

DSC III - Liquid Protein Formulation Studies

Ahrer K., Buchacher A., Iberer G., and Jungbauer A. (2006) Thermodynamic stability and formation of aggregates of human immunoglobulin G characterised by differential scanning calorimetry and dynamic light scattering. *J Biochem Biophys Methods* **66**, 73-86.

Abstract: The final process step of polyclonal human immunoglobulin G is formulation with agents such as sugars, polyols, amino acid and salts. Often the most stable formulations were empirically identified. Physicochemical methods, such as differential scanning calorimetry and dynamic light scattering, provide a deeper insight on the biophysical properties of such a protein solution. The combination of these methods proved to be sensitive enough to detect fine differences in the properties relevant for the development of stable protein solutions. The influence of additives, such as maltose and glycine in combination with water or low concentrations of salts, on human immunoglobulin preparations was analysed. Differential scanning calorimetry illustrated that 0.2 M glycine had better stabilising effects compared to 10% maltose. Dynamic light scattering and differential scanning calorimetry revealed that solutions preventing aggregation were not optimal in terms of thermodynamic stability. Aggregation was minimised with increasing ionic strength, shown by dynamic light scattering, whereas thermodynamic stability for heat sensitive parts of human immunoglobulin G, analysed with differential scanning calorimetry, was decreased.

Alden M. and Magnusson A. (1997) Effect of temperature history on the freeze-thawing process and activity of LDH formulations. *Pharm Res* **14**, 426-430.

Abstract: **PURPOSE:** The purpose of the study was to investigate the effect of freeze-thawing processes with different temperature histories on thermal transformations and on protein activity of lactate dehydrogenase (LDH) formulations. Polyethylene glycol (PEG 6000) and maltodextrin were used as cryoprotectants. **METHODS:** The thermal characterization was made by oscillating DSC (ODSC). LDH activity assays were performed spectrophotometrically. **RESULTS:** The crystallization of the solutions and the melting of the frozen samples occurred at fairly constant heat of crystallisation and heat of fusion values and temperatures. The main difference between the two investigated temperature cycles was an exothermic peak at -45 degrees C, which might reflect the transition between the cubic and hexagonal ice structures. When PEG was added to the system an additional endothermic peak appeared at -15 degrees C in the heating program. It was transformed into the shape of a glass transition at the same temperature when the heating rate was increased. The degree of crystallinity of the samples was evaluated as the quota between the Cp component of heat of transformation and the total heat of transformation values. Only minor differences between the two temperature histories and between the samples were observed. The cp component of the melting endotherm revealed a complex melting process with two overlapping endothermic transformations. The good protein protecting ability of PEG obtained when cooling and heating rate was low, was greatly reduced with increasing rate. The addition of maltodextrin to PEG-containing solutions lowered the activity recovery. **CONCLUSIONS:** The endothermic transformation of a PEG-ice structure at -15 degrees C in the heating process is strongly correlated to the protective ability of PEG 6000 in the freeze-thawing process of LDH. To obtain the highest protein activity after the freeze-thawing process, the formulation shall be transformed by a low cooling and heating rate. The crystallinity of the system melting at about 2 degrees C is independent of temperature history. The cp component of the melting endotherm, however, shows a complex transformation, where two phases of different crystallinity and stability might be involved.

Anraku M., Kouno Y., Kai T., Tsurusaki Y., Yamasaki K., and Otagiri M. (2007) The role of N-acetyl-methioninate as a new stabilizer for albumin products. *Int J Pharm* **329**, 19-24.

Abstract: Sodium octanoate (Oct) and N-acetyl-L-tryptophanate (N-AcTrp) are widely used as stabilizers during the pasteurization of albumin products. However, N-AcTrp has a possible side effect of intracerebral disease. To provide safe and risk-free albumin products, we validated N-acetyl-methioninate (N-AcMet) as a new stabilizer for albumin products. The effect of N-AcMet on oxidation was examined using 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) as an oxidizing agent. Carbonyl content in the presence of N-AcMet, as well as that in the presence of N-AcTrp after 24h (), was significantly decreased. The effect of AAPH on the oxidative status of 34-Cys on human serum albumin was also studied by HPLC. It was found that N-AcMet as well as N-AcTrp, has a large protective effect on the sulfhydryl group after 1h. Further, N-AcMet was found to be a superior radical scavenger to N-AcTrp using 1,1'-diphenyl-2-

picrylhydrazyl (DPPH) radicals. The thermal stabilizing role of N-AcMet manifested as an increase in denaturation temperature and calorimetric enthalpy, as determined by differential scanning calorimetry (DSC). In the present study, we suggest that use of N-AcMet in albumin preparation is safe and free of risk of side effects.

Badkar A., Yohannes P., and Banga A. (2006) Application of TZERO calibrated modulated temperature differential scanning calorimetry to characterize model protein formulations. *Int J Pharm* **309**, 146-156.

Abstract: The objective of this study was to evaluate the feasibility of using T(ZERO) modulated temperature differential scanning calorimetry (MDSC) as a novel technique to characterize protein solutions using lysozyme as a model protein and IgG as a model monoclonal antibody. MDSC involves the application of modulated heating program, along with the standard heating program that enables the separation of overlapping thermal transitions. Although characterization of unfolding transitions for protein solutions requires the application of high sensitive DSC, separation of overlapping transitions like aggregation and other exothermic events may be possible only by use of MDSC. A newer T(ZERO) calibrated MDSC model from TA instruments that has improved sensitivity than previous models was used. MDSC analysis showed total, reversing and non-reversing heat flow signals. Total heat flow signals showed a combination of melting endotherms and overlapping exothermic events. Under the operating conditions used, the melting endotherms were seen in reversing heat flow signal while the exothermic events were seen in non-reversing heat flow signal. This enabled the separation of overlapping thermal transitions, improved data analysis and decreased baseline noise. MDSC was used here for characterization of lysozyme solutions, but its feasibility for characterizing therapeutic protein solutions needs further assessment.

Bedu-Addo F. K., Johnson C., Jeyarajah S., Henderson I., and Advant S. J. (2004) Use of biophysical characterization in preformulation development of a heavy-chain fragment of botulinum serotype B: evaluation of suitable purification process conditions. *Pharm Res* **21**, 1353-1361.

Abstract: PURPOSE: The purpose of this study was to investigate the physicochemical and structural characteristics of recombinant botulinum serotype B (rBoNTB(Hc)) under various conditions and to use the information in evaluating suitable purification process conditions. METHODS: The solubility of rBoNTB(Hc) was evaluated at pH 4, 5, 6 7.5, 8, and 9. Secondary structure was evaluated using circular dichroism, and conformational stability was monitored using high sensitivity differential scanning calorimetry. Hydrophobic interaction chromatography, size exclusion chromatography-high performance liquid chromatography (SEC-HPLC), sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE), peptide mapping, and UV spectroscopy were used to monitor stability under the various conditions. RESULTS: The secondary structure of rBoNTB(Hc) consists predominantly of beta-sheets. Solubility of rBoNTB(Hc) was lowest at its pI and highest at low and high pH. In the presence of NaCl, however, solubility decreased with increase in pH. Conformational and chemical stability are improved below pH 7.5. In the presence of 150 mM NaCl at high pH, conformational and chemical stability of rBoNTB(Hc) are further decreased. The study suggests that the purification process should minimize exposure of rBoNTB(Hc) to high pH and salt conditions. CONCLUSIONS: Optimal stability of rBoNTB(Hc) is achieved at low pH. The biophysical and analytical studies provide us with an understanding of rBoNTB(Hc) stability behavior in solution and assists in developing efficient purification conditions.

Bonacci G., Sanchez M. C., Gonzalez M., Ceschin D., Fidelio G., Vides M. A., and Chiabrande G. (2000) Stabilization of homogeneous preparations of pregnancy zone protein lyophilized in the presence of saccharose. Structural and functional studies. *J Biochem Biophys Methods* **46**, 95-105.

Abstract: Human pregnancy zone protein (PZP) is a macromolecule of 360 kDa, organized as a disulfide-linked homodimer of two 180 kDa subunits, with an amino acid sequence and structure remarkably similar to that of human alpha2-Macroglobulin. Homogeneous PZP samples undergo fast aging forming oligomeric aggregates of high molecular weight. This aged PZP loses its ability to interact with proteinases and consequently, non-recognition of receptors occurs. In the present work, we assessed the effect of saccharose on the stability of native PZP on lyophilized samples kept for a long period of time. Herein, we demonstrate that the addition of 0.25 M saccharose to homogeneous PZP and further lyophilization is enough to prevent aging and preserve functional activity for more than 1 year. Hence, high quality samples, in terms of purity, stability and functional activity will allow to develop biochemical studies in order to

know the PZP role in physiological and pathological states where the protein levels are increased, such as pregnancy and tumoral disorders.

Branchu S., Forbes R. T., York P., and Nyqvist H. (1999) A central composite design to investigate the thermal stabilization of lysozyme. *Pharm Res* **16**, 702-708.

Abstract: PURPOSE: The formulation and processing of protein drugs requires the stabilization of the native, biologically active structure. Our aim was to investigate the thermal stability of a model protein, lysozyme, in the presence of two model excipients, sucrose and hydroxypropyl-beta-cyclodextrin (HP-beta-CD). METHODS: We used high sensitivity differential scanning calorimetry (HSDSC) in combination with a central composite design (CCD). As indicators of protein thermal stability, the measured responses were the unfolding transition temperature (T_m), the onset temperature of the denaturation (T_o), and the extrapolated onset temperature ($T_{o,e}$). RESULTS: A highly significant (F probability <0.001) statistical model resulted from analysis of the data. The largest effect was due to pH (over the range 3.2-7.2), and the pH value that maximized T_m was 4.8. Several minor but significant effects were detected that were useful for mechanistic understanding. In particular, the effects of protein concentration and cyclodextrin concentration on T_m and $T_{o,e}$ were found to be pH-dependent. This was indicative of the partially hydrophilic nature of protein-protein interactions and protein-cyclodextrin interactions, respectively. CONCLUSIONS: Response surface methodology (RSM) proved efficient for the modeling and optimization of lysozyme thermal stability as well as for the physical understanding of the protein-sugar-cyclodextrin system in aqueous solution.

Breen E. D., Curley J. G., Overcashier D. E., Hsu C. C., and Shire S. J. (2001) Effect of moisture on the stability of a lyophilized humanized monoclonal antibody formulation. *Pharm Res* **18**, 1345-1353.

Abstract: PURPOSE: To determine the effect of moisture and the role of the glass transition temperature (T_g) on the stability of a high concentration, lyophilized, monoclonal antibody. METHODS: A humanized monoclonal antibody was lyophilized in a sucrose/histidine/polysorbate 20 formulation. Residual moistures were from 1 to 8%. T_g values were measured by modulated DSC. Vials were stored at temperatures from 5 to 50 degrees C for 6 or 12 months. Aggregation was monitored by size exclusion chromatography and Asp isomerization by hydrophobic interaction chromatography. Changes in secondary structure were monitored by Fourier transform infrared (FTIR). RESULTS: T_g values varied from 80 degrees C at 1% moisture to 25 degrees C at 8% moisture, there was no cake collapse and there were no differences in the secondary structure by FTIR. All formulations were stable at 5 degrees C. High moisture cakes had higher aggregation rates than drier samples if stored above their T_g values. Intermediate moisture vials were more stable to aggregation than dry vials. High moisture samples had increased rates of Asp isomerization at elevated temperatures both above and below their T_g values. Chemical and physical degradation pathways followed Arrhenius kinetics during storage in the glassy state. Only Asp isomerization followed the Arrhenius model above the T_g value. Both chemical and physical stability at $T > \text{ or } = T_g$ were fitted to Williams-Landel-Ferry (WLF) kinetics. The WLF constants were dependent on the nature of the degradation system and were not characteristic of the solid system. CONCLUSION: High moisture levels decreased chemical stability of the formulation regardless of whether the protein was in a glassy or rubbery state. In contrast, physical stability was not compromised, and may even be enhanced, by increasing residual moisture if storage is below the T_g value.

Buera M. P., Rossi S., Moreno S., and Chirife J. (1999) DSC confirmation that vitrification is not necessary for stabilization of the restriction enzyme EcoRI dried with saccharides. *Biotechnol Prog* **15**, 577-579.

Abstract: The glass transition temperature (T_g) of preparations of the restriction enzyme EcoRI, vacuum-dried in the presence of sucrose, trehalose, or raffinose, was determined using differential scanning calorimetry. T_g values were well below those expected for low-moisture sucrose, trehalose, or raffinose, and this was attributed to the presence of glycerol (a plasticizer), which was a main component of the restriction enzyme preparation. This was verified by determining the glass transition temperature of glycerol, which was found to be (onset value) -77 degrees C. Present results confirmed that vitrification (i.e., glass formation) was not necessary for enzyme protection in present low-moisture saccharide systems. As shown in previous work, enzyme EcoRI was very stable stored at 37/45 degrees C in spite of the fact that sugar matrices were completely rubbery, as unequivocally demonstrated in the present work.

Burton L., Gandhi R., Duke G. and Paborji M. (2007) Use of microcalorimetry and its correlation with size exclusion chromatography for rapid screening of the physical stability of large pharmaceutical proteins in solution. *Pharm. Dev. Technol.* **12**, 265-273.

Abstract: The utility of microcalorimetry as a rapid screening tool for assessing the solution stability of high molecular weight pharmaceutical proteins was evaluated by using model recombinant antibodies, Protein I and Protein II. Changes in the transition midpoint, $T(m)$, were monitored as a function of pH and/or in the presence of excipients, and results were compared with traditional accelerated stability data from samples that were analyzed by size exclusion chromatography (SEC). The data from microcalorimetry were well correlated with those from SEC for predicting both optimal solution pH as well as excipient effects on solution stability. These results indicate that microcalorimetry can be an efficient screening tool useful in identifying optimal pH conditions and excipients to stabilize pharmaceutical proteins in solution formulations.

Capelle M. A., Gurny R. and Arvinte T. (2007) High throughput screening of protein formulation stability: practical considerations. *Eur J Pharm. Biopharm.* **65**, 131-148.

