

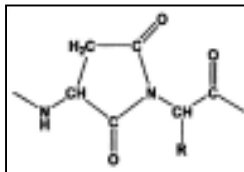
IN THIS ISSUE

Measuring Succinimide Formation	1
Sequence Effects on Protein Solubility	1
Extrinsic Fluorescence and ANS	2
Trehalose Destabilization?	2
Photostability of Protein Biologics	3
Protein Deposition on Solid Surfaces.....	3
Protein Stability in a Biodegradable Polymer.	3
A High Concentration Lyophilized MAb	4
Factors Controlling Membrane Fouling	4
Polyols as Stabilizers	5

CHEMICAL STABILITY

Xiao et al., ¹⁸O Labeling Method for Identification and Quantification of Succinimide in Proteins.

Analytical Chemistry **2007**, *79*: 2714-2721.



Abstract: Hydrolysis of succinimide, the intermediate in both deamidation and Asp-isoAsp interconversion, was monitored using electrospray time-of-flight mass spectrometry. Using ¹⁸O-labelled water, the 2 Dalton mass difference is easily detectable. This new method was used to examine the degradation of a monoclonal antibody, where Asp³⁰ of the light chain undergoes interconversion. The extent of degradation was found to be 21% after storage for 8 weeks at 45° C (pH 5.0). Using this new technique, the half-life of succinimide hydrolysis was measured to be about 6 hours under native but less than 5 seconds under denaturing conditions (at pH 8.2). Finally, the ratio of isoAsp to Asp after hydrolysis was found to be 3.5:1 under native conditions.

Analysis: While deamidation appears to be the most common hydrolytic pathway for degradation in protein pharmaceuticals, there are an increasing number of examples of interconversion of Asp residues to isoAsp. Both reactions proceed through the same intermediate called succinimide (also known as cyclic imide). Monitoring and quantifying these processes has been a challenge. This new

paper from a group at Amgen describes measuring the uptake of labeled water into the protein using mass spectrometry. The work provides some of the most detailed measurements of succinimide conversion to isoAsp and Asp in a large protein.

PROTEIN SOLUBILITY

Su et al., The Acidity of Protein Fusion Partners Predominantly Determines the Efficacy to Improve the Solubility of the Target Proteins Expressed in



Escherichia coli. *J. Biotechnol.* **2007**, *129*: 373-382.

Abstract: A set of fusion proteins expressed in *E. coli* was evaluated for their level of expression as soluble species. The sequences were analyzed using metrics found in previous studies (e.g., Wilkinson and Harrison, *Bio/technology* **1991**, *9*: 443-448), who focused on molecular weight and the percentage of Asn, Gly, Pro and Ser as indicators of solubility. To this, more factors have been added, including net negative charge average, the pI and the net charge at pH 7.0. These new parameters were intended to assess the impact of protein acidity on solubility. The role of hydrophilicity in increasing protein solubility is also discussed.

Analysis: Occasionally, there are papers that point us in the right direction, even with negative evidence. Despite the title, the data in this paper do not support the contention that protein acidity has a significant impact on solubility. However, this paper does present many of the factors that have been considered in the past for improving protein solubility. Given the increased demand to develop high concentration formulations, knowledge about protein solubility is at a premium. This paper provides some insight into what aspects of primary structure may increase or decrease solubility, at least in these types of systems. Much more work remains to be done before an overall understanding of protein solubility is achieved. Papers like this will help pave the way, even if the findings within it are not as conclusive as the title leads one to believe.

SPECTROSCOPIC METHODS

Gasymov and Glasgow, ANS Fluorescence: Potential to Augment the Identification of the External Binding Sites of the Protein. *Biochim. Biophys. Acta* **2007**, 1774: 403-411.

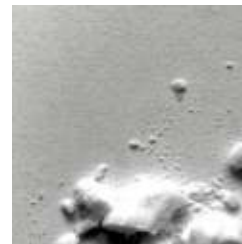


Abstract: Extrinsic fluorescence studies of proteins can provide information on the overall conformational state. The most commonly used fluorescent probe is ANS. Originally, it was thought that ANS bound to hydrophobic patches, with a corresponding increase in fluorescent intensity. However, it then became clear that some of the binding was electrostatic in nature. This study measures the strength of interaction using titrating calorimetry. While electrostatics plays some role in the binding to proteins, it cannot account for all of the binding strength. Clearly, short-range forces, such as van der Waal's interactions/hydrophobic interactions contribute as well.

Analysis: For many years, there has been an effort to find extrinsic fluorescent probes whose spectral properties change upon binding to proteins. One of the original probes described in the literature is ANS, which was presumed to bind to hydrophobic patches on the surface of proteins. The interest in ANS grew when it was used to identify molten globule states, which have been considered as important protein folding intermediates. However, it discovered that much of the interaction between proteins and ANS was due to electrostatic interactions. This new paper explores the relative contributions of electrostatics and other short-range forces to the binding constant. In short, while electrostatic interactions are important, there is clearly a hydrophobic interaction component as well.

