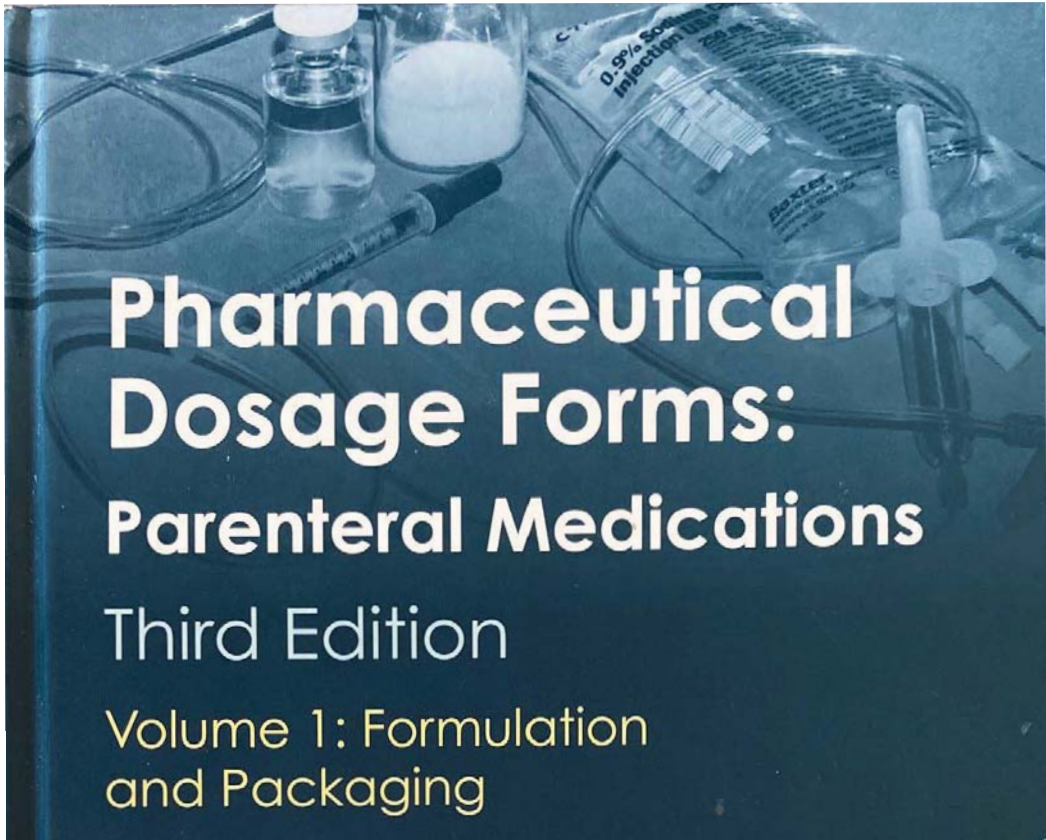




# Pharmaceutical Dosage Forms: Parenteral Medications

Third Edition

Volume 1: Formulation  
and Packaging



**informa**  
healthcare

Edited by  
Sandeep Nema  
John D. Ludwig

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**Volume 1  
Formulation and Packaging**

**Edited by**

**Sandeep Nema**

*Pfizer, Inc.*

*Chesterfield, Missouri, U.S.A.*

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New York London

First published in 1984 by Marcel Dekker, Inc., New York, New York.  
This edition published in 2010 by Informa Healthcare, Telephone House, 69-77 Paul Street, London EC2A 4LQ, UK.

Simultaneously published in the USA by Informa Healthcare, 52 Vanderbilt Avenue, 7th Floor, New York, NY 10017, USA.

Informa Healthcare is a trading division of Informa UK Ltd. Registered Office: 37-41 Mortimer Street, London W1T 3JH, UK. Registered in England and Wales number 1072954.

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A CIP record for this book is available from the British Library.

Library of Congress Cataloging-in-Publication Data available on application

ISBN-13: 9781420086430

ISBN-13: 9781420086539 (three-volume set)

Orders may be sent to: Informa Healthcare, Sheepen Place, Colchester, Essex CO3 3LP, UK

Telephone: +44 (0)20 7017 5540

Email: [CSDhealthcarebooks@informa.com](mailto:CSDhealthcarebooks@informa.com)

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Typeset by MPS Limited, A Macmillan Company  
Printed and bound in India

*We dedicate this work to those who have inspired us.  
To my parents Walter and Ruth Ludwig and my wife Sue Ludwig  
To my parents Hari and Pratibha Nema and my wife Tina Busch-Nema*

## Foreword

I was a faculty member at the University of Tennessee and a colleague of Dr. Kenneth Avis when he conceived, organized, and edited (along with H.A. Lieberman and L. Lachman) the first edition of this book series that was published in 1984. It was so well received by the pharmaceutical science community that an expanded three-volume second edition was published in 1992. Dr. Avis did not survive long enough to oversee a third edition, and it was questionable whether a third edition would ever be published until two of his graduate students, Drs. Nema and Ludwig, took it upon themselves to carry on Dr. Avis' tradition.

Their oversight of this third edition is work that their mentor would be highly pleased and proud of. From 29 chapters in the second edition to 43 chapters in this new edition, this three-volume series comprehensively covers both the traditional subjects in parenteral science and technology as well as new and expanded subjects. For example, separate chapter topics in this edition not found in previous editions include solubility and solubilization, depot delivery systems, biophysical and biochemical characterization of peptides and proteins, container-closure integrity testing, water systems, endotoxin testing, focused chapters on different sterilization methods, risk assessment in aseptic processing, visual inspection, advances in injection devices, RNAi delivery, regulatory considerations for excipients, techniques to evaluate pain on injection, product specifications, extractables and leachables, process analytical technology, and quality by design.

The editors have done an outstanding job of convincing so many top experts in their fields to author these 43 chapters. The excellent reputations of the authors and editors of this book will guarantee superb content of each chapter. There is no other book in the world that covers the breadth and depth of parenteral science and technology better than this one. In my opinion, the editors have achieved their primary objectives—publishing a book that contains current and emerging sterile product development and manufacturing information, and maintaining the high standard of quality that readers would expect.

*Michael J. Akers  
Baxter BioPharma Solutions  
Bloomington, Indiana, U.S.A.*

## Preface

*Pharmaceutical Dosage Forms: Parenteral Medications* was originally published in 1984 and immediately accepted as a definitive reference in academic institutions and the pharmaceutical industry. The second edition was published in 1993. The ensuing years have produced incredible technological advancement. Classic small-molecule drugs are now complemented by complex molecules such as monoclonal antibodies, antibody fragments, aptamers, antisense, RNAi therapeutics, and DNA vaccines. There have been significant innovations in delivery devices, analytical techniques, in-silico modeling, and manufacturing and control technologies. In addition, the global regulatory environment has shifted toward greater emphasis on science-based risk assessment as evidenced by the evolving cGMPs, quality by design (QbD), process analytical technology (PAT), continuous processing, real time release, and other initiatives. The rapidly changing landscape in the parenteral field was the primary reason we undertook the challenging task of updating the three volumes. Our objectives were to (i) revise the text with current and emerging sterile product development and manufacturing science and (ii) maintain the high standard of quality the readers expect.

The third edition not only reflects enhanced content in all the chapters, but also more than half of the chapters are new underscoring the rapidly advancing technology. We have divided the volumes into logical subunits—volume 1 addresses formulation and packaging aspects; volume 2, facility design, sterilization and processing; and volume 3, regulations, validation and future directions. The authors invited to contribute chapters are established leaders with proven track records in their specialty areas. Hence, the textbook is authoritative and contains much of the collective experience gained in the (bio)pharmaceutical industry over the last two decades. *We are deeply grateful to all the authors who made this work possible.*

Volume 1 begins with a historical perspective of injectable drug therapy and common routes of administration. Formulation of small molecules and large molecules is presented in depth, including ophthalmic dosage forms. Parenteral packaging options are discussed relative to glass and plastic containers, as well as elastomeric closures. A definitive chapter is provided on container closure integrity.

Volume 2 presents chapters on facility design, cleanroom operations, and control of the environment. A chapter discussing pharmaceutical water systems is included. Key quality attributes of sterile dosage forms are discussed, including particulate matter, endotoxin, and sterility testing. The most widely used sterilization techniques as well as processing technologies are presented. Volume 2 concludes with an in-depth chapter on lyophilization.

Volume 3 focuses on regulatory requirements, risk-based process design, specifications, QbD, and extractables/leachables. In addition, we have included chapters on parenteral administration devices, siRNA delivery systems, injection site pain assessment, and control, PAT, and rapid microbiology test methods. Volume 3 concludes with a forward-looking chapter discussing the future of parenteral product manufacturing.

These three volumes differ from other textbooks in that they provide a learned review on developing parenteral dosage forms for *both* small molecules and biologics. Practical guidance is provided, in addition to theoretical aspects, for how to bring a drug candidate forward from discovery, through preclinical and clinical development, manufacturing, validation, and eventual registration.

The editors wish to thank Judy Clarkston and Lynn O'Toole-Bird (Pfizer, Inc.) for their invaluable assistance and organizational support during this project, and Sherri Niziolek and Bianca Turnbull (Informa Healthcare) for patiently leading us through the publishing process.

We also acknowledge the assistance of Pfizer, Inc. colleagues Lin Chen and Min Huang for reviewing several of the chapters.

We would like to express special gratitude to the late Kenneth E. Avis (University of Tennessee College of Pharmacy) for his dedication to teaching and sharing practical knowledge in the area of parenteral medications to so many students over the years, including us. Finally, we acknowledge the contributions of Dr Avis, Leon Lachman, and Herbert A. Lieberman who edited the earlier editions of this book series.

Sandeep Nema  
John D. Ludwig

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## 9 Formulation of protein- and peptide-based parenteral products

Gaozhong Zhu and Y. John Wang

### INTRODUCTION

Since the early 1970s, scientific advances in molecular biology and genetic engineering have led to enormous success in protein- and peptide-based therapeutics for the treatment of many human diseases. They cover almost all therapeutic categories, including cardiovascular hemostasis, antineoplastic, diabetes and endocrinology, anti-infective, neuropharmacological, enzyme replacement, wound healing, respiratory, and bone cartilage. Protein-based therapeutics are emerging as a major class of new molecular entities in the pharmaceutical industry. Over 200 biotechnology and pharmaceutical companies are developing protein-based therapeutics. More than 150 biologics are currently marketed, and over 400 are in advanced stages of testing and clinical trials (1).

Unlike small molecules, which are typically synthesized through chemical processes, proteins are produced in living systems. The main technology used to produce proteins utilizes recombinant DNA techniques to produce protein molecules in a host cell. Several types of host cells have been employed, including *Escherichia coli*, yeast, mammalian cells [e.g., Chinese hamster ovary (CHO) cells and human fibroblasts], and plant-derived cells. Several other technologies are also used to produce therapeutic proteins. Small proteins and peptides, such as calcitonin, may be produced by chemical synthesis. Most human serum albumin is sourced from human blood, urokinase from urine, and streptokinase from fungi. Recombinant human antithrombin (ATryn<sup>®</sup>), a new product approved by the FDA in 2009, is produced by transgenic animals.

### CHARACTERISTICS OF PROTEINS AND PEPTIDES

Compared with small-molecule drugs, protein-based pharmaceuticals are not only larger in molecular weight, but they also contain more complex compositions and higher order structures. Intrinsically, most proteins have poor stability and a very short half-life in vivo. Because of their poor oral bioavailability, most proteins require parenteral administration routes. In some cases, they require specific delivery systems targeting the specific site of action to achieve sufficient efficacy. Therefore, formulating these proteins as therapeutic agents with proper efficacy and safety profiles has been a challenging task. For successful product development, one needs to have a thorough understanding of the protein's physicochemical and biological characteristics, including stability, immunogenicity, and pharmacokinetic properties. The characterization of proteins is therefore an important step in formulation development.

### Molecular Composition, Structure, and Heterogeneity

A protein, or polypeptide, is formed through the linkage of peptide bonds of amino acids. Generally, protein structures are described at four levels: primary, secondary, tertiary, and quaternary. Details about these can be found in the preceding chapter of this volume.

Because of their complex manufacturing process, from cell culture to downstream purification, protein products generally contain multiple species in terms of molecular weight or size, which could be due to various modifications to the polypeptide side chains or glycans, reversible or irreversible formation of oligomers by either noncovalent or covalent linkages, and formation of large soluble and/or insoluble aggregates. It is important to characterize and quantify all species, as they may directly affect product efficacy, safety, and immunogenicity.

