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Excipient Selection for Protein Stabilization

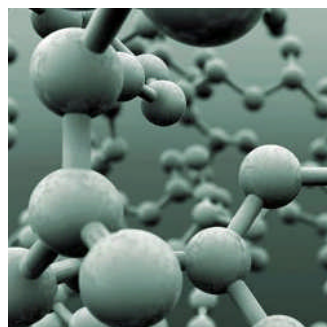
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The complex task of stabilizing proteins is made more challenging due to the limited number of approved excipients.



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Biopharmaceuticals, while highly effective medicines, are susceptible to aggregation upon manufacturing, distribution, and storage, which can result in immunogenicity and/or reduced efficacy. Stabilization of proteins and other biologic drugs is, therefore, crucial for ensuring both safety and efficacy.

The molecular structures of therapeutic proteins and the critical sites within these proteins that are prone to oxidation, deamidation, hydrolysis, etc., and can result in aggregation and fragmentation, are well known. The development process, therefore, begins with efforts to stabilize therapeutic proteins using protein engineering. However, the improvement in stability is generally limited because of the unstable nature of proteins; the

stabilization energy for the native state is typically between 5 and 20 kcal/mol, which is equivalent to that of a few hydrogen bonds. Because the folded state is only marginally more stable than the unfolded state, any changes in the protein environment may trigger protein degradation, aggregation, and or inactivation.

The use of excipients is, therefore, common during the entire manufacturing process and in the final formulation to achieve protein stabilization through retardation of chemical degradation processes and prevention of aggregation, according to Kristina Kemter, project leader in the Research and Development department of Leukocare. Selection of appropriate stabilization agents, however, is challenging, and made more so by the limited choice of substances that have been approved for this use.

Aggregation and other stability issues

Protein stability is a result of achieving a balance between destabilizing and stabilizing forces. The destabilizing forces are mainly due to the large increase in entropy of unfolding; the stabilizing forces are provided by intra-protein and protein-solvent interactions. Disruption of these interactions will shift the balance and destabilize a protein, and many stress factors are known to disrupt this delicate balance and affect protein stability.

These stress parameters include temperature, pH, ionic strength, metal ions, surface adsorption, shearing, shaking, additives, solvents, protein concentration, purity, morphism, pressure, and freeze/thaw-drying, according to Kemter. As a result, a variety of physical and chemical processes can lead to aggregation of proteins. She also notes that there are significant differences between freeze-dried and liquid protein products in terms of the stress exposures upon manufacturing (freeze drying) and during storage of the final products.

Liquid formulations in general have a higher risk for physical and chemical instability during storage than dried formulations due to their high mobility and the increased likelihood of chemical reactions and physical instability. For liquid-protein preparations, stability can be enhanced by selection of pH-buffering salts, and often amino acids can also be used. It is often interactions at the liquid/air interface or liquid/solid interface (with the packaging) that lead to aggregation following adsorption and unfolding of the protein, according to Jens T. Bukrinski, a formulation scientist with Novozymes. Shear stresses during spray drying can also lead to exposure of hydrophobic protein regions upon spray drying that allow initiation of aggregation, according to Kemter.

Stability, particularly thermal stability, can be increased greatly when the protein is dried. For example, lyophilized formulations are generally more shelf-life stable than liquid formulations; however, protein molecules can potentially form large amounts of aggregates during the lyophilization process, according to Jay Kang, director of analytical and formulation development at Patheon. In the freezing phase, the formation of numerous ice crystals



creates a large water/solid surface, a highly concentrated solution, and often an altered pH environment. All these conditions can cause protein aggregation. During drying, removal of water puts significant stresses on protein molecules and can induce aggregate formation.

