PROTEIN STABILITY STUDIED BY CHARACTERIZING PROTEIN
CONFORMATION, AGGREGATION AND PARTICLE FORMATION

by

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Protein Stability Studied by Characterizing Protein Conformation, Aggregation and Particle Formation

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ABSTRACT

Therapeutic proteins are emerging as one of the most important categories of treatments in modern medicine. However, the development of protein drugs presents inherent challenges. Proteins can easily aggregate due to marginal conformational and colloidal stability, even in optimal formulations, and are sensitive to various stresses such as temperature and pH excursions, mechanical shock and agitation, and photo-degradation etc. The aggregation of therapeutic proteins can also result in the loss of drug efficacy and an increase in immunogenicity, outcomes which affect the successful treatment of patients. Therefore, it is critical to understand protein stability and to minimize protein aggregation. To achieve this goal, characterization of protein conformation, aggregation and particle formation is indispensable during therapeutic protein drug development. In this work I evaluated a new approach, using concomitant Raman spectroscopy and dynamic light scattering (DLS) to characterize the conformational stability and aggregation propensity of therapeutic proteins at high concentration. The combined DLS-Raman technique was then employed to study the aggregation behavior of three insulin analogues at high concentration. Also, an emerging particle characterization technique, nanoparticle tracking analysis, was evaluated for the quantification and sizing of submicron particles in therapeutic protein samples. Finally, in an effort to improve protein stability by the addition of an excipient, I
found that polysorbate 20 could effectively reduce aggregation and sub-visible particle formation of intravenous immunoglobulin in freeze-dried formulations.

The form and content of this abstract are approved. I recommend its publication.

Approved: John F. Carpenter
I dedicate this work to my parents Wei Zhou and Huijie Zhang, and my sister Yan Zhou.
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CHAPTER I

INTRODUCTION

Therapeutic proteins are one of the most important categories of modern medicines. They are especially effective in treating diseases and conditions such as cancer, diabetes, hemophilia, multiple sclerosis, Crohn’s disease and rheumatoid arthritis. There have been more than 200 therapeutic protein drugs on the market up until 2015 and even more are under development or in clinical trials. However, it is still challenging to develop protein drugs due to a number of reasons, foremost the inherent instability of protein molecules. Proteins are only marginally stable even in formulation conditions where the protein native state is thermodynamically favored. As a macromolecule with complex 3-dimensional structure, in most cases a protein has to retain both native secondary and tertiary structures to be therapeutically effective. However, the free energy of unfolding is ~5-20 kcal/mol, which is only equal to the bond energy of a few hydrogen bonds. The protein’s native conformation can easily be perturbed under conditions such as high or low temperatures, extreme pH or exposure to hydrophobic surfaces, converting native protein molecules to unfolded or partially unfolded species, which have the potential to promote protein aggregation. Protein molecules are therefore extremely unstable and susceptible to aggregation.

In addition, therapeutic protein may be exposed to various stresses through their lifetime: manufacturing, packaging, shipping, storage and delivering to patients. For example, during manufacturing, freeze-thawing of the bulk drug substance is often a necessary step after final protein purification. However, freeze-thawing has been reported to be a stress condition that results into protein aggregation. Other stresses that therapeutic proteins are exposed to during their lifetime include but are not limited to: temperature
fluctuation, pH excursion, agitation, light exposure, cavitation, mechanical shock, contact with various surfaces such as glass, stainless steel, rubber, silicone oil, plastic, air-water interfaces\textsuperscript{9-12}. These stresses may function synergistically to promote protein aggregation.

The levels and characteristics of protein aggregates are important product quality attributes. Because therapeutic proteins are commonly administrated intravenously or subcutaneously\textsuperscript{13}, aggregates with the size equal or larger than 10 µm might cause occlusion of small capillaries of the circulatory system\textsuperscript{14}. Therefore therapeutic protein drug products are required to follow the specific guidelines for the level of particles in the size range of ≥ 10 and ≥25 µm in final drug product (United States Pharmacopeia <787> and <788>). More importantly, during the past several years there has been increasing concern that aggregated protein species including subvisible protein particles are potentially immunogenic in patients\textsuperscript{15-18}. An immunogenic reaction after drug administration might cause resistance to drug and could further result in life-threatening complications such as anaphylaxis, and neutralization of life-saving therapies or even endogenous proteins with important functions\textsuperscript{17}. Many studies have been conducted to understand the immunogenicity of therapeutic protein aggregates\textsuperscript{19-25}. For example, aggregate-dependent immunogenicity has been described in several mouse models for recombinant growth hormone and human interferon alpha 2b \textsuperscript{26,27}. Moreover, a link between aggregates and subvisible particles in marketed interferon products to the clinical immunogenicity rates in patients has also been reported\textsuperscript{28}. Despite these efforts, gaps remain in understanding the impact of protein aggregates on immunogenicity in patients, including both the analytical difficulties in characterizing the subvisible particles and insufficient understanding of the mechanism and the factors causing immunogenicity in patients. Therefore it is critical to understand protein
stability and aim to minimize protein aggregation at all stages of the protein therapeutics product. To achieve this, characterization of protein conformation, stability and aggregation profiles are indispensable during therapeutic protein drug development.

During the development history of a therapeutic protein product, it is necessary to rapidly assess protein structure, aggregation and thermal stability. Among the biophysical characterization techniques, far-UV circular dichroism (CD) and Fourier transformation infrared (FTIR) spectroscopies are conventionally used to measure the secondary structure of protein; whereas near-UV CD and intrinsic/extrinsic fluorescence spectroscopy are used for tertiary structure measurements.\(^\text{29}\)

CD spectroscopy can measure the unequal absorption of right-handed and left-handed circularly polarized light due to protein chirality.\(^\text{30}\) Far-UV spectroscopy in the range of 170-250 nm usually provides secondary structure information (especially sensitive for alpha helix); and near-UV CD spectroscopy in the range of 250-350 nm can provide tertiary structure information (signature bands for aromatic amino acids). The application of CD spectroscopy is limited by the existence of large protein aggregates due to light scattering and some buffer components.

FTIR spectroscopy measures the vibrational absorption of energy by protein molecules in the middle infrared region (400-4000 cm\(^{-1}\)). In FTIR spectra of proteins, the amide I band of protein in the 1600-1700 cm\(^{-1}\) range is mainly due to the C=O stretching of the peptide bonds, and indicates different vibrational signals for different secondary structural components.\(^\text{31}\) The interference of water absorbance in the amide I region, and the requirements for a low moisture environment have limited FTIR spectroscopy applications. And it is a generally low through-put technique in practice.\(^\text{32}\)
Fluorescence spectroscopy measures the fluorescence emission by either intrinsic aromatic residues (e.g. tryptophan) or extrinsic dyes (e.g. 8-anilino-1-naphthalenesulfonate) interacting with protein molecules\textsuperscript{33}. The fluorescence spectrum can provide information about the environment of the fluorophore and thus reflect the alteration of tertiary structure.

To evaluate protein thermal stability, separate analysis needs to be done using far and near-UV CD spectroscopy, fluorescence spectroscopy and differential scanning calorimetry (DSC) to analyze the structural or thermal attributes during thermal ramping. Furthermore, to characterize protein aggregation, there are multiple techniques available such as size exclusion chromatography (SEC) and analytical ultracentrifugation (AUC), UV-VIS spectroscopy, static or dynamic light scattering (SLS or DLS) for estimation of soluble and insoluble aggregates. In addition, particle analysis has gained popularity in characterization of protein aggregation during the last decade\textsuperscript{28,34}.