Depending on its size and the nature of its associations, several analytical techniques can be used to characterize a protein's size. Routinely, electrophoretic and chromatographic (with multiangle light-scattering detector) techniques have been used to estimate protein size up to

oligomers. By combining a denaturing electrophoretic technique (sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE]) with size-exclusion high-performance liquid chromatography (HPLC) or a native electrophoretic technique (Native PAGE), the size of proteins and the nature of their associations (covalent vs. noncovalent) in native and denatured states can also be estimated. To more accurately determine the size of proteins, mass spectrometry, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) or liquid chromatography mass spectrometry (LC-MS), is often used. However, because of the matrix effect and the high energy applied, the molecular weight or size determined by this technique may not be the true size in solution.

To measure the size of a protein in solution up to 100 nm, several biophysical techniques may be feasible, including analytical ultracentrifugation (AUC), field flow fractionation (FFF), and dynamic light scattering. It should be noted that the size distribution of proteins in solution, especially for reversible association, may be highly dependent on the solution properties, including pH, salt concentration, and protein concentration. Therefore, the mobile phase used in these analyses is preferably the same as the formulation vehicle, and the impact of the dilution factor during analysis should be assessed.

Insoluble aggregates or particles larger than 100  $\mu\text{m}$  can be observed by visual inspection with the unaided eye. Their size can be estimated by microscopy. Subvisible insoluble aggregates between 10 and 100  $\mu\text{m}$  in size can be quantified and sized either by a light obscuration test or by a microscopic particle count test per USP method <788>. It is still technically challenging to accurately quantify and size particles between 0.1 and 10  $\mu\text{m}$ . A technique using Micro-Flow<sup>TM</sup> imaging (MFI) has been used for particles as small as 0.75  $\mu\text{m}$  (2).

#### Isoelectric Point

Proteins that contain both positively and negatively charged amino acids are amphoteric molecules. One property that characterizes a protein's charge profile is its isoelectric point, or pI. The pI of a protein is the pH at which it carries no net electrical charge. At a pH below its pI, a protein is positively charged; above its pI, it is negatively charged.

The pI may be approximately calculated from the amino acid composition data, that is,  $\text{pI} = (\text{pK}_1 + \text{pK}_2 + \text{pK}_3 \dots + \text{pK}_n)/n$  for  $n$  ionizable groups. However, because the dielectric constant in the immediate vicinity of an ionizable group depends on protein structure, and because hydrogen bonding may alter dissociation constants ( $K_a$ ), the true pI can differ significantly from the calculated one. Several websites provide theoretical estimations of pI for proteins (e.g., <http://www.scripps.edu/~cdputnam/protcalc.html>, [http://www.expasy.ch/tools/pi\\_tool.html](http://www.expasy.ch/tools/pi_tool.html), and <http://www.nihilnovus.com/Palabra.html>).

Some proteins have multiple species with different charge profiles, and each species has its own pI, so these proteins appear to have more than one pI. Some glycoproteins in particular exhibit complicated pI patterns because of the heterogeneity in their glycan composition. Also, some proteins comprise multiple deamidation species, which also results in complicated charge profiles that could be characterized by several techniques, including isoelectric focusing (IEF), ion exchange chromatography (IEX), and capillary electrophoresis (CE).

Proteins show a broad range of pIs, mostly in the range of 2 to 12. The pI of a protein may play an important role in solubility and stability. In general, protein solubility is at its minimum when the pH is near its pI. Also, because zero net charge at pI should presumably allow maximum interaction between salt bridges and exert the least interaction between protein molecules, it could be expected to be the most stable condition for conformation. However, studies have shown that the optimal pH for conformational stability can be quite different from the pI and in many cases is found at a pH corresponding to a large net charge of the protein (3).

#### Solubility

The varieties of functional groups (charged, hydrophobic, etc.) on the side chain of amino acids and glycans (for glycoproteins) make protein solubility dependent on the pH, salt concentration, and polarity of the solvent. The overall size of the protein does not necessarily influence solubility. For example, antibodies, which have molecular weights of approximately 150 kDa, can often achieve aqueous solubility greater than 100 mg/mL.

The factors that determine a protein's solubility include its intrinsic properties and the composition of the solvent. The intrinsic properties are the composition of amino acids, the folded structure, and for glycoproteins, the composition and structure of glycans. Generally, a protein made of a large proportion of hydrophobic amino acids such as Phe, Tyr, and Trp will have low water solubility, and adding glycans increases water solubility. The solvent properties, including pH, salt concentration, and specific ligands, can also significantly affect the solubility. Protein solubility as a function of pH is typically in the shape of a U or V, where the minimum is at the pI. However, there are exceptions. The solubility of a protein at low ionic strength generally increases with the salt concentration, which is called the salting-in effect. As the salt concentration increases, the additional counter-ions shield the ionic charge and thereby increase the protein solubility. As salt concentration continues to increase, protein solubility decreases (the salting-out effect). At high salt concentration, the salts begin to compete with the ionic moieties of the protein for the solvation of the polar solvent, which results in decreasing solubility. A specific ligand or stabilizer that binds to the protein may also influence solubility. For example, increased solubility of fibroblast growth factor was observed in the presence of heparin or heparin-like substances (5). Also, alteplase solubility was increased by the addition of arginine (6). However, one needs to assess whether the ligand or excipient is acceptable for the intended clinical use before adding it into the final formulation.

**Thermal transition important.** Because native proteins exhibit folded structure in solution, they can undergo transition from native form to unfolded or denatured form with increasing temperature. The thermal transition midpoint ( $T_m$ ), defined as the temperature at which equal amounts of native and denatured forms exist in equilibrium, is an important characteristic of proteins, measuring their thermal stability. Generally, a higher  $T_m$  value indicates better thermal stability.

Measurement of  $T_m$  has been widely used in preformulation and formulation development. The profile of  $T_m$  as a function of pH provides important information in selecting the optimal pH for formulation. This method has also been used in screening different stabilizers, as an excipient that elevates  $T_m$  is expected to be a potential stabilizer (7). However, it should be noted that in choosing the formulation, one also needs to consider other information, as  $T_m$  alone is only indicative of thermal stability.

Proteins in solid state also exhibit thermal transitions upon heating. These are typically determined by DSC. However, it is difficult to measure the true thermal transitions of solid protein, because in most cases other components present in the solid dosage form also contribute to the overall thermal transition. Recently, glass transition temperatures ( $T_g$ ) for proteins have been estimated by extrapolating excipient concentration to zero using  $T_g$  values measured at a very fast scanning rate in binary mixtures of protein and another glass form excipient, such as sucrose, over a range of excipient concentrations (8).

**Instability: Key Degradation Pathways**

The structural complexity of proteins makes them susceptible to processing and handling conditions that can result in structural and functional modifications. A protein can undergo a variety of covalent and noncovalent reactions or modifications, which may be generally classified into two main categories: (i) physical or non-covalent bond degradation pathways and (ii) chemical or covalent bond degradation pathways. Common physical degradation pathways include denaturation or unfolding, adsorption, and aggregation due to noncovalent forces. Chemical degradation pathways include covalent-bonded aggregation, disulfide exchange, deamidation, isomerization, racemization, fragmentation, oxidation,  $\beta$ -elimination, Maillard reaction, diketopiperazine formation, and so on. Oftentimes, physical degradation facilitates chemical degradation, and vice versa. The fundamentals of these degradation pathways have been extensively described in several review articles and book chapters (9-14). A brief description of each degradation pathway, the factors responsible for degradation in some proteins, and remedies are presented below.

**Denaturation**

Denaturation is the process of altering protein structure (i.e., secondary, tertiary, or quaternary structures) from its native folded state. Denaturation may result in an unfolded state, which could further facilitate other physical and chemical degradations. Because a specific structure is required for proteins to exert physiological and pharmacological activities, denaturation causes loss of efficacy and incurs the risk of safety such as immunogenicity.

Many times, the denaturation process can be described as  $N \leftrightarrow I \leftrightarrow D$ . The folded native structure (N) unwinds and passes through a partially unfolded or intermediate state (I) to a denatured state (D). This process may be reversible or irreversible, depending on conditions. For reversible denaturation, the unfolded protein will regain its native state once the denaturing condition is removed.

Many factors can cause denaturation, including heat, freezing, extreme pHs, organic solvents, high salt concentration, lyophilization, surface adsorption, and mechanical stress. These denaturing conditions disrupt a protein's higher order structure, which is held together by intramolecular forces including hydrogen bonding, salt bridges or electrostatic forces, hydrophobic interactions, and van der Waals forces.

Hydrogen bonds are critical in determining overall protein conformation, since they are the major forces that stabilize the secondary  $\alpha$ -helices and  $\beta$ -sheets, as well as the overall folded structure. Water, the nearly ubiquitous medium for proteins, contributes to this hydrogen bonding. Cosolvents such as ethanol and acetone and chaotropic agents such as urea and guanidine hydrochloride disrupt the hydrogen bonds and thus readily denature proteins.

The ionic side chains of aspartic acid, glutamic acid, lysine, arginine, and histidine, normally found on the surface of the protein, contribute to the stability of the native conformation by forming salt bridges. The pH of the solvent will determine the charge of the side chains on these amino acids and the extent of ionic bonding. Therefore, an extreme pH shift can disrupt these salt bridges and lead to denaturation. Furthermore, organic solvents will reduce dielectric constant and increase ionic forces or salt bridges, so inappropriate exposure to organic solvents can also result in denaturation.

Because hydrophobic side chains (i.e., phenyl, indole, and hydrocarbon chains) are usually tucked inside the protein's globular structure, significant stabilizing effects result from their hydrophobic interactions. These interactions, too, are sensitive to the effects of solvents. Disruption of hydrophobic interactions is also considered the mechanism of denaturation by surfactant, extreme temperature, and mechanical stress, all of which commonly occur during manufacturing processes.

**Adsorption**

Proteins are amphiphilic polyelectrolytes, so they tend to adsorb at liquid-solid, liquid-gas, and liquid-liquid interfaces. When adsorption of proteins occurs, the molecules exchange their interactions with the solvent and other solutes for interactions with the surface. Two mechanisms are primarily responsible for protein adsorption. One mechanism is charge-charge

or electrostatic interaction. For example, salmon calcitonin, as a positively charged protein, strongly binds to the negative potential of a glass surface through electrostatic interaction (15). The other mechanism is hydrophobic interaction. One example is bovine serum albumin, which near its isoelectric point has shown the highest affinity to the hydrophobic surface of polystyrene through hydrophobic interactions (16). Other interactions, including charge-dipole, dipole-dipole, and van der Waals forces, may also contribute to the adsorption.

These interactions may lead to altered structures, including secondary, tertiary, and quaternary structures, which could further facilitate other physical and chemical degradation, including aggregation and covalently bonded modification. Therefore, depending on the nature of the protein and of the contact surface, interfacial adsorption can significantly impact a protein drug's potency, stability, and safety, particularly in a low-concentration dosage form.

The key strategy to minimize or inhibit protein adsorption is either to adjust formulation parameters or to modify or avoid certain contact surfaces. The formulation parameters that potentially control adsorption include protein concentration, pH, ionic strength, and addition of specific excipients such as surfactants or albumin. For example, modification of the contact surface of siliconized vials has minimized interferon adsorption on the glass surface (17). When these approaches do not prevent significant adsorption, alternative contact surfaces should be considered during process or storage. In some cases, when the level of adsorption can be predicted, overage is required in the vials.

#### *Aggregation by Noncovalent Linkage*

Non-covalently linked aggregation often results from some degree of denaturation of proteins, since unfolding leads to the exposure of hydrophobic moieties previously buried in the protein interior, which is followed by the association of unfolded molecules via noncovalent interactions to form aggregates. Non-covalently linked aggregation can be a reversible or an irreversible process, depending on conditions.

Reversible aggregation is highly dependent on protein concentration, pH, salt concentration, and other formulation components. Generally, proteins tend to form high-molecular weight species (HMWS) at high protein concentration. Upon dilution, these HMWS or oligomers may dissociate into monomers or dimers. This self-association phenomenon may be characterized by AUC (18) or by static light scattering (19).

Irreversible aggregates can be soluble or insoluble, depending on the size and nature of the molecules. Generally, these aggregates can be induced by single or multiple stress conditions, including heat, extreme pH, mechanical pumping, high pressure, shaking or agitation, freezing, and freeze-drying. For example, acidic pH and a temperature of 37°C have resulted in irreversible aggregation of albumin (20).

To minimize aggregation, besides tight control of the process parameters, adjusting formulation parameters, such as adding sucrose, should be assessed. Sucrose and other polyols maintain protein molecules in a native compact form, so as to be resistant to external stress.

#### *Aggregation by Covalent Linkage*

The most commonly observed protein aggregation by covalent linkage occurs through intermolecular disulfide linkage, also called disulfide bond formation and scrambling. This intermolecular aggregation may occur to any protein containing cysteine or cystine.