Abstract: The formulation of protein drugs is a difficult and time-consuming process, mainly due to the complexity of protein structure and the very specific physical and chemical properties involved. Understanding protein degradation pathways is essential for the success of a biopharmaceutical drug. The present review concerns the application of high throughput screening techniques in protein formulation development. A protein high throughput formulation (HTF) platform is based on the use of microplates. Basically, the HTF platform consists of two parts: (i) sample preparation and (ii) sample analysis. Sample preparation involves automated systems for dispensing the drug and the formulation ingredients in both liquid and powder form. The sample analysis involves specific methods developed for each protein to investigate physical and chemical properties of the formulations in microplates. Examples are presented of the use of protein intrinsic fluorescence for the analysis of protein aqueous properties (e.g., conformation and aggregation). Different techniques suitable for HTF analysis are discussed and some of the issues concerning implementation are presented with reference to the use of microplates.

Chan H. K., Au-Yeung K. L., and Gonda I. (1996) Effects of additives on heat denaturation of rhDNase in solutions. *Pharm Res* **13**, 756-761.

Abstract: PURPOSE. To study the thermal stability of recombinant human deoxyribonuclease I (rhDNase) in aqueous solutions. METHODS. Differential scanning calorimetry (DSC) was used to measure the denaturation or melting temperature (T_m) and enthalpy (H_m) of rhDNase. The effects of denaturants (guanidine HCl and urea) and additives (mainly divalent cations and disaccharides) were investigated at pH 6-7. RESULTS. The T_m and H_m of rhDNase in pure water were measured as 67.4 degrees C and 18.0 J/g respectively, values typical of globular proteins. The melting peak disappeared on re-running the sample after cooling to room temperature, indicating that the thermal denaturation was irreversible. The latter was due to the occurrence of aggregation accompanying the unfolding process of rhDNase. Size exclusion chromatography indicated that during heat denaturation, rhDNase formed soluble high molecular weight aggregates with a molecular size >300kD estimated by the void volume. Of particular interest are the divalent cations: $Ca(2+)$ stabilizes rhDNase against thermal denaturation and elevates T_m and H_m while $Mg(2+)$, $Mn(2+)$ and $Zn(2+)$ destabilize it. Sugars also stabilize rhDNase. As expected, denaturants destabilize the protein and lower the T_m and H_m . All destabilization of rhDNase can be prevented by adding $Ca(2+)$ to the solutions. CONCLUSIONS. $CaCl_2$ and sugars were found to stabilize rhDNase against thermal denaturation while divalent cations, urea and guanidine HCl destabilize the protein. The effects could be explained by a mixture of mechanisms. For $Ca(2+)$ the protective effect is believed to be due to an ordering of the rhDNase structure in its native state, and by prevention of breaking of a disulfide bridge, thus making it less susceptible to unfold under thermal stress.

Chang S. L., Hofmann G. A., Zhang L., Deftos L. J., and Banga A. K. (2003) Stability of a transdermal salmon calcitonin formulation. *Drug Deliv* **10**, 41-45.

Abstract: This study was designed to monitor the stability of salmon calcitonin during storage conditions, under the electric fields generated during iontophoresis and electroporation, in contact with transdermal glass diffusion cells, and during transport through skin. The formulation in a citrate buffer (pH 4.0) was stable in storage for short-term studies but degraded significantly on extended storage. Albumin was able to minimize adsorption in contact with glass surfaces, and aprotinin was able to minimize proteolytic

degradation in contact with skin. The formulation was stable under electric field, but there was a loss due to adsorption if salt bridges were used.

Cueto M., Dorta M. J., Munguia O., and Llabres M. (2003) New approach to stability assessment of protein solution formulations by differential scanning calorimetry. *Int J Pharm* **252**, 159-166.

Abstract: A central composite rotatable second order design was used to evaluate chicken egg-white lysozyme (lysozyme) thermal stability at different pH, and lysozyme, sucrose and 2-hydroxypropyl-beta-cyclodextrin (HPbetaCD) concentrations, by means of differential scanning calorimetry (DSC). Four measurements were used to characterize the thermogram: the calorimetric enthalpy ($\Delta H(\text{cal})$), the temperature at maximum heat flux (T_m), the ratio of maximum heat flux over thermogram area ($C(pT(m))/\text{area}$), and the ratio of calorimetric enthalpies from the second heating cycle to the first enthalpy ($R(\Delta H(\text{cal}))$). These parameters were interpreted using the three step equilibrium model for protein degradation (irreversible degradation following reversible unfolding). In addition to degradation, increased lysozyme concentration leads to a sizeable decrease in $\Delta H(\text{cal})$ and area ratio, showing how it causes protein aggregation; which in turn promotes protein degradation. $\Delta H(\text{cal})$ and T_m reach maxima at pH 5, $R(\Delta H(\text{cal}))$ at pH 4.19, while $C(pT(m))/\text{area}$ increases linearly with pH, revealing a specific base catalysis of the irreversible degradation step. The role of sucrose concentration in lysozyme stabilization is linked to the stabilization of the unfolded moiety; it neither affects $\Delta H(\text{cal})$ nor $C(pT(m))/\text{area}$, but increases both T_m and $R(\Delta H(\text{cal}))$. No influence of HPbetaCD on the stability of lysozyme was observed, probably due to low concentrations employed.

Cui L., Du G., Zhang D. and Chen J. (2008) Thermal stability and conformational changes of transglutaminase from a newly isolated *Streptomyces hygrosopicus*. *Bioresour. Technol.* **99**, 3794-3800.

Abstract: Thermal stability and conformational changes of transglutaminase (TGase) from a newly isolated *Streptomyces hygrosopicus* were investigated in this study. The inactivation kinetics of the microbial transglutaminase (MTGase) was fitted using one-step inactivation model. It was much more stable under 40 degrees C. The half-lives for the MTGase at 50 degrees C and 60 degrees C were only 20 min and 8 min, respectively. Spectroscopic studies of the enzyme suggested conformational transition from ordered secondary structural elements (alpha/beta-protein) to unordered structure during thermal denaturation. Some polyols could improve the thermal stability of the enzyme. Among the polyols examined, the prolonged half-lives of 40 min at 50 degrees C and 20 min at 60 degrees C were gained by adding 10% glycerol. The results of differential scanning calorimetric (DSC) analysis showed a distinct transition peak with a significant greater T_m and ΔH for the MTGase mixed with polyols in comparison with the control, which indicated that the polyols could maintain the natural structure of the enzyme to some extent. The SDS-PAGE electrophoresis of cross-linked casein confirmed that the stabilizers could protect the MTGase from thermal denaturation

Derrick T., Grillo A. O., Vitharana S. N., Jones L., Rexroad J., Shah A., Perkins M., Spitznagel T. M., and Middaugh C. R. (2007) Effect of polyanions on the structure and stability of repifermintrade mark (keratinocyte growth factor-2). *J Pharm Sci* **96**, 761-766.

Abstract: The interaction of several of the fibroblast growth factors (FGFs) with polyanions is thought to be of physiological significance and has been exploited to create more stable pharmaceutical formulations of FGF-1 and -2. The extent of such phenomena throughout the 23-member FGF family is, however, unknown. In these studies, we examine the effect of several polyanions on the structure and stability of keratinocyte growth factor 2 (KGF-2, FGF-10), a candidate for use as a wound-healing agent. Employing a variety of methods sensitive to the protein's structure including circular dichroism (CD), intrinsic fluorescence, derivative near-UV absorption spectroscopy, bis-ANS (4,4'-dianilino-1,1'-binaphthyl-5,5-disulfonic acid) fluorescence, differential scanning calorimetry (DSC), and dynamic light scattering (DLS), we find that a variety of polyanions (e.g., heparin, sucrose octasulfate (SOS), and inositol hexaphosphate (IHP)) stabilize KGF-2 by increasing the thermal-unfolding temperature by approximately 9-15 degrees C. Negatively charged liposomes produce a similar effect, arguing for relatively nonspecific interactions of polyanions with KGF-2. Unlike some other FGFs, no evidence for the presence of a molten globule state is found during thermal perturbation of this growth factor. The generality of this polyanion/protein interaction is discussed as well as its potential role in various cellular events such as protein folding and transport. (c) 2006 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci*.

Ejima D., Tsumoto K., Fukada H., Yumioka R., Nagase K., Arakawa T. and Philo J. S. (2007) Effects of acid exposure on the conformation, stability, and aggregation of monoclonal antibodies. *Proteins* **66**, 954-962.

Abstract: Exposure of antibodies to low pH is often unavoidable for purification and viral clearance. The conformation and stability of two humanized monoclonal antibodies (hIgG4-A and -B) directed against different antigens and a mouse monoclonal antibody (mIgG1) in 0.1M citrate at acidic pH were studied using circular dichroism (CD), differential scanning calorimetry (DSC), and sedimentation velocity. Near- and far-UV CD spectra showed that exposure of these antibodies to pH 2.7-3.9 induced only limited conformational changes, although the changes were greater at the lower pH. However, the acid conformation is far from unfolded or so-called molten globule structure. Incubation of hIgG4-A at pH 2.7 and 3.5 at 4 degrees C over the course of 24 h caused little change in the near-UV CD spectra, indicating that the acid conformation is stable. Sedimentation velocity showed that the hIgG4-A is largely monomeric at pH 2.7 and 3.5 as well as at pH 6.0. No time-dependent changes in sedimentation profile occurred upon incubation at these low pHs, consistent with the conformational stability observed by CD. The sedimentation coefficient of the monomer at pH 2.7 or 3.5 again suggested that no gross conformational changes occur at these pHs. DSC analysis of the antibodies showed thermal unfolding at pH 2.7-3.9 as well as at pH 6.0, but with decreased melting temperatures at the lower pH. These results are consistent with the view that the antibodies undergo limited conformational change, and that incubation at 4 degrees C at low pH results in no time-dependent conformational changes. Titration of hIgG4-A from pH 3.5 to 6.0 resulted in recovery of native monomeric proteins whose CD and DSC profiles resembled those of the original sample. However, titration from pH 2.7 resulted in lower recovery of monomeric antibody, indicating that the greater conformational changes observed at this pH cannot be fully reversed to the native structure by a simple pH titration.

Fan H., Ralston J., Dibiase M., Faulkner E., and Russell M. C. (2005) Solution behavior of IFN-beta-1a: an empirical phase diagram based approach. *J Pharm Sci* **94**, 1893-1911.

Abstract: An empirical phase diagram approach has been developed as a practical tool to aid macromolecular preformulation/formulation studies. This method employs an eigenvector based procedure to visualize and interpret complex data sets. Human Interferon-beta-1a, an important therapeutic protein, was used to further develop the method and test its utility. The protein was characterized in solution as a function of pH (2-8), temperature (10 degrees C-85 degrees C) and ionic strength (I = 0.1 and 1.0) using intrinsic and ANS fluorescence, Far-UV circular dichroism (Far-UV CD), Fourier Transform Infrared spectroscopy (FTIR) and derivative UV absorbance spectroscopies, as well as differential scanning calorimetry (DSC) to supplement spectroscopic thermal stability studies. Derivative UV absorbance data were initially used to construct a pH-temperature phase diagram at each ionic strength. Three distinctive phases at I = 0.1 and two major phases at I = 1.0 were identified corresponding to different conformation/aggregation states of the protein. For the first time, heterogeneous data sets (i.e., data from different techniques) including Far-UV CD, fluorescence and UV absorbance results were used to generate empirical phase diagrams. Results from different data sets are compared; precautions in applying the method and its overall utility are discussed.

Fan H., Vitharana S. N., Chen T., O'Keefe D. and Middaugh C. R. (2007) Effects of pH and polyanions on the thermal stability of fibroblast growth factor 20. *Mol Pharm.* **4**, 232-240.

Abstract: Fibroblast growth factor 20 (FGF20) is a member of the FGF family with potential for use in several different therapeutic categories. In this work, we provide the first structural characterization of FGF20 using a wide variety of approaches. Like other members of the FGF family, FGF20 appears to possess a beta-trefoil structure. The effect of pH on the conformation and thermal stability of FGF20 is evaluated using far-UV circular dichroism (CD), intrinsic and ANS fluorescence, and high-resolution derivative UV absorption spectroscopy. Empirical phase diagrams are constructed to describe the solution behavior of FGF20 over a wide pH and temperature range. The protein appears to be unstable at pH <5, with aggregation and precipitation observed during dialysis. A major heat-induced conformational change also causes aggregation and precipitation of FGF20 at elevated temperatures. The highest thermal stability is observed near neutral pH (T_m ~55 degrees C at pH 7). The effect of several high- and low-molecular mass polyanions on the thermal stability of FGF20 is also examined using CD, intrinsic fluorescence, and DSC analysis. Among these ligands, heparin exhibits the greatest stabilizing effect on FGF20, increasing the T_m by more than 10 degrees C.

Faria T. Q., Mingote A., Siopa F., Ventura R., Maycock C. and Santos H. (2008) Design of new enzyme stabilizers inspired by glycosides of hyperthermophilic microorganisms. *Carbohydr. Res* **343**, 3025-3033.

Abstract: In response to stressful conditions like supra-optimal salinity in the growth medium or temperature, many microorganisms accumulate low-molecular-mass organic compounds known as compatible solutes. In contrast with mesophiles that accumulate neutral or zwitterionic compounds, the solutes of hyperthermophiles are typically negatively charged. (2R)-2-(alpha-D-Mannopyranosyl)glycerate (herein abbreviated as mannosylglycerate) is one of the most widespread solutes among thermophilic and hyperthermophilic prokaryotes. In this work, several molecules chemically related to mannosylglycerate were synthesized, namely (2S)-2-(1-O-alpha-D-mannopyranosyl)propionate, 2-(1-O-alpha-D-mannopyranosyl)acetate, (2R)-2-(1-O-alpha-D-glucopyranosyl)glycerate and 1-O-(2-glyceryl)-alpha-D-mannopyranoside. The effectiveness of the newly synthesized compounds for the protection of model enzymes against heat-induced denaturation, aggregation and inactivation was evaluated, using differential scanning calorimetry, light scattering and measurements of residual activity. For comparison, the protection induced by natural compatible solutes, either neutral (e.g., trehalose, glycerol, ectoine) or negatively charged (di-myo-inositol-1,3'-phosphate and diglycerol phosphate), was assessed. Phosphate, sulfate, acetate and KCl were also included in the assays to rank the solutes and new compounds in the Hofmeister series. The data demonstrate the superiority of charged organic solutes as thermo-stabilizers of enzymes and strongly support the view that the extent of protein stabilization rendered by those solutes depends clearly on the specific solute/enzyme examined. The relevance of these findings to our knowledge on the mode of action of charged solutes is discussed

Faria T. Q., Knapp S., Ladenstein R., Macanita A. L., and Santos H. (2003) Protein stabilisation by compatible solutes: effect of mannosylglycerate on unfolding thermodynamics and activity of ribonuclease a. *Chembiochem* **4**, 734-741.