One common drawback of most of analytical techniques mentioned above (except FTIR spectroscopy) is that they may not be viable for analysis of high concentration samples with sample dilution, which should be avoided in studying protein structure, stability and aggregation because protein’s physical properties and behavior are often concentration dependent. However, high protein concentration (>100 mg/mL) at low volumes (<1.5 mL) has been prevalent for therapeutic protein drug formulations, especially for monoclonal antibody products packaged in prefilled syringes.

In a competitive market where the timelines to develop a drug product are short, it would be helpful to have analytical techniques that can give information about a variety of biophysical attributes in a single measurement. The short analysis time would allow not only for rapidly screening protein drug candidates and perform pre-formulation studies, but also
for late stage studies such as comparability studies after manufacturing changes\textsuperscript{35-39}.
Moreover, in many cases due to the limited quantity of protein available for analysis it is particularly valuable to obtain as much information as possible from a given sample by simultaneous measurements on the same sample. Simultaneous measurements also assure that critical solution and processing conditions are identical and that variations in sample handling and instrument operations are minimized. However for these studies, multiple techniques are traditionally used to characterize protein conformation, aggregation and thermal stability, and the results are then combined to evaluate the overall physicochemical properties of the protein.

In chapter II, I addressed these different analytical challenges by using combined, parallel measurements of the same sample with Raman spectroscopy and DLS to study the structure, thermal stability and aggregation of model therapeutic proteins, human serum albumin (HSA) and intravenous immunoglobulin (IVIG), at high concentrations. DLS is robust for qualitatively analyzing particle size and sample polydispersity, and Raman spectra provides secondary and tertiary structural information\textsuperscript{40-44}. Raman spectroscopy is advantageous not only because it is ideally suited to studying protein at high concentrations but also due to the minimal sample preparation and less sensitivity to water interference compared with IR spectroscopy\textsuperscript{45,46}. By combining the DLS and Raman spectroscopy systems, I was able to characterize the size distribution and protein conformation at the same time for the same sample. Also, by acquiring Raman spectra and light scattering data during real-time heating studies and during isothermal incubations at various temperatures, I was able to compare directly, the effects of temperature or time of incubation on protein secondary and tertiary structure, as well as on protein aggregation. In my experiments,
thermal stability of HSA at pH 7 was first studied to evaluate instrument capability and performance, at various protein concentrations and heating intervals. The effects of pH (pH 3, 5, and 8) on HSA conformation, thermal stability and aggregation were characterized, where I found that the thermal stability followed the trend of pH 3 < 5 < 8 while aggregation propensity had an opposite trend. As an example of the effects of chemical degradation on protein structure and thermal stability, HSA was oxidized by exposure to H$_2$O$_2$, which induced significant protein aggregation but no structural perturbation except that the methionine was oxidized into sulfoxide. Further, a thermal ramping study showed that the thermal stability of HSA was severely reduced and aggregation propensity was promoted by H$_2$O$_2$ treatment. Finally, I studied high concentration formulations of IVIG during real-time heating experiments, and a comprehensive characterization of heating-induced structural perturbations and aggregation was obtained. In conclusion, the Raman-DLS system offers unique physical insights into the properties of high concentration protein samples by providing comprehensive data on protein tertiary and secondary structures and hydrodynamic size during real-time heating or isothermal incubation experiments.

To further take advantage of the Raman-DLS system, in chapter III I characterized the sizes of aggregates of insulin analogs, lispro, aspart and glulisine, and the conformations of the constituent protein molecules. In commercial formulations of insulin analogues, meta-cresol/phenol are included to promote native state assembly of the insulin molecules and suppress microbial contamination$^{47-49}$. However, when an insulin analog is delivered to patients with a pump and catheter system, there is a loss of meta-cresol/ phenol from the formulation$^{50-56}$, which can lead to aggregation and even precipitation of insulin molecules in the pump reservoir and tubing$^{54,56,57}$. In a recent study, it was observed that quiescent
incubation of lispro or aspart samples at 37°C - after depletion of meta-cresol and phenol - resulted into the formation of soluble protein aggregates rather than fibrils\textsuperscript{58}. In the case of insulin fibrils formation, native alpha helix structure is commonly found to be converted to non-native beta sheet structure\textsuperscript{59-64}. However, the conformational transition of insulin molecules upon the conversion to soluble aggregates has not been investigated. In this chapter, I addressed this issue by analyzing samples of the lispro, aspart and glulisine with concomitant Raman spectroscopy and DLS. To generate aggregates, samples of three insulin analogs, lispro, aspart and glulisine, were incubated without phenolic preservatives for 30 days at 37°C. As a function of incubation time, aggregate levels were quantified with size exclusion chromatography, and the sizes of aggregates and the conformations of the constituent molecules were characterized with concomitant dynamic light scattering and Raman spectroscopy. During incubation samples of lispro were progressively converted to soluble aggregates with hydrodynamic diameters of ca. 15 nm, and at the end of the 30-day incubation 95% of the native protein had aggregated. Raman spectroscopy documented that formation of the aggregates resulted in conversion of a large fraction of native alpha helix secondary structure into non-native beta sheet structure and a distortion of disulfide bonds. In contrast, in samples of aspart and glulisine only about 20% of the native proteins was converted into aggregates after 30 days, and minimal structural perturbations were detected. Also, consistent with the relative rates of aggregation during isothermal incubation, with Raman spectroscopy it was observed that during heating the onset temperature for secondary structural perturbations of lispro occurred 7-10 °C lower in temperature than those for aspart or glulisine. Overall this study demonstrates that as in the case with formation of amyloid
fibrils from insulin, formation of soluble aggregates of lispro results in a high level of conversion of alpha helix into beta sheet.

Not only soluble aggregates but also subvisible particles, including both micron particles with size ≤ 100 µm and submicron particles, may play important roles in therapeutic protein product quality, stability and adverse effects in patients\textsuperscript{14,16,28,65}. There are a variety of techniques available for subvisible particle characterization. One difficulty is that no single analytical method or instrument can cover the entire size range of particles from submicron to micron-sized to visible\textsuperscript{5,66,67} (Figure 1), and the measured results are not comparable in between different techniques which are based on different principles. In the submicron size range, DLS and emerging techniques such as nanoparticle tracking analysis (NTA) are commonly used. Resonant mass measurement can count and size particles in the size range of ~200 nm to ~5 µm (with the detention limit dependent on the specific sample), and is uniquely capable of differentiating dense particles from lighter ones, such as protein particles versus silicone oil droplets/air bubbles. Electrical sensing zone covers the size range from ~500 nm to 1000 µm. In the micron size range, both light obscuration method and flow imaging microscopy are capable of counting and sizing particles from 1 to 100 µm.

Also, each approach has key limitations and is critically affected by instrument parameters and sample characteristics. These factors must be evaluated rigorously in order to maximize utility of the method for analysis of therapeutic proteins samples. For example, the particle counts of samples with high protein concentrations can be significantly reduced in light-dependent techniques such as light obscuration and flow imaging microscopy due to the decreased contrast between particles and solutions\textsuperscript{67}. 
Figure 1. Working size range of analytical techniques for protein particles (adapted from Sarah Zolls et al.).