Generally, proteins with a free thiol group tend to form aggregates more easily through disulfide bonds, especially when the free thiol group is solvent-exposed on the surface of the protein. Free thiol groups buried within the tertiary structure are less reactive. The formation of disulfide bonds in protein aggregates with free thiol groups can take place either through the two free thiol groups available on the surface of each of two protein molecules, or through thiol-disulfide exchange, whereby a reactive thiol group in one molecule attacks an existing disulfide bond in another molecule to form a new disulfide bond between the two molecules.

Proteins without free thiol groups may still form aggregates by disulfide bond scrambling through intermolecular disulfide exchange, especially in alkaline conditions. A cystine or disulfide bond in one molecule can be reduced into two free cysteines, which can react with cysteine or cystine in another molecule to form a new disulfide bond.

Aggregates formed through disulfide bonds may, through multiple disulfide scrambling reactions, result in high-molecular-weight aggregates, which could eventually precipitate from solution. In addition, the formation of a new disulfide bond may change the native conformation to a denatured form, which could further aggregate through noncovalent hydrophobic interactions due to exposed hydrophobic residues.

In neutral or alkaline pH conditions, disulfide-bonded aggregation generally becomes more severe as the thiol group becomes more reactive. However, extremely acidic pH may also cause disulfide-bonded aggregation (21).

To prevent or minimize disulfide-bonded aggregates, the main formulation parameter is pH. A slightly lower pH (e.g., pH 5) may significantly reduce aggregation. The addition of reducing agents such as cysteine, or of stabilizers that alter conformation such that free cysteine or reactive cystine becomes more buried into tertiary structure, may also minimize aggregation. For proteins with severe disulfide aggregation, lyophilized formulation should be considered, as reactivity in the solid state is reduced significantly.

Nonreducible aggregates through nondisulfide linkages have also been reported. The reactions involving these covalent linkages include (i) oxidation-induced reactions through Trp or Tyr linkage (22); (ii) reaction through transamidation, whereby an amino group of amino acids (e.g., lysine residue or N-terminal of a protein) in one molecule forms an isopeptide bond with the carbonyl group of either Asn or Gln in another molecule [examples are insulin (23) and lyophilized ribonuclease A (24); and (iii) reaction through a reactive dehydroalanine generated from  $\beta$ -elimination at alkaline pH, which forms nonreducible cross linkages with other amino acids such as Tyr, Lys, His, Arg, and Cys.

#### *Intramolecular Disulfide Exchange*

Disulfide exchange can also take place within a protein molecule when a cystine (disulfide) bond is reduced into two cysteines; one of the cysteine residues then reacts with another cysteine to form a new disulfide bond. Improper linkages of disulfide bonds were responsible for a reduction in biological activity of interleukin-2 (IL-2) (25). There are three cysteines in IL-2 at positions 58, 105, and 125. The native protein forms a disulfide linkage between the two cysteines at 58 and 105. The cleavage of this disulfide in IL-2 and the subsequent formation of two less active isomers with disulfide bonds at incorrect positions (Cys<sup>58</sup>-Cys<sup>125</sup> and Cys<sup>105</sup>-Cys<sup>125</sup>) are promoted by high pH and copper ions (25). Intramolecular disulfide exchange has also been reported for monoclonal antibodies.

To minimize this type of degradation, it is important to select a low formulation pH and minimize any impurities, such as peroxides or heavy metals, known to promote redox reactions.

#### *Deamidation/Isomerization*

Deamidation refers to the removal of ammonia from the amide (RCONH<sub>2</sub>) moiety of an Asn or Gln side chain, resulting in a carboxylic acid. Deamidation is a major cause of instability of proteins and peptides in aqueous solution. In lyophilized solid state, the deamidation rate is slower than in solution.

Deamidation occurs through different pathways at different pH levels. In an acidic pH of 2 to 5, deamidation occurs by direct hydrolysis, which causes Asn or Gln residue to change into Asp or Glu residue, respectively. The type of neighboring amino acids does not affect the deamidation rate. Hydrolytic mechanisms in neutral or alkaline pHs are more complex, however. Under these conditions, the side chain carbonyl group on the Asn or Gln residue reacts with the nitrogen atom on the peptide backbone to form a cyclic imide (succinimide) intermediate (Asu). Depending on which bond in the cyclic imide breaks, the reaction product can be (i) the des-amido peptide (Asp), (ii) the isopeptide (IsoAsp), or (iii) D-isomers. The formation of isopeptides is called isomerization, or sometimes referred to as transpeptidation, because an extra methylene group is inserted to the peptide backbone. When deamidation occurs, the IsoAsp to Asp ratio is typically 3. Detailed descriptions of deamidation and isomerization can be found in a review by Wakankar and Borchardt (26).

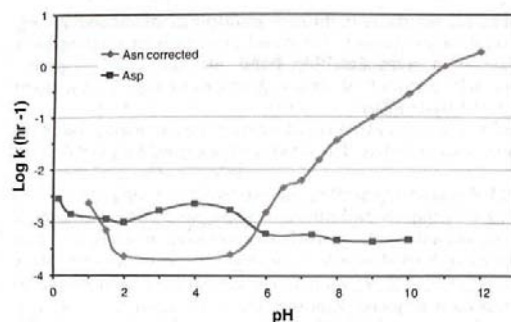


**Figure 1** Comparison of Asn deamidation (diamond) and Asp dehydration (square) rates in a model peptide (Val-Tyr-Pro-Asx-Gly-Ala) at 37°C. Dehydration is the rate-limiting step for isomerization. Source: From Refs. 104 and 105.

At neutral to alkaline pHs, the rate of deamidation is significantly affected by the size of the amino acid on the C-terminal side of the Asn or Gln residue. In general, Asn is more labile than Gln and is most labile when adjacent to glycine, which is least obstructive to the formation of a cyclic imide. Since Asn-Gly is most susceptible to deamidation, protein engineers make significant efforts to avoid constructing a protein drug candidate with such a hot spot.

The deamidation rate profile as a function of pH is V-shaped, usually with a minimum rate at a pH of about 4 to 5 (Fig. 1). In a number of synthetic peptides, the half-life of deamidation reactions of Asn residues at 37°C in pH 7.2 phosphate buffer ranges from two days to nine years (27). Not all Asn residues are equally labile; those buried within the interior portion of a protein are inaccessible to water and thus less reactive. Secondary and tertiary structures play an important role in determining the site and the rate of deamidation. In insulin, for example, there are three asparagines. At acidic pH, the prevailing deamidated species was monodesamido-(A21)-insulin. At neutral pH, deamidation occurred to Asn at the B3 position (28). For growth hormone containing nine asparagines, deamidation occurred primarily at the Asn-149 position (29). These proteins and others—pramlintide (30), epidermal growth factor (31), IgG (32), IgG1 (33)—represent a small fraction of proteins that have shown deamidation.

Isomerization at Asp goes through the same cyclic imide intermediate, Asu. Because of the effect of pH on the leaving group (-OH), the rate-pH profile is significantly different from that of deamidation of Asn. Examples of protein drugs that undergo IsoAsp formation include insulin aspart (34), hirudin (35), and porcine somatotropin (36). On the basis of the study of two monoclonal antibodies (37), the degradants from isomerization were detected by hydrophobic interaction chromatography.

The best way to avoid deamidation and isomerization is to mutate Asn-Gly or Asp-Gly sequence in the solvent-exposed region of the protein, if the mutation at these sites does not affect the biological activity. Otherwise, an appropriate pH (in the range of 5–6) should be selected to minimize the degradation rate. As the formation of cyclic imide intermediates does not depend on water, these reactions may occur even in anhydrous conditions such as 100% dimethyl sulfoxide (DMSO) (38).

#### Racemization

The racemization reaction is catalyzed by both acid and base. Racemization of peptides and proteins results in the formation of diastereomers. Racemization under basic conditions is hypothesized to proceed by abstraction of the  $\alpha$ -proton from an amino acid in a peptide to yield a negatively charged planar carbanion. A proton can then be returned to this optically inactive intermediate, thus producing a mixture of D- and L-enantiomers for the individual amino acid. Since a peptide is composed of multiple chiral centers, the product formed is a diastereomer. Racemization is biologically significant because a peptide composed of D-amino acids is generally metabolized much more slowly than a naturally occurring peptide made

only of L-amino acids. For this reason, many new synthetic peptides, both agonists and antagonists, incorporate D-amino acids. A pH dependency for racemization was demonstrated in an aqueous degradation study of a decapeptide, RS-26306 (39), which found that at neutral and alkaline pHs, racemization contributed to more degradation than did deamidation.

#### Fragmentation

The peptide bond ( $\text{RNH}-\text{CO}-\text{R}$ ) can undergo hydrolysis, resulting in peptide fragments. Generally, most peptide bonds are relatively stable. For example, oxytocin injection was reported to be stable at room temperature for five years (40). Protirelin, a tripeptide (PyrGlu-His-Pro), is stable for 20 hours at  $80^\circ\text{C}$  at both pH 3.3 and pH 6 (41).

The formulation factor that most influences the hydrolytic rate is solution pH. The rate of hydrolysis is in direct proportion to the activity of hydronium or hydroxide ions, when in acidic or alkaline pHs, respectively. Generally, the reaction becomes much faster in either extremely acidic or extremely alkaline conditions. Fragmentation of therapeutic peptides, including nafarelin (42), secretin (43), captopril, (44), and urokinase (45), has been reported in various pH conditions.

The type of neighboring amino acids also affects the susceptibility of the linkage to fragmentation. For example, the bond between aspartic acid and proline is sensitive to acid hydrolysis. A cleavage at Asp-Pro was found in basic fibroblast growth factor (bFGF) (46) and macrophage colony-stimulating factor (47). The resultant products are peptides with aspartic acid at the C-terminus. The C-terminal peptide bond adjacent to serine is also a reactive one, because of the neighboring-group effect of the alcohol on serine (48).

Another example of fragmentation is the cleavage at hinge regions of antibodies resulting in a Fab fragment. The hinge region in IgG1 heavy chain comprises about eight amino acids, including two cysteines responsible for the two disulfide linkages between the two heavy chains. Cleavage takes place at multiple sites, with the majority between Asp and Lys and between His and Thr (49). The extreme flexibility at the hinge region and the solvent exposure are the driving forces for such cleavage. The rate of hydrolysis at hinge region is minimal at pH 6 (50).

Besides chemically induced fragmentation, protein hydrolysis can also be mediated by some residual proteases remaining from production. The cleavage site in this case is dependent on the type of proteases present. As the proteolytic activity of proteases is typically pH dependent, the degradation rate is also dependent on solution pH. For example, a cathepsin D protease derived from CHO was identified as being responsible for degradation of an Fc-fusion recombinant protein. This protease belongs to an aspartic protease family and is preferentially active at acidic pH (51).

#### Oxidation

Several amino acid residues including Cys, Met, Trp, His, and Tyr are potential sites of oxidation. These residues can be oxidized by atmospheric oxygen or by peroxide impurities from a number of raw materials including formulation excipients such as polysorbates. Oxidation can also be induced by exposure to light or catalyzed by transition metal ions such as  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$ . The most commonly observed oxidations in therapeutic peptides and proteins are described below.

**Oxidation of cysteine.** Under neutral or basic conditions, the free thiol ( $-\text{SH}$ ) group of a cysteine is the most reactive moiety of all amino acid components. The disulfide ( $-\text{S}-\text{S}-$ ) bond formed from the oxidation of two thiol groups results in significant changes in conformation both intramolecularly and intermolecularly.

Oxidation of the thiol group is promoted at both neutral and basic pH. The rate-pH profile for captopril, a quasi-dipeptide, shows an increase in oxidation rate starting at pH 5 (44). This reaction can also be catalyzed by heavy metals. For example, this reaction was effectively retarded by the addition of a metal chelating agent such as EDTA in FGF formulation (5).

**Oxidation of methionine.** The thioether ( $-\text{CH}_2-\text{S}-\text{CH}_3$ ) moiety on methionine is susceptible to oxidation to form sulfoxide ( $-\text{CH}_2-\text{SO}-\text{CH}_3$ ) derivatives. The susceptibility of methionine to

oxidation is highly dependent on its site in a protein. For example, of the three methionines in human growth hormone, Met-125 is most reactive, Met-14 is less, and Met-170 is not reactive at all (52). The reactive methionine is likely to be the one exposed on a protein surface, and the unreactive one buried within the core. Air in the headspace of formulated and freeze-dried growth hormone can cause 40% of the growth hormone molecules to be oxidized during a six-month storage period (53). Examples of other pharmaceutical proteins exhibiting Met oxidation are listed below.

- Interleukin-2 (54)
- Murine antibody (Orthoclone OKT3) (55)
- Herceptin<sup>®</sup> (trastuzumab) (56)
- Recombinant interferon  $\gamma$  (Actimmune) and recombinant tissue plasminogen activator (alteplase, Activase<sup>®</sup>) (57)
- Recombinant human granulocyte colony-stimulating factor (58,59)
- Parathyroid hormone (60)

As methionine can be easily oxidized by atmospheric oxygen, replacing oxygen with nitrogen or argon during manufacturing or in the headspace of the final product container is a common practice to minimize oxidation. In addition, adding free methionine as an antioxidant is also an effective approach to reducing oxidation (61).