EXCIPIENTS: EXCLUDED SOLUTES

Habib et al., Thermal Destabilization of Stem Bromelain by Trehalose. *The Protein Journal* **2007**, 26: 117-124.

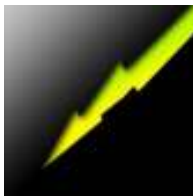


Abstract: Trehalose is a naturally occurring osmolyte that is known to stabilize a wide variety of biomolecules, acting as an excluded solute. Unexpectedly, trehalose lowers the T_m for bromelain, an enzyme from pineapple, by 7° C. In addition, the enzyme inactivated more quickly at 60° C in the presence of 1 M trehalose. Similar results were seen with sucrose as well. The explanation appears to be greater preferential exclusion with the denatured state than with the native state.

Analysis: When an article states that trehalose acts to destabilize a protein, it deserves some consideration. It is universally accepted that, to some degree, excluded solutes stabilize proteins to denaturation. In fact, disaccharides, such as sucrose and trehalose, are usually considered as the best examples of excipients that behave in this fashion. In this case, the T_m values actually decrease in the presence of both trehalose and sucrose. While they are not the most detailed unfolding curves, the reported trend in T_m does appear to be correct. Moreover, the enzyme appears to inactivate more quickly in the presence of disaccharides. These findings appear to contradict the current view of sugars as stabilizers in aqueous solution. The explanation put forward by the authors is of 'preferential hydration' of the denatured state. It is known that stabilization via the excluded solute effect does presume that (a) the denatured state is larger in surface area than the native state and (b) there is lack of binding by the sugar to both states. If either of these two assumptions is not true here, then it could lead to the reported results. It is an important lesson for formulation scientists. Not all denatured states are the same. They depend on the protein and on the stress used to unfold the protein. Furthermore, there can be specific interactions or binding in some cases (and this can be concentration dependent—note that guanidinium is preferentially excluded at low concentrations but not at high concentration, where it becomes a chaotrope by binding to protein surfaces).

PHOTOSTABILITY

Kerwin and Remmele, Protein from Light: Photodegradation and Protein Biologics. *J. Pharm. Sci.* **2007**, 96: 1468-1479.



Abstract: Exposure of proteins to light can cause photooxidation of aromatic and cysteine side chains. These chemical modifications can possibly alter structure, reduce potency, and increase immunogenicity. The major pathways of photodegradation are delineated and the current warnings about light exposure for marketed products are provided.

Analysis: At last, a detailed review on photodegradation of protein pharmaceuticals is available. Despite having ICH guidelines in place for photostability testing for a while now, there was precious little literature about possible decomposition pathways. This review article summarizes the most sensitive amino acids and the likely pathways for photodegradation. The figures are particularly useful, as many are drawn from more than one reference, giving a comprehensive overview of the possible redox pathways.

SURFACE ADSORPTION

Kroslak et al., Effect of Temperature, pH and Salt Concentration on β -Lactoglobulin Deposition Kinetics Studied by Optical Waveguide Lightmode Spectroscopy. *Biomacromolecules* **2007**, 8: 963-970.



Abstract: Deposition kinetics for β -lactoglobulin were studied between 61 and 83° C after primary deposition was conducted at 25° C to condition the surface. Optical waveguide lightmode spectroscopy (OWLS) is a fast on-line technique that can detect and quantify multiple layer formation up to tens of nanometers thick. Deposition kinetics increased as the pH was raised from 5.5 to 7.4, a trend similar to that seen for aggregation in solution. Activation energies for deposition were determined, ranging from 340 kJ/mole at pH 5.5 to 230 kJ/mole at pH 7.4.

Analysis: Adsorption of proteins to surfaces is an important physical process. Unfortunately, it is also one that is difficult to measure and quantify. Kroslak and co-workers describe a new method for monitoring the deposition of β -lactoglobulin on solid surfaces. Whether OWLS could be an important PAT tool remains to be seen. It appears from the description in this article that the technique is somewhat invasive and can be easily fouled. Moreover, calibration issues are not discussed. Despite these limitations, it appears that OWLS can be used to determine adsorption kinetics of proteins at solid-liquid interfaces, which is valuable. The fact that the kinetics could be mapped as a function of pH and temperature indicates it may find utility during preformulation work on container compatibility.

CONTROLLED RELEASE

Gu et al., Maintenance of Vascular Endothelial Growth Factor and Potentially other Therapeutic Proteins Bioactivity during a Photo-initiated Free-Radical Cross-linking Reaction Forming Biodegradable Elastomers. *Eur. J. Pharm. Biopharm.* **2007**, 66: 21-27.



