate controls can be put in place throughout product life cycle to ensure their safety and efficacy.

### 4.4.1 Manufacturing Controls to Ensure Therapeutic Protein Product Quality

#### Identity

Historically various methods have been used as an identity test. The current regulatory expectation is that an HPLC chromatography method interrogating digested peptide patterns be used at drug substance release. The landmark peptide peaks should be identified by mass spectrometry analysis during product development. A robust test that provides an unambiguous identification is particularly important for products manufactured in a multiproduct manufacturing facility to prevent potential misidentification of products.

#### Purity

Because therapeutic proteins are heterogeneous, purity of a product is generally measured by multiple methods. The impurities should be interrogated by size, charge, and hydrophobicity. Each minor peak or band representing impurities, degradation products, or product variants should be identified and quantified based on manufacturing history.

#### Potency

In biological product testing, in vitro potency assays are considered a surrogate to clinical efficacy and are therefore an integral component of product quality testing as well as to monitor the consistency of the product throughout the life cycle of the product. Although by convention, potency assays are interpreted as a measurement of the biological activity of the product, such as the enzymatic activity of an enzyme, a well-designed potency assay should encompass all aspects of biological characteristics that collectively define the mechanism of action. Depending on the

class of products, the scope of potency assays also varies depending of the factors involved in the product to act. Therefore, in addition to biological activity assays, potency assays should also include testing for affinity to ligands, such as receptors and substrates as well as tests for attributes that impact circulation half-life.

### Enzymatic Assays

Enzyme activities are impacted by two integral factors: the affinity of the enzyme to its substrate and the ability of the enzyme to catalyze the conversion of its substrate to the final product. Therefore, enzyme activity assays should cover both these aspects of any given enzyme product. Generally, this can be accomplished with an enzyme kinetic assay. In addition, to better represent the efficacy of the enzyme for the indication, the substrate(s) should resemble the property and structure of the natural substrate relevant in the indicated disease(s).

For the majority of currently approved enzyme products, enzyme activity assays are performed using a small-molecule synthetic substrate conjugated to a colorimetric function group or a fluorochrome. The assays are commonly conducted at saturating levels of substrate that generally do not represent levels of the natural substrate under the indicated disease conditions. Furthermore, the small molecules generally only represent the linkage or group specificity but lack the structural representation of natural substrates of the enzymes; the results therefore may not be representative of the in vivo enzymatic activity. Numerous studies have demonstrated that the $K_M$ and catalytic efficiency ($k_{cat}/K_M$) obtained from the synthetic substrates can differ significantly from these obtained using natural substrates [178, 179]. Whereas enzymatic assay measuring the end point product of enzymatic reaction using a synthetic substrate may be appropriate for a rapid control for process intermediates, an enzymatic kinetics assay using a substrate that resembles the structure and linkage specificity is expected for release and stability testing for both drug substance and final drug product. Enzyme kinetic assays are also expected in post-licensure comparability and similarity studies.

### Cell-Based and In Vivo Potency Assays (Bioassays)

For growth hormones and cytokines, the potency bioassays are aimed at measuring biological activity of the product in vitro using mammalian or other relevant cell culture systems, as well as in vivo in animals. For certain products, in vitro cell-based potency assays alone are insufficient to demonstrate clinical efficacy as they do not take into account product attributes affecting pharmacokinetics, organ distribution, or metabolism. For example, in addition to the ability to stimulate red blood cell production, clinical performance of recombinant erythropoietin relies heavily on the glycan structure on the molecule [180]. As a result, the potency assay for many erythropoietin products is still performed as mouse-based in vivo reticulocyte proliferation assays. However, in cases where biological activity and attributes

affecting half-life are well known, the in vivo potency assay can be replaced by a combination of assays independent testing these attributes. For example, a combination of in vitro cell-based potency assay and glycan analysis has been shown to be adequate as a control for erythropoietin products in lieu of the reticulocyte proliferation in vivo assay [181].