**Oxidation of tryptophan.** Oxidation of tryptophan can generate multiple oxidized species. Stability studies of Trp amino acid alone in aqueous solution (62) and Trp residues in small peptides and lysozymes (63) and in bovine  $\alpha$ -crystallin (64) clearly identified the main degradants as 5-hydroxy-Trp, oxy-indole alanine, kynurenine, and *N*-formylkynurenine. There are very few articles reporting the oxidation of Trp in therapeutic proteins. Davies et al. reported oxidized bovine serum albumin with oxygen radicals generated from cobalt radiation (65); Trp oxidation in monoclonal antibodies was recently reported by Yang et al. (66) and Wei et al. (67), and ozone and UV irradiation were used as stress conditions in these studies. That Trp oxidation has not been studied in depth is perhaps due to the fact that no model oxidizing condition has been adopted, and a system that promotes Trp oxidation is not easy to reproduce. In the case of Met oxidation, tert-butyl hydroperoxide (tBHP) and  $H_2O_2$  are commonly used reagents to generate oxidized Met species. Most recently, a free radical generator, AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) was found to effectively oxidize tryptophan in peptides or large proteins (68). By using AAPH as an oxidation stressing agent, one can predict the vulnerability of a protein or the specific Trp residue in a protein.

As Trp oxidation appears to be mediated through oxygen radicals because of light exposure or peroxide residues from excipients, it is important for mitigation of Trp oxidation to limit direct light exposure and to use high-quality excipients.

#### *$\beta$ -Elimination*

The disulfide bond between two peptide chains can be cleaved disproportionally, catalyzed by hydroxide ions to produce dehydroalanine and thiocysteine through the mechanism of  $\beta$ -elimination. The thiolate ion ( $HS^-$ ) is very reactive and will continue to react with other disulfide bonds, causing chain reactions. This is one of the operative mechanisms for the covalent aggregates in solid phase for bovine serum albumin (69) and ANP, a 25-amino acid peptide (70).  $\beta$ -elimination has also been attributed to the fragmentation of hinge regions in antibodies (71).

#### *Maillard Reaction*

The first substantial investigation of the reaction of reducing sugars with amino acids was carried out about 75 years ago by Maillard. The chemical reactions involved are, first, the reversible formation of a Schiff base between the aldehydic function group of reducing sugars (e.g., glucose) and the amino group of lysine residues in proteins, followed by a relatively

slow, but essentially irreversible, Amadori rearrangement, with the formation of ketoamines, which forms the hemiketal structure. As the Schiff base may also be involved in cross-linking, this reaction commonly leads to nonenzymatic browning, also called a browning reaction. This reaction occurs most readily in neutral to weakly alkaline conditions.

In protein formulation, reducing sugars such as glucose, maltose, fructose, and lactose should be avoided to prevent potential Maillard reactions. In addition, one needs to be concerned with acidic pH, which may cause hydrolysis of sucrose into glucose and fructose, leading to glycation of the protein. One example is freeze-dried bFGF. When the cake collapsed at elevated temperature, the acidity from its buffer, citric acid, caused hydrolysis of sucrose, which resulted in glycation of the protein with glucose (46).

#### *Diketopiperazine Formation*

Rearrangement of the N-terminal dipeptide results in the splitting off of a cyclic diketopiperazine at high pH. Proline and glycine in the N-terminal promote the reaction. Aspartame degrades through a similar mechanism (72), with the cyclization of aspartame minimal at a pH of 6 to 7, moderate at 7 to 8.5, and rapid above 8.5. Diketopiperazine formation has also been reported in the case of vascular endothelial growth factor (73).

### PHARMACEUTICAL DEVELOPMENT PRINCIPLES

Because of their poor permeability to epithelium cell membranes and instability in gastrointestinal tract, proteins and peptides have very poor oral bioavailability and are therefore primarily formulated as injectable or parenteral products for intravenous (IV) infusion or subcutaneous (SC), intramuscular (IM), or intrathecal (IT) administration. Some proteins are also delivered through noninvasive administration routes, such as nasal and pulmonary formulations, to improve patient compliance. For local therapeutic effect, proteins such as growth factors are formulated for topical application and bone matrices.

Most protein products are in aqueous solution or in solid, freeze-dried form. Some are also formulated as suspensions in a crystalline form or in other lipid- or polymer-based delivery systems such as microspheres, liposomes, and nanoparticles. Rarely, some proteins, such as bovine growth hormone, are formulated in oleaginous vehicle.

The formulation development process for products that involve lipids or polymers as carriers is more complicated than the process for simple conventional dosage forms of liquid and lyophilized formulations. The principles described below focus mainly on the development process for the conventional dosage forms, some of which have also been discussed in several book chapters and review articles (74–79).

#### **General Formulation Development Process in Industry**

Generally, the manufacturing process for most protein products consists of two steps. One is the manufacture of drug substance, *aka* the active pharmaceutical ingredient (API), which is typically stored in bulk containers, such as plastic or stainless steel containers, in frozen condition for long-term storage purposes. The other step is the manufacture of the final drug product from drug substance. This is the final presentation to patients and healthcare professionals, typically stored in glass vials, prefilled syringes, or injection devices, at refrigerated condition for convenient use and distribution.

Formulations for drug substance and drug product may not be the same. Typically, the drug substance formulation is developed earlier, as it is the first step of the manufacturing process. However, drug substance should ideally be in a formulation vehicle that can be readily further formulated into drug product. Therefore, it is important to develop the drug product formulation as early as possible, so that the manufacturing process from drug substance to drug product will be harmonized and straightforward.

Prior to formulation development, it is important to conduct preformulation development activities, which serve to identify the key degradation pathways of the molecule and to develop stability-indicating assays to support formulation development studies. In general, preformulation activities should evaluate the biophysical and biochemical properties of the molecule under pharmaceutically relevant stress conditions, which include chemical-related

factors (such as extremes of pH, salts, buffers, oxygen, and peroxides) and process-related factors (such as high temperature, freezing, thawing, light, agitation, pressure, and shear stress). In these studies, biophysical characterization assays such as UV-visible spectroscopy, DSC, CD, Fourier transform infrared spectroscopy (FT-IR), AUC, and fluorescence spectroscopy are often used to determine which conditions result in the highest thermal stability and the least conformational changes. Biochemical methods such as size-exclusion HPLC, reversed-phase HPLC, hydrophobic interaction chromatography, capillary electrophoresis, peptide map, gel electrophoresis, ion-exchange HPLC, and potency assays are used to identify the key chemical degradation pathways, primarily through covalent bond changes, under these stress conditions.

Results from preformulation studies should identify the potential sources of the instability of the molecules and the key stability-indicating assays to monitor the degradation products, as these are needed for formulation screening and optimization studies. Once the leading formulation(s) are identified from the screening studies, appropriate container closure for final product presentation should be evaluated. In addition, the manufacturing process should be developed.

The choice of formulation, dosage form, and final product presentation is dependent on several factors. These choices not only need to offer the best stability and safety profiles, but they must also be easy to scale up for manufacturing and convenient to use during administration. The decisions are also dependent on the development stage of the product. At preclinical and early clinical stages, the goal of the project is to evaluate the proof of concept in product efficacy and safety as early as possible, so the shelf-life of early clinical products may only need to be 12 months or even less, and the storage or shipping conditions may not require the refrigerated or ambient temperature that is typically used for commercial products. Therefore, frozen or lyophilized formulations are typically chosen for preclinical and early clinical studies. At late clinical and commercial stages, there are more stringent requirements. At these stages, not only stability but also cost and marketing competitiveness are very important in making the final choice of the formulation. A very critical factor in a field of many competing, similar biotech products is patient compliance and acceptance, so prefilled syringes and auto-injection devices are in many cases essential for the success of the product's introduction to the market. As a result, evaluation of these dosage forms and devices must be initiated during phase III or pivotal clinical trials.

Overall, formulation development is a critical and evolving process step in product development. It is important to initiate the development effort as early as possible. It is also important to have input from multiple functional areas before finalizing the formulation choice.

#### **Evaluation of Critical Formulation Parameters**

To prepare a stable formulation suitable for patient use, the critical formulation parameters should be evaluated, including protein concentration, pH, buffers, stabilizers, tonicity modifiers, bulk agents (for lyophilized products only), and preservatives (for multidose products only). A discussion of evaluating each formulation parameter follows.

##### *Protein Concentration*

Protein concentration not only serves as a critical parameter in finalizing product presentation, but is also a key parameter in product stability. The following considerations are important in the selection of a viable protein concentration in a formulation:

- It should be below the protein's solubility in the selected formulation vehicle. Since protein solubility varies in different formulation vehicles and at different temperatures, the selected protein concentration should be below the "true" or thermodynamic solubility in the vehicle at the intended long-term storage temperature. However, measurement of true protein solubility is challenging, so this is an empirical exercise where real-time data needs to be evaluated on potential precipitation of the protein at the selected concentration and stored for the desired duration. This

assessment should be made for both drug substance and drug product before a protein concentration is finalized.

- It should maintain the protein's stability during long-term storage. Generally, proteins at high concentrations may lead to aggregation and precipitation. On the other hand, low protein concentrations may lead to significant loss of protein content due to adsorption onto various product-contacting surfaces (e.g., container closures during storage, filter membranes and silicone tubing during processing). Also, a higher relative ratio of any reactive impurities (such as traces of heavy metals, peroxides from surfactants, and sterilants used in the aseptic filling process) to the protein could cause degradation.
- It should be operable under manufacturing conditions. High protein concentrations achieved at lab-scale may not be operable at manufacturing scale. For example, a formulation with a high protein concentration may have high viscosity, requiring an (UFDF) step. On the other hand, at low concentrations there may be significant protein loss from the filtration membrane due to the larger surface area at manufacturing scale. Therefore, it is critical to assess the scalability of the manufacturing process before choosing a protein concentration.
- It should minimize product waste during the manufacturing process, testing, and clinical use. Material losses during manufacturing (e.g., line loss), sample testing, and dose preparation for clinical use (e.g., residual in containers) are all volume based, so a high protein concentration will result in a large amount of protein waste. Therefore, the lowest protein concentration that delivers the required dose and maximizes production yield should be identified.
- It should meet the requirements for product presentation. Product presentation is selected on the basis of factors such as dose (size and frequency), administration route, convenience of dose preparation, patient weight distribution (for weight-based dosing schemes), number of manufacturing lots desired or manageable per year, and commercial considerations of cost and product differentiation. From these analyses, the amount of protein per vial is specified, and subsequently, the optimal volume and corresponding protein concentration is determined on the basis of factors such as solubility, stability, minimal protein waste, and manufacturability.
- It should take into account manufacturing process cycle time and cost. For lyophilized products, the major contributor to manufacturing cost is the lyophilization time. Reducing the fill volume by increasing protein concentration can significantly reduce the lyophilization time.

In summary, protein concentration, as a critical formulation parameter, should be chosen on the basis of multiple factors, including solubility, stability, manufacturability, cost, product presentation, and commercial considerations.

#### pH

As proteins containing both positively and negatively charged amino acids are amphoteric molecules, solution pH has a direct impact on the overall stability of proteins. When solution pH is far from the pI of the protein, electrostatic repulsions between like charges in the protein increase, causing a tendency to unfold. In addition to electrostatic interactions, pH also affects other interactions within proteins. Therefore, changing pH directly affects the conformational stability of proteins and their solubility in aqueous solution. In addition, since certain chemical reactions are highly pH dependent, solution pH also affects the chemical stability of proteins. The following areas should be assessed when selecting an optimal pH for a protein formulation:

- Profiles of conformational stability, chemical stability, and solubility as functions of pH in solution may not overlap each other for a given protein. The pH at maximum solubility may not be the same as the pH at maximum conformational stability or

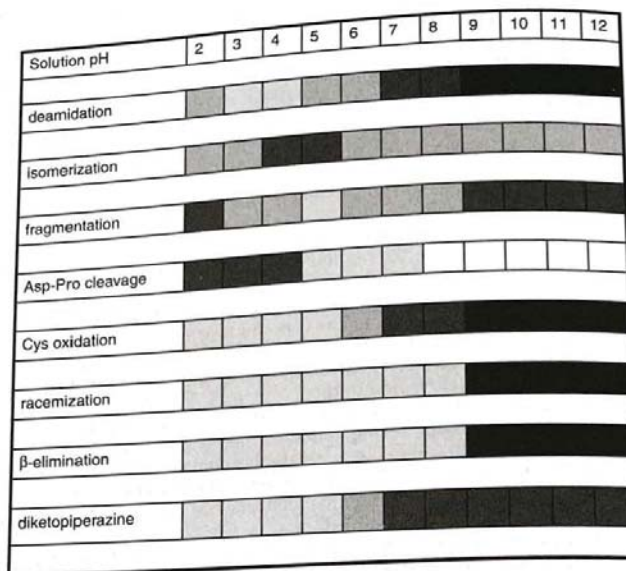


Figure 2 Reaction rates as a function of pH. (Darker color indicates a faster reaction rate at designated pH range.)

chemical stability. It is important to define an optimal pH that is a good compromise among all these characteristics.