For the submicron particle characterization (30-1000 nm), NTA provides both particle counts and size distributions. Motions of individual nanoparticles in solution are tracked by a microscope when they are illuminated by the integrated laser (Figure 2). The size of each nanoparticle can be calculated from its recorded trajectory based on Stokes-Einstein equation and the concentration of nanoparticles can be obtained because the particles are individually
tracked. There has been limited published research on the impact of operational and sample parameters on data acquisition, analysis and robustness. In chapter IV, I used a model

![Diagram of NTA principle](image1)

![Example of tracked nanoparticles](image2)

Figure 2. A. Illustration of the principle of NTA (adapted from nanosight.com); B. Example of tracked nanoparticles during data analysis by NTA, the white dots are nanoparticles, the red traces are the trajectories of the particles made during data collection, and the white curve represent the real time size distribution of nanoparticle during analysis.
therapeutic protein and synthetic nanoparticles to critically evaluate such factors with the NanoSight LM20 NTA instrument. I found that to obtain proper size distributions complete tracking numbers of at least 200 and 400 were required for latex nanobeads and protein nanoparticles respectively. In addition, when set at suboptimal values the minimum expected particle size parameter led to inaccurate sizing and counting for all particles types investigated. A syringe pump allowed for higher sampling volumes, and results were reproducible for nanoparticle sizing and counts at flow rates $\leq 7 \mu$L/min. Finally, because therapeutic protein products are often formulated at relatively high protein concentrations, I investigated the effects of protein concentration on nanoparticle characterization. With high protein concentrations, nanoparticle sizing was not affected whereas particle concentrations were significantly reduced. Linear relationships between particle count and dilution factor were obtained when a high protein concentration formulation was diluted into particle-free solutions at the same protein concentrations, but not when dilutions were made into buffer.

In addition to monitoring drug quality to meet regulation expectations, subvisible particle characterization is also valuable in detecting trace amount of protein aggregates during formulation and process developments. Particle counting methods provide a much more sensitive means by which to detect and quantify protein aggregation than traditional methods such as SEC. In two recent examples, particle formation was observed after mAbs were subjected to freeze-thawing or pumping, but no loss of monomer or formation of soluble aggregates could be detected with SEC. Although much work on subvisible particle formation has been done with aqueous solution formulations, limited studies have been performed on particle formation of reconstituted lyophilized formulations. A single published
study found that addition of sucrose and sorbitol significantly reduced the amount of subvisible particles observed in reconstituted lyophilized IgG1 formulations.\(^73\)

Lyophilization is an important formulation strategy for developing stable therapeutic protein dosage forms. Up to 2012, nearly half of the therapeutic protein drugs on the market were in the lyophilized dosage form. During lyophilization, however, proteins are subjected to stress conditions that reduce protein stability and promote aggregation. For example, during freezing due to the low temperature and formation of ice crystals, proteins are subject to cold denaturation, ice-water interface, increased salt concentration and potential pH shifts.\(^75\) Because of the amphiphilic nature, protein molecules could adsorb to the ice-water interface during freezing and later on be exposed to the solid-air interface that is formed after the removal of the ice crystals during drying. There have been studies shown that the amount of protein exposed on the glass solid-air interface determined protein stability during long-term storage for both keratinocyte growth factor (KGF) and recombinant human growth hormone (rhGH).\(^76,77\) Furthermore, an annealing step after freezing which reduced the fraction of protein exposed at the solid-air interface was demonstrated to reduce protein aggregation significantly, which further confirms the unstable nature of protein molecules at the solid-air interface.

The amount of protein at the solid-air interface can not only be affected by the freeze-drying procedures such as pre-drying annealing but also be potentially impacted by formulation compositions. Non-ionic surfactants such as polysorbate 20 (ps20) have been proved to effectively compete with protein for adsorption onto hydrophobic surfaces.\(^78,80\) In chapter V, as an effort to improve protein stability in lyophilized formulation, both annealing and addition of ps20 were investigated for their role in protecting IVIG as a model protein in
lyophilization. I also took advantage of the sensitivity of subvisible particle characterization in detecting protein aggregation for the lyophilized formulations in this study. Immediately after lyophilization, the specific surface area, surface fraction of protein or protein secondary structure in the lyophilized cake did not change significantly in the presence of ps20. After reconstitution, no significant amount of aggregation were found for each formulation based on SEC characterization. However, particle analysis by both flow imaging microscopy and resonant mass measurement showed that ps20 reduced the amount of protein aggregates to a large extent. During the 16-week long storage study at 50°C, formulations without trehalose showed gradual loss of monomer and increase in aggregated protein species based on SEC analysis, whereas formulations with trehalose had no significant aggregation. Particle analysis showed that without trehalose protein particles were generated quickly during storage, but with trehalose IVIG was stable up to 8 weeks and only slight increase of particle levels was found in week 16. The formulation with trehalose plus ps20 formulation had the lowest level of protein particles among all the formulations at any given time point. Finally, it was found that during reconstitution, the inclusion of ps20 in the rehydration medium could significantly reduce the formation of protein particles.
CHAPTER II

CONCOMITANT RAMAN SPECTROSCOPY AND DYNAMIC LIGHT SCATTERING FOR CHARACTERIZATION OF THERAPEUTIC PROTEINS AT HIGH CONCENTRATIONS

Introduction

There are several points during the development history of a therapeutic protein product where there is a need to assess rapidly protein structure, aggregation and thermal stability. For example, during early development of a therapeutic monoclonal antibody, relative physical stability (e.g., during heating) and aggregation propensity are often compared between several candidate variants of the antibody. This early testing allows for choice of the variant with the most favorable physical pharmaceutical properties. Similar testing can be performed with the chosen product candidate during pre-formulation studies and formulation development, in which the effects of solution conditions such as pH and different excipients on protein unfolding/aggregation are determined. These studies are also often conducted under so-called “accelerated degradation” conditions of heating or exposure to other stresses such as forced oxidation. And even after commercial launch of a therapeutic protein, there may be a need to compare its thermal stability and aggregation before and after a manufacturing change. Such studies are a vital part of the characterization that is required to provide assurance that the product made by the new manufacturing approach is comparable to the protein made by the original process.

1 Portions of this chapter are published with permission from Analytical Biochemistry volume 472, March 2015, pages: 7-20.
Traditionally for such studies multiple techniques have been utilized separately to characterize protein conformation, aggregation and thermal stability, with the results from different instruments combined to provide an overall assessment of the protein’s physicochemical properties. To characterize size and aggregation, size-exclusion chromatography (SEC) is widely and routinely used to quantify the amount of monomer, dimer, trimer and higher-order oligomers. Other techniques such as field flow fraction (FFF) and analytical ultracentrifugation (AUC) are used for orthogonal confirmation of SEC results. Dynamic light scattering (DLS), static light scattering (SLS) and turbidity measurements are also used to monitor protein aggregation. For studies of protein structure combined with determination of relative stability (e.g., during heating), optical spectroscopic techniques with varied structural resolution and sensitivity are commonly used: intrinsic/extrinsic fluorescence, UV absorbance, far- and near-UV circular dichroism (CD) and infrared (IR).