### Receptor/Ligand Binding Affinity Assays

Enzymes, cytokines, and hormones generally act in targeted tissues or organs through binding to their corresponding receptors on the surface of cells. Proper control of the binding affinity of these proteins to their respective receptors is critical to ensure clinical performance of these protein products. The affinity of these products to their receptors should be well characterized during product development and tested at product release and during stability testing. Whereas cell-based biological activity assays provide some information for the binding of the products to their receptors, due to the inherent variability of these types of assays, they are generally suboptimal in monitoring alterations of binding affinity that may occur after manufacturing changes. An alternative method that directly assesses the affinity of the products to their respective receptors is necessary to measure this important aspect. This can generally be accomplished by in vitro binding assays where the equilibrium dissociation constant ($K_D$) of the product and its receptor/ligand is measured. Recent advances in surface plasmon resonance (SPR) technology make this analytical technique among the most commonly used method for measuring protein affinity to their targets [182, 183]. These assays are expected to be part of release and stability testing at the time of licensure. Sponsors of biological products are encouraged to develop them early in product development cycle and ideally implement a validated receptor/ligand binding assay for phase III clinical materials.

### Assays Measuring Attributes Affecting Half-Life

There are many factors acting either independently or collectively to affect serum circulating half-life of a biologic product, many of which have been exploited by manufacturers to achieve better therapeutic effects. These include managing cell culture conditions to control for glycosylation, covalent conjugation of polyethylene glycol (PEG) molecule(s) to proteins, and fusion of various protein tags to proteins. This section discusses expected testing to confirm the consistency of these attributes.

## Sialylation

The majority of therapeutic proteins and monoclonal antibodies expressed in mammalian cell lines are glycosylated to variable extent, at select Asn residues. The terminal monosaccharide of these glycans may be capped by sialic acid to various degrees. Numerous studies have shown that the total sialic acid content and, more profoundly, the structure of sialic acid-capped *N*-glycans significantly affect glycoprotein absorption to and clearance from serum [184–186]. Characterization and quantitation of sialylation are, therefore, crucial in maintenance of product consistency. Because the majority of therapeutic proteins contain multiple *N*-glycosylation sites, and each site is generally glycosylated at different degrees, the overall glycan and sialic acid content on protein molecules can be extremely heterogeneous. More importantly, levels of sialylation can be affected by many cell culture parameters such as pH, level and composition of nutrients, cell culture additives, cell growth rate, dissolved oxygen, and temperature [187]. Due to all these factors, from a product life cycle management perspective, analysis of sialic acid and sialylated glycan structures should be implemented as early as possible in product development. The tests for sialylation generally include relative sialic acid content (i.e., mol sialic acid/mol of protein) and quantitation of sialylated glycan structures. Due to sialylated glycans being negatively charged (with the net charge proportional to the number of sialic acid on each glycan), the latter often consists of quantitation of neutral, mono-, di-, tri-, and tetra-sialylated glycans cleaved from the protein molecules. Glycans are generally separated by capillary electrophoresis or anion exchange HPLC followed by mass spectrometry identification of the contents of each individual peak. Extensive charge profiling is expected as part of product characterization, whereas quantitation of critical glycan group(s) and sialic content should be part of release testing. However, for release testing, manufacturers can opt for a method that is highly reproducible, precise, and easy to operate in a QC environment.

## PEGylation

For small proteins and proteins expressed from prokaryotic expression systems, conjugation of one or more polyethylene glycol (PEG) molecules can effectively increase half-life and reduce immunogenicity of the products [188]. However, PEGylation, especially multiple covalent attachments of PEG molecules to primary amines on a protein molecule, can generate undesirable effects including reduction in biological activity, reduction in affinity to receptors, and lot-to-lot variations for some products. To minimize these undesirable effects, the majority of manufacturers have moved away from random PEGylation to site-specific mono-PEGylation [189]. Owing to continuous efforts in optimization in PEG production, linker development, and PEGylation reaction conditions, PEGylation of therapeutic proteins has evolved into a very robust and reliable process, and the resulting PEGylated products are fairly homogenous and stable. The characterization

generally includes conjugation site identification by mass spectrometry and quantitation of non-, mono-, and di/multi-PEGylated species by a column chromatography.