- As chemical stability involves several different degradation pathways and each degradation pathway may have a different stability profile as a function of pH in solution (Fig. 2), it is important to balance all the degradation profiles before finalizing the optimal pH for the formulation. The degradation pathways that lead to significant loss in potency or biological activity or that cause immunoreactivity should be kept to a minimum when selecting the pH.
- The optimal pH selected in solution may not be optimal for proteins in solid dosage forms, so it is important to reassess the effect of pH when the protein dosage form changes from liquid to lyophilized form. For example, opposite trends in pH-dependent stability were observed for lyophilized and liquid formulations (80).
- The selected pH should not have any impact on the stability of other excipients in the formulation. Certain excipients may not be stable in a certain pH range under long-term storage conditions. For example, acidic pH has caused the hydrolysis of sucrose, a commonly used stabilizer in protein formulations (81,82).
- The selected pH should be compatible with product-contacting surfaces during manufacturing and storage. It has been reported that acidic pH caused corrosion of stainless steel in the presence of chloride ions, which generated iron ions that catalyzed methionine oxidation in a monoclonal antibody (83). Also, high or low pH may cause more leachables from stoppers, which serve as primary container closure.
- The selected pH needs to be safe to use for its intended administration route. A certain pH range may be suitable for IV injection but cause side effects when used for SC, IM or IT injection.

For most protein formulations, the selected pH is in the range of 6 to 7, as this range is close to the physiological pH and also provides the optimal stability for most proteins. For

peptides, formulation pH is mostly in the acidic range of 4 to 5, as this pH range provides better aqueous solubility and less adsorption.

**Buffers**  
Buffers are often used to control the formulation pH, keeping it within a narrow range to prevent small changes that can affect the stability and solubility of proteins. The selection of a proper buffer type and concentration for proteins should be based on the following considerations:

- The buffer species and concentration should not cause protein instability. For example, sodium phosphate buffer may result in a significant pH drop upon freezing, which has been found to cause instability of some proteins, particularly at high buffer concentration or low protein concentration conditions (84).
- The buffer species should have a  $pK_a$  near the target pH, preferably within one pH unit. Table 1 lists the pH control ranges for some commonly used buffer species.
- When buffer concentration has no effect on the protein stability, it should be kept to a minimum, but high enough to provide sufficient buffering capacity to control the formulation pH. High buffer concentration may cause some pain or discomfort upon SC or IM injection. In addition, for lyophilized product, a high concentration of buffer species such as sodium phosphate or citrate may lead to a lower glass transition temperature, which would require a longer lyophilization cycle.
- Buffers, like salts of ionic compounds, contribute to the overall ionic strength of the formulation solution. Therefore, buffer concentration also influences other properties that are dependent on ionic strength, such as protein solubility and stability.
- Certain buffer species, besides controlling solution pH, may also serve as a stabilizing agent in some protein formulations. For example, citrate may serve as a chelating agent to remove any heavy metals that potentially catalyze oxidation. Histidine also has an antioxidant effect (85).
- The buffer species and concentration should be safe to use for its intended administration route. Some buffer species and concentrations may be suitable for IV injection but may not be compatible when used for SC, IM, or IT injections. For example, citrate was found to cause more pain than histidine as a buffer when administered by SC injection (86).

It should be noted that as zwitterions, proteins have their own buffering capacity, especially at high concentration, so a buffer may not be required in formulations if the pH can be maintained by the protein itself. It has been reported that monoclonal antibodies at 60 mg/mL have a strong self-buffering capacity and that the long-term stability of self-buffered formulations is comparable to that of conventionally buffered formulations (87).

#### Ionic Strength/Salt Concentration

As proteins carry both negatively and positively charged groups, ionic strength in formulation solution may directly affect the solubility and stability. Ionic strength in parenteral formulation

Table 1 Buffers for Protein Formulations

| Buffer    | Acid                 | Base               | pH range control  | Examples             |
|-----------|----------------------|--------------------|-------------------|----------------------|
| Phosphate | Monosodium phosphate | Disodium phosphate | 5.8–7.8           | Elaprase®, Remicade® |
| Acetate   | Acetic acid          | Sodium acetate     | 3.8–5.8           | Avonex®, Neupogen®   |
| Citrate   | Citric acid          | Sodium citrate     | 3.0–7.4           | Amevive®, Rituxan®   |
| Succinate | Succinic acid        | Sodium succinate   | 3.3–6.6           | Actimmune®           |
| TRIS      | TRIS HCl             | TRIS               | 7.1–9.1           | Wellferon®, Enbrel®  |
| Histidine | Histidine HCl        | Histidine          | 5.1–7.0           | Xolair®, Raptiva®    |
| Carbonate | Sodium bicarbonate   | Sodium carbonate   | 5.4–7.4, 9.3–11.3 | Fuzeon™              |

is mainly adjusted using NaCl. NaCl affects electrostatics in a protein either by nonspecific (Debye-Huckel) electrostatic shielding or by specific ion binding to the protein. At low concentration, salts affect electrostatic shielding and weaken ionic repulsion/attraction as counter-ions, so this shielding effect may be stabilizing when there are major repulsive interactions leading to protein unfolding, or could be destabilizing when there are major stabilizing salt bridges or ion pairs in the proteins. At high concentrations, electrostatic shielding is saturated. The dominant effect of salt, as of other additives, is on the solvent properties of the solution. The stabilizing salts seem to increase surface tension at the water-protein interface and strengthen hydrophobic interaction by keeping hydrophobic groups away from water molecules, inducing preferential hydration of proteins (13). Therefore, ionic strength or salt concentration affects both the solubility and the stability of proteins. The following areas should be assessed when selecting a proper ionic strength or salt concentration:

- Similar to the effect of pH, the profiles of solubility and stability as functions of ionic strength may not overlap each other for a specific protein, so the optimum ionic strength at maximum solubility may not be the same as at maximum stability. It is important to define an optimal ionic strength that is a good compromise between these two characteristics.
- Ionic strength or NaCl concentration has been reported to have an impact on the viscosity of formulations, especially at high protein concentrations (88). It may therefore serve an important factor in adjusting the viscosity of the product. Viscosity is an important parameter for syringeability of high-protein concentration formulations used in SC and IM administration.
- Ionic strength or NaCl concentration should be compatible with other excipients in the formulation and with product-contacting surfaces. It has been reported that high salt concentration combined with acidic pH may cause rusting of stainless steel, resulting in an elevated level of iron ions responsible for oxidation of the protein (83).
- Ionic strength or NaCl is certainly a key contributor to the overall tonicity of the formulation, and it is important to keep the concentration or tonicity suitable for the intended administration routes.

#### Stabilizers

When adjusting the parameters discussed above—protein concentration, pH, buffer, and ionic strength—still does not result in sufficient protein stability, the addition of stabilizers should be considered. Several types of stabilizers, listed in Table 2, are commonly used in protein formulations to stabilize proteins against various stresses. It is important to consider the following aspects when choosing a stabilizer:

- The choice of stabilizer type and concentration should be rational. An experimental laboratory-scale model should be developed to screen the stabilizer type and concentration for specific degradation against specific stress. For example, to identify a stabilizing excipient against shaking stress, surfactants and concentration ranges should first be tested through an established shaking model. To find a stabilizer against freezing/thawing stress, an appropriate freeze/thaw stress model should be used to screen various cryoprotectants and their concentration ranges.
- The number of stabilizers in a single formulation should be minimal and based on needs. An ideal stabilizer inhibits or minimizes multiple degradation pathways. For example, conformational stabilizers, such as sucrose, which enhance conformational stability, minimizes not only the aggregation but also other chemical degradations such as oxidation and fragmentation that occur when the reactive sites are exposed in absence of sucrose.
- The type and concentration of stabilizers should be compatible with other excipients in the formulation. For example,  $\text{Ca}^{2+}$  may be a good stabilizer, but if the buffer is phosphate and the pH is above neutral, precipitation of calcium phosphate may occur.
- Any stabilizers that may cause interference with protein assays should be avoided. For examples, polymers and albumins used as stabilizers in formulations may interfere

**Table 2** Commonly Used Stabilizers in Protein Formulations

| Type                   | Hypothesized stabilizing mechanism(s) (reference number)                                                                                                                                                                                                             | Examples                                                                                                                                                                                                                                                                                                                                                     |
|------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Sugars                 | Stabilize proteins by preferential hydration in solution; serve as cryoprotectant and/or lyoprotectant; certain sugars such as glucose may chelate heavy metals, thus serving as antioxidants (89).                                                                  | Sucrose: Follistim <sup>®</sup> , Panglubulin <sup>®</sup> , Ovidrel <sup>®</sup> , Xigris <sup>™</sup><br>Trehalose: Advate, Herceptin <sup>®</sup><br>Lactose: Factrel <sup>®</sup> , Glucagon <sup>®</sup><br>Glucose: Gammagard <sup>®</sup> S/D<br>Maltose: Bexxar <sup>®</sup> , Gamimune <sup>®</sup>                                                 |
| Polyols                | Stabilize proteins by preferential hydration in solution; may serve as cryoprotectant and/or lyoprotectant; certain polyols such as mannitol may also serve as antioxidants by chelating metal ions (89).                                                            | Mannitol: DigiFab <sup>™</sup> , Fabrazyme <sup>®</sup> , Cerezyme <sup>®</sup><br>Sorbitol: Digibind <sup>®</sup> , Neulasta <sup>™</sup> , Neupogen <sup>®</sup><br>Glycerol: Humalog <sup>®</sup> , Humulin <sup>®</sup> R                                                                                                                                |
| Surfactants            | Reduce agitation-induced aggregation by reducing surface tension; facilitate refolding by specific or nonspecific binding to protein; minimize adsorption and prevent other degradation by preferentially binding to interfaces (air-liquid, ice or solid surfaces). | Polysorbate 20: Neulasta <sup>™</sup> , Replagal <sup>®</sup> , Raptiva <sup>®</sup><br>Polysorbate 80: PEG-Intron, Remicade <sup>®</sup> , WinRho SDF <sup>®</sup><br>Poloxamer 188: Elitek <sup>™</sup>                                                                                                                                                    |
| Amino acids            | Suppress protein aggregation and protein-protein or protein-surface interactions; arginine increases the surface tension of water, thus favorably interacting with most amino acid side chains and peptide bonds (90).                                               | Glycine: Gamunex <sup>®</sup> , Synagis <sup>®</sup> , Neumega <sup>®</sup><br>Arginine: Activase <sup>®</sup> , TNKase <sup>®</sup><br>Cysteine: Acthrel <sup>®</sup> , SecreFlo <sup>®</sup><br>Histidine: BeneFIX <sup>®</sup>                                                                                                                            |
| Metal chelators        | Chelate heavy metals to prevent metal-ion-catalyzed oxidation of cysteine and methionine residues in proteins.                                                                                                                                                       | EDTA: Kineret <sup>™</sup> , Ontak <sup>®</sup>                                                                                                                                                                                                                                                                                                              |
| Divalent metal cations | Stabilize protein conformation by specific binding to certain sites of protein.                                                                                                                                                                                      | Ca <sup>++</sup> : Pulmozyme <sup>®</sup> , ReFracto <sup>®</sup> , Kogenate <sup>®</sup><br>Zn <sup>++</sup> : Nutropin Depot <sup>™</sup> , Aralast <sup>™</sup>                                                                                                                                                                                           |
| Polymers or proteins   | Stabilize proteins by mechanisms similar to those of surfactants; serve as cryoprotectant or lyoprotectant.                                                                                                                                                          | Albumin: Intron <sup>®</sup> A, Rebif <sup>®</sup> , Zevalin <sup>™</sup> , Procrit <sup>®</sup><br>PEG: Autoplex <sup>®</sup> T, Hemofir <sup>®</sup> M, Monarc-M <sup>™</sup> , Prolastin <sup>®</sup><br>Carboxymethylcellulose: Plenaxis <sup>™</sup><br>Heparin: Thrombate III <sup>®</sup> , Autoplex <sup>®</sup> T<br>Dextran: Mylotarg <sup>™</sup> |

with protein assays, particularly in UV, HPLC methods and gel electrophoresis, creating complications for release and stability testing.