Mechanisms of destabilization

Temperature and pH have the greatest influence on both physical and chemical protein stability. High temperatures can lead to thermal denaturation and subsequent aggregation as well as accelerate chemical degradation pathways (i.e., side chain oxidation, hydrolysis, and deamidation) that may result in aggregation. In addition, most proteins are generally stable only in a narrow pH range, and physical and chemical pathways to aggregation are possible outside of that window, according to Kemter. Furthermore, the effect of pH on the chemical stability of a protein can be altered in the presence of excipients.

For instance, the effect of salts strongly depends on the pH of the solution, which dictates the charged state of ionizable groups in the protein. "Salts may stabilize, destabilize, or have no effect on proteins depending on the type and concentration of salt, the nature of ionic interactions, and the presence and amount of charged residues in proteins on fully exposed surfaces and/or in fully or partially buried interior sections," Kemter says.

Depending on the type and concentration, metal ions may also stabilize or destabilize a protein because their interactions with proteins are highly protein-dependent. Metal ions may also significantly affect protein stability without affecting much of its secondary structure. "Trace amounts of metal ions in protein formulations may catalyze the oxidation of proteins, namely via the Fenton pathway, targeting in particular the residues methionine, cysteine, histidine, tryptophan, tyrosine, proline, arginine, lysine, or threonine. The catalysis depends on the concentration of the metal ions, and the metal-catalyzed reaction can be facilitated in the presence of reducing agents such as ascorbate or sulfhydryl (RSH) compounds," Kemter explains. Oxidation of proteins by metal ions, oxygen, or reducing agents can lead to aggregation.

Similarly, chelating agents such as ethylenediaminetetraacetic acid (EDTA) and citric acid can bind and destabilize proteins or bind harmful metal ions and prevent oxidation. The net effect depends on the metal ions, oxidation mechanism, and the type of chelating agent, according to Kemter.

Protein concentration can also influence aggregation and in some cases chemical degradation, depending on the mechanism. "More biopharmaceuticals are being formulated at very high concentrations today. Higher concentrations create more chances for unwanted self-association, and consequently high viscosities, which can make both processing and injection more difficult," Bukrinski observes. High pressure can also cause protein unfolding during freeze-drying or spray-freeze drying.

Chemical degradation pathways include not only oxidation, hydrolysis, and deamidation, but also isomerization, succinimidation, disulfide bond formation and breakage, non-disulfide crosslinking, and deglycosylation, and often occur simultaneously, according to Kemter. She notes that deamidation of asparagine and glutamine residues is most common, and the rate, mechanism, and location of deamidation are all pH-dependent. Oxidation is another important chemical degradation mechanism, particularly the oxidation of the thio groups in methionine and cysteine residues. The formation of disulfide bond linkages or thio-disulfide exchanges can result in protein aggregation or polymerization.

"All of these influences can occur—either simultaneously or separately—when different types of stresses are applied to a protein, such as during isolation and purification of a protein; drying of a protein by lyophilization, spray-drying, spray-freeze drying or foam drying; storage of a protein in solution; after drying; as well as reconstitution after drying," states Kemter.

Types of stabilizing excipients

Stabilizing excipients are added to formulations to slow down or prevent protein aggregation through different mechanisms, including strengthening of protein-stabilizing forces, destabilization of the denatured state, and direct binding to the protein, which are applied during isolation and purification, drying (lyophilization, spray-drying, spray-freeze drying, foam-drying), storage in solution or after drying, and reconstitution after drying, according to Kemter.

Six categories of excipients are commonly used to stabilize proteins against aggregation, according to Kang: buffers, salts, amino acids, polyols/disaccharides/polysaccharides, surfactants, and antioxidants. These excipients prevent aggregation through several mechanisms. "First, pH is critical to protein stability and must be controlled to an optimal value through the use of appropriate buffers. Salts and amino acids increase the ionic strength of solutions while minimizing electrostatic interactions between protein molecules."