Abstract: Differential scanning calorimetry, optical spectroscopy, and activity measurements were used to investigate the effect of mannosylglycerate, a negatively charged osmolyte widely distributed among thermophilic and hyperthermophilic archaea and bacteria, on the thermal unfolding of ribonuclease A (RNase A). For comparison, assays in the presence of trehalose, a canonical solute in mesophiles, and potassium chloride were also carried out. A thermodynamic analysis was performed by using differential scanning calorimetry data. The changes in the heat capacity for unfolding were similar for the different solutes examined. Mannosylglycerate was an efficient thermostabiliser of RNase A and induced an increase of 6 degrees C mole⁻¹ in the melting temperature. Moreover, the performance of mannosylglycerate as a stabiliser depended on the net charge of the molecule, with the maximal effect being observed at pH values above 4.5. Analysis of the enthalpic and entropic contributions to unfolding, derived from calorimetric data, revealed that the stabilisation rendered by mannosylglycerate is primarily achieved through a decrease in the unfolding entropy. Also, the number of protons taken up by RNase A upon denaturation in the presence of mannosylglycerate was considerably higher than with other solutes, a result consistent with a more rigid structure of the native protein. Mannosylglycerate (potassium salt) inhibited the activity of RNase A, albeit to a smaller extent than KCl, and acted as an efficient suppressor of aggregation of the denatured protein, thereby having a remarkable beneficial effect on the inactivation of RNase A upon thermal denaturation. The results are discussed in view of the physiological role of this charged compatible solute.

Faria T. Q., Lima J. C., Bastos M., Macanita A. L., and Santos H. (2004) Protein stabilization by osmolytes from hyperthermophiles: effect of mannosylglycerate on the thermal unfolding of recombinant nuclease a from *Staphylococcus aureus* studied by picosecond time-resolved fluorescence and calorimetry. *J Biol Chem* **279**, 48680-48691.

Abstract: 2-O-alpha-Mannosylglycerate, a negatively charged osmolyte widely distributed among (hyper)thermophilic microorganisms, is known to provide notable protection to proteins against thermal denaturation. To study the mechanism responsible for protein stabilization, pico-second time-resolved fluorescence spectroscopy was used to characterize the thermal unfolding of a model protein, *Staphylococcus aureus* recombinant nuclease A (SNase), in the presence or absence of mannosylglycerate. The fluorescence decay times are signatures of the protein state, and the pre-exponential coefficients are used to evaluate the molar fractions of the folded and unfolded states. Hence, direct determination of equilibrium constants of unfolding from molar fractions was carried out. Van't Hoff plots of the equilibrium constants provided reliable thermodynamic data for SNase unfolding. Differential scanning calorimetry

was used to validate this thermodynamic analysis. The presence of 0.5 m potassium mannoglycerate caused an increase of 7 degrees C in the SNase melting temperature and a 2-fold increase in the unfolding heat capacity. Despite the considerable degree of stabilization rendered by this solute, the nature and population of protein states along unfolding were not altered in the presence of mannoglycerate, denoting that the unfolding pathway of SNase was unaffected. The stabilization of SNase by mannoglycerate arises from decreased unfolding entropy up to 65 degrees C and from an enthalpy increase above this temperature. In molecular terms, stabilization is interpreted as resulting from destabilization of the denatured state caused by preferential exclusion of the solute from the protein hydration shell upon unfolding, and stabilization of the native state by specific interactions. The physiological significance of charged solutes in hyperthermophiles is discussed.

Fatouros A., Osterberg T., and Mikaelsson M. (1997) Recombinant factor VIII SQ--inactivation kinetics in aqueous solution and the influence of disaccharides and sugar alcohols. *Pharm Res* **14**, 1679-1684.

Abstract: PURPOSE: To investigate the influence of various nonreducing disaccharides and sugar alcohols on the inactivation kinetics of recombinant factor VIII SQ (r-VIII SQ) in aqueous solution not containing albumin as a stabiliser. METHODS: The stability of r-VIII SQ was followed using measurement of activity (VIII:C) and HPLC gel filtration at different temperatures. The thermal stability was investigated using differential scanning calorimetry (DSC). RESULTS: The decline in VIII:C followed pseudo-first order kinetics. However, the Arrhenius plot was not linear for formulations without carbohydrate, demonstrating a distinct, reproducible curvature. The reaction rate at 5 degrees C was faster than expected from the Arrhenius kinetics. The energy of activation (Ea) for formulations without added carbohydrates, derived from the linear part of the Arrhenius plot, varied between 77 and 86 kJ/mole in the temperature range 20-37 degrees C. The addition of 600 mg/ml sucrose increased the Ea to 104 kJ/mole. DSC measurements showed that Tm' was 64.2 +/- 0.2 degrees C for r-VIII SQ without stabiliser. This value increased linearly with increasing concentrations of carbohydrate. This stabilising effect is most probably explained by the theory of preferential hydration. CONCLUSIONS: The inactivation kinetics of r-VIII SQ in aqueous solution without addition of carbohydrates followed pseudo-first order kinetics but the Arrhenius plot was nonlinear. Sucrose and sorbitol both had highly stabilising effects on r-VIII SQ at concentrations above 300 mg/ml. The preparation containing 600 mg/ml sucrose was stable for at least 12 months at 5 degrees C and 6 months at 25 degrees C.

Fransson J., Hallen D., and Florin-Robertsson E. (1997) Solvent effects on the solubility and physical stability of human insulin-like growth factor I. *Pharm Res* **14**, 606-612.

Abstract: PURPOSE: The solubility and physical stability of human Insulin-like Growth Factor I (hIGF-I) were studied in aqueous solutions with different excipients. METHODS: The solubility of hIGF-I was determined by UV-absorption and quantification of light blocking particles. The physical stability of hIGF-I was studied with differential scanning calorimetry (DSC) and circular dichroism (CD) spectroscopy. RESULTS: Human IGF-I precipitated at low temperature in the presence of 140 mM benzyl alcohol and 145 mM sodium chloride. CD data showed that the tertiary structure of hIGF-I during these conditions was perturbed compared to that in 5 mM phosphate buffer. In the presence of benzyl alcohol 290 mM mannitol stabilized hIGF-I. Sodium chloride or mannitol by themselves had no effect on either the solubility or the tertiary structure. Benzyl alcohol was attracted to hIGF-I, whereas sodium chloride was preferentially excluded. The attraction of benzyl alcohol was reinforced by sodium chloride leading to salting-out of hIGF-I. The CD-data indicated interactions of benzyl alcohol with phenylalanine in hIGF-I. Thermal denaturation of hIGF-I occurred in all solutions with sodium chloride, whereas mannitol or benzyl alcohol had no effect on the thermal stability. The thermal stability of hIGF-I was thus decreased in 145 mM sodium chloride although it was excluded from hIGF-I. CONCLUSIONS: The self-association and thermal aggregation of hIGF-I is driven by hydrophobic interactions. Benzyl alcohol is attracted to hIGF-I and induces changes in the tertiary structure causing hydrophobic attraction of the protein at low temperatures.

Gaisford S. (2005) Stability assessment of pharmaceuticals and biopharmaceuticals by isothermal calorimetry. *Curr Pharm Biotechnol* **6**, 181-191.

Abstract: The assessment of stability (of actives, excipients and/or formulated products) is an important, and often time-consuming, part of pharmaceutical product development. Conventionally, HPLC is used to quantify the concentrations of a parent compound and any degradation products as a function of storage time. HPLC, however, is relatively insensitive to small changes in concentration and it is often the case that

stability assays are conducted under stress conditions, in order to accelerate any degradation processes. The Arrhenius relationship is then employed to give an initial prediction of stability under storage conditions while long-term studies, under storage conditions, are conducted to confirm these predictions. The properties of isothermal calorimetry, such as its intrinsic sensitivity to small changes in heat and invariance to the physical form of a sample, make it ideally suited for stability assessment because it obviates the need for an Arrhenius analysis. In addition, the ability to conduct titration or gas perfusion experiments vastly increases its range of applications. Recent advances in instrumental design and data analysis have made it easier to analyse data quantitatively for complex systems. It is the purpose of this review to highlight some of these developments, discuss them in the context of pharmaceutical and biopharmaceutical examples and explore some of the future challenges and applications of the technique.

Garidel P., Hegyi M., Bassarab S. and Weichel M. (2008) A rapid, sensitive and economical assessment of monoclonal antibody conformational stability by intrinsic tryptophan fluorescence spectroscopy. *Biotechnol. J* **3**, 1201-1211.

Abstract: Steady-state intrinsic tryptophan fluorescence spectroscopy is used as a rapid, robust and economic way for screening the thermal protein conformational stability in various formulations used during the early biotechnology development phase. The most important parameters affecting protein stability in a liquid formulation, e. g. during the initial purification steps or preformulation development, are the pH of the solution, ionic strength, presence of excipients and combinations thereof. A well-defined protocol is presented for the investigation of the thermal conformational stability of proteins. This allows the determination of the denaturation temperature as a function of solution conditions. Using intrinsic tryptophan fluorescence spectroscopy for monitoring the denaturation and folding of proteins, it is crucial to understand the influence of different formulation parameters on the intrinsic fluorescence probes of proteins. Therefore, we have re-evaluated and re-assessed the influence of temperature, pH, ionic strength, buffer composition on the emission spectra of tryptophan, phenylalanine and tyrosine to correctly analyse and evaluate the data obtained from thermal-induced protein denaturation as a function of the solution parameters mentioned above. The results of this study are a prerequisite for using this method as a screening assay for analysing the conformational stability of proteins in solution. The data obtained from intrinsic protein fluorescence spectroscopy are compared to data derived from calorimetry. The advantage, challenges and applicability using intrinsic tryptophan fluorescence spectroscopy as a routine development method in pharmaceutical biotechnology are discussed

Gombotz W. R., Pankey S. C., Phan D., Drager R., Donaldson K., Antonsen K. P., Hoffman A. S., and Raff H. V. (1994) The stabilization of a human IgM monoclonal antibody with poly(vinylpyrrolidone). *Pharm Res* **11**, 624-632.

Abstract: An IgM anti-group B Streptococcus monoclonal antibody (4B9) was found to undergo irreversible heat-induced aggregation at 50 degrees C. A variety of excipients was tested for their ability to inhibit antibody aggregation. The amount of 4B9 aggregation, which was determined by analysis on a size-exclusion HPLC, was significantly reduced in the presence of low concentrations [between 0.1 and 1.0% (w/v)] of poly(vinylpyrrolidone) (PVP) molecules ranging in molecular weight from 10 to 40 kDa. When the PVP concentration was greater than 1.0%, antibody aggregation was enhanced, and with the highest molecular weight PVP, antibody precipitation occurred. HPLC was used to show that more PVP was associated with the 4B9 at 50 degrees C than at 25 degrees C. Differential scanning calorimetry revealed that PVP concentrations greater than 2.0% decreased the antibody thermal transition temperature. Enzyme-linked immunosorbent assays were used to assess the effects of PVP on the antigen binding capacity of 4B9 and on 4B9 quantitation. At 4 degrees C, PVP solutions of up to 5.0% had no effect on either 4B9 quantitation or antigen binding. At 50 degrees C, however, less 4B9 was detected in the 5.0% PVP solution. The heat stabilization of the 4B9 antibody by low concentrations of PVP can be explained by a weak binding of PVP to the native protein. The PVP may sterically interfere with protein-protein interactions, thus reducing aggregation. Higher concentrations of PVP lead to protein aggregation and precipitation, probably by a volume-exclusion mechanism.(ABSTRACT TRUNCATED AT 250 WORDS).

Gonzalez M., Murature D. A., and Fidelio G. D. (1995) Thermal stability of human immunoglobulins with sorbitol. A critical evaluation. *Vox Sang* **68**, 1-4.

Abstract: The effect of the additive sorbitol on the thermal stabilization of human IgG was investigated by differential scanning calorimetry and size exclusion chromatography. In the presence of 33% sorbitol, the

temperature at which denaturation of IgG began (T_i) was increased from 52 to 65 degrees C. Similarly, the temperature of the maximum heat capacity (T_{max}) was increased from 69 to 76 degrees C. Sorbitol also decreased dimer aggregation and the extent of oligomerization during heating compared with IgG dissolved in phosphate buffer. Sorbitol at 33% prevented massive protein denaturation but a 10-15% of oligomerization of high molecular weight aggregates with turbidity could not be avoided when heating for 10 h at 60 degrees C. The use of sorbitol 33% to avoid heat denaturation of human IgG during viral inactivation did not prevent protein aggregation or the appearance of turbidity. Consequently, further processing will be required to achieve a product suitable for pharmaceutical use.

Gupta S. and Kaisheva E. (2003) Development of a multidose formulation for a humanized monoclonal antibody using experimental design techniques. *AAPS PharmSci* **5**, E8.

Abstract: The purpose of this study was to identify optimal preservatives for a multidose formulation of a humanized monoclonal antibody using experimental design techniques. The effect of antimicrobial parenteral preservatives (benzyl alcohol, chlorobutanol, methylparaben, propylparaben, phenol, and m-cresol) on protein stability was assessed using size-exclusion chromatography, differential scanning calorimetry, right-angle light scattering, UV spectroscopy, and potency testing using a cell-based fluorescence-activated cell sorting method. A quick, cost-effective preservative screening test was designed. Combinations of preservatives were examined using an I-optimal experimental design. The protein was most stable in the presence of methylparaben and propylparaben, and was compatible with benzyl alcohol and chlorobutanol at low concentrations. Phenol and m-cresol were not compatible with the protein. The I-optimal experimental design indicated that as an individual preservative, benzyl alcohol was promising. The model also indicated several effective combinations of preservatives that satisfied the antimicrobial efficacy and physical stability constraints. The preservative screening test and the experimental design approach were effective in identifying optimal concentrations of antimicrobial preservatives for a multidose protein formulation; (1) benzyl alcohol, and (2) the combination of methylparaben and chlorobutanol were screened as potential candidates to satisfy the regulatory requirements of various preservative efficacy tests.