- Any stabilizers that may introduce potential contaminants, especially animal or human derived, should be avoided. Albumin, for example, is an excellent stabilizer for many therapeutic proteins and was widely used in early products; however, because of concerns about blood source contamination, it has seldom been used as a stabilizer in recent products. However, availability of pharmaceutical grade recombinant human serum albumin may change this dynamics.
- Stabilizers of high quality from a reputable vendor should be used for lab screening studies. Like any excipients, stabilizers may contain different levels and types of impurities when they are made from different sources or processes. These impurities

could cause inconclusive results from screening studies, as they may result in instability even while the stabilizer itself has a stabilizing effect. A case in point is the peroxide level in polysorbates: varying peroxide levels in polysorbates used in formulation studies often confound the study results.

- The type and concentration of stabilizers chosen should be safe to use for intended administration routes. It is important to assess the safety and toxicity of any new excipient prior to clinical studies.

As shown, choosing the proper type and concentration of stabilizer is a challenging process. It is important to consider all aspects, including solubility, stability, compliance, safety, and operational challenges (such as posing analytical difficulties).

#### *Tonicity Modifiers*

For parenteral administration, the final product is generally formulated to be isotonic or iso-osmotic, which is equivalent to 0.9% or 150 mM NaCl with an osmolality of 289 mOsm/kg. The following aspects should be considered when finalizing the type and concentration of tonicity modifiers in the final product:

- Commonly used tonicity modifiers in protein formulations include NaCl, mannitol, and sorbitol. An excipient already selected for the formulation, such as salt or stabilizer, is the preferred choice when increasing concentration does not have an effect on the overall properties of the formulation.
- The requirement of isotonicity depends on the administration route and dose preparation. If the product is diluted into IV fluid such as normal saline solution prior to administration, the formulation may not be required to be isotonic. However, if the product is directly injected without any dilution, isotonicity is preferred, particularly for SC, IM, and IT injections.
- For lyophilized product, the formulation prior to lyophilization may not be required to be isotonic even when the reconstituted solution is required to be isotonic. Isotonicity in final reconstituted solution can be achieved by choosing the proper type and volume of diluent to reconstitute the lyophilized product.

It should be noted that some recent studies have shown that infusion of solutions with iso-osmolality but hypotonicity may cause some adverse effects (91). This suggests that although the terms "isotonic" and "isoosmotic" have been used interchangeably, they may have different effects on safety, particularly for products that will be infused in large quantity.

#### *Bulking Agents (for Lyophilized Product Only)*

For lyophilized product, bulking agents are required to provide enough solids to maintain good cake structure during lyophilization and long-term storage. To choose the proper type and amount of bulking agent, the following aspects should be considered:

- The type and amount of bulking agent added to the formulation should be compatible with the protein and other excipients. It should not cause significant protein instability during lyophilization or storage. An excipient already selected for the formulation, such as a stabilizer (sucrose or trehalose), should be preferred as the bulking agent when increasing the concentration does not have an effect on the overall properties of the formulation (e.g., high sucrose concentration may require an extremely long lyophilization cycle or result in partially collapsed cakes).
- Mannitol and glycine are commonly used bulking agents as they provide better cake appearance and do not require a longer lyophilization cycle. However, because of their crystalline nature, they may cause phase separation during storage, leading to stability issues. In addition, a high content of mannitol may lead to vial breakage during freezing (92) due to volume expansion during crystallization. This vial breakage phenomenon has been also observed with NaCl crystallization during lyophilization (93).

**Table 3** Preservatives for Protein Formulation

| Type           | Example(s)                                                   | Comments                                                                                    |
|----------------|--------------------------------------------------------------|---------------------------------------------------------------------------------------------|
| Phenol         | Antivenin<br>Apliso®                                         | Air and light sensitive; may act as a reducing agent.                                       |
| Benzyl alcohol | Nutropin AQ®<br>Epogen®<br>Nutropin®<br>Pegasys®<br>Factrel® | Usually in diluent for reconstitution of lyophilized product.                               |
| m-Cresol       | Humatrope<br>NovoLog®                                        | Used in both liquid multiuse products and diluents for lyophilized products.                |
| Thimerosal     | Antivenin                                                    | Not commonly used for recent products because of mercury-related toxicity.                  |
| Chlorobutanol  | Desmopressin                                                 | Widely used preservative in pharmaceuticals, including injectables. Typically used at 0.5%. |

- Polymeric bulking agents [e.g., hydroxyethyl starch (HES) or dextran] and proteins (e.g., albumin) may significantly reduce the length of lyophilization cycle by raising  $T_g'$ , however, some of them may interfere with certain protein assays. Therefore, potential complications in analytical testing should be considered when choosing this type of bulking agents.
- For high-protein-concentration formulations, bulking agents may not be required, as a protein itself serves as a bulking agent to provide good cake structure.

#### Preservatives (for Multidose Products)

Most injectable protein products are intended for single-dose injection, which does not require inclusion of antimicrobial preservatives in the formulation. However, some products are intended for multidose administration, which requires preservatives in the formulation to prevent any microbial growth from the time the product is opened for use to the time the last dose is administered.

Table 3 lists the preservatives that have been used for protein formulations. To choose the appropriate type and concentration of preservatives in a formulation, the following aspects should be considered:

- The type and concentration of preservatives selected for a formulation should make the final product meet the antimicrobial effectiveness testing required by USP and BP/EP at the time of product release and at the end of shelf-life and last dosing. One needs to be aware that requirements in BP/EP are more stringent than those in USP (94).
- Adding preservatives generally results in protein instability. This is not surprising, as the bactericidal or bacteriostatic effect is derived from the preservative's interaction with proteins or DNA in microorganisms. It is important to screen for a compatible type of preservative for specific formulations or proteins.
- Minimizing the contact time between preservative and protein is a general approach to reducing the preservative's stability impact. In this approach, preservatives are typically added to the diluent (for lyophilized product) or to the product upon preparation for the first dose.

#### General Strategies for Formulation Screening and Optimization

As discussed above, protein formulation has multiple parameters, including the protein itself, buffers, pH, stabilizers, and other excipients, and each parameter has its own functions. Some of these parameters may interact with each other; for example, pH and stabilizers both affect

**Table 4** Formulation Parameters, Evaluation Models, and Critical Attributes for Design of Experiments

| Formulation parameters               | Evaluation models  | Critical attributes |
|--------------------------------------|--------------------|---------------------|
| Protein concentration                | Thermal stress     | Appearance          |
| pH                                   | Freeze/thaw cycle  | Content             |
| Buffer type/concentration            | Peroxides exposure | Aggregation         |
| Stabilizer type/concentration        | Light exposure     | Fragmentation       |
| Tonicity modifier type/concentration | Shaking/agitation  | Oxidation           |
| Preservative type/concentration      |                    | Deamidation         |
|                                      |                    | Potency             |

the stability of the molecule. In addition, the product has to be exposed to multiple process-related stresses during manufacturing, storage, and handling, such as extreme temperatures, freeze/thaw cycles, agitation, and pressure. Therefore, it is challenging to develop a stable formulation containing many parameters against various process-related stresses.

Design of experiments (DOE) has proven an effective tool in dealing with such a complicated development process involving multiple variables. DOE, as part of the concept of quality by design (QbD) recently introduced by regulatory agencies for pharmaceutical development, is a tool to establish the design space through statistical analysis. The design space forms a link between development and manufacturing design (ICH Q8, Pharmaceutical Development). For formulation development, the design space refers to the defined range of formulation parameters and quality attributes that have been demonstrated to provide assurance of quality.

Formulation development using a DOE approach typically has two stages: formulation screening and optimization/robustness studies. The goal of the screening study is to identify the key formulation parameters, while the optimization/robustness study defines the optimal or robust range of the selected key parameters.

To design a proper space for statistical DOE studies, it is important to collect all the information from preformulation development activities and any prior knowledge on the protein. This information helps in identifying the critical formulation parameters and the key degradation pathways that potentially affect the critical quality attributes (CQAs) of the product for the design space, and in selecting a proper evaluation model that can be used for screening or optimization of formulations. In addition, the target product profile (TPP) should also be established prior to DOE studies.

Table 4 lists the parameters and attributes for DOE studies. Once the study is completed and data are collected, statistical analysis should be performed to establish the design space. This established design space can not only justify the choice of formulation ranges and help identify the robust region of the formulation, but can also enable study of the interactions between each formulation parameter. Several case studies using the DOE concept have been described in a book chapter written by Ng and Rajagopalan (95).

#### Choice of Container and Closure System

Because proteins may interact with the contact surfaces, the compatibility of immediate packaging material with protein product needs to be evaluated during selection of the container closure system.

As described in section "General Formulation Development Process in Industry," most protein products are formulated as drug substance and drug product, which are typically stored at frozen and refrigerated conditions, respectively. In choosing an appropriate container closure system for drug substance, the following aspects should be considered:

- Commonly used container closure systems for drug substance include plastic bottles or bags and stainless steel vessels. Various types of plastics have been used in packaging protein drug substance, including Teflon, polyolefin, glycol-modified polyethylene terephthalate (PETG), polypropylene (PP), polycarbonate (PC), polyvinylchloride (PVC), polyvinyl alcohol (PVA), and polyethylene (PE). It is important to

evaluate whether the product remains stable under intended storage conditions. For example, hydrophobic proteins tend to adsorb more on hydrophobic polymers such as Teflon. In addition, the material's gas permeability and leachables should also be evaluated. Plastics with high gas permeability may affect product stability during long-term storage if the product is oxygen sensitive. Plastics such as PVC that contain a substantial amount of plasticizer may generate more leachables than other plastics. When stored in a stainless steel vessel, acidic pH and chloride ions may cause corrosion. With an increased amount of dissolved metal ions, protein oxidation is a concern (83).

- The material should retain its function at the intended storage condition. Since most protein drug substances are stored frozen, it is important to assess the brittleness point of the plastics at the intended storage temperature. If the brittleness point is above the intended storage temperature, container closure integrity may be compromised. In addition, breakage of container closure may occur upon impact, such as an accidental drop. Therefore, PC and Teflon are preferred because of their lower brittleness points.
- The size of the containers should be selected on the basis of product stability, potential expansion of product, and cost effectiveness. For example, a sufficient amount of headspace should be allowed to accommodate volume expansion upon freezing for frozen drug substance. If the product is stored as a liquid, then minimal headspace should be considered to minimize the potential instability caused by agitation upon handling and shipping. With large containers, it may be difficult to control the freezing process. Stainless steel cryo-vessels with temperature-controlling systems have many advantages over plastic containers, such as controlled freezing and thawing rates and nonbreakable characteristics. However, they are expensive and need to undergo cleaning validation for multiuse purposes. In addition, they may need frequent passivation to retain resistance to potential corrosion.

To choose an appropriate container closure system for drug product, the following aspects should be considered:

- Unlike drug substance, most drug products are stored in glass vials with rubber stopper systems. Some products are also packaged in prefilled syringes, cartridges, and dual chamber Lyo-Ject<sup>®</sup> syringes, which all consist of glass barrels or tubing and rubber stoppers. Plastic vials or tubing have also recently been introduced. It is important to evaluate the product's compatibility with various types of glass, plastics, and rubber stoppers, as protein adsorption and other degradations may occur.
- Container closure integrity should be retained to ensure the product's sterility throughout its shelf-life.
- Most glass vials are washed and then depyrogenated prior to use. It has been reported that siliconized vials may minimize adsorption of the product (17). However, the possibility of silicone oil causing protein aggregation also needs to be examined. For prefilled syringes, the glass barrels should be siliconized for proper syringeability. It is important to recognize, however, this step may affect not only syringeability but also product stability. For lyophilized product, the stoppers may need to be dried in an oven or autoclave following steam sterilization, because retained moisture may be released to the lyophilized cake during long-term storage and cause instability issues, which are critical for moisture-sensitive products.
- The type of container closure system should be decided on the basis of the product's stability, the development stage, the intended use (indication and administration route), and marketing competitiveness. For an early clinical development stage, a vial and stopper system is often chosen, as it involves less technical complexity and thus requires less development time. For late development or commercial stages, the performance of a container closure system—such as improving ease of administration, minimizing drug wastage, and conforming with patient needs (e.g., self-administration)—becomes more important. Prefilled syringes, self-injection devices using cartridges, and dual chamber lyo-ject syringes may help the drug product to gain a greater market share because of their convenience of use, which leads to better patient compliance.

- The size of container closure should be decided on the basis of several factors, including dosing regimen, product stability, cost effectiveness, and compliance for patient use. For example, vial size affects the headspace, which is a critical parameter for most liquid products as it may impact product stability upon agitation during shipping and handling. For prefilled syringes, the size of the syringe also affects the headspace and the movement of the stoppers upon exposure to reduced pressure during airplane shipping. Cartridges tend to have less headspace.

For both drug substance and drug product, the suppliers of the container closure system should be reputable and well established. Suppliers that have established Drug Master Files (DMFs) for the packaging components should be preferred. In addition, for commercial products, a second source for the container closure system may need to be established in case issues arise with the primary source.