Another source of variability in PEGylated products derives from the heterogeneous composition of the PEG starting material, so particular focus early in the development is placed on the qualification of a consistent PEG supply. As a critical raw material for PEGylated products, the qualification of PEG should be comprehensive and should include tests for purity, functional group activity, polydispersity, and stability testing. In some cases where PEGs from different suppliers are used, in addition of comparison of PEG manufacturing process and lot release data, the qualification of each PEG may require some nonclinical study comparing protein PEGylated with the PEGs from different sources.

In many instances, conjugation of even one PEG molecule interferes with critical attributes such as enzymatic activity or receptor binding affinity. Manufacturers are expected to fully characterize and control for site of PEGylation by mass spectrometry and quantitate single-, di-, multi-, and non-PEGylated proteins through SEC- or EX-HPLC. The manufacturers are also expected to characterize hydrolysis of PEG moiety from the protein under relevant storage conditions. These quantitative assays should be included in release and stability programs.

#### Polypeptide Tags

Another alternative used by manufacturers to extend serum half-life of protein biologics has been to add another protein or polypeptide tag at the amino- or carboxy- terminus or both termini. Such tags include carboxyl terminal peptide [190], human serum albumin [191], and XTEN [192]. They generally fold as a stand-alone unit separated structurally and functionally from the therapeutic protein, often not contributing or contributing little to biophysical characteristics of the chimeric construct other than size. The major control for these products focuses on the integrity of the tags in manufacturing process and during storage as the junction of the two separately folded structures tends to be targeted for degradation.

### 4.4.2 Posttranslation Modifications (PTMs) on Protein Products

Glycosylation occurs as part of protein biosynthesis, and, for the majority of protein products, e.g., products manufactured using eukaryotic cell expression systems, is considered the most important of the PTMs since differences in glycosylation have been shown to impact critical attributes including circulation half-life, affinity to receptors, and effector functions. The glycans collectively affect the structure and function of the protein, and even relatively small variations in overall glycosylation profile can have drastic effects on the performance of therapeutic proteins [193]. Due to the high degree of heterogeneity, the function of each individual glycan is hard to measure. However, mapping of overall glycan profile can provide some

details on glycosylation for the purpose of maintaining product consistency during process development. Such analyses include monosaccharide composition, overall glycan profiling, and site-specific glycan analysis and are expected to be included as part of characterization studies and incorporated as part of comparability studies to support post-licensure manufacturing changes. Recent advances in mass spectrometry have enabled identification of microheterogeneity and relative abundance of glycans on specific glycopeptides [194, 195].

Among the various glycan forms, glycans bearing mono-mannose-6-phosphate (M6P) or di-M6P (bis-M6P) are of particular importance for products used as enzyme replacement therapy (ERT) for lysosomal storage diseases [196]. M6P- and bis-M6P-containing glycans serve as primary mechanism in targeting these enzymes to lysosomes through binding to cation-dependent and cation-independent M6P receptors [197, 198]. The presence of M6P residues on the *N*-glycans of these recombinant therapeutic proteins is a critical quality attribute since increasing levels of M6P on ERT products has been observed to positively correlate with more efficient uptake and subsequent treatment efficacy for lysosomal storage diseases both in vitro and in vivo [199, 200]. Despite a decade of efforts, characterization of mono- and especially bis-M6P-bearing glycans still poses a considerable analytical challenge. Many of the methods used so far are too complex and require highly purified material and extensive post-testing data analysis, which has significantly limited their utility in upstream process optimization and monitoring. A recent study employed a combination of ultra-performance liquid chromatography (UPLC) and capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) for a rapid separation of *N*-glycans. The method presents a novel yet relatively simple approach for the qualitative and semiquantitative structural characterization of M6P-containing oligosaccharides on therapeutic enzymes [197]. Regardless which method is chosen, for all enzyme replacement therapy products for lysosomal storage diseases, the total M6P content, relative bis-M6P content, and the $K_D$ of the product to MPRs are expected to be included as release specifications as these attributes correlate directly with clinical efficacy and can vary by even subtle changes in manufacturing process and levels of product-related impurities.