Polyols/disaccharides/polysaccharides, on the other hand, stabilize protein molecules through preferential hydration or preferential exclusion, in which the excipient is excluded from the protein surface, allowing the rearrangement and stabilization of a stabilizing hydrate shell around the protein surface and preventing protein molecules from interacting. Surfactants, meanwhile, protect proteins against interaction with hydrophobic surfaces such as air/water interfaces and container surfaces, and antioxidants protect proteins against oxidation.

Specific examples include trehalose and sucrose (disaccharides); mannitol and sorbitol (sugar alcohols); histidine, glycine, and arginine (amino acids); polysorbate 20, polysorbate 80, and proteins like human serum albumin (surfactants); sodium chloride (salt); and dextran and polyethylene glycol (polymers). Each type has a completely different stabilizing mechanism.

Alkylsaccharides as alternatives to polysorbates

The quality of the excipients can have a dramatic impact on protein stability. For example, common surfactants such as polysorbates are well known to contain trace amounts of peroxides that can become significant during storage and consequently compromise protein stability through oxidation of susceptible amino acids, particularly exposed methionine, according to Kang. "A good practice is to use compendial excipients in the development study and control the quality/storage of the excipients from the very beginning, so no surprises are encountered in GMP manufacturing and subsequent long-term storage," he says.

The issues with polysorbates have been a focus of Aegis Therapeutics. "The oxidative damage to biotherapeutic proteins can directly reduce their efficacy, but perhaps more importantly introduce unwanted immunogenicity, which can give rise to neutralization of activity and in more serious cases, neutralization of any residual biological activity associated with the patient's own corresponding proteins," observes Edward T. Maggio, president and chief executive officer of the company.

The polysorbates do an excellent job of preventing aggregation and permitting the creation of high-concentration formulations of biotherapeutics, but even though they are ideally highly purified when first incorporated, Maggio notes that formation of chemically reactive species occurs immediately upon any contact with oxygen during manufacturing or storage of the final product.

Aegis has shown that alkylsaccharides are as effective, and in some cases, more effective than the polysorbates for the prevention of aggregation, and they are completely free of any oxidative damage problems. Comprising a sugar attached to a long chain fatty acid, alkylsaccharides break up rapidly in the body into a sugar, typically glucose, and a fatty acid. They are essentially nontoxic food components, and are considered generally recognized as safe (GRAS) for food purposes. Both Big Pharma and smaller biotech companies (e.g., Roche and Bidel) are investigating the use of alkylsaccharides as replacements for polysorbates, according to Maggio. He also notes that there is extensive safety and toxicity study data in the drug master file covering the alkylsaccharides to which licensees of the technology receive a right of reference for their regulatory filings.

A blending approach

To assist formulators of biologic drugs with the stabilization and protection of therapeutic proteins in dry and liquid formulations, Leukocare developed its Stabilizing and Protecting Solutions (SPS) platform of excipient formulations. The proprietary SPS platform has been proven to stabilize different biological macromolecules during freeze drying and spray drying, during storage at increased temperature in dry or liquid formulations, and even during sterilization processes, according to Kempter. She adds that the modular concept of the SPS facilitates the possibility to adjust excipient compositions to the specific requirements of the biomolecule to protect from various types of stress exposures.

SPS formulations solely comprise excipients (mainly based on amino acids) listed in pharmacopeia that are also in general listed by FDA as inactive ingredients. Interactions between multiple functional groups of the excipients in solution and particularly during drying result in a strong amorphous character of the dried product, according to Kemter. The osmolytic excipients of the compositions may also support stabilization of the hydration shell around proteins in liquid formulations according to the preferential exclusion theory. In dried formulations, and according to the theory of water replacement, the excipients may substitute the stabilizing hydrogen bonds between proteins and water through the formation of stabilizing electrostatic interactions or hydrogen bonds with the proteins upon drying.