#### **Manufacturing Process Development**

The next step after selection of an appropriate formulation and container closure system is to develop a manufacturing process that maintains protein stability under process conditions such as mixing, filtration, filling, and lyophilization. Instability of proteins under these conditions is often observed, and therefore it is important to evaluate and define the optimal process conditions prior to cGMP manufacturing. From these studies, the critical process parameters and acceptable operation ranges should be defined using a DOE approach similar to that used in formulation development. The CQAs impacted by these process conditions should be evaluated. The following aspects should be considered when developing a robust and suitable manufacturing process:

- All product-contacting surfaces during manufacturing should be compatible with the formulation. Generally, the product-contacting surfaces during drug product manufacturing include silicone tubing, a Teflon-coated stir bar, a stainless steel tank or impeller mixer, rubber gaskets, plastic connectors, plastic housing and filter membranes, and other glass or plastic containers. It is important to evaluate the compatibility of the protein with these contact surfaces prior to the start of manufacturing using these materials.
- The container closure system should be compatible with the fill line at the manufacturing site. Typically, machinability needs to be conducted prior to cGMP manufacturing to ensure that the filling and stoppering operations run smoothly, with low rejection rates, and that the final container closure system meets the container closure integrity test criteria (integrity is typically tested by dye leak or vacuum decay method).
- The mixing condition should not result in product degradation. If mixing by a magnetic stir bar causes protein precipitation, alternative mixing methods such as an impeller should be considered.
- If the product is sensitive to dissolved oxygen in solution, several manufacturing process steps should be designed appropriately. Degassing the solutions and overlaying inert gas (nitrogen or argon) in the headspace of vials may be required to minimize oxidation due to dissolved oxygen in the product.
- The filter size for sterile filtration should be large enough not to give high back pressure during filtration. For aqueous protein formulations, a polyvinylidene fluoride (PVDF) membrane is the most commonly used. A filter sizing study should be performed to define the proper size of filter for cGMP manufacturing.
- The filling conditions should be compatible with the product. For solution filling, several filling machines are commonly used, including peristaltic pump, stainless steel piston syringe, ceramic piston syringe, rolling diaphragm, and rotary time pressure filling systems. While a syringe-filling system typically provides better accuracy, it applies high shear stress between the piston and barrel during movement, which could lead to precipitation of proteins. In addition, the filling speed needs to be controlled to avoid foaming or splashing during filling.

- The lyophilization parameters developed at laboratory scale should be robust enough to produce consistent product quality. Sometimes when the lyophilization cycle developed for the laboratory-scale lyophilizer is directly transferred to the production-scale lyophilizer, product quality may not be the same. This could be caused by the poor robustness of lyophilization cycle, as different lyophilizer designs may lead to different levels of heat and mass transfer, which could cause changes in product quality, such as cake collapse, if the selected lyophilization parameters are on the edge of the process design.
- Hold conditions (time and temperature) for all process intermediates should be established to meet the needs of routine production operations and to support potential excursions. An operation deviation may result in longer hold time for the process intermediate than during routine manufacturing; examples could be deviations prior to the formulation step or filling into final product, or after lyophilization. Supporting data for defining acceptable hold conditions need to address product quality from both chemical stability and microbiological perspective. Assessment of the microbiological acceptability of a process intermediate hold time is tied to manufacturing process operations and to the microbial growth potential of the intermediate composition. Support for extended or cumulative hold times generally comes from development-scale studies, while support for microbiological properties comes from manufacturing-scale studies.

#### Stability Studies

After the manufacture of drug substance and drug product, the following stability studies are generally conducted to support the shelf-life during long-term storage and the product quality during distribution and use at clinics:

- *Long-term stability studies to support shelf-life.* Several ICH guidelines outline the content and testing requirements for stability studies supporting shelf-life at long-term storage conditions (ICH Q1A, Q1C, Q1D, Q1E, and Q5C). Depending on the intended long-term storage condition, accelerated and stress conditions are often required for the clinical stability program and lots made during process validation. For postapproval commercial stability programs, one lot of drug substance and one lot of drug product are generally required to be placed on stability annually, and only at the long-term storage condition.
- *Temperature cycling studies to support excursions during distribution.* From completion of manufacturing to the time when the product is dosed into patients, the drug product experiences exposure to various temperatures, different from the intended long-term storage temperature range, which is typically the refrigerated temperature. It is important to conduct studies to evaluate the effect of these temperature variations on product stability. The design of these studies has been recommended in the Parenteral Drug Association (PDA) Technical Report No. 39 (96).
- *Shipping studies to support the exposure to vibration and reduced pressure during air and ground transportation.* It is important to recognize that shaking studies conducted at laboratory scale may not be representative of the actual shipping conditions to which the product is exposed, since the shaking studies may not have vibration amplitudes and frequencies similar to those generated during shipping. In addition, reduced pressure occurs during air shipment. This is a concern particularly for prefilled syringes, as stopper movement has been observed during multiple cycles of reduced pressure, which may affect the sterility of the product (97). Therefore, it is important to demonstrate that the product remains intact or within the designed space using actual or simulated shipping conditions, including representative secondary packaging and product orientation.
- *Confirmatory photostability studies to support exposure to light.* Sensitivity to light is highly dependent on the composition, structure, and formulation of the product. Most proteins and peptides are sensitive to intense light, particularly UV light, which

typically results in oxidation. However, they are relatively stable under normal indoor light. In addition, protein products are typically stored in refrigerated condition, in which there is no direct exposure to light. Commercial products normally have secondary packages, which prevent the product's exposure to light. To demonstrate that the product remains stable, it is important to conduct a confirmatory photostability study using representative commercial packaging per the ICH guideline Q1B.

- *In-use and compatibility studies with the administration system to support product stability and to assess compatibility with product-contacting surfaces and stresses during administration.* For IV infusion, some products may need to be diluted in an IV bag and then infused into patients through an IV infusion apparatus by a pump or other mechanism. It is critical to assess product stability after dilution prior to dosing, as well as the compatibility of the product in contact with the surfaces of the administration apparatus and infusion system, such as pumps. For lyophilized products, the stability of reconstituted solution should be evaluated to ensure the product remains stable during the ambient exposure period after reconstitution.

#### FORMULATIONS OF MARKETED PROTEIN PRODUCTS

In the United States, by law all marketed injectable products must disclose quantitative formulation, so the details on each marketed product are public. Sources on prescription information include the Physicians' Desk Reference (PDR) and numerous websites, including the FDA's <http://www.accessdata.fda.gov/scripts/cder/drugsatfda> and websites for specific products (which commonly take the form [www.tradenamename.com](http://www.tradenamename.com)—e.g., [www.simponi.com](http://www.simponi.com)). The formulations of discontinued products can be found in older editions of the PDR.

The excipients used in parenteral products were first reviewed and collated by Wang and Kowal in 1980 (98). Subsequently, there were reviews by Nema et al. (99) and Powell et al. (100). Specifically for biotech products, they were first reviewed by Wang and Hansen (101), and recently by Gokarn et al. (102).

The types of excipients used in protein formulations have evolved over time. For example, in the early years (1980s–1990s), human serum albumin (HSA) was commonly used as a stabilizer in many protein formulations, particularly low-concentration, high-potency products such as interferons, Factor VIII, and other growth factors. However, because of concerns about potentially contaminated blood that might compromise the quality of albumin, most products have been reformulated into HSA-free formulations. For example, Eprex® (epoetin  $\alpha$ ), originally containing albumin, was reformulated to use polysorbate 80 as an HSA-free product in prefilled syringes. Avonex® (interferon  $\beta$ ), originally having albumin in lyophilized form, was reformulated to a polysorbate-20-containing liquid formulation in prefilled syringes.

Another example of evolving parenteral formulation excipients is the type of polysorbate. Because of concerns about potential auto-oxidation of the unsaturated double bond in polysorbate 80 (103), used predominantly in the early days of protein formulations, the trend seems to be moving toward the use of polysorbate 20. For example, Neupogen® is formulated with polysorbate 80, but the surfactant in its newer version, PEGylated protein (Neulasta®) was changed to polysorbate 20. Polysorbate 80 included in the Activase® formulation was also changed to polysorbate 20 in its newer variant TNKase®.

To show how formulations have evolved over time, examples of recombinant human growth hormones and monoclonal antibodies (including Fab, Fab-PEG, and Fc-fusion proteins) are listed in Tables 5 and 6, respectively. For human growth hormone, changes of formulation have been minimal since its first approval in 1985 to one recently approved in 2008. For lyophilized monoclonal antibodies, the buffer species used has changed from phosphate in early approved products to histidine in recently approved products. Sucrose is the most commonly used sugar in lyophilized formulations, and only a few products use trehalose or maltose. In addition, amino acids including arginine and glycine are used in both liquid and lyophilized formulations. For liquid monoclonal antibodies, sodium phosphate appears to be the most commonly used buffer, likely because of its good buffering capacity

Table 5 Formulations for Human Growth Hormone (in order of approval year)

| Trade Name       | Approval year | Dosing route | Presentation        | Dosage form | Formulation                                                                                                                                                                                        |
|------------------|---------------|--------------|---------------------|-------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Protropin®       | 1985          | SC           | Vial/stopper        | Lyophilized | 5 mg vial <sup>a</sup> : 40 mg mannitol, 0.1 mg monobasic sodium phosphate, 1.6 mg dibasic sodium phosphate, pH 4.5–7.0                                                                            |
| Humatrope®       | 1987          | SC/IM        | Vial/stopper        | Lyophilized | 5 mg/5 mL vial: 25 mg mannitol, 5 mg glycine, 1.13 mg dibasic sodium phosphate, pH 7.5                                                                                                             |
| Norditropin®     | 1987          | SC           | Pen cartridge       | Lyophilized | 6 mg/2 mL cartridge <sup>a</sup> : same formulation as in vial/stopper                                                                                                                             |
|                  |               |              | Pen cartridge       | Liquid      | 5 mg/1.5 mL cartridge <sup>a</sup> : 1 mg histidine, 4.5 mg Poloxamer 188, 4.5 mg phenol, 60 mg mannitol                                                                                           |
| Nutropin®        | 1993          | SC           | Vial/stopper        | Lyophilized | 5 mg/1 mL vial <sup>a</sup> : 45 mg mannitol, 0.4 mg sodium phosphate monobasic, 1.3 mg sodium phosphate dibasic, 1.7 mg glycine, pH 7.4                                                           |
|                  |               |              | Vial/stopper        | Liquid      | 10 mg/2 mL vial: 17.4 mg sodium chloride, 5 mg phenol, 4 mg polysorbate 20, 10 mM sodium citrate, pH 6.0                                                                                           |
| Tev-Tropin®      | 1995          | SC           | Pen cartridge       | Liquid      | 5 mg/1 mL cartridge <sup>a</sup> : same formulation as in vial/stopper                                                                                                                             |
| Genotropin®      | 1995          | SC           | Vial/stopper        | Lyophilized | 5 mg/5 mL vial: 30 mg mannitol, pH 7–9                                                                                                                                                             |
|                  |               |              | 2-chamber cartridge | Lyophilized | 5 mg/1 mL cartridge <sup>a</sup> : 5.8 mg somatotropin, 2.2 mg glycine, 1.8 mg mannitol, 0.32 mg sodium dihydrogen phosphate anhydrous, 0.31 mg disodium phosphate anhydrous, pH 6.7               |
| Salzen/Serostim® | 1996          | SC           | Vial/stopper        | Lyophilized | 4 mg vial <sup>a</sup> : 27.3 mg sucrose, 0.9 mg phosphoric acid, pH 6.5–8.5                                                                                                                       |
| Zorbtive®        | 2003          | SC           | Vial/stopper        | Lyophilized | 4 mg vial <sup>a</sup> : 27.3 mg sucrose, 0.9 mg phosphoric acid, pH, 7.4–8.5                                                                                                                      |
| Omnitrope®       | 2006          | SC           | Vial/stopper        | Lyophilized | 1.5 mg/1.13 mL vial <sup>a</sup> : 0.88 mg disodium hydrogen phosphate heptahydrate, 0.21 mg sodium dihydrogen phosphate dihydrate, 27.6 mg glycine                                                |
|                  |               |              | Pen cartridge       | Liquid      | 5 mg/1.5 mL cartridge <sup>a</sup> : 1.3 mg disodium hydrogen phosphate heptahydrate, 1.6 mg sodium dihydrogen phosphate dihydrate, 3.0 mg poloxamer 188, 52.5 mg mannitol, 13.5 mg benzyl alcohol |
| Valtropin®       | 2007          | SC           | Vial/stopper        | Lyophilized | 5 mg vial/1 mL: 10 mg glycine, 45 mg mannitol, 0.22 mg monobasic sodium phosphate, 2.98 mg dibasic sodium phosphate, pH 6.0                                                                        |
| Accretropin®     | 2008          | SC           | Vial/stopper        | Liquid      | 5 mg vial: 0.75% NaCl, 0.34% phenol, 0.2% Pluronic F-68, 10 mM sodium phosphate, pH 6.0                                                                                                            |