The capacity to perform different measurements in parallel on the same sample is particularly valuable. Often material for studies is limited, especially during the early comparisons between variants of a given monoclonal antibody product. Obtaining more than one data type from a given sample (e.g., UV absorbance spectra and turbidity measurements or fluorescent spectra and SLS measurements) helps conserve precious protein. Also, making simultaneous measurements on a sample assures that critical solution and processing conditions are identical and that variations in sample handling and instrument operations are minimized.

This consistency is particularly important for real-time studies of protein structure and/or aggregation during heating. Real-time heating studies are useful for relatively rapid
characterization of protein stability, but in most cases the protein will aggregate during heating. Because this is an irreversible process, many different factors -- such as protein concentration, heating rate and dwell time at a given temperature for data acquisition -- can affect the thermal transition temperatures; and, hence, the parameters used to assess relative protein stability. Therefore, often thermal transition temperatures for the same protein obtained on two different instruments will not agree, unless extreme care is taken to match all relevant conditions. Sometimes it is not physically possible to obtain the requisite matches between instruments, and then the thermal transition temperatures for different processes such as protein secondary structural change due to unfolding and protein assembly due to aggregation cannot be compared rigorously.

The high concentration formulations used for many modern monoclonal antibody therapeutics and some older products (e.g., HSA and IVIG) make analytical assessment more complicated, even when a single method is used. At the tens of milligrams concentrations found in many of these formulations, certain methods such as fluorescence and far-UV CD spectroscopies may not be viable without sample dilution. Dilution should be avoided in studying protein structure and aggregation because a protein’s physical properties and behavior in a dilute solution often do not match those occurring at the actual protein concentration in the product.

In the current study, we addressed these different analytical challenges by using combined, parallel measurements of the same sample with Raman spectroscopy and DLS to study the structure, thermal stability and aggregation of model therapeutic proteins (HSA and IVIG) at high concentrations. DLS, based on the time-dependent correlation of light intensity fluctuation due to Brownian motion of particles, is robust for qualitatively analyzing particle
size and sample polydispersity for particle diameters from several nm to a few μm. The 173° backscattering detector minimizes interference from multiple scatterings and enables the collection of size distribution data of high concentration protein samples. Raman spectra provide secondary and tertiary structural information through analysis of peak positions and ratios of spectral features that characterize Amide I, Amide III and other backbone vibrations (used to characterize the secondary structure both qualitatively and quantitatively); as well as peaks for vibrations of aromatic side chains such as tyrosine (Tyr) and tryptophan (Trp) (used to monitor protein tertiary structure). Compared to IR spectroscopy, Raman spectroscopy requires minimal sample preparation, and is less sensitive to water vibrations that interfere with the Amide I band, making it less difficult to subtract this water contribution. Raman spectroscopy is also ideally suited to studying proteins at high concentration.

By combining the DLS and Raman spectroscopy systems, we were able to characterize the size distribution and conformation at the same time for the same protein sample. This approach avoids variation issues arising from sample to sample, as well as instrumental and experimental conditions. Also, by acquiring Raman spectra and light scattering data during real-time heating studies and during isothermal incubations at various temperatures, we were able to compare directly, the effects of temperature or time of incubation on protein secondary and tertiary structure, as well as aggregation.

In our experiments, thermal stability of human serum albumin (HSA) at pH 7 was first studied to evaluate instrument capability and performance, with varied protein concentrations and heating intervals. Then, the effects of pH (pH 3, 5, and 8) on HSA conformation, thermal stability and aggregation were characterized. In addition, during
isothermal incubation experiments, HSA aggregation and structural perturbation kinetics were studied at pH 7. As an example of the effects of chemical degradation on protein structure and thermal stability, HSA was oxidized by exposure to H$_2$O$_2$, and the effects on structure, aggregation and thermal stability were compared to undamaged HSA during real-time heating experiments. Finally, we studied high concentration formulations of IVIG during real-time heating experiments. Together these experiments provide insights into the uniquely valuable data that can be obtained with combined study of protein structure, aggregation and thermal stability with concomitant Raman spectroscopy and DLS.

Materials and Methods

Materials

HSA (Albuminar®-5, CSL Behring LLC) and IVIG (Gammagard® Liquid, Baxter HealthCare Corp) were purchased from the University of Colorado at Boulder’s Wardenburg Pharmacy. All other chemicals were purchased from Fisher Scientific (Hampton, NH) and were of reagent grade or higher quality.

Instrument configuration, experimental methods and data analysis

For the Raman-DLS studies, a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, UK) was combined with a Kaiser Raman RxN1 spectrometer (RxN-1 Kaiser Optical Systems, Inc., Ann Arbor, MI). The instrument is a prototype system developed by the Malvern Bioscience Development Initiative (Columbia, MD). A 785 nm laser with a ~280 mW laser power source was used for Raman spectroscopy. DLS data were collected at 632 nm with a 173° backscattering detector which minimizes interference from multiple
scatterings and enables the collection of size distribution data of high concentration protein samples \(^{67}\). As shown in Figure 3, a computer controlled moveable mirror enables data collection to be switched between Raman spectroscopy and DLS. In a typical experiment, a \(~110 \mu L\) sample was placed into a 3×3 mm quartz cuvette, which was then loaded into a Peltier temperature-controlled sample compartment. To avoid potential problems due to sample evaporation, the sample cuvette was tightly sealed with a Teflon stopper. Unless otherwise noted, Raman spectra were collected with 12 co-additions of a 10 s exposure (See reference \(^{85}\)).

For experiments studying the effects of heating, data were acquired from 20°C to 90°C at 1°C intervals, unless otherwise noted. At each data acquisition temperature, Raman and DLS data were acquired sequentially. Then the temperature was increased at about 6°C/minute to the next data acquisition temperature. The sample was allowed to equilibrate briefly before the data set was acquired and saved, a process that required about 4 minutes. This process was repeated until the experimental temperature range was covered. An automated routine in the software from Malvern was used for the set up and running of the heating experiments; thus, during these runs operator input and monitoring were not required.
To obtain background Raman spectra for the corresponding buffer without protein, Raman spectra only were acquired during the same temperature ramping and data acquisition protocol. The buffer spectrum at each temperature was subtracted from the spectrum for the protein sample at the same temperature.

Raman spectral data analysis and determination of thermal transition temperatures were performed with a prototype software package provided by the Malvern Biosciences Development Initiative (Columbia, MD). The Raman spectra midpoint temperature (T_m) and onset temperature (T_onset) were calculated by fitting the data with the sigmoidal function:

$$y = \frac{A}{1 + \exp \left( \frac{T_m - x}{B} \right)} + C,$$

Equation 1

Where y is the fitted parameter such as peak center of Amide I or contents of alpha helix/beta sheet, and x is the temperature, and A, B, C are constants. To obtain the T_onset
values, the second derivative of the fit was used to find the point of maximum curvature on
the rising edge of the transition.

For DLS results obtained during heating experiments, the correlation function data
acquired at each temperature were examined and the z-averaged size was obtained from the
zetasizer analysis software. Z-averaged size was calculated based on the cumulants analysis
of correlation function \( G(\tau) \), where \( \tau \) is the decay time\(^{86} \). The log of \( G(\tau) \) is fitted as
polynomial function of \( \tau \) as follows:

\[
\ln [G(\tau)] = a + b\tau + c\tau^2 + \ldots \quad \text{Equation 2}
\]

where the value of \( b \) is the z-averaged diffusion coefficient. The z-averaged size can then be
calculated based on Stokes-Einstein equation.