Guo J., Harn N., Robbins A., Dougherty R., and Middaugh C. R. (2006) Stability of helix-rich proteins at high concentrations. *Biochemistry* **45**, 8686-8696.

Abstract: A number of techniques, including circular dichroism, FTIR, front face fluorescence, and UV absorption spectrophotometries, dynamic light scattering, and DSC, were used to directly measure the colloidal and conformational stability of proteins in highly concentrated solutions. Using bovine serum albumin (BSA), chicken egg white lysozyme, human hemoglobin A0, and bovine fibrinogen as model proteins, the thermal transition temperatures of proteins in dilute and concentrated solutions were compared. At 10 degrees C, no significant differences in both secondary and tertiary structures were detected for proteins at different concentrations. When temperature was introduced as a variable, however, hemoglobin and fibrinogen demonstrated higher transition midpoints ($T(m)$ s) in concentrated rather than in dilute solutions ($\Delta T(m)$ approximately 2-10 degrees C). In contrast, lysozyme and BSA in concentrated solutions exhibit a lower $T(m)$ than in dilute solutions ($\Delta T(m)$ approximately 2-20 degrees C). From these studies, it appears that a variety of factors determine the effect of high concentrations on the colloidal and conformational stability of a particular protein. While the prediction of excluded volume theory is that high concentrations should conformationally stabilize proteins, other factors such as pH, kinetics, protein dynamics, and intermolecular charge-charge effects may affect the overall stability of proteins at high concentrations under certain conditions.

Harn N., Allan C., Oliver C. and Middaugh C. R. (2007) Highly concentrated monoclonal antibody solutions: direct analysis of physical structure and thermal stability. *J Pharm. Sci* **96**, 532-546.

Abstract: Virtually all current analytical methods employed in the development of highly concentrated monoclonal antibody (MAb) formulations require dilution of the sample before acquiring data. Thus, there is an unmet need for methods to study proteins directly at high concentration, since extrapolation of stability indicating parameters obtained from dilute studies may not be representative of the high concentration solution. Only slight or no modifications of biophysical methods including fluorescence, UV absorbance, circular dichroism, and FTIR (ATR and transmittance) spectroscopies as well as differential scanning calorimetry (DSC) are described here that permit the direct study of immunoglobulins (and other proteins) at high concentrations. Although FTIR spectra show differences that are dependent upon

sampling geometry, other spectroscopic data from two different recombinant MAbs suggests that structure of each antibody exists in a physically similar state in the concentration range of 0.1-190 mg/mL in 40 mM pH 6 citrate-phosphate buffer. Upon thermally stressing these proteins, spectroscopic techniques that probe tertiary structure demonstrate a decrease in the apparent thermal melting temperature of approximately 5-20 degrees C of both proteins with increasing concentration. In contrast, DSC thermograms and CD thermal experiments suggest a minor degree of stabilization (approximately 2 degrees C) for both antibodies although protein association could be responsible for these observations. Empirical phase diagrams produced from spectroscopic data also suggest (1) the existence of similar structural states at low temperatures independent of concentration and (2) a decrease in the temperature at which phase changes are observed with increasing concentration. The decrease in structural stability observed in these studies is probably the result of aggregation or self-association of the recombinant MAbs upon heating in crowded solutions and not due to a decrease in the intrinsic structural stability of the MAbs

Hattori M., Nagasawa K., Ohgata K., Sone N., Fukuda A., Matsuda H., and Takahashi K. (2000) Reduced immunogenicity of beta-lactoglobulin by conjugation with carboxymethyl dextran. *Bioconj Chem* **11**, 84-93.

Abstract: We prepared two beta-lactoglobulin (beta-LG)-carboxymethyl dextran (CMD) conjugates (Conj. 10A and Conj. 10B) by using a water-soluble carbodiimide to decrease the immunogenicity of beta-LG. The molar ratios of beta-LG to CMD in the conjugates were 5:1 (Conj. 10A) and 2:1 (Conj. 10B). The beta-LG-CMD conjugates maintained the retinol-binding activity of native beta-LG. Intrinsic fluorescence study indicated that shielding of the surface of beta-LG by CMD occurred in each conjugate, which was eminent in Conj. 10B. A local conformational change around (125)Thr-(135)Lys (alpha-helix) in each conjugate was detected by ELISA with monoclonal antibodies. The denaturation temperature of beta-LG evaluated by differential scanning calorimetry was greatly enhanced in each conjugate. The anti-beta-LG antibody response was markedly reduced after immunization with the beta-LG-CMD conjugates in BALB/c, C57BL/6, and C3H/He mice. We determined the B cell epitopes of beta-LG and each conjugate recognized in these mice and found that the linear epitope profiles of the beta-LG-CMD conjugates were similar to those of beta-LG, while the antibody response for each epitope was dramatically reduced. The reduced immunogenicity of beta-LG was most marked in the case of Conj. 10B, which contained more CMD than Conj. 10A, and was effectively shielded by CMD. We concluded that masking of epitopes by CMD is responsible for the decreased immunogenicity of the beta-LG in these conjugates.

Heller M. C., Carpenter J. F., and Randolph T. W. (1999) Protein formulation and lyophilization cycle design: prevention of damage due to freeze-concentration induced phase separation. *Biotechnol Bioeng* **63**, 166-174.

Abstract: Hemoglobin has been previously shown to unfold during freeze drying when lyophilized from formulations that undergo freeze-concentration induced phase separation (Heller et al. 1997. *Biotechnol Prog* 13:590-596). In this report, we show that such damage may be avoided using kinetic strategies to arrest the phase separation. By rapidly cooling samples during liquid nitrogen spray-freeze drying, the time that the formulation spends in temperature regimes (ca. -3 to -23 degrees C) in which phase separation is both thermodynamically favorable and kinetically realizable is minimized. Increased protein damage with decreasing cooling rates and/or longer annealing periods at -7 degrees C is observed by FTIR spectroscopy. Phase separation and concomitant protein damage may also be avoided by addition of mannitol at concentrations sufficient to cause crystallization. Mannitol crystals segregate the freeze concentrated solution into microscopic domains that block propagation and nucleation of phase separating events. Addition of noncrystallizing sugars, such as sucrose and trehalose, or nonionic surfactants, such as Tween 80 and Triton X-100, has little protective effect against phase separation induced damage during freezing drying.

Jones L. S., Peek L. J., Power J., Markham A., Yazzie B., and Middaugh C. R. (2005) Effects of adsorption to aluminum salt adjuvants on the structure and stability of model protein antigens. *J Biol Chem* **280**, 13406-13414.

Abstract: The effect of adsorption onto aluminum salt adjuvants on the structure and stability of three model protein antigens was studied using fluorescence and Fourier transform infrared spectroscopies, as well as isothermal titration and differential scanning calorimetric techniques. Lysozyme was preferentially adsorbed to aluminum phosphate (Adju-Phos), whereas ovalbumin and bovine serum albumin were better

adsorbed to aluminum hydroxide (Alhydrogel). A linearized Langmuir adsorption isotherm was used to obtain information regarding the binding interactions between proteins and adjuvants. Binding energetics and stoichiometry data obtained from isothermal titration calorimetry measurements were complex. Based on the spectroscopic and differential scanning calorimetry studies, the structure of all three proteins, when adsorbed to the surface of an aluminum salt, was altered in such a way as to render the proteins less thermally stable. Besides the pharmaceutical significance of this destabilization, we consider the possibility that this phenomenon may facilitate the presentation of antigens and thus contribute to the adjuvant activity of the aluminum salts.

Kang F., Jiang G., Hinderliter A., DeLuca P. P., and Singh J. (2002) Lysozyme stability in primary emulsion for PLGA microsphere preparation: effect of recovery methods and stabilizing excipients. *Pharm Res* **19**, 629-633.

Abstract: PURPOSE: To investigate the conformational stability of a model protein, lysozyme, in the primary emulsion phase of the microsphere preparation process. METHODS: The conformational stability of lysozyme during primary emulsification was assessed by differential scanning calorimetry (DSC) and enzymatic activity assay. PEG 400 was used to separate lysozyme from water-in-oil (w/o) emulsion containing poly(lactide-co-glycolide) (PLGA). RESULTS: No significant changes in the recovery of lysozyme were observed due to increasing sonication time from 20 to 60 s at 40 W or increasing intensity from 40 to 60 W for 20 s. By using the method involving PEG 400, lysozyme recovery in the presence of PLGA was increased from 11.8% to 709%. Hydroxypropyl-beta-cyclodextrin (HP-beta-CD) increased lysozyme recovery from 35% to 70% at low lysozyme concentration (20 mg/ml), and from 70% to 77% at high lysozyme concentration (100 mg/ml) in the presence of PLGA. Sugars such as trehalose and mannitol failed to increase lysozyme recovery. DSC results suggested the retention of the conformational structure of the recovered lysozyme, which was supported by an enzymatic activity assay. CONCLUSIONS: HP-beta-CD was found to be a promising stabilizer that protected lysozyme during the primary emulsification. Protein recovery method with the help of PEG 400 allowed the study of protein stability in w/o emulsions in the presence of PLGA. DSC provided supplementary information on the conformational changes of lysozyme during emulsification.

Kang F. and Singh J. (2003) Conformational stability of a model protein (bovine serum albumin) during primary emulsification process of PLGA microspheres synthesis. *Int J Pharm* **260**, 149-156.

Abstract: The goal of this study was to investigate the conformational stability of a model protein, bovine serum albumin (BSA), during the primary emulsification process of poly(D,L-lactide-co-glycolide) (PLGA) microspheres preparation. Differential scanning calorimeter (DSC) was utilized to assess the conformational structure of BSA during primary emulsification in the presence and absence of PLGA. Three excipients [i.e. mannitol, hydroxypropyl-beta-cyclodextrin (HP-beta-CD) and sodium dodecyl sulfate (SDS)] were investigated for their stabilizing effect on BSA during emulsification process. The DSC profile of intact BSA was best fitted by a non-2-state model with two peaks, which have midpoint temperatures ($T(m1)$, 60.9 +/- 0.4 degrees C and $T(m2)$, 66.4 +/- 1.0 degrees C), respectively, and a total calorimetric enthalpy $\Delta H(\text{tot})$ of 599 +/- 42 kJ/mol. After emulsifying BSA aqueous solution with methylene chloride, an additional apparent peak at a higher temperature was observed. The T_m of this peak was 77.4 +/- 0.8 degrees C. HP-beta-CD was able to suppress the occurrence of an additional peak, whereas mannitol failed. SDS increased the thermal stability of BSA dramatically. Furthermore, HP-beta-CD increased BSA recovery from 72 +/- 8% to 89 +/- 7% after extraction from w/o in the presence of PLGA. These results provided evidence that HP-beta-CD could be a promising excipient for conformational stability of BSA during synthesis of PLGA microspheres.

Kasraian K., Kuzniar A., Earley D., Kamicker B. J., Wilson G., Manion T., Hong J., Reiber C., and Canning P. (2001) Sustained in vivo activity of recombinant bovine granulocyte colony stimulating factor (rbG-CSF) using HEPES buffer. *Pharm Dev Technol* **6**, 441-447.

Abstract: The purpose of this study was to develop a long-acting injectable formulation of bG-CSF for veterinary use. However, in order to achieve sustained in vivo activity it was first necessary to stabilize the protein at the injection site. Preformulation studies, as well as literature, suggest that bG-CSF aggregates at neutral pH ranges (i.e., pH 6-8) and at temperatures of approximately 40 degrees C. Therefore, bG-CSF will not retain its activity for an extended period of time at the injection site. During this study we determined that HEPES buffer has a very significant impact on protein stability as well as on biological

performance. Recombinant bovine granulocyte colony stimulating factor (rbG-CSF) was formulated in 1 M HEPES buffer for subcutaneous injection into cows. bG-CSF formulated in 1 M HEPES buffer resulted in sustained in vivo activity of bG-CSF compared to the "control" formulation (control formulation: 5% mannitol, 10 mM acetate buffer, 0.004% tween-80, pH 4). White blood cell (WBC) count was used as a marker to evaluate in vivo activity of the formulation. WBC numbers remained above a threshold value for only 24-30 h for the control formula. However, when bG-CSF was formulated in 1 M HEPES, the WBC remained above threshold for 3 days or 72 h. Formulating bG-CSF in 1 M HEPES at pH 7.5 also resulted in greater solution stability. This was surprising since bG-CSF is intrinsically not stable at neutral pH. The effect of 1 M HEPES on the TM (temperature at maximum heat flow on calorimetry scan) of bG-CSF was determined by microcalorimetry. In the absence of 1 M HEPES buffer the TM was 48 degrees C (onset approximately 40 degrees C), while bG-CSF formulated in 1 M HEPES buffer has a TM of 59 degrees C (onset approximately 50 degrees C). Similar organic buffers, such as MOPS, HEPPS, TES, and tricine, also resulted in improved solution stability as well as in sustained in vivo activity. The dramatic effect of these buffers on stability and biological performance of bG-CSF is not well understood. One hypothesis is that the electrostatic interaction between the zwitterionic form of these buffers and bG-CSF provides stabilization against denaturation.

Katakam M., Bell L. N., and Banga A. K. (1995) Effect of surfactants on the physical stability of recombinant human growth hormone. *J Pharm Sci* **84**, 713-716.