Abstract: The stability of the protein, vascular endothelial cell growth factor (VEGF) during photo-initiated free radical crosslinking of a biodegradable star polymer was evaluated. While significant loss of bioactivity of VEGF is seen with UV irradiation in the presence of a free radical initiator, it appears that the macromer protects the protein. Addition of bovine serum albumin provides some additional protection as well. In short, it appears that conditions can be identified that allow formation of delivery system consisting of a bioactive protein within a biodegradable polymeric matrix through *in situ* photopolymerization

Analysis: As more proteins are evaluated for inclusion into drug delivery systems, there needs to be an assessment of their stability in these systems. In this case, the polymeric matrix is formed while the protein is present. As it is a free radical polymerization, there is significant chance of damage occurring to the protein. Furthermore, the polymerization is initiated by UV light, which in itself could cause

photooxidation. This group finds that the protein can be protected to some degree, although it is clear that some cross-linking of the protein to the matrix occurs under all conditions. Therefore, the general utility of such an approach is probably limited. However, it is useful to have more and more studies on alternative methodologies for preparing controlled release dosage forms of biopharmaceuticals. Only in doing so will the best techniques for manufacturing such systems will be identified.

LYOPHILIZATION

Colandene et al., Lyophilization Cycle Development for a High-Concentration Monoclonal Antibody Formulation Lacking a Crystalline Bulking Agent. *J. Pharm. Sci.* **2007**, 96: 1598-1608.



Abstract: A lyophilization cycle was optimized for a monoclonal antibody (MAb) at 80 mg/ml using experimental design methodology. Analysis of the solid-state properties by DSC and lyoscopy indicated that as the protein concentration increased, the T_g' and T_c (collapse temperature) began to deviate from each other. At 80 mg/ml, the T_c is about 5-7° C higher than T_g' . Primary drying could be shorted significantly by using a product temperature well above T_g' without any apparent detrimental effects on the product.

Analysis: High concentration formulations are the subject of intense focus within many companies, especially for monoclonal antibodies. Until recently, reports on high concentration formulations were rare. This new work from Human Genome Sciences illustrates an important factor in developing such formulations. As the protein concentration increases, the solid-state properties will become dominated by the protein rather than the excipients. Furthermore, the protein can now act as its own bulking agent. This is consistent with our previous understanding about developing stable lyophilized formulations. What is more surprising in this study is that the T_g' and T_c begin to deviate as the protein concentration increases. At 80 mg/ml, the two are different by 5-7° C. This means that one can conduct primary drying above T_g' without risk of collapse, reducing run times significantly. While there are many aspects of the formulation that become

more difficult as the protein concentration increases, here is one benefit. The authors hypothesize the behavior is explained by differences in viscous flow at higher protein concentrations. Future work will show whether this is correct.

MEMBRANE FOULING

Salgin, Effects of Ionic Environments on Bovine Serum Albumin Fouling in a Cross-Flow Ultrafiltration System. *Chem. Eng. Technol.* **2007**, 30: 255-260.

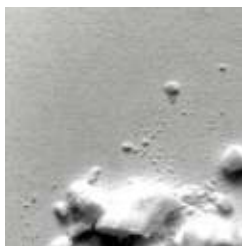


Abstract: The effects of electrostatic interactions on membrane fouling by BSA during ultrafiltration (UF) were investigated. The transmembrane pressure was measured from pH 3.48 to 7.46 (on either side of the pI of BSA) and from 0.001 M to 0.1 M KCl. Furthermore, the zeta potentials of the protein and membrane were measured under the same conditions. The cake layer resistance decreased with increasing pH and ionic strength.

Analysis: Loss of proteins on membranes is a significant problem, whether during aseptic processing, diafiltration, ultrafiltration, or nanofiltration. Therefore, any insight that can be gained on the mechanism of fouling and methods for reducing losses is important. In this study, Salgin provides data that the zeta potential of the protein plays an important role in controlling membrane fouling. Our own analysis of the data indicates that the zeta potential of the protein is markedly more important than that of the membrane. This suggests that colloidal stability of proteins is an important aspect of reducing membrane fouling, at least with hydrophilic membranes, such as polyether sulfone (PES). Therefore, evaluation of colloidal stability could be important in process development as well as in designing the final formulation.

POLYOLS AS STABILIZERS

Chanasattru et al.,
Modulation of Thermal
Stability and Heat-
Induced Gelation of β -
Lactoglobulin by High
Glycerol and Sorbitol
Levels. *Food Chemistry*
2007, 103: 515-520.



Abstract: The effect of both glycerol and sorbitol on the thermally-induced gelation of β -lactoglobulin was investigated. Addition of sorbitol increased the T_m from 74° C to 86° C (at 50%), while glycerol only raised T_m by 2° C. Coincidentally, β -lactoglobulin did not gel when heated in the presence of sorbitol, but it did in the presence of glycerol. The stabilization appears to be due to preferential exclusion of sorbitol from the native protein, resulting in a marked increase in the conformational stability of the protein. The effect of increased viscosity on collisional frequency was also considered.

Analysis: Gelation of peptides and proteins is a common physical instability, and one of special importance in the food industry. Therefore, much of our understanding of thermally-induced gelation comes from that discipline, including work from this group. As with many other protein systems, an increase in the conformational stability can slow aggregation. This group also discusses protein-protein interactions (colloidal stability) and viscosity effects on gelation rates as well. Interestingly, glycerol, while an excluded solute, is relatively important when compared to sorbitol.