It was observed that there were high quality fits with relatively low error until sample
temperatures well exceeded the onset temperature for an increase in the z-averaged size. For
some of these data sets, size distributions were determined and are presented in the Results
section. At higher temperatures, the fits were poor because the protein solutions had become
gelled and/or grossly precipitated, as assessed by visual observation.

The aggregation onset temperature \( (T_{\text{agg}}) \) during each heating experiment was
determined as the temperature where the z-averaged size exceeded the base value by more
than 25%. The base value was calculated as the mean value of the hydrodynamic size at the
five initial data points.

In the initial thermal stability study of HSA, the errors for thermal transition values
were the results from fitting errors to the data sets for a single sample. For all of the other
experiments, $T_m$, $T_{onset}$ and $T_{agg}$ errors were for the standard deviations from the means of values obtained for three or more independent samples ($n \geq 3$).

**Preparation of therapeutic protein samples**

HSA was dialyzed in Slide-A-Lyzer® Dialysis Cassettes with a 10 kDa molecular weight (MW) cutoff (Pierce, Rockford, Illinois) against 20 mM sodium phosphate-citrate buffer at 4°C; with three changes of the external solution, the volume of which exceeded sample volume by 1000-fold. Unless otherwise noted, pH 7 buffer (20 mM sodium phosphate-citrate) was used with 150 mM NaCl. Protein concentration was determined by UV absorbance at 280 nm, using an extinction coefficient of 0.53 cm$^{-1}$•mL•mg$^{-1}$. IVIG was used without dialysis, in the commercial formulation (250 mM glycine buffer, pH 5). The protein concentration was determined by UV absorbance 280 nm, using an extinction coefficient of 1.357 cm$^{-1}$•mL•mg$^{-1}$.

**Initial thermal stability study of HSA**

A dialyzed HSA sample at pH 7 was diluted to a protein concentration 39 mg/mL. Then, Raman spectra and DLS data were collected during heating from 20 to 90°C, with a data acquisition interval of 1°C.

**Effects of protein concentration and data acquisition interval on HSA thermal stability**

To study effects of protein concentration, a dialyzed HSA sample at pH 7 was diluted to 10, 15, 20 and 39 mg/mL. Then, Raman spectra and DLS data were collected during heating of samples at each protein concentration. A data acquisition interval of 1°C was used.
In a separate study, the effects of data acquisition interval were investigated. HSA (41 mg/mL) was heated from 20 to 90°C with data acquisition intervals of 0.5, 1, 2 and 5°C.

**Isothermal incubation of HSA**

HSA (31 mg/mL) at pH 7 was incubated at 60, 63 and 65°C. Data were collected from time 0 to ~200 minutes, at time intervals of about 4 minutes.

**Effects of pH on HSA structure and thermal stability**

HSA was dialyzed at 4°C against 20 mM sodium phosphate-citrate buffer with 150 mM NaCl at three pH values: 3, 5, and 8. After dialysis, it was found that there was a significant amount of insoluble protein aggregates in the pH 3 sample, as assessed visually and by size exclusion chromatography (data not shown). Therefore, the sample was centrifuged at 194,000 × g for 1 hour, using a sw55Ti rotor in a Beckman Optima LE-80k ultracentrifuge. The resulting supernatant was used for the experiments. Protein concentration of samples at all three pH’s was adjusted to 37 mg/mL. For comparison of effects of pH on structure and HSA size, DLS data and Raman spectra were first obtained at 20°C. Then, data were acquired at an interval of 1°C during heating from 20 to 90°C.

**Effects of oxidation with H₂O₂ on HSA structure, aggregation and thermal stability**

HSA (in 20 mM sodium phosphate-citrate buffer at pH 7 with 150 mM NaCl) at protein concentration ~46 mg/mL was incubated at 37°C with and without 3% H₂O₂ (v/v) for five days. After incubation, the HSA samples that had been incubated -- with or without H₂O₂ -- were dialyzed at 4°C against 20 mM sodium phosphate-citrate buffer (pH 7 with 150
mM NaCl); with three changes of the external solution, the volume of which exceeded sample volume by 1000-fold. Protein concentration was adjusted to 18 mg/mL. For comparison of effects of oxidation on HSA structure and size, DLS data and Raman spectra for oxidized and control samples were first obtained at 20°C.

The oxidized and control HSA samples were also analyzed by size exclusion chromatography (SEC) to quantify levels of soluble aggregates. An Agilent 1100 (Santa Clara, CA) and a Tosoh (San Francisco, CA) TSK G3000SWXL column (7.8 × 300 mm²) were used. The mobile phase consisted of 100 mM sodium phosphate (pH 7), 100 mM sodium sulfate and 0.05% w/v NaN₃, and the flow rate was 1 mL/min.

Prior to evaluation of the thermal stability of H₂O₂-treated HSA, protein aggregates were removed from the samples by centrifugation at 194,000 ×g for 3 hours with a sw55Ti rotor in a Beckman Optima LE-80k ultracentrifuge. The control sample, which had not been treated with H₂O₂, was also processed by ultracentrifugation. The protein concentrations in the supernatants of both samples were adjusted to 18 mg/mL. Thermal stability was studied with Raman spectroscopy and DLS during heating from 20°C to 90°C with a heating interval of 1°C.

**Thermal stability of IVIG**

To remove any aggregates and particles, IVIG from the product vial was centrifuged at 194,000 ×g for 3 hours with a sw55Ti rotor in a Beckman Optima LE-80k ultracentrifuge. The resulting supernatant was removed, and the protein was diluted to 51 mg/mL with the IVIG product formulation buffer (0.25 M Glycine, pH 5). DLS data and Raman spectra were acquired at an interval of 1°C during heating from 20 to 90°C.
Results and Discussion

Initial thermal stability study of HSA

The initial study investigated structural transitions and aggregation during heating of a high concentration (39 mg/mL) solution of HSA. Aggregation was monitored with DLS, and structural transitions were assessed based on temperature-dependent changes in the Raman spectra for the protein. Furthermore, as a relative measure of protein aggregation propensity and thermal stability, temperatures at which there were transitions in measured parameters by either method were calculated and compared.

There are several structural elements in proteins for which bands in the Raman spectrum can be assigned, and such assignments have been made in the literature for HSA. For the HSA spectrum shown in Figure 4, the peak from around 1650 to 1680 cm\(^{-1}\) was assigned to Amide I, which results from the protein backbone’s C=O stretching coupled with minor contributions from C-N stretching and N-H in-plane bending [18, 26]. The Amide I band is sensitive to alterations in a protein’s secondary structure [40, 93]. At 20°C, the peak position of the Amide I band was located at 1653 cm\(^{-1}\), and this band was assigned to alpha helix [92, 94]. As temperature increased to 65°C, no significant peak shifting was observed for the Amide I band. However, by the time that the temperature reached 70°C there was peak broadening, and a shoulder appeared at 1670 cm\(^{-1}\) (Figure 4). At 80°C, the peak shifted more toward 1670 cm\(^{-1}\). The Amide I peak shifting and broadening during heating were consistent with the gradual loss of alpha helix structure and concomitant formation of beta sheet and random coil [90, 91, 95].
To resolve more clearly the component Raman spectral bands within the Amide I region and temperature-dependent changes, the second derivative for this region was calculated (Figure 4 upper-right inset). As temperature increased, there was a clear loss of peak intensity at 1653 cm\(^{-1}\) that was accompanied by an increase of peak intensity at 1670 cm\(^{-1}\). Thus, as noted above, during heating of HSA there is a loss of native alpha helix and formation of non-native beta sheet and random coil \(^{90,91,95}\). An isosbestic point between the two peaks further confirmed the direct transition of native to non-native secondary structural elements in HSA during heating (Figure 4 upper-right inset).