### Modifications on Amino Acid Residues

Certain amino acid residues in protein products are susceptible to undergoing some form of modification during manufacturing process or upon long-term storage. These include oxidation, deamidation, and atypical disulfide formation. Many factors, including pH, temperature, and ionic strength of buffers, can contribute to the formation of these modifications. In many instances, due to heterogeneity of biological products, it is hard to assess the impact of each individual modification on product safety and potency. It is therefore critical to characterize the degree of each modification early in product development and to optimize and control for condition to minimize change of certain modified forms over product development

and after licensure. Currently, some of these modifications are monitored at release and on stability by various chromatography methods. However, such modifications generally only slightly change the elution profile on chromatograms, severely impact the method's ability to resolute, and accurately quantify the impurity. Depending on how the area under the curve is calculated, these methods tend to over- or underestimate the actual content of the impurities. A more robust and accurate analysis of these impurities would be to focus on how to better resolve the impurity peaks away from the main product. It is expected that the manufacturers identify potential amino acid modifications that occur during manufacturing process and upon long-term storage conditions. For practical reasons, identification of amino acid residual modifications can be demonstrated by mass spectrometry analyses of digested peptides of products stored under relevant stressed or accelerated storage conditions. Peptides containing modifications observed in these analyses should be controlled for at release and on stability using a chromatography method or methods where the peptides are identified incontrovertibly and quantitated relative to their unmodified counterparts.

### Dimers, Oligomers, and Aggregates

Due to their difference in size, the formation of dimers, oligomers, and aggregates can be easily identified by size-exclusion chromatography (SEC). It is important to stress that, due to heterogeneity of these species, especially for aggregates, an orthogonal method would be needed during product characterization to confirm that the SEC method is suitable to quantitate these impurities.

Some products on the market contain a protein stabilizer, such as HSA, in their final formulation. The presence of the stabilizing protein limits the use of SEC as a method to quantitate these impurities. In such cases, it is expected that such testing has been performed prior to formulation and a separate method to detect and quantitate product-specific oligomerization in product release and on stability. Due to the interference of the stabilizer, a semi-quantitation method, such as Western blot analyses both under reduced and non-reduced conditions, may be acceptable.

### Truncated Forms

Endogenous proteases may cleave the amino or carboxyl terminus of a protein in a site-specific or random manner during cell culture process. Additionally, co-purifying proteases may further cleave protein products during manufacturing process when intermediates are held between unit operations. Whereas the integrity of the termini can be demonstrated by amino or carboxyl terminal sequencing, this method does not provide much quantitative information on the differentially processed forms. In cases where the differentially processed forms contribute to safety and potency, each form should be quantitated through a method that

indisputably resolves each form. Depending on the complexity of the product, the method can vary significantly. The manufacturer is expected to identify the impact of the variants on product quality and develop an appropriate product-specific method to quantify each form.

### Charge Variant

The net charge of a protein is a fundamental physical property of any protein [201]. In addition to affecting intrinsic structure and thus solubility and stability of a protein, the charges also influence biological activity as well as their binding affinity to receptors or ligands. For therapeutic proteins, besides charges from the amino acid residues, charged glycans also account for a significant portion of the overall charge status of a protein. Because sialylated and phosphorylated glycans contribute significantly to the negative charges, a well-characterized charge profile not only ensures consistency of solubility and stability of a product but also provides controls for critical attributes affecting half-life and receptor binding. Generally, a quantitative method, such as capillary isoelectric focusing (cIEF) or ion-exchange HPLC should be implemented to provide quantitative limits for each peak or each group of peaks.

Non-monoclonal antibody protein therapeutics, as a product class, represent a very diversified group of proteins, each of which bears a unique series of critical attributes that collectively define a specific mechanism of action. The variety of expression hosts and combinations of chromatography steps also introduce a different profile of process-related, product-related impurities and product variants for each product. Consequently, besides several known attributes common to the majority protein products, characteristics to evaluate stability of each product can also vary. The characteristics discussed in this section summarize studies from a large list of currently approved non-monoclonal antibody protein products; they do not necessarily represent critical attributes defining the potency, purity, and safety profile of a particular product. A comprehensive characterization of a new product relies on the understanding of the product and accumulation of knowledge on how the manufacturing process impact the critical attributes throughout product development.

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