Recombinant human serum albumin (rHSA)

rHSA is the most common protein found in human plasma and is known for ligand binding and colloidal stabilization, properties that are also ideal for the stabilization of proteins and other biologics. In addition, because rHSA is a natural protein in the body, it is safer than other alternative surfactant excipients, such as synthetic polymers, because it has a low risk for immunogenicity and there is a natural pathway for its elimination from the body.



In addition to preventing protein aggregation by blocking undesired adsorption onto vial surfaces or air/liquid interfaces, rHSA has also been shown to prevent self-association of protein drugs through preferential hydration or preferential exclusion effects and to prevent the formation of micrometer-sized particles to increase the solubility of poorly soluble peptide-based drugs. Furthermore, rHSA exhibits antioxidant properties used for the stabilization of radiolabeled drugs. "As a result of this multifunctionality, in some cases, it is possible to reduce the number of excipients required to stabilize a biologic drug," Bukrinski says. He also notes that through covalent linking of a biologic API to rHSA or through encapsulation in albumin particles, it is possible to extend the circulatory half-life and control the release of drugs. Novozymes offers recombinant HSA products specifically produced for the biopharmaceutical industry.

Choosing the right stabilizing excipients

Because every protein is different and reacts differently with different types of excipients, the selection of the proper excipients for a given formulation is a complex and challenging task. Some excipients may be effective in one formulation and cause protein degradation and aggregation in another. "For instance, commonly used polysorbate detergents often disrupt the protein complexes in virus-like particles, and therefore an alternative such as recombinant human serum albumin or a carbohydrate-based polymer is needed," Bukrinski comments.

The choice of the right excipients for stabilization of a desired protein must take into account the very complex physical and chemical degradation behaviors and the potential multifunctional influences that each excipient can have on protein stability, according to Kemter. "The formulation scientist must have a thorough knowledge of several factors: how to optimize the physical and chemical stability of the active ingredient; how, rationally, to include specific excipients in the formulation; how to obtain the optimum conditions for stability; how to prevent stability issues during up-scaling; and, finally, how to design a formulation that is suitable for the intended route of administration, that is, one that allows the absorption barrier to be overcome."

Unfortunately, the structural differences among different proteins are so significant that generalization of universal stabilization strategies has not been successful to date. In addition, while it is desirable to keep the number of functional excipients as low as possible, the vast amount of potential destabilizing effects often necessitates the addition of several excipients.

Many scientists select excipients empirically, which takes a long time and puts a strain on resources, according to Kang. "It is more effective to perform a preformulation study to identify degradation pathways and stability-indicating analytical methods before testing major categories of excipients for their ability to protect protein molecules against specific degradations. Further study using a design-of-experiment (DoE) approach enables determination of the optimal levels of the most effective excipients and their interactions for the final formulation," he asserts. In fact, Patheon has been able to shorten the development timeline by designing formulations based on scientific rationale and the screening of large quantities of excipients using DoE.

Even so, Kemter believes that there remains a significant need for improved methods for the folding of proteins and the prevention of unfolding during production and storage and that can be generally applied to the majority of biopharmaceuticals. "Such technology would eliminate the need for so many individual investigations," she notes.

Evaluation of stability

Typical studies for determination of the stability of biopharmaceutical formulations include determination of the level of aggregates and insoluble particles of various sizes; detection of increased immunogenicity; determination of the level of amino acid oxidation, in particular, methionine and tryptophan; and evaluation of the fragmentation of polypeptide chains, all for samples subjected to both stressed and unstressed conditions, according to Maggio.

Most of the tests are accelerated aging studies, such as heating at elevated temperatures for 1 to 4 weeks, shaking at high temperature with an air/liquid interface for several days, and freezing and thawing for multiple cycles. Temperature ramping experiments are also common for determination of the melting and aggregation temperatures under different conditions, but Bukrinski has on several occasions observed that results from such studies do not always correlate well with shelf-life stability.