Figure 4. Effects of heating on Raman spectra of HSA and selected spectral elements. HSA was studied at pH 7 and a concentration of 39 mg/mL. The main figure shows Raman spectra with normalized intensities acquired at 20, 65, 70 and 80°C. The vertical dash lines were drawn to highlight the change of characteristic Raman bands along with the change of temperature. The left-upper inset shows the intensity of the skeleton vibration at \(\sim941\) cm\(^{-1}\) (red rectangle), the Amide III band at 1245 cm\(^{-1}\) (green triangle) and the intensity ratio of Tyr at 850 and 830 cm\(^{-1}\) (blue circle) as a function of temperature. The upper-right inset has second derivative of the Amide I region acquired at various temperatures during heating.
The Amide III band at ~1245 cm\(^{-1}\), another indicator for secondary structure, is attributed to the in-plane bending of N-H and stretching of C-N\(^{40,91}\). The increased intensity of the 1245 cm\(^{-1}\) peak with temperature also can be assigned to the formation of beta sheet structure during heating (Figure 4 upper-left inset).

The Raman band at 941 cm\(^{-1}\) (Figure 4) is usually referred to as the Amide skeleton band and results from the N-C\(\alpha\)-C stretching of the peptide backbone\(^{40,93}\). For the Raman spectrum of HSA, this band is a marker of alpha helix structure\(^93\). The reduction in intensity at 941 cm\(^{-1}\) during heating further confirms the loss of native alpha helix structure of HSA.

By monitoring the Fermi doublets at 850 and 830 cm\(^{-1}\) information was obtained about heating-induced changes in HSA’s tertiary structure. These spectral features are assigned to the vibrational frequencies of tyrosine, and a change in the intensity ratio of the two peaks (I\(_{850}/I_{830}\)) reflects a change in the microenvironment around tyrosine \(^{40,44}\). A higher value indicates that the residue is either in a hydrophobic environment or in a polar environment but only moderately hydrogen bonded. In contrast, the value becomes lower if the phenolic hydroxyl oxygen is ionized or becomes a strong hydrogen bond donor \(^{42,96}\). Therefore, the decrease of I\(_{850}/I_{830}\) for HSA from 1.4 to 0.8 during heating (Figure 4) suggests there was a tertiary structural change in which Tyr residues as donors were more strongly hydrogen bonded to a negative-charge receptor such as a carboxylate ion \(^{42,96}\).

As an example of comparisons that can be made between the heating-induced changes in markers of secondary and tertiary structure, the intensity of the Amide skeleton band 941 cm\(^{-1}\) and the values for I\(_{850}/I_{830}\) were plotted versus temperature (Figure 4, left inset). For secondary structure, there was not a substantial change until the temperature reached about 67\(^{\circ}\)C, above which there was a relative steep drop in band intensity.
Conversely, the tertiary structure as reflected in the values for I_{850}/I_{830} began to be altered gradually as temperature increased above about 35°C and then underwent a more dramatic change starting at about 67°C. Thus, prior to secondary structural transitions, heating leads to perturbation of tertiary structure, and then at higher temperatures it appears that secondary structural changes result in further tertiary structural alterations.

To assess quantitatively heating-induced changes in HSA secondary structure, the Raman spectra were analyzed with two different approaches. In the first, from the second derivative spectra in the Amide I region the intensities were determined for the alpha helix band at 1653 cm^{-1} and the beta sheet band at 1670 cm^{-1}. The values were plotted versus temperature, and curves were fit to the resulting plots (see Materials and Methods for details). Based on the fit curves, T_{onset} and T_m values were determined (Figure 5A). For the alpha helix band, these were 67.0±0.2°C and 68.1±0.4°C, respectively. For the beta sheet band the values were 66.0±0.2°C and 69.5±0.2°C. Therefore, the thermal transitions for loss of alpha helix band intensity and the gain in beta sheet occurred at essentially the same temperatures. This observation was consistent with the conclusion that during heating of HSA there was a direct conversion of alpha helix to beta sheet.

In the second approach, a database was provided by Malvern that contained Raman spectra of 18 proteins with known secondary structure contents. Also, a partial least squares (PLS) analysis routine for this spectral database was developed by Malvern. Based on PLS analysis of the HSA spectrum, at 20°C, the protein contained 68% alpha helix and 0% beta sheet, which matches well with values obtained by x-ray crystallography. The percentages of alpha helix and beta sheet were then plotted versus temperature, and curves were fit to the plots using a sigmoidal model as described in the Materials and Methods (Figure 5A). Based
on the fit curves, $T_{\text{onset}}$ and $T_{\text{m}}$ values were determined. For the alpha helix content, these were $67.0 \pm 0.1^\circ\text{C}$ and $70.2 \pm 0.2^\circ\text{C}$, respectively. For the beta sheet content the values were $67.0 \pm 0.2^\circ\text{C}$ and $70.3 \pm 0.2^\circ\text{C}$. Therefore, with both methods of determining thermal transition temperatures consistent values were obtained, and the results documented that loss of alpha helix in HSA during heating was concomitant with an increase in beta sheet content.

The underlying cause of this structural conversion was revealed by examining the DLS data (Figure 5B) for HSA that were obtained during the same heating experiments as were the Raman spectra. At $20^\circ\text{C}$, the z-averaged hydrodynamic diameter was 7.3 nm which is consistent with values for HSA in the literature. At $20^\circ\text{C}$ the intensity distribution was monodispersed and it remained this way during heating until $63^\circ\text{C}$, at which point a peak with ~200 nm size was observed, indicating the onset of heating-induced aggregation (Figure 5B). At $65$ and $70^\circ\text{C}$, a peak for intermediate-sized aggregates was observed, and at $70^\circ\text{C}$ there was an additional peak centered at around 400 nm. At temperatures greater than $74^\circ\text{C}$, the sample was observed visually to be grossly precipitated, and the fits to the correlation function data were relatively poor. Thus, size distributions could not be determined above this temperature.
Figure 5. A. Two ways to characterize the alteration of secondary structure occurring during heating of HSA. In the first approach, the percentages of alpha helix and beta sheet were calculated from the Raman spectra using a partial least square model as described in the Methods. In the second approach, the relative intensities of the alpha helix and beta sheet bands in the second derivative spectra were plotted. The solid lines shown are those resulting from sigmoidal fitting of the data points used for each plot. B. DLS measured intensity distributions for HSA obtained during heating are shown for selected temperatures. C. The z-averaged hydrodynamic size of HSA (39 mg/mL) vs temperature.