Abstract: The physical stability of a human growth hormone (hGH) formulation upon exposure to air/water interfaces (with vortex mixing) and to nonisothermal stress [determined by differential scanning calorimetry (DSC)] was investigated. The effect of these stresses on the formation of soluble and insoluble aggregates was studied. The aggregates were characterized and quantified by size exclusion-HPLC and UV spectrophotometry. Vortex mixing of hGH solutions (0.5 mg/mL) in phosphate buffer, pH 7.4, for just 1 min caused 67% of the drug to precipitate as insoluble aggregates. These aggregates were noncovalent in nature. Non-ionic surfactants prevented the interfacially induced aggregation at their critical micelle concentration (cmc) for Pluronic F-68 (polyoxyethylene polyoxypropylene block polymer) and Brij 35 (polyoxyethylene alkyl ether) and above the cmc for Tween 80 (polyoxyethylene sorbitan monooleate). However, the same surfactants failed to stabilize hGH against thermal stress in DSC studies. Higher concentrations of surfactants actually destabilized hGH as evidenced by the decrease in the onset temperature for the denaturation endotherm.

Kissmann J., Ausar S. F., Foubert T. R., Brock J., Switzer M. H., Detzi E. J., Vedvick T. S. and Middaugh C. R. (2008) Physical stabilization of Norwalk virus-like particles. *J Pharm. Sci* **97**, 4208-4218.

Abstract: Virus-like particles (VLPs) used as vaccine antigens often elicit strong immune responses due to their intrinsic repetitive, high-density display of epitopes, and the fact that the mammalian immune system is highly attuned to recognizing particles in the size range of viruses (20-150 nm). To retain these immunogenic qualities, vaccines that utilize virus-like particle (VLP) antigens should be formulated to stabilize both native conformational epitopes and the overall particulate nature of the VLP. This work describes a systematic approach for identifying potential stabilizers for formulation of Norwalk VLPs (NV-VLPs) in aqueous suspension. A number of excipients were screened for their ability to inhibit aggregation of NV-VLPs under conditions known to induce aggregation. Those compounds shown to inhibit aggregation were further evaluated under conditions of thermal stress and the NV-VLP structure was monitored using biophysical techniques such as CD, ANS fluorescence, and DSC to provide insight into the mechanisms by which stability was conferred. Increased thermal stability in the presence of chitosan glutamate, sucrose, and trehalose was correlated with stabilization of secondary and tertiary structural elements of NV-VLPs. These excipients may be useful for formulation of a stable NV-VLP vaccine

Kumar V., Sharma V. K. and Kalonia D. S. (2008) Effect of polyols on polyethylene glycol (PEG)-induced precipitation of proteins: Impact on solubility, stability and conformation. *Int J Pharm (epublication)*.

Abstract: Effect of polyols on the solubility of bovine serum albumin (BSA) in the presence of polyethylene glycols (PEGs) was investigated in order to strengthen the understanding of the observed effects of polyols and PEGs on protein properties in solution. Effect of polyols and/or PEGs on the thermodynamic (conformational) stability of BSA was measured using DSC and circular dichroism (CD). Glucose, sucrose, raffinose, glycerol and sorbitol, all reduced the extent of protein precipitation. Solubility of BSA in the presence of ethylene glycol increased in the case of PEG 1450 and PEG 8000, but was

unaffected in the case of PEG 400. DSC studies indicated that smaller PEGs have destabilizing influence on protein structure. CD studies showed that smaller PEGs (ethylene glycol) induce subtle unfolding while stabilizing polyols induce subtle compaction. Results show that, effect of polyols on the apparent solubility of the protein correlates with their effect on the thermodynamic stability of the protein, smaller PEGs are not appropriate for estimating the activity of proteins in saturated solutions, and subtle changes in protein conformation can significantly affect protein precipitation. Though smaller PEGs have weak attractive interactions with protein molecules, perturbation of protein structure by PEGs can be balanced by utilizing appropriate stabilizing solutes

Li B., O'Meara M. H., Lubach J. W., Schowen R. L., Topp E. M., Munson E. J., and Borchardt R. T. (2005) Effects of sucrose and mannitol on asparagine deamidation rates of model peptides in solution and in the solid state. *J Pharm Sci* **94**, 1723-1735.

Abstract: Asparagine (Asn) degradation kinetics in two model peptides, Gly-Gln-Asn-Gly-Gly (GQNGG) and Val-Tyr-Pro-Asn-Gly-Ala (VYPNGA), were studied at 50 degrees C in pH 7 buffer solutions in the presence and absence of 5% (w/v) sucrose or mannitol and at 50 degrees C and 30% relative humidity in solid samples lyophilized from these solutions. Solid formulations were characterized using Karl Fischer coulometric titration, thermal gravimetric analysis (TGA), differential scanning calorimetry (DSC), Fourier-transform infrared spectrometry (FTIR), and solid-state nuclear magnetic resonance (NMR) spectroscopy. GQNGG and VYPNGA showed similar pseudo first-order deamidation rates in solution in the absence of sucrose and mannitol. Adding 5% sucrose or mannitol decreased the rates by no more than 17%. The model peptides degraded 2- to 80-fold more slowly in the solid formulations of sucrose and mannitol than in 5% solutions of these carbohydrates. Ratios of deamidation rates of the model peptides depended upon the solid matrix. In the mannitol solid, the ratio of deamidation rates of GQNGG and VYPNGA (GQNGG:VYPNGA) was approximately 8, while in the sucrose solid, the model peptides deamidated at similar rates (GQNGG:VYPNGA congruent with 1). DSC showed the mannitol formulations to be largely amorphous immediately after lyophilization with some ordered, crystalline-like structure; the extent of ordered structure increased during storage as shown by FTIR and ssNMR. In contrast, the sucrose formulation was largely amorphous after lyophilization and remained so during storage. Together, the results showed that 5% sucrose or mannitol in solution does not significantly change the rates of Asn deamidation of the model peptides, while sucrose stabilizes the model peptides against deamidation more than mannitol in the solid state.

Liao Y. H., Brown M. B., Nazir T., Quader A., and Martin G. P. (2002) Effects of sucrose and trehalose on the preservation of the native structure of spray-dried lysozyme. *Pharm Res* **19**, 1847-1853.

Abstract: PURPOSE: To investigate the effects of sucrose, trehalose, sucrose/ dextran mixtures, and sucrose/trehalose mixtures on the preservation of the native structure of spray-dried lysozyme in the solid state. METHODS: The intensity of the alpha-helical band and the melting enthalpies (ΔH_m) of spray-dried lysozyme in the dried form and in aqueous solution were obtained using second derivative FTIR and differential scanning calorimetry (DSC) respectively. RESULTS: The intensity of the alpha-helical band and the ΔH_m of spray-dried lysozyme obtained were linearly correlated and both suggest that the stabilization of lysozyme in the dried form was excipient concentration-dependent with a close to maximum stabilization being conferred by sucrose or trehalose at a mass ratio 1-2 (sugar:enzyme). Sucrose appeared to be more effective than trehalose on a weight by weight basis whilst stabilizing effects of dextran/sucrose or trehalose/ sucrose mixtures were found to be additive. CONCLUSION: Dehydration during spray drying was considered the main stress to the denaturation of lysozyme. A major effect of the sugars in protecting lysozyme against dehydration was attributable to hydrogen bonding between the sugar and protein molecules, which lead to an increase in the change in the negative value of the free energy between native and denatured states.

Liao Y. H., Brown M. B., Quader A., and Martin G. P. (2003) Investigation of the physical properties of spray-dried stabilised lysozyme particles. *J Pharm Pharmacol* **55**, 1213-1221.

Abstract: The aim of this study was to investigate the effect of the composition of formulations on the physical properties, including glass-transition temperatures (T_g) and aerodynamic-related characteristics, of spray-dried lysozyme particles. The T_g , as determined by differential scanning calorimetry, of spray-dried lysozyme formulations was found to be dependent upon the type and amount of excipient(s) included in the formulation. In addition, the T_g of sucrose-containing particles appeared to be raised markedly by the

inclusion of trehalose, but not by dextran. The surfaces of all spray-dried particles were shown by scanning electron microscopy to be smooth with some containing characteristic dimples, typical of spray-dried material, and the morphology appeared to be independent of variation in excipient composition. However, the volume median diameters (VMD) of spray-dried powders, as determined by laser diffraction, were found to depend upon the amounts of excipients. The fine particle fraction of enzyme delivered to the lower stage of a twin-stage impinger from lysozyme-trehalose 1:1 powders appeared to be greater than that from lysozyme-sucrose 1:1 particles (22.5% vs 15.9%) when dispersed via a Rotahaler although a similar dispersibility of the two formulations (39.6% vs 36.7%) was found from a glass inhaler. In general, spray-drying was demonstrated to be feasible to produce respirable particles of the stabilised model protein, with Tg of the formulations being > 30 degrees C higher than room temperature.

Lueckel B., Helk B., Bodmer D., and Leuenberger H. (1998) Effects of formulation and process variables on the aggregation of freeze-dried interleukin-6 (IL-6) after lyophilization and on storage. *Pharm Dev Technol* **3**, 337-346.

Abstract: This study assessed the impact of residual moisture, Tg, and excipient physical state of different formulations on the "in-process" and shelf-life stability of freeze-dried interleukin-6 (IL-6). The effect of an annealing procedure was also evaluated. Characterization of the lyophilizates was done by Karl Fischer titration, differential scanning calorimetry (DSC), and x-ray measurements. Analysis of protein stability was carried out by size exclusion chromatography (SEC), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and turbidity measurements. During freeze-drying, the most effective protection against aggregation was provided by completely amorphous formulations consisting of trehalose or sucrose either alone or in combination with glycine or mannitol. Other amorphous formulations like those of sucrose with lysine-HCl or dextran could not provide comparable stabilization. In lyophilizates containing a crystallized excipient such as glycine or mannitol, IL-6 suffered destabilization, which was less pronounced if an additional amorphous excipient was present. For the completely amorphous formulations, aggregation was prevented during a 9-month storage at 25 and 40 degrees C as long as the storage temperature did not exceed the Tg value of the lyophilizate, otherwise severe damage occurred. Formulations containing amorphous dextran or lysine-HCl could not effectively stabilize IL-6 even when stored below Tg. Annealing helped to improve cake robustness and appearance, but for lyophilizates containing an excipient crystallized by annealing an increase of IL-6 aggregation was observed despite a storage below Tg. Thus, the amorphous state of the excipients and a high Tg can be considered necessary conditions for preventing aggregation of freeze-dried IL-6. Whether the conditions are also sufficient depends on the choice of excipients. Destabilization can occur with some excipients despite their amorphous state as well as in the presence of crystallized excipients despite a storage below Tg. Compared to sucrose, trehalose is a more favorable excipient for protein lyophilization because it exhibits a higher Tg, possesses better stabilizing properties, and can reduce protein aggregation which may have been caused by annealing.

Maiorella B. L., Ferris R., Thomson J., White C., Brannon M., Hora M., Henriksson T., Triglia R., Kunitani M., Kresin L., and . (1993) Evaluation of product equivalence during process optimization for manufacture of a human IgM monoclonal antibody. *Biologicals* **21**, 197-205.

Abstract: We have developed a battery of tests to characterize monoclonal antibodies and assess the effect of potential manufacturing process changes. Tryptic peptide mapping, molecular weight determination by HPLC and classical light scattering, isoelectric focussing, oligosaccharide mapping by HPAE-PAD chromatography, circular dichroism spectra and differential scanning calorimetry were applied as sensitive assays of antibody structure. Biological activity was assessed by measurement of specific antigen binding activity, binding spectrum and opsonic activity. Pharmacokinetics was assessed by clearance rate studies in rats. The sensitivity of this battery of assays was demonstrated by the ability to readily detect differences between a human monoclonal antibody (IgM-2) produced by in vitro cell culture versus ascites culture. These same tests support equivalence of a second monoclonal antibody (IgM-1) produced before and after in vitro cell culture process improvements which resulted in a twofold increase in product titer.

Mattern M., Winter G., Kohnert U., and Lee G. (1999) Formulation of proteins in vacuum-dried glasses. II. Process and storage stability in sugar-free amino acid systems. *Pharm Dev Technol* **4**, 199-208.

Abstract: The purpose of this research was to investigate the freeze- and vacuum-drying behavior of L-amino acids of current/potential use as adjuvants for formulating proteins. The analytical methods used

were wide-angle x-ray diffraction, differential scanning calorimetry, and scanning electron microscopy. Protein analysis was performed either as an activity assay (lactate dehydrogenase [LDH]) or by size-exclusion chromatography (granulocyte colony-stimulating factor [rhG-CSF]). After samples were freeze-dried, only the four basic amino acids (arginine, lysine, histidine, and citrulline) formed amorphous solids, which, however, were partially crystalline. The remaining amino acids all formed fully crystalline solids. After samples were vacuum-dried, (20 degrees C, 0.1 mbar, 1 ml fill volume in 2-ml vials) fully crystalline solids were formed by all of the amino acids. For arginine, the addition of either HCl, H₃PO₄, or H₂SO₄ sufficient to form the respective salt produced amorphous solids after vacuum-drying, but they had high residual water contents and low glass transition temperatures (T_g). Addition of phenylalanine to arginine base inhibited crystallization of the latter at low concentrations during vacuum-drying procedure, leading to formation of a pure rubbery solid. At higher concentrations the phenylalanine crystallized, producing dry products with glass transition temperatures of > 60 degrees C. The process and storage stability of LDH and rhG-CSF in the vacuum-dried phenylalanine/arginine glasses was greatly improved at temperatures up to 40 degrees C compared with the unprotected proteins. Uptake of moisture during storage was, however, a complicating factor, reducing T_g, promoting crystallization, and leading to decreased protein stability. The PO₄ salt of arginine produced especially high glass transition temperatures after it was vacuum-dried. These sugar-free amino acid formulations thus are potential stabilizers for proteins.

Matheus S., Friess W., and Mahler H. C. (2006) FTIR and nDSC as Analytical Tools for High-Concentration Protein Formulations. *Pharm Res* **23**, 1350-1363.