<sup>a</sup>Indicates a product that has multiple dose strengths, but only the lowest strength is listed

**Table 6** Formulations for Antibodies, Fc Fusion, and Fab Conjugates (in order of approval year)

| Trade name        | Nonproprietary name                   | Approval year | Dosing route | Presentation | Dosage form | Formulation                                                                                                                                                                                                      |
|-------------------|---------------------------------------|---------------|--------------|--------------|-------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Orthoclone OKT-3® | Muromonab-CD3                         | 1986          | IV           | Ampule       | Liquid      | 5 mg/5 mL ampule: 2.25 mg monobasic sodium phosphate, 9.0 mg dibasic sodium phosphate, 43 mg sodium chloride, 1.0 mg polysorbate 80, pH 6.5–7.5                                                                  |
| Reopro®           | Abciximab (Chimeric Fab)              | 1994          | IV           | Vial/stopper | Liquid      | 10 mg/5 mL vial: 0.01 M sodium phosphate, 0.15 M sodium chloride, 0.001% polysorbate 80, pH 7.2                                                                                                                  |
| Rituxan®          | Rituximab                             | 1997          | IV           | Vial/stopper | Liquid      | 100 mg/10 mL vial <sup>a</sup> : 9 mg/mL sodium chloride, 7.35 mg/mL sodium citrate dihydrate, 0.7 mg/mL polysorbate 80, pH 6.5                                                                                  |
| Enbrel®           | Etanercept                            | 1998          | SC           | Vial/stopper | Lyophilized | 25 mg/0.5 mL vial: 40 mg mannitol, 10 mg sucrose, 1.2 mg tromethamine, pH 7.1–7.7                                                                                                                                |
|                   |                                       |               |              | PFS          | Liquid      | 25 mg/0.5 mL PFS <sup>b</sup> : 1% sucrose, 100 mM sodium chloride, 25 mM L-arginine hydrochloride, 25 mM sodium phosphate, pH 6.1–6.5                                                                           |
| Remicade®         | Infliximab                            | 1998          | IV           | Autoinjector | Liquid      | Same as Liquid in PFS                                                                                                                                                                                            |
|                   |                                       |               |              | Vial/stopper | Lyophilized | 100 mg/10 mL vial: 500 mg sucrose, 0.5 mg polysorbate 80, 2.2 mg monobasic sodium phosphate monohydrate, 6.1 mg dibasic sodium phosphate dihydrate, pH 7.2                                                       |
| Simulect®         | Basiliximab                           | 1998          | IV           | Vial/stopper | Lyophilized | 10 mg/2.5 mL vial <sup>a</sup> : 3.61 mg monobasic potassium phosphate, 0.5 mg disodium hydrogen phosphate (anhydrous), 0.80 mg sodium chloride, 10 mg sucrose, 40 mg mannitol, 20 mg glycine                    |
| Synagis®          | Palivizumab                           | 1998          | IM           | Vial/stopper | Liquid      | 50 mg/0.5 mL vial <sup>a</sup> : 1.9 mg histidine, 0.06 mg glycine, 0.2 mg chloride, pH 6.0                                                                                                                      |
| Herceptin®        | Trastuzumab                           | 1998          | IV           | Vial/stopper | Lyophilized | 440 mg/20 mL vial: 400 mg α,α-trehalose dihydrate, 9.9 mg L-histidine HCl, 6.4 mg L-histidine, 1.8 mg polysorbate 20, pH 6.0                                                                                     |
| Zenapax®          | Daclizumab                            | 1999          | IV           | Vial/stopper | Liquid      | 25 mg/5 mL vial: 3.6 mg sodium phosphate monobasic monohydrate, 11 mg sodium phosphate dibasic heptahydrate, 4.6 mg sodium chloride, 0.2 mg polysorbate 80, pH 6.9                                               |
| Mylotarg®         | Gemtuzumab ozogamicin (calicheamicin) | 2000          | IV           | Vial/stopper | Lyophilized | 5 mg/5 mL vial: dextran 40, sucrose, sodium chloride, monobasic and dibasic sodium phosphate <sup>a</sup>                                                                                                        |
| Campath®          | Alemtuzumab                           | 2001          | IV           | Vial/stopper | Liquid      | 30 mg/1 mL vial: 8.0 mg sodium chloride, 1.44 mg dibasic sodium phosphate, 0.2 mg potassium chloride, 0.2 mg monobasic sodium phosphate, 0.1 mg polysorbate 80, 0.0187 mg disodium edetate dihydrate, pH 6.8–7.4 |

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|                                                     |                                             |      |              |              |             |                                                                                                                                                                                                                                                 |
|-----------------------------------------------------|---------------------------------------------|------|--------------|--------------|-------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Humira® (United States)<br>Trudexa (European Union) | Adalimumab                                  | 2002 | SC           | PFS          | Liquid      | 40 mg/0.8 mL in PFS: 4.93 mg sodium chloride, 0.69 mg monobasic sodium phosphate dihydrate, 1.22 mg dibasic sodium phosphate dihydrate, 0.24 mg sodium citrate, 1.04 mg citric acid monohydrate, 9.6 mg mannitol, 0.8 mg polysorbate 80, pH 5.2 |
| Zevalin®                                            | Ibritumomab tiuxetan ( <sup>90</sup> Y)     | 2002 | IV           | Vial/stopper | Liquid      | 3.2 mg/2 mL vial: 0.9% NaCl, 50 mM sodium acetate                                                                                                                                                                                               |
| Amevive®                                            | Alefacept                                   | 2003 | IV/IM        | Vial/stopper | Lyophilized | 7.5 mg/0.5 mL vial <sup>a</sup> : 12.5 mg sucrose, 5 mg glycine, 3.6 mg sodium citrate dihydrate, 0.06 mg citric acid monohydrate, pH 6.9                                                                                                       |
| Bexxar®                                             | Tositumomab (IgG and IgG- <sup>131</sup> I) | 2003 | IV           | Vial/stopper | Liquid      | 35 mg/2.5 mL vial <sup>b</sup> : 10% maltose, 145 mM sodium chloride, 10 mM phosphate, pH 7.2                                                                                                                                                   |
| Xolair®                                             | Omalizumab                                  | 2003 | SC           | Vial/stopper | Lyophilized | 202.5 mg/1.4 mL vial: 145.5 mg sucrose, 2.8 mg L-histidine hydrochloride monohydrate, 1.8 mg L-histidine, 0.5 mg polysorbate 20                                                                                                                 |
| Raptiva®                                            | Efalizumab                                  | 2003 | SC           | Vial/stopper | Lyophilized | 150 mg/1.3 mL vial: 123.2 mg sucrose, 6.8 mg L-histidine hydrochloride monohydrate, 4.3 mg L-histidine, 3 mg polysorbate 20, pH 6.2                                                                                                             |
| Avastin®                                            | Bevacizumab                                 | 2004 | IV           | Vial/stopper | Liquid      | 100 mg/4 mL vial <sup>a</sup> : 240 mg α,α-trehalose dihydrate, 23.3 mg sodium phosphate (monobasic, monohydrate), 4.8 mg sodium phosphate (dibasic, anhydrous), 1.6 mg polysorbate 20, pH 6.2                                                  |
| Erbix®                                              | Cetuximab                                   | 2004 | IV           | Vial/stopper | Liquid      | 100 mg/50 mL vial <sup>b</sup> : 8.48 mg/mL sodium chloride, 1.88 mg/mL sodium phosphate dibasic heptahydrate, 0.41 mg/mL sodium phosphate monobasic monohydrate, pH 7.0–7.4                                                                    |
| Tysabri®                                            | Natalizumab                                 | 2004 | IV           | Vial/stopper | Liquid      | 300 mg/15 mL vial: 123 mg sodium chloride, 17.0 mg sodium phosphate monobasic monohydrate, 7.24 mg sodium phosphate dibasic heptahydrate, 3.0 mg polysorbate 80, pH 6.1                                                                         |
| Vectibix™                                           | Panitumumab                                 | 2005 | IV           | Vial/stopper | Liquid      | 100 mg/50 mL vial <sup>a</sup> : 29 mg sodium chloride, 34 mg sodium acetate, pH 5.6–6.0                                                                                                                                                        |
| Orencia®                                            | Abatacept                                   | 2005 | IV           | Vial/stopper | Lyophilized | 250 mg/10 mL vial: 500 mg maltose, 17.2 mg monobasic sodium phosphate, 14.6 mg sodium chloride, pH 7.2–7.8                                                                                                                                      |
| Lucentis®                                           | Ranibizumab                                 | 2006 | Intravitreal | Vial/stopper | Liquid      | 0.5 mg/0.05 mL vial: 10 mM histidine HCl, 10% α,α-trehalose dihydrate, 0.01% polysorbate 20, pH 5.5                                                                                                                                             |

FORMULATION OF PROTEIN- AND PEPTIDE-BASED PARENTERAL PRODUCTS

(continued)

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**Table 6** Formulations for Antibodies, Fc Fusion, and Fab Conjugates (in order of approval year) (*continued*)

| Trade name | Nonproprietary name | Approval year | Dosing route | Presentation | Dosage form | Formulation                                                                                                                                                        |
|------------|---------------------|---------------|--------------|--------------|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Soliris®   | Eculizumab          | 2007          | IV           | Vial/stopper | Liquid      | 300 mg/30 mL vial: 13.8 mg sodium phosphate monobasic, 53.4 mg sodium phosphate dibasic, 263.1 mg sodium chloride, 6.6 mg polysorbate 80, pH 7.0                   |
| Cimzia®    | Certolizumab pegol  | 2008          | SC           | Vial/stopper | Lyophilized | 200 mg/1 mL vial: 100 mg sucrose, 0.9 mg lactic acid, 0.1 mg polysorbate, pH 5.2                                                                                   |
| Arcalyst®  | Rilonacept          | 2008          | SC           | Vial/stopper | Lyophilized | 160 mg/2.3 mL vial: histidine, arginine, polyethylene glycol 3350, sucrose, glycine, pH 6.2–6.8 <sup>b</sup>                                                       |
| Nplate®    | Romiplostim         | 2008          | SC           | Vial/stopper | Lyophilized | 250 µg/0.72 mL vial <sup>a</sup> : 30 mg mannitol, 15 mg sucrose, 1.2 mg L-histidine, 0.03 mg polysorbate 20, sufficient HCL to bring pH to 5.0                    |
| Simponi®   | Golimumab           | 2009          | SC           | PFS          | Liquid      | 50 mg/0.5 mL in PFS: 50 mg golimumab antibody, 0.44 mg L-histidine and L-histidine monohydrochloride monohydrate, 20.5 mg sorbitol, 0.08 mg polysorbate 80, pH 5.5 |
| Ilaris®    | Canakinumab         | 2009          | SC           | Vial/stopper | Lyophilized | 180 mg/1 mL vial: sucrose, L-histidine, L-histidine HCL monohydrate, polysorbate 80 <sup>b</sup>                                                                   |

<sup>a</sup>Indicates a product that has multiple dose strengths, but only the lowest strength is listed

<sup>b</sup>Quantitative formulation is not disclosed, not consistent with 21 CFR 201.100 (b) (5) iii.

Abbreviations: SC, subcutaneous; IM, intramuscular; IV, intravenous.

around pH 6 to 7, the pH at which most monoclonal antibodies are formulated. Polysorbate 80 or 20 is also present in many monoclonal antibody formulations.

### CONCLUSION

In this book chapter we have attempted to provide an overview of formulation development for peptide- and protein-based therapeutics. For successful formulation development, it is important first of all to understand and characterize the unique characteristics of the protein or peptide, including molecular composition, structures, size, charge profile (pI), solubility, thermal transition midpoint, and key degradation pathways. Preformulation activities are also critical in identifying the key instability issues and potential pharmaceutically relevant sources responsible for specific degradation pathways. This chapter provides general principles and examples of major pharmaceutical development activities, including evaluation of critical formulation parameters, selection of container closure, development of the manufacturing process, and stability studies to support shelf-life and clinical use conditions. The results from these development activities are generally required in completing the pharmaceutical development sections of regulatory filings. Finally, trends in the evolution of formulation development since the early 1980s are described on the basis of several examples, including human growth hormone and monoclonal antibodies.

### ACKNOWLEDGMENTS

The authors would like to thank Drs. Zahra Shahrokh and Jamie Tsung (both at Shire Human Genetic Therapies, Inc., Cambridge, MA) and Dr. Wei Wang (Pfizer, St. Louis, MO) for their valuable comments, and also Mr. Dusan Djordjevic for editing the manuscript.

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