A whole host of advanced techniques are employed to analyze the results obtained in the above tests. Some examples include polyacrylamide-gel electrophoresis (PAGE), particle-size analysis using micro-flow imaging (MFI), dynamic and static light scattering, size-exclusion chromatography (SEC), ion-exchange chromatography (IEX), and reversed-phase (RP) high-pressure liquid chromatography (HPLC), liquid chromatography/mass spectrometry (LC/MS), peptide and glycan mapping, circular dichroism (CD), Fourier transform infrared spectroscopy, and other advanced MS methods.



A newer method that, according to Bukrinski, is increasingly used as an orthogonal method for the validation of SEC results is field flow fractionation (FFF). Instead of passing through a packed column, the fluid passes through a narrow channel and a force applied perpendicular to the channel causes separation of the particles based on their different mobilities.

Not all methods will work equally well for each biologic API. Therefore, the first step in any study is to determine which methods provide accurate results for the protein in question. "It is very important to first determine the appropriate methods for stability evaluation, followed by optimization of the pH and ionic strength for the formulation. It is only at that point that screening for appropriate excipients can be effective," Bukrinski says.

Because so many stability tests must be performed for the development of an effective biopharmaceutical formulation, he also notes that the ability to perform rapid, high-throughput screening is imperative. "We perform as many analytical methods as we can using robotics systems that can evaluate small quantities of samples in microplates. Often times, however, tight timelines will dictate a less optimal formulation development process," notes Bukrinski.

"Two of the most efficient screening tools include biophysical characterization and high-throughput degradation analysis under stressed conditions," adds Kang. "Biophysical tools, such as differential scanning calorimetry (DSC) and CD, directly measure the effect of excipients on the higher order structure of the proteins, which is often a good indicator of protein stability. High-throughput methods such as plate-based dynamic light scattering (DLS) can screen a large amount of excipients in a short period time."

Compared to small molecules, however, protein degradation involves multiple pathways, and a simple Arrhenius equation is generally not applicable, according to Kang. It is, therefore, difficult to predict long-term real-time stability through stressed and accelerated stability studies. "New mathematical models are currently being developed to establish a correlation between protein stability at different storage temperatures, and if successful, will greatly enhance formulation development and shelf-life prediction for protein drugs," he states.

Automated stability determination using chemical denaturation

Because temperature has such a significant influence on protein aggregation, it is important to obtain accurate thermodynamic data on proteins. However, temperature denaturation is often irreversible and accompanied by aggregation and precipitation and, therefore, not suitable for thermodynamic analysis.

On the other hand, isothermal chemical denaturation is generally a reversible process for most biologic drugs and has been proven to provide reliable thermodynamic data of use for the evaluation of protein stability. AVIA Biosystems has developed an automated protein denaturation system for protein stability analysis that uses intrinsic (or extrinsic) fluorescence to monitor the protein conformational changes associated with protein unfolding (denaturation). Using the instrument, the identification of solvent conditions that maximize the structural stability of biologics can be completely automated, according to the company. Proteins and other compounds can also be ranked by their stability and propensity to aggregate.

Few approved excipients creates challenges

Although the large number of stability tests and analyses required for the development of stable protein formulation present difficulties for biopharmaceutical companies, one of the biggest challenges for formulators is the limited number of known and approved excipients for protein stabilization, according to Kemter. In addition, many biopharmaceutical manufacturers will only use excipients that are approved internally, and generally only consider other excipients that are approved but have not been previously used by the company if no solution is possible with the existing choices, according to Bukrinski. "New excipients—whether just new to the company or compounds that have not been used as excipients before—will only be used if they have significantly high value and can compensate for the extended approval process. There must be a justification for the additional cost and risk," he says.

Kang agrees that the biggest challenge in developing new stabilizing excipients for biologics is the additional safety data required to introduce a novel excipient to a pharmaceutical product, particularly a parenteral formulation, which is the form in which most protein drugs are formulated. "The resources and time associated with this requirement makes formulation scientists hesitant to try new excipients," he observes.

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