Abstract: PURPOSE: The aim of the study is to evaluate Fourier-transform infrared spectroscopy (FTIR) as an analytical tool for high-concentrated protein formulations. METHODS: FTIR is used to determine the melting temperature (T_m (FTIR)) of various proteins, such as bovine serum albumin (BSA), immunoglobulin (IgG(1)), beta-lactoglobulin (beta-LG), and lysozyme (HEWL), at different protein concentrations (5-100 mg/mL), where four data interpretation methods are discussed. The obtained T_m (FTIR) values are further compared to the T_m measured by the nanodifferential scanning calorimetry (nDSC) technique. RESULTS: The T_m (FTIR) values of IgG(1) and beta-LG showed strong consistency and corresponded to the nDSC results irrespective of the method of data interpretation and the protein concentration applied. In contrast, the T_m (FTIR) of BSA and HEWL is characterized by significant deviations. Only the midpoint of the second-derivative intensity-temperature curve of the intermolecular beta-sheet mode measured at a concentration of 100 mg/mL is consistent with the nDSC results. CONCLUSIONS: Determination of a T_m (FTIR) is feasible by the midpoint of the intensity-temperature plot of the arising intermolecular beta-sheet band. More significant results are obtained for proteins, which are predominantly composed of intramolecular beta-sheet elements as well as at higher protein concentrations. A further study was started to assess the predictability of long-term protein stability by T_m (FTIR).

Peek L. J., Martin T. T., Elk N. C., Pegram S. A. and Middaugh C. R. (2007) Effects of stabilizers on the destabilization of proteins upon adsorption to aluminum salt adjuvants. *J Pharm. Sci* **96**, 547-557.

Abstract: Excipients for protein-based vaccines are currently identified by evaluating the stability of the protein in solution. In most cases, however, the protein is adsorbed to the surface of an aluminum salt adjuvant in the final vaccine formulation. Previous studies showed that model protein antigens may be structurally altered and less thermally stable upon adsorption to aluminum salt adjuvants [Jones LS, Peek LJ, Power J, Markham A, Yazzie B, Middaugh CR, 2005, *J Biol Chem* 280:13406-13414]. The work presented herein provides evidence that compounds that stabilize the protein in solution also stabilize the adsorbed protein; however, the stability of the adsorbed protein in the presence of the stabilizer remains lower than that of the protein in solution. Potential implications of the reduced stability on the approach used to select excipients during formulation development are discussed.

Pereira P., Kelly S. M., Cooper A., Mardon H. J., Gellert P. R. and van der Walle C. F. (2007) Solution formulation and lyophilisation of a recombinant fibronectin fragment. *Eur J Pharm. Biopharm.* **67**, 309-319.

Abstract: The 9th-10th type III fibronectin domain pair shows promise in tissue engineering and tumour vasculature targeting. Calorimetry and structure-function analysis were used to investigate the effects of solution formulation and lyophilisation of a mutant ((9-10)FNIII-P). A single endothermic transition for (9-10)FNIII-P in solution was observed at pH<8, irrespective of addition of sucrose or PEG. The temperature

at the maximum heat capacity ($T(m)$) and enthalpy (ΔH) of the transition increased for increasing sucrose concentrations but decreased for increasing PEG concentrations. The transition was fitted to a single two-state unfolding mechanism (in contrast to unfolding in guanidine. x HCl) and was partially reversible only at pH 4, with increasing concentrations of sucrose causing a marked fall in ΔH between scans. Circular dichroism spectra for the thermal unfolding of (9-10)FNIII-P at pH 4 showed loss of native beta-sheet structure and loss of aromatic contributions to the peak centred around 226 nm yielding an intermediate conformation, which in the presence of sucrose was more disordered. Despite a glass transition ($T(g')$) for (9-10)FNIII-P(aq) of -70 degrees C, primary drying at -30 degrees C did not perturb its conformation upon reconstitution or its biological activity following lyophilisation; the addition of sucrose or PEG had no influence on structure or activity. The main consideration in the formulation of (9-10)FNIII-P was therefore pH.

Perico N., Purtell J., Dillon T. M. and Ricci M. S. (2008) Conformational implications of an inversed pH-dependent antibody aggregation. *J Pharm. Sci (epublication)*.

Abstract: Antibody formulation development relies on accelerated stability data at elevated temperatures to optimize formulation parameters. However, the pH- and temperature-dependence of aggregation is complicated for antibody formulations. In this study, a human monoclonal IgG2 antibody exhibited typical pH-dependent dimer formation under normal storage conditions (4 and/or 29 degrees C). However, an inversed pH-dependence was discovered for high molecular weight aggregate formation at elevated temperatures (37 degrees C). The different stability profiles exhibited at the various storage conditions resulted in nonlinearity of the Arrhenius kinetics. Thermal unfolding at or below 37 degrees C was not evident by differential scanning calorimetry. Enriched populations of the structural isoforms of the IgG2 subclass were tested for their unique temperature and pH-dependence of aggregation. The Arrhenius kinetics of aggregation for each of the individual IgG2 isoforms was also nonlinear. However, the temperature-dependence of clipping suggested that clip-mediated aggregation was responsible for the increased higher order aggregates at low pH and elevated temperatures. Unique clip species resulting from the conformational differences between the IgG2 isoforms lead to increased aggregation. These results have implications on the mechanisms of antibody aggregation and on the validity of accelerated data to predict shelf-life accurately. (c) 2008 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci*

Petersen S. B., Jonson V., Fojan P., Wimmer R., and Pedersen S. (2004) Sorbitol prevents the self-aggregation of unfolded lysozyme leading to and up to 13 degrees C stabilisation of the folded form. *J Biotechnol* **114**, 269-278.

Abstract: We present a calorimetric investigation of stabilisation of hen egg-white lysozyme with sorbitol in the pH range 3.8-10.5. Differential scanning calorimetry and steady-state fluorescence were used to determine the denaturation temperatures of lysozyme as a function of sorbitol concentration. The fluorescence data were collected in the presence of 2M urea to lower the melting point of the protein to an observable range of the instrument. The effect of sorbitol on the activation energy of unfolding was investigated by scanrate studies. The effect of sorbitol lysozyme interaction was investigated using isothermal titration calorimetry. The titration experiments were performed with folded as well as unfolded lysozyme to investigate in more detail the nature of the interaction. The data obtained in those experiments show a remarkable stabilisation effect of sorbitol. We observed a 4.0 degrees C increase in the T_m for 1 M sorbitol in the pH range 3.8-8.5 by scanning calorimetry. The effect increases dramatically at pH 9.5 where we observe a 9.5 degrees C stabilisation. An increase in the sorbitol concentration to 2 M stabilises lysozyme by 11.3-13.4 degrees C in the pH range 9.5-10.5. In the absence of urea, no significant effects of sorbitol were observed on the activation energy for unfolding for lysozyme at pH 4.5. This indicates together with the results from the titration experiments that sorbitol may stabilise the folded form of lysozyme by destabilising the unfolded form of lysozyme. At pH values at and above lysozyme's pI (approximately 9.3), the unfolding of the protein is accompanied with a substantial amount of self-aggregation seen in the calorimetry experiments in the ratio of $\Delta H_{cal}/\Delta H_{vH}$. In the presence of sorbitol, the self-aggregation was counterbalanced by higher sorbitol concentrations. These results strongly suggest a negative influence of sorbitol on the unfolded form of lysozyme and thereby stabilising the native form.

Pikal M. J., Rigsbee D., Roy M. L., Galreath D., Kovach K. J., Wang B., Carpenter J. F. and Cicerone M. T. (2008) Solid state chemistry of proteins: II. The correlation of storage stability of freeze-dried human growth hormone (hGH) with structure and dynamics in the glassy solid. *J Pharm. Sci* **97**, 5106-5121.

Abstract: This research presents storage stability of human growth hormone, hGH, in lyophilized disaccharide formulations. Stability via HPLC assay was assessed at 40 and 50 degrees C. Structure of the protein in the solids was assessed by infrared spectroscopy. Molecular mobility was characterized by structural relaxation times estimated from DSC data and by measurement of atomic motion on a nanosecond time scale by neutron scattering. Very large stability differences were observed among the various formulations, with both chemical and aggregation stability showing the same qualitative trends with formulation. Near the T(g), T(g) appeared to be a relevant stability parameter, but for storage well below T(g), stability seems unrelated to T(g). Stability (chemical and aggregation) was weakly correlated with secondary structure of the protein, and there was a partial quantitative correlation between degradation rate and the structural relaxation time. However, at equivalent levels of disaccharide relative to protein, sucrose systems were about a factor of two more stable than trehalose formulations, but yet had greater mobility as measured by structural relaxation time. Secondary structure was equivalent in both formulations. Neutron scattering results documented greater suppression of fast dynamics by sucrose than by trehalose, suggesting that well below T(g), fast dynamics are important to stability

Pikal M. J., Rigsbee D. and Roy M. L. (2008) Solid state stability of proteins III: calorimetric (DSC) and spectroscopic (FTIR) characterization of thermal denaturation in freeze dried human growth hormone (hGH). *J Pharm. Sci* **97**, 5122-5131.

Abstract: This research is a study of the changes in secondary structure (Fourier transform infrared spectroscopy, FTIR), aggregation, and loss of the magnitude of the heat of denaturation upon scanning to and partially through the temperature range of the thermal denaturation peak of a model protein, human growth hormone (hGH). We study two formulations, a system of essentially pure protein (with a trace of phosphate buffer) and a system formulated with trehalose in a 3:1 trehalose:hGH weight ratio. The extent of denaturation is measured by loss of secondary structure by FTIR, the loss of heat of denaturation by differential scanning calorimetry (DSC), and the fraction of protein aggregated by HPLC. We examine loss of structure on heating to the DSC onset of thermal denaturation and restoration of structure by cooling below the denaturation temperature and holding to (nominally) allow time for refolding, and we also examine restoration of structure upon dissolving and refreeze drying samples heated to selected temperatures in the denaturation range. We find that denaturation occurs only above the glass transition temperature, is highly cooperative, and is only reversible by redissolving the "denatured" formulated (trehalose) solid. Further, all measures of the extent of denaturation are in essential agreement

Pikal M. J., Rigsbee D. and Akers M. J. (2008) Solid state chemistry of proteins IV. what is the meaning of thermal denaturation in freeze dried proteins? *J Pharm. Sci*.

Abstract: This research addresses the thermodynamic significance of the denaturation endotherm observed during differential scanning calorimetry (DSC) scans of proteins in dry formulations, such as freeze dried solids. Human growth hormone formulations are the chosen representative examples. We employ observations of denaturation temperature, glass transition temperature, and the differences between estimated molecular mobilities to argue that unfolding is under partial thermodynamic control. Further, unfolding during a DSC scan is simulated using a three state kinetic model, which is a two state unfolding model followed by aggregation. Kramers-type rate constants are used, where the preexponential term is dominated by viscous forces. Simulation results are in qualitative agreement with experiment, and clearly show that while the denaturation endotherm is impacted by irreversibility, caused by nonzero scan rate and aggregation, the position of the endotherm peak is changed only slightly. Thus, the denaturation peak is a good approximation for the thermodynamic denaturation temperature. Using data for denaturation temperature, heat of denaturation, and heat capacity of denaturation, free energy versus temperature curves were calculated. We find that even formulations with added saccharides are thermodynamically unstable near ambient temperature; significant denaturation in the solid state is prevented by low mobility. (c) 2008 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci*

Remmele R. L. (2005) Microcalorimetric approaches to biopharmaceutical development *in* Analytical techniques for biopharmaceutical development, Rodriguez-Diaz, R., Wehr, T. Tuck, S., eds., Marcel Dekker, New York NY, pp. 327-381.

Piedmonte D. M., Summers C., McAuley A., Karamujic L. and Ratnaswamy G. (2007) Sorbitol crystallization can lead to protein aggregation in frozen protein formulations. *Pharm. Res* **24**, 136-146.
Abstract: PURPOSE: This work examines the cause of aggregation of an Fc-fusion protein formulated in sorbitol upon frozen storage for extended periods of time at -30 degrees C. MATERIALS AND METHODS: We designed sub-ambient differential scanning calorimetry (DSC) experiments to capture the effects of long-term frozen storage. The physical stability of formulation samples was monitored by size exclusion high performance liquid chromatography (SE-HPLC). RESULTS: DSC analysis of non-frozen samples shows the expected glass transitions (T_g) at -45 degrees C for samples in sorbitol and at -32 degrees C in sucrose. In time course studies where sorbitol formulations were stored at -30 degrees C and analyzed by DSC without thawing, two endothermic transitions were observed: a melting endotherm at -20 degrees C dissipated over time, and a second endotherm at -8 degrees C was seen after approximately 2 weeks and persisted in all later time points. Protein aggregation was only seen in the samples formulated in sorbitol and stored at -30 degrees C, correlating aggregation with the aforementioned melts. CONCLUSIONS: The observed melts are characteristic of crystalline substances and suggest that the sorbitol crystallizes over time. During freezing, the excipient must remain in the same phase as the protein to ensure protein stability. By crystallizing, the sorbitol is phase-separated from the protein, which leads to protein aggregation.

Remmele, R.L., and Gombotz, W.R. (2000) Differential Scanning Calorimetry: A practical tool for elucidating the stability of liquid biopharmaceuticals. *BioPharm* **13**, 36-46.

Remmele R. L., Jr., Nightlinger N. S., Srinivasan S., and Gombotz W. R. (1998) Interleukin-1 receptor (IL-1R) liquid formulation development using differential scanning calorimetry. *Pharm Res* **15**, 200-208.
Abstract: PURPOSE: To elucidate the solution conditions that confer stability of aqueous IL-1R using differential scanning calorimetry (DSC). METHODS: Optimal pH conditions were determined by monitoring degradation products encountered during accelerated studies (at elevated temperatures) using SDS-PAGE. At the pH optimum, DSC screened for excipients that enhanced thermal stability by shifting the T_m to higher values. Using SEC the relationship between thermal unfolding and stability was investigated by considering if lower T_m 's in the presence of preservatives correlated with degradation products at 37 degrees C over time. The degree of aggregation relative to that of a control determined the level of stability achieved. RESULTS: Circular dichroism (CD) measurements confirmed molecular modeling studies showing IL-1R to be about 39% beta-sheet. Two major transitions characterized the DSC data with T_m 's observed near 47 degrees C and 66 degrees C. Among 21 excipients screened, NaCl exhibited the greatest stabilizing influences based on shifting the low temperature transition to 53 degrees C. The low temperature transition was later found to comprise two transitions, yielding a total of three melting transitions for IL-1R. High T_m 's arising from the presence of preservatives correlated with the order of stability (i.e., 0.065% phenol > 0.1% m-Cresol > 0.9% benzyl alcohol). CONCLUSIONS: The three melting transitions are consistent in origin with the cooperative unfolding of three unique immunoglobulin-like domains of IL-1R. Optimal stability was achieved in 20 mM sodium citrate at pH 6 with sufficient NaCl to attain the tonicity of human serum. A correlation between the predicted ranking of stability and the extent of aggregation was demonstrated using DSC.