The z-average values for HSA size were plotted as a function of temperature. As heating progressed to 62°C, there was no observable change, but above this temperature there was a large increase. A curve was fit to the plot of values versus temperature, and the T_{agg} value was determined (Figure 5C). The T_{agg} value was lower than T_{onset} for secondary structural changes, 63.5°C vs ~66°C. The discrepancy is probably due to the fact that DLS is
very sensitive to large particles because the scattering intensity is proportional to the sixth power of the particle diameter. Thus, the size measured will be skewed to a larger value when even a minute amount of large aggregates is present, i.e., less than 1% in mass. With Raman spectroscopy, like all the other spectroscopic techniques, the spectra represent the average structure of all sub-populations and are not sensitive to relatively small amounts of structurally perturbed protein molecules. Thus, even though conversion of a few percent of HSA molecules into large aggregates would have a large effect on DLS z-average values, the corresponding Raman spectra would not be altered detectably from that observed for the fully native protein.

Overall, the data from Raman spectroscopy and DLS showed that during heating of HSA the first detectable change was perturbation of tertiary structure. As the temperature increased further, the protein aggregated, and it was the process of aggregation that caused the loss of native alpha helix and the concomitant increase in non-native, intermolecular beta sheet. This conclusion is consistent with the commonly found phenomenon that during heating partial unfolding of native structure results in protein aggregation, which in turn is associated with a loss of native secondary structure and formation of intermolecular beta sheet \(^{45,99-102}\). These results show the importance of having an independent, but concomitant, measure of protein aggregation when studying heating-induced structural transitions of proteins.

**Effects of protein concentration and data acquisition interval on HSA thermal stability**

To assess the effect of protein concentration on HSA thermal stability, samples of 10, 15, 20 and 39 mg/mL were analyzed at pH 7. At 20°C, there were no detectable differences
between the Raman spectra of HSA at the different concentrations (see Figure 6). However, the spectral quality increased in direct proportion to the protein concentration. During heating, the peak centers of Amide I spectra for HSA at all concentrations studied increased from ~1653 cm\(^{-1}\) to ~1661 cm\(^{-1}\). The \(T_{\text{onset}}\) and \(T_m\) values calculated from the sigmoidal fitting of the temperature-dependent position of Amide I are shown in Table 1. Based on both values, under the conditions of our experiments, protein concentration did not affect the stability of HSA during heating (Table 1).

The \(T_{\text{agg}}\) values obtained from the temperature-dependent z-averaged hydrodynamic size also did not vary with protein concentration (Table 1). However, the greater the protein concentration was, the larger the z-averaged size was for the sample after the aggregation onset (inset Figure 7C). More specifically, at 67°C, the HSA sample at 10 mg/mL showed two unresolved peaks with z-averaged size of 18 nm, whereas for the sample at 39 mg/mL, two peaks at 10 and 100 nm were well resolved with z-averaged size of 55 nm.

The heating step is an operational parameter that can potentially affect thermal stability evaluations for proteins. To gain insight into the effects of heating step interval on protein thermal stability, HSA at 41 mg/mL was evaluated with a heating interval of 0.5, 1, 2 or 5°C. There were no discernable differences in the heating-induced shifts in the amide I band (Figure 8A) or in the calculated values for \(T_{\text{onset}}\) and \(T_m\) (Table 1). Temperature-dependent DLS data (Figure 8B; Table 1) showed that the \(T_{\text{agg}}\) values also were not affected by heating interval. But the sizes of aggregates obtained were slightly larger for samples studied in experiments with the smaller heating intervals than for those observed with larger intervals (Figure 8B inset). This effect was probably because the sample was exposed to
higher temperatures for longer time during the experiments with the smaller data acquisition intervals.

Figure 6. The Raman spectra of HSA with protein concentration of 10, 15, 20 and 39 mg/mL at 25°C. There are no discernable differences among the protein concentration gradient.

Figure 7. A. The effect of protein concentration on the Raman spectra Amide I peak centers during heating of HSA. The protein was studied (pH 7) at protein concentrations of 10, 15, 20 and 39 mg/mL. B. The effect of protein concentration on the z-averaged hydrodynamic size of HSA during heating. Inset shows the intensity distribution of sizes measured by DLS at 67°C. Error bars denote standard deviation for values obtained for independent samples (n ≥ 2).
Table 1. Onset and mid-point temperatures for structural changes and aggregation onset during heating.

<table>
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<tr>
<th>Experimental Parameter Tested</th>
<th>T&lt;sub&gt;onset&lt;/sub&gt; (°C)</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; (°C)</th>
<th>T&lt;sub&gt;agg&lt;/sub&gt; (°C)</th>
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<td>IVIG Aromatic Side Chain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr 830 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>61±1.4</td>
<td>68±1.7</td>
<td>N/D</td>
</tr>
<tr>
<td>Tyr 850 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>60.0±0.8</td>
<td>66±1.7</td>
<td>N/D</td>
</tr>
<tr>
<td>Trp 1550 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>59±2.8</td>
<td>69±2.5</td>
<td>N/D</td>
</tr>
<tr>
<td>Trp 1340/1360 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>62.7±1.2</td>
<td>68.1±0.9</td>
<td>N/D</td>
</tr>
</tbody>
</table>
Figure 8. A. The effect of heating step size on the Raman spectra Amide I peak centers during heating of HSA (41 mg/mL). B. The effect heating step size on the z-averaged hydrodynamic size of HSA during heating. Inset shows the intensity distribution of sizes measured by DLS at 65°C. Error bars denote standard deviation for values obtained for 3 independent samples.

Isothermal incubation of HSA

The kinetics of structural alterations and aggregation for HSA were evaluated during isothermal incubation at 60, 63 and 65°C, which were near the onset temperatures for the thermal transitions observed during heating experiments. In Figure 9A, the Raman Amide I band position was plotted against the duration of incubation. At the 20°C starting point, the values were 1654.3, 1654.3 and 1654.7 cm⁻¹ for HSA incubated at 60, 63 and 65°C, respectively. At 65°C the shift to higher wave numbers was faster and to a greater degree than that observed at 63°C; there was almost no change for Amide I peak center at 60°C. After 200 mins incubation, the Amide I peak was centered at 1654.5, 1655.4 and 1656.8 cm⁻¹ for 60, 63 and 65°C, respectively. As a direct reflection of protein secondary structure, the Amide I peak shifting indicates the structural perturbation rate and extent were both positively correlated with the temperature. As expected, structure perturbation also positively correlated with the increase of z-averaged size (Figure 9B). With starting points of 7.9 nm,
8.4 nm and 9.9 nm for HSA incubated at 60, 63 and 65°C, respectively, the z-averaged size increased to 9.9 nm, 34 nm and 116.5 nm respectively after 200 mins isothermal incubation. The protein aggregated the fastest and to the greatest extent at 65°C.