Remmele R. L., Jr., Bhat S. D., Phan D. H., and Gombotz W. R. (1999) Minimization of recombinant human Flt3 ligand aggregation at the T_m plateau: a matter of thermal reversibility. *Biochemistry* **38**, 5241-5247.

Abstract: This study elucidates the importance of thermal reversibility as it pertains to the minimization of recombinant human Flt3 ligand aggregation and its potential role for determining solution conditions that can achieve the greatest long-term storage stability. Both thermal reversibility and T_m were evaluated as microcalorimetric parameters of stability within the range extending from pH 6 to 9, where the T_m was shown to plateau near 80 degrees C. Within this region, the reversibility was shown to decrease from 96.6% to 15.2% while the pH was increased from 6 to 9, respectively. Accelerated stability studies conducted at 50 degrees C exhibited rates of aggregation augmented by pH that inversely correlated with the thermal reversibility data. Namely, high thermal reversibility at the T_m plateau correlated with slower rates of aggregation. Enthalpic calorimetric to van't Hoff ratios ($\Delta H_1/\Delta H_v$) yielded results close to unity within the plateau region, suggesting that the unfolding of rhFlt3 ligand was approximately two-state. Evidence that unfolding preceded the formation of the aggregate was provided by far-UV CD data of a soluble isolate of

the aggregated product exhibiting a 28% loss of alpha-helix offset by a 31% gain in beta-sheet. This information combined with the thermal reversibility data provided compelling evidence that unfolding was a key event in the aggregation pathway at 50 degrees C. Minimization of aggregation was achieved at pH 6 and corroborated by evidence acquired from sodium dodecyl sulfate-polyacrylamide gel electrophoresis and size exclusion data. Correspondingly, the bioactivity was found to be optimal at pH 6. The findings link thermal reversibility to the propensity of Flt3 ligand to aggregate once unfolded in the T_m plateau region and provide a basis for relating the reversibility of thermal denaturation to the prediction of long-term storage stability in aqueous solution.

Remmele R. L., Jr., Zhang-van Enk J., Dharmavaram V., Balaban D., Durst M., Shoshitaishvili A., and Rand H. (2005) Scan-rate-dependent melting transitions of interleukin-1 receptor (type II): elucidation of meaningful thermodynamic and kinetic parameters of aggregation acquired from DSC simulations. *J Am Chem Soc* **127**, 8328-8339.

Abstract: The role of thermal unfolding as it pertains to thermodynamic properties of proteins and their stability has been the subject of study for more than 50 years. Moreover, exactly how the unfolding properties of a given protein system may influence the kinetics of aggregation has not been fully characterized. In the study of recombinant human Interleukin-1 receptor type II (rhuIL-1R(II)) aggregation, data obtained from size exclusion chromatography and differential scanning calorimetry (DSC) were used to model the thermodynamic and kinetic properties of irreversible denaturation. A break from linearity in the initial aggregation rates as a function of 1/T was observed in the vicinity of the melting transition temperature (T_m) approximately 53.5 degrees C), suggesting significant involvement of protein unfolding in the reaction pathway. A scan-rate dependence in the DSC experiment testifies to the nonequilibrium influences of the aggregation process. A mechanistic model was developed to extract meaningful thermodynamic and kinetic parameters from an irreversibly denatured process. The model was used to simulate how unfolding properties could be used to predict aggregation rates at different temperatures above and below the T_m and to account for concentration dependence of reaction rates. The model was shown to uniquely identify the thermodynamic parameters DeltaC(P) (1.3 +/- 0.7 kcal/mol-K), DeltaH(m) (74.3 +/- 6.8 kcal/mol), and T_m with reasonable variances.

Royall P. G. and Gaisford S. (2005) Application of solution calorimetry in pharmaceutical and biopharmaceutical research. *Curr Pharm Biotechnol* **6**, 215-222.

Abstract: In solution calorimetry the heat of solution (Delta(sol)H) is recorded as a solute (usually a solid) dissolves in an excess of solvent. Such measurements are valuable during all the phases of pharmaceutical formulation and the number of applications of the technique is growing. For instance, solution calorimetry is extremely useful during preformulation for the detection and quantification of polymorphs, degrees of crystallinity and percent amorphous content; knowledge of all of these parameters is essential in order to exert control over the manufacture and subsequent performance of a solid pharmaceutical. Careful experimental design and data interpretation also allows the measurement of the enthalpy of transfer (Delta(trans)H) of a solute between two phases. Because solution calorimetry does not require optically transparent solutions, and can be used to study cloudy or turbid solutions or suspensions directly, measurement of Delta(trans)H affords the opportunity to study the partitioning of drugs into, and across, biological membranes. It also allows the in-situ study of cellular systems. Furthermore, novel experimental methodologies have led to the increasing use of solution calorimetry to study a wider range of phenomena, such as the precipitation of drugs from supersaturated solutions or the formation of liposomes from phospholipid films. It is the purpose of this review to discuss some of these applications, in the context of pharmaceutical formulation and preformulation, and highlight some of the potential future areas where solution calorimetry might find applications.

Salnikova M. S., Joshi S. B., Rytting J. H., Warny M. and Middaugh C. R. (2008) Preformulation studies of Clostridium difficile toxoids A and B. *J Pharm. Sci* **97**, 4194-4207.

Abstract: To enhance the physical stability of Clostridium difficile toxoids A and B, screening for stabilizing compounds was performed. The screening of 30 GRAS compounds at various concentrations and in several combinations was performed in two parts. First, a high-throughput aggregation assay was used to screen for compounds which delayed or prevented aggregation of toxoids under stress conditions (toxoids at pH 5-5.5 were incubated at 55 degrees C for 55 or 75 min). Compounds which stabilized both proteins were further studied for their ability to delay unfolding under conditions leading to a presumably

native-like folded state (pH 6.5). The thermal stability of the toxoids on the surface of Alhydrogel was monitored with DSC and also showed significant improvement in the presence of certain excipients. This study has generated information concerning the free and adjuvant bound toxoids behavior under a range of conditions (temperature, solutes) that can be used to design pharmaceutical formulations of enhanced physical stability

Salnikova M. S., Middaugh C. R. and Rytting J. H. (2008) Stability of lyophilized human growth hormone. *Int J Pharm.* **358**, 108-113.

Abstract: To evaluate relationships between the extent of protein-excipient interactions, structural relaxation of an amorphous matrix, and the physico-chemical stability of a protein, human growth hormone (hGH) was lyophilized with sucrose and trehalose in a 1:2 weight ratio. The protein-excipient interactions were analyzed immediately after lyophilization with isoperibol solution calorimetry (ISC), water sorption analysis (WSA), differential scanning calorimetry (DSC) and Fourier Transform Infrared Spectroscopy (FTIR). The physical and chemical stability of hGH during storage at 50 degrees C was monitored by reverse phase (RP)-HPLC, SEC-HPLC and UV absorption spectroscopy. The hGH formulation containing sucrose demonstrated greater protein-excipient interactions and faster initial relaxation times compared to the trehalose formulation. Although both formulations had similar chemical stability (rate of deamidation), physical stabilities (e.g. degree of aggregation) were different. The hGH/sucrose formulation manifested a higher rate and lower extent of insoluble aggregate formation. The decreased amount of aggregation in the sucrose formulation could be correlated with a greater extent of protein-excipient interactions and the presence of a more homogeneous mixture. In contrast, the higher rate of aggregation in the sucrose formulation could be directly correlated with the higher molecular mobility of the matrix

Sathish H. A., Kumar P. R. and Prakash V. (2007) Mechanism of solvent induced thermal stabilization of papain. *Int J Biol Macromol.* **41**, 383-390.

Abstract: In the present study an attempt is made to elucidate the effects of various cosolvents, such as sorbitol, sucrose, xylose and glycerol, on papain. The stabilizing effects of these cosolvents on the structure and function of papain is determined by the activity measurements, fluorescence spectroscopy and differential scanning calorimetry (DSC). The enzyme activity measurements indicate several fold increase in the thermal stability of the enzyme in all the cosolvents used. The thermal denaturation studies of papain in presence of various concentrations of cosolvents indicated a shift in the apparent thermal denaturation temperature (app T_m) suggesting increased thermal stability of papain in presence of cosolvents. The app T_m shifted from a control value of 83±1 degrees C to a value of >90±1 degrees C in presence of 40% sorbitol. The DSC thermogram for native papain can be clearly deconvoluted into two transitions corresponding to left and right domain and in presence of cosolvents both transitions A and B shift to higher temperature. Maximum stabilization was seen in case of 30% sorbitol where the thermal transition temperatures increased compared to control. The results from partial specific volume measurements of papain in presence of cosolvents suggest that the preferential interaction parameter (xi₃) was negative in all cosolvents and maximum hydration was observed in the case of glycerol where the preferential interaction parameter was 0.165g/g. These above results suggest that there is a considerable increase in the thermal stability of papain in presence of these cosolvents as a result of preferential hydration.

Schwartz D., Sofia S., and Friess W. (2006) Integrity and stability studies of precipitated rhBMP-2 microparticles with a focus on ATR-FTIR measurements. *Eur J Pharm Biopharm* **63**, 241-248.

Abstract: A major obstacle in the development of protein drug formulations is the need to maintain the native, active protein structure both during the formulation process and upon long time storage. Controlled precipitation was evaluated for its potential to supply stable microparticulate formulations of bone-regenerating recombinant human Bone Morphogenetic Protein-2 (rhBMP-2). Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) did provide insight into the protein formulation and stability. Temperature dependent ATR-FTIR measurements and DSC measurements allow for the study of changes in the protein structure during melting. To address the question of isomerization, peptide mapping was performed, and protein aggregation was monitored by size exclusion chromatography (SEC). It could be demonstrated by ATR-FTIR that controlled precipitation did not harm the protein and the process is fully reversible. DSC measurements further confirmed these findings. No changes in the transition temperature and process were observed after precipitation and redissolution. Upon storage, isomerization and aggregation could be detected, but to a lower extent in the precipitated formulation as compared to a

solution reference. Thus, controlled precipitation of rhBMP-2 is fully reversible and has the potential as alternative formulation tool for the generation of a microparticulate drug delivery system.

Sellers S. P. and Maa Y. F. (2005) Principles of biopharmaceutical protein formulation: an overview. *Methods Mol Biol* **308**, 243-263.

Sharma V. K. and Kalonia D. S. (2003) Temperature- and pH-induced multiple partially unfolded states of recombinant human interferon-alpha2a: possible implications in protein stability. *Pharm Res* **20**, 1721-1729.

Abstract: PURPOSE: To study the effect of solution conditions on the structural conformation of recombinant human interferon-alpha2a (IFNalpha2a) to investigate its tendency to form partially unfolded intermediates. METHODS: The structural properties of IFNalpha2a were studied at various pH values (2.0-7.4) and temperatures (5 degrees C-80 degrees C) using Trp fluorescence emission, fluorescence quenching, near- and far-UV circular dichroism (CD) spectroscopy, and DSC. RESULTS: Fluorescence intensity measurements as a function of temperature indicated the onset of the thermal unfolding of IFNalpha2a, denoted by Td, around 60 degrees C above pH 4.0. Td was not observed at pH 3.5 and below. Acrylamide and iodide quenching studies indicated partial unfolding of protein with decrease in pH and with increase in temperature up to 50 degrees C. Near-UV CD studies indicated a significant loss in the tertiary structure of protein on increase in temperature from 15 degrees C to 50 degrees C at all solution pHs. DSC scans supported results obtained from fluorescence and CD studies at pH 4.0 and below. DSC, however, was insensitive to changes that occurred at moderate temperatures at pH 5.0 and 7.4. CONCLUSIONS: IFNalpha2a has a tendency to acquire multiple partially unfolded states with structural conformations sensitive to solution pH and temperature. These states were formed at moderate temperatures, and it is speculated that these partially unfolded states could play an important role in the aggregation of proteins during the long-term storage of aqueous protein formulations.

Shiraki K., Kudou M., Nishikori S., Kitagawa H., Imanaka T., and Takagi M. (2004) Arginine ethylester prevents thermal inactivation and aggregation of lysozyme. *Eur J Biochem* **271**, 3242-3247.

Abstract: Arginine is a versatile additive to prevent protein aggregation. This paper shows that arginine ethylester (ArgEE) prevents heat-induced inactivation and aggregation of hen egg lysozyme more effectively than arginine or guanidine. The addition of ArgEE decreased the melting temperature of lysozyme. This data could be interpreted in terms of ArgEE binding to unfolded lysozyme, possibly through the ethylated carboxyl group, which leads to effective prevention of intermolecular interaction among aggregation-prone molecules. The data suggest that ArgEE could be used as an additive to prevent inactivation and aggregation of heat-labile proteins.

Singh S. and Singh J. (2003) Effect of polyols on the conformational stability and biological activity of a model protein lysozyme. *AAPS PharmSciTech* **4**, E42.

Abstract: The purpose of this study was to investigate the stabilizing action of polyols against various protein degradation mechanisms (eg, aggregation, deamidation, oxidation), using a model protein lysozyme. Differential scanning calorimeter (DSC) was used to measure the thermodynamic parameters, mid point transition temperature and calorimetric enthalpy, in order to evaluate conformational stability. Enzyme activity assay was used to corroborate the DSC results. Mannitol, sucrose, lactose, glycerol, and propylene glycol were used as polyols to stabilize lysozyme against aggregation, deamidation, and oxidation. Mannitol was found to stabilize lysozyme against aggregation, sucrose against deamidation both at neutral pH and at acidic pH, and lactose against oxidation. Stabilizers that provided greater conformational stability of lysozyme against various degradation mechanisms also protected specific enzyme activity to a greater extent. It was concluded that DSC and bioassay could be valuable tools for screening stabilizers in protein formulations.

Singh S. and Singh J. (2004) Controlled release of a model protein lysozyme from phase sensitive smart polymer systems. *Int J Pharm* **271**, 189-196.

Abstract: The purpose of this study was to investigate the suitability of phase sensitive smart polymer-based protein formulations in order to deliver a model protein, lysozyme, in a conformationally stable and biologically active form at a controlled rate over extended period of time. Four different formulations, using D,L-poly(lactide) (D,L-PLA) and a solvent mixture of different ratios of benzyl benzoate (BB) and

benzyl alcohol (BA), were prepared. Conformational stability and biological activity of lysozyme were studied by differential scanning calorimeter and enzyme activity assay, respectively. We found a significant ($P < 0.05$) increase in burst and rate of release of incorporated lysozyme from formulations containing greater proportion of BA. In order to increase the conformational stability and biological activity of lysozyme, we incorporated mannitol as stabilizer into formulations. Mannitol increased the conformational and biological activity of lysozyme in comparison to the control formulation prepared without mannitol. In conclusion, phase sensitive smart polymer-based delivery systems were able to deliver a model protein, lysozyme, in a conformationally stable and biologically active form at a controlled rate over extended period of time.

Tadeo X., Pons M. and Millet O. (2007) Influence of the Hofmeister anions on protein stability as studied by thermal denaturation and chemical shift perturbation. *Biochemistry* **46**, 917-923.

Abstract: The influence of external cosolutes on the thermal stability of the B1 domain of protein L (ProtL) has been studied by circular dichroism, fluorescence spectroscopy, and differential scanning calorimetry. The thermal denaturation midpoint is effectively modulated by the addition of a suite of anions and follows the Hofmeister series. The maximum increase in thermostability (corresponding to 14 degrees C) was observed in the presence of 1 M sodium sulfate. After conversion of the experimental data into the change in the virial coefficient, a mechanistic model was used to estimate the relative contributions from excluded volume and preferential anion solvation for each anion. As expected, the excluded volume term stabilizes the native conformation of ProtL for all the cosolutes, but opposite effects on protein stability arise from the anion's solvation depending on their tendency to interact with or to become excluded from the protein surface. This behavior is in agreement with the results of independent NMR experiments: the anions that strongly interact with the protein surface produce significant perturbations in the amide protein chemical shift ($\Delta d_{23}(\text{HN})$). A correlation obtained between $\Delta d_{23}(\text{HN})$ and the temperature coefficients for the different amide protons provides qualitative information about the structural determinants for the interaction between the protein surface and the cosolute.

Tiwari A. and Bhat R. (2006) Stabilization of yeast hexokinase A by polyol osmolytes: correlation with the physicochemical properties of aqueous solutions. *Biophys Chem* **124**, 90-99.

Abstract: Osmolytes of the polyol series are known to accumulate in biological systems under stress and stabilize the structures of a wide variety of proteins. While increased surface tension of aqueous solutions has been considered an important factor in protein stabilization effect, glycerol is an exception, lowering the surface tension of water. To clarify this anomalous effect, the effect of a series of polyols on the thermal stability of a highly thermolabile two domain protein yeast hexokinase A has been investigated by differential scanning calorimetry and by monitoring loss in the biological activity of the enzyme as a function of time. A larger increase in the $T(m)$ of domain 1 compared with that of domain 2, varying linearly with the number of hydroxyl groups in polyols, has been observed, sorbitol being the best stabilizer against both thermal as well as urea denaturation. Polyols help retain the activity of the enzyme considerably and a good correlation of the increase in $T(m)$ ($\Delta T(m)$) and the retention of activity with the increase in the surface tension of polyol solutions, except glycerol, which breaks this trend, has been observed. However, the $\Delta T(m)$ values show a linear correlation with apparent molal heat capacity and volume of aqueous polyol solutions including glycerol. These results suggest that while bulk solution properties contribute significantly to protein stabilization, interfacial properties are not always a good indicator of the stabilizing effect. A subtle balance of various weak binding and exclusion effects of the osmolytes mediated by water further regulates the stabilizing effect. Understanding these aspects is critical in the rational design of stable protein formulations.

Tsai P. K., Volkin D. B., Dabora J. M., Thompson K. C., Bruner M. W., Gress J. O., Matuszewska B., Keogan M., Bondi J. V., and Middaugh C. R. (1993) Formulation design of acidic fibroblast growth factor. *Pharm Res* **10**, 649-659.

Abstract: The design of an aqueous formulation for acidic fibroblast growth factor (aFGF) requires an understanding of the type of compounds that can either directly or indirectly stabilize the protein. To this end, spectrophotometric turbidity measurements were initially employed to screen the ability of polyanionic ligands, less specific compounds, and variations in solution conditions (temperature and pH) to stabilize aFGF against heat-induced aggregation. It was found that in addition to the well-known protection of aFGF by heparin, a surprisingly wide variety of polyanions (including small sulfated and phosphorylated

compounds) also stabilizes aFGF. These polyanionic ligands are capable of raising the temperature at which the protein unfolds by 15-30 degrees C. Many commonly used excipients were also observed to stabilize aFGF in both the presence and the absence of heparin. High concentrations of some of these less specific agents are also able to increase the temperature of aFGF thermal unfolding by as much as 6-12 degrees C as shown by circular dichroism and differential scanning calorimetry. Other compounds were found which protect the chemically labile cysteine residues of aFGF from oxidation. Aqueous formulations of aFGF were thus designed to contain both a polyanionic ligand that enhances structural integrity by binding to the protein and chelating agents (e.g., EDTA) to prevent metal ion-catalyzed oxidation of cysteine residues. While room-temperature storage (30 degrees C) leads to rapid inactivation of aFGF in physiological buffer alone, several of these aFGF formulations are stable in vitro for at least 3 months at 30 degrees C. Three aFGF topical formulations were examined in an impaired diabetic mouse model and were found to be equally capable of accelerating wound healing.

Twomey C., Doonan S., and Giartosio A. (1995) Thermal denaturation as a predictor of stability on long-term storage of a protein. *Biochem Soc Trans* **23**, 369S.

Vemuri S., Beylin I., Sluzky V., Stratton P., Eberlein G., and Wang Y. J. (1994) The stability of bFGF against thermal denaturation. *J Pharm Pharmacol* **46**, 481-486.

Abstract: The influence of sulphated ligand and pH on thermal denaturation of basic fibroblast growth factor (bFGF) was investigated by differential scanning calorimetry (DSC), and verified by fluorescence spectrophotometry. Purity of bFGF before and after heat denaturation was assessed by SDS-PAGE analysis. In DSC studies the samples were heated to 95 degrees C. The midpoint of the temperature change in the thermogram was designated as T_m. Sulphated ligand experiments were undertaken in potassium phosphate (pH 6.5) and sodium acetate buffers. Control thermograms (with no ligand) showed a T_m at 59 degrees C in potassium phosphate buffer. Higher T_m values were noted as sulphated ligand concentration was increased. Similarly when heparin was added, the T_m moved to a higher temperature. A ratio as low as 0.3:1 of heparin to bFGF, increased the T_m to 90 degrees C, which is a 31 degrees C shift in T_m. The effect of pH on thermal denaturation of bFGF was studied in a citrate-phosphate-borate buffer system. A shift in T_m from 46 to 65 degrees C was observed as the pH is changed from 4 to 8. Changes in protein conformation as a function of pH were monitored by fluorescence spectroscopy. It was found that a pH range from 5 to 9 is optimal for the stability of bFGF formulations. In a stability study it was noted that heparin protected bFGF from thermal denaturation only at high temperature.

Vidanovic D., Milic A. J., Stankovic M., and Poprzen V. (2003) Effects of nonionic surfactants on the physical stability of immunoglobulin G in aqueous solution during mechanical agitation. *Pharmazie* **58**, 399-404.

Abstract: The objective of this study was to evaluate the influence of nonionic surfactants in the presence of glycine and sodium chloride on the physical stability of immunoglobulin G (IgG) in aqueous solution. Among surfactants suitable for parenteral preparation, Polysorbate 80 (Tween 80) and Polyoxyl 35 Castor Oil (Cremophor EL) were selected. The physical stability of IgG in the absence and in the presence of excipients was investigated in aqueous solution during mechanical agitation (concentration of IgG 15%; pH 7.1; temperature 6 +/- 2 degrees C). Suitable concentrations of Tween 80 and Cremophor EL were experimentally determined by surface tension measurements at 6 +/- 2 degrees C. Glycine and sodium chloride were used in different concentrations. The influence of the excipients on the physical stability of IgG in solution has been examined by surface tension measurements, protein content assay (Kjeldahl and HPLC) and differential scanning calorimetry (DSC). Based on the results of the investigations, it was found that Tween 80 and Cremophor EL, used in experimentally determined critical micelle concentration (cmc), decreased the physical stability of IgG in solution. Tween 80 and Cremophor EL in the presence of glycine (1.5 g/l) could stabilize the IgG in solution during mechanical agitation. The comparison of the effects of Tween 80 and Cremophor EL on the physical stability of IgG, showed that Tween 80 had better stabilization effects on IgG in solution under the experimental conditions selected.

Volkin D. B., Sanyal G., Burke C. J., and Middaugh C. R. (2002) Preformulation studies as an essential guide to formulation development and manufacture of protein pharmaceuticals. *Pharm Biotechnol* **14**, 1-46.

Wakankar A. A., Borchardt R. T., Eigenbrot C., Shia S., Wang Y. J., Shire S. J. and Liu J. L. (2007) Aspartate isomerization in the complementarity-determining regions of two closely related monoclonal antibodies. *Biochemistry* **46**, 1534-1544.

Abstract: The aspartic acid residues (Asp) present in the complementarity-determining regions (CDRs) of the light chains of two recombinant monoclonal antibodies (MAbs), MAb I and MAb II, are highly susceptible to isomerization due to the presence of glycine residues (Gly) on their C-terminal ends. Asp isomerization in these MAbs leads to formation of the isoaspartate (IsoAsp) and the cyclic imide (Asu) variants of these MAbs. Both MAb I and MAb II, employed in this study, elicit their pharmacological responses through binding human IgE. The formation of the MAb variants as a result of Asp isomerization significantly reduces the binding affinities of these antibodies to IgE, thereby reducing their potencies. Here we report on significant differences in the susceptibility of the MAb I and the MAb II to Asp isomerization. The molecular basis for these differences in rates of Asp isomerization was elucidated. The effect of primary sequence on Asp isomerization was evaluated using pentapeptide models of the MAbs, which included the labile Asp residues and their neighboring amino acid residues. The separation of the parent MAbs and pentapeptides from their isomerization products was achieved using hydrophobic interaction chromatography (HIC) and rp-HPLC, respectively. Structural characterization of the MAbs was performed using differential scanning calorimetry (DSC), circular dichroism (CD), and X-ray crystallography. Our investigations demonstrate that the differences in the Asp isomerization rates between MAb I and MAb II can be attributed to structural factors including the conformational flexibility and the extent of solvent exposure of the labile Asp residue.

Wakankar A. A., Liu J., Vandervelde D., Wang Y. J., Shire S. J. and Borchardt R. T. (2007) The effect of cosolutes on the isomerization of aspartic acid residues and conformational stability in a monoclonal antibody. *J Pharm. Sci* **96**, 1708-1718.

Abstract: The aspartate residue (Asp 32) located in the complementarity-determining region (CDR) of a recombinant humanized monoclonal antibody (MAb I) is highly susceptible to the isomerization reaction. The modification of Asp 32 residue due to the isomerization reaction results in a significant reduction in the binding affinity of MAb I to IgE. The binding of a MAb I therapeutic to IgE is important for its desired pharmacological effect. In earlier investigations, we demonstrated that the conformational flexibility and residue exposure are factors that are responsible for the observed reactivity of Asp 32 in MAb I. This report explores the role of cosolutes such as glycerol and sucrose in the modulation of Asp 32 reactivity in MAb I. These cosolutes are routinely incorporated in injectable pharmaceutical formulations. The reactivity of the Asp residue in MAb I in these different cosolute-based formulations was compared to its reactivity in a peptide model VDYDG comprising residues 29-33 of MAb I. The formulations of MAb I and VDYDG containing varying concentrations of glycerol and sucrose were incubated at 50 degrees C for a period of 5-7 days. The isomerization of the Asp residue in VDYDG and MAb I was monitored using rp-HPLC and hydrophobic interaction chromatography (HIC), respectively. Structural analysis of MAb I using differential scanning calorimetry (DSC) demonstrated that the structural stability of MAb I was increased in formulations containing glycerol and sucrose. However, the stability of Asp 32 in MAb I was significantly decreased in these formulations. This research suggests that a formulation approach that relies purely on enhancing the structural stability of proteins through addition of these cosolutes could result in problems associated with the chemical stability of these biomolecules.

Wimmer H., Olsson M., Petersen M. T., Hatti-Kaul R., Peterson S. B., and Muller N. (1997) Towards a molecular level understanding of protein stabilization: the interaction between lysozyme and sorbitol. *J Biotechnol* **55**, 85-100.

Abstract: The paper is investigating the mechanism of stabilization of proteins by polyols at the molecular level. It is addressing the interactions of sorbitol, a polyol commonly used as a protein stabilizing agent, with hen egg white lysozyme, a well studied protein. Differential scanning calorimetry shows an increase in denaturation temperature of lysozyme upon addition of sorbitol at a concentration of 250 mM and above. Increasing sorbitol concentration also caused an increase in signal intensity of the CD spectrum of lysozyme in the wavelength region of 280-300 nm. Two-dimensional nuclear magnetic resonance spectroscopy was used to examine interactions between lysozyme and sorbitol. Most significant changes are manifest in the anomalous relaxation properties of Ala and Thr methyl groups indicating modifications of local motions and possibly compression of the entire structure. This is further corroborated by new intra-protein nuclear Overhauser effects in the presence of sorbitol. There is also evidence that water is displaced

from the enzyme surface close to Ile-88 upon addition of sorbitol. In combination these results reveal a complex interplay of different interactions. Comparison to NMR-spectra of lysozyme with a bound inhibitor (tri-N-acetyl-glucosamine) shows that the interaction with sorbitol affects spatially disparate regions of the protein.