![Figure 9. Effect of temperature on Raman spectra Amide I peak centers. A. and z-averaged hydrodynamic size. B. during isothermal incubation of HSA (31 mg/mL HSA at pH 7). Error bars denote standard derivation for values obtained for 3 independent samples. In some cases error bars are not visible because they were smaller than the symbols.](image)

Notably for the sample incubated at 63°C, the magnitude of the increase in z-averaged size was substantially greater than that for the increase in Amide I peak position. As discussed above, conversion of a small fraction of the protein population into larger aggregates could result in a substantial increase in the z-average value, because of the great increase in light scattering with size. In contrast, even if HSA molecules in the aggregates had greatly perturbed secondary structure, with a relatively small fraction converted into aggregates the impact on Raman spectra would be minor. This is because under these conditions the overall average secondary structure of all protein molecules in the sample would be only slightly altered.
**Effects of pH on HSA structure and thermal stability**

The effects of pH on the structure and thermal stability of HSA have been characterized by various techniques such as CD spectroscopy, DSC and fluorescence spectroscopy, but the earlier studies were performed at relatively low protein concentrations. Here we utilized the Raman-DLS system to study effects of pH on the structure and thermal stability of HSA at the relatively high concentration of 37 mg/mL. At 20°C, as compared to the Raman spectra for the protein at pH 5 and 8, at pH 3 the second derivative Amide I region showed a slight intensity loss at 1654.5 cm⁻¹ and gain at 1670 cm⁻¹ (Figure 10A). This change is attributed to a reduction in alpha helix content of HSA at pH 3. There were also decreases in the intensities at 941 cm⁻¹ and at 1320 cm⁻¹, which further confirmed the helical structure decrement (Figure 11). It has previously been shown that HSA has a compact conformation between pH 4.3 and 10 and converts to a more open structure with reduced helix content -- in a process referred to as “acid expansion” -- below pH 4.3. Consistent with this effect of acidic pH on HSA, at 20°C the z-averaged size of HSA was ~7, ~8 and 9 nm at pH 8, 5 and 3, respectively.

For the thermal stability study, Raman spectra were collected for HSA samples at each pH from 20 to 90°C (Figure 12). For samples at pH 5 and 8, HSA started with 68% helix content, and this value did not change substantially until 65°C (Figure 10B). Then the content dropped relatively steeply with temperature at pH 5 compared to the temperature-induced structural change in HSA observed at pH 8. The HSA sample at pH 3 started with slightly lower helix content, 66%, and went through a broader, more gradual temperature-induced transition, but which had an earlier onset temperature. However, by the point the
samples reached 90°C, HSA helix contents were similar with values of 35, 32 and 35% at pH 3, 5 and 8, respectively.

Figure 10. Effect of pH on HSA (37 mg/mL) structure and size. A. Second derivative of Amide I region of the Raman spectra acquired at 20°C. B. Alpha helix percentage during heating. Helix content was calculated from the Raman spectra as described in the Methods. C. Z-averaged size during heating. D. Z-averaged size and Raman spectra tertiary structure marker, Tyr 850 cm⁻¹ band position, at pH 3, 5 and 8. The first twenty data points from 20°C to 39°C during heating were chosen. In panels B and C error bars denote standard derivation for values obtained for 3 independent samples.
Figure 11. The Raman spectra of 37 mg/mL HSA at pH 3, 5, 8 measured at 20°C.

The $T_{\text{onset}}$ and $T_m$ of secondary structural transitions were determined, and the values of both followed the trend of pH 3 < pH 5 < pH 8 (Table 1). These results demonstrated that HSA thermal stability is substantially reduced at pH 3.

During heating at pH 3, the z-averaged size of HSA started at 9 nm (20°C) and increased gradually with temperature (Figure 10C). Aggregation was first observed at 45.7±0.9°C, as evidenced by the increase of the z-averaged size to ~11 nm. By the time the sample reached 90°C the z-averaged size was about 70 nm. Macroscopically at this point the sample was visually a transparent, diffuse gel. For HSA at pH 5, there was a sharp transition to larger z-average size starting at 54.8±0.5°C, and after heating the aggregated sample had formed a white precipitate. At pH 8, the transition occurred at 61.8±0.3°C, and during heating the sample formed an opaque gel. For HSA at both pH’s, the last size data with correlation coefficient higher than 0.85 indicated a z-averaged size of 287.7 nm for pH 5 at 61°C, and 198.2 nm for pH 8 at 74°C.
The Raman spectra of HSA at selective temperatures during thermal ramping at A. pH 3, B. pH 5 and C. pH 8. The vertical dash lines were drawn to highlight the change of characteristic Raman bands along with the change of temperature.

The smaller size increase for HSA at pH 3 during heating could be due to highly charged protein molecules and charge-charge repulsion between protein molecules and/or nanoparticles formed during heating. Previously it has been observed that HSA at pH 2 aggregated less than the protein at pH 7 during heating from 25°C to 90°C\textsuperscript{107}. Thus, even though HSA has a perturbed conformation at low pHs, the high positive charge of the molecules limits the magnitude of aggregation during heating; conformational stability of HSA is reduced but colloidal stability is increased.

Finally, in Figure 10D for each pH studied, the z-averaged size for HSA is plotted against the Tyr 850 cm\textsuperscript{-1} peak center at low temperatures (20°C to 39°C) where no significant aggregation or denaturation had taken place. It is clear from this plot that HSA at pH 3 has a
perturbed tertiary structure and expanded monomer size compared to HSA at pH 5 and 8. Although the secondary structure differences were small between pH 3, 5 and 8 (Figure 10A), the combination of size and tertiary structure marker Tyr 850 cm$^{-1}$ could be used to capture and amplify the small differences in the protein’s structure.

**Effects of oxidation with H$_2$O$_2$ on HSA structure, aggregation and thermal stability**

Oxidation of therapeutic proteins can occur during production in cell cultures due to dissolved oxygen, and during downstream processes. This damage can also occur in the final drug container due to light exposure and/or impurities in excipients and their degradation$^{108}$. Furthermore, often the oxidation of a therapeutic protein causes decreased conformational stability and increased aggregation$^{108-113}$. To evaluate the effects of oxidation on HSA structure and aggregation, we first employed an accelerated degradation approach in which experimental samples were exposed to 3% (v/v) H$_2$O$_2$ at 37°C over 5 days and then analyzed for aggregation by DLS and structural alterations by Raman spectroscopy. SEC was also used to characterize and quantify protein aggregates. Control samples that were incubated at 37°C without H$_2$O$_2$ were similarly analyzed. For the control HSA sample, even after the 5-day incubation no significant aggregation occurred relative to that found in the starting material, as assessed by both DLS and SEC (data not shown). However, for H$_2$O$_2$-treated HSA, significant aggregation was observed (Figure 13). DLS intensity percentage results for the control HSA sample had a monodispersed peak with a center close to 7 nm. In contrast, for H$_2$O$_2$-treated HSA sample there was an additional prominent peak at 30 nm and the z-average size greatly increased (Figure 13A, C). When DLS results of the H$_2$O$_2$-treated HSA were converted to volume percentage for the main peak, there was a tailing in the >10 nm
size range, indicating that a fraction of the protein population had aggregated (Figure 13B). For the control HSA sample, the majority of the protein was monomeric with a minute fraction of high molecular weight species. For the H_2O_2-treated sample, as assessed by SEC analysis, there was a substantial decrease in the amount of monomeric HSA and a concomitant increase in high molecular weight species (Figure 13D). Thus, oxidative stress of HSA under accelerated degradation conditions fostered aggregation of the protein, and DLS results plotted as intensity percentage provided a sensitive means by which to detect the aggregates.

Figure 13. Effects of incubation at 37°C, with or without 3% H_2O_2 (v/v), on HSA (18 mg/mL) size. A. DLS intensity distribution. B. DLS volume distribution. C. DLS z-averaged hydrodynamic size. D. SEC chromatograms. In panels A, B and C error bars denote standard derivation for values obtained for 3 independent incubated samples.
In the Raman spectra of the H$_2$O$_2$-treated HSA, there was only slight peak shifting and broadening in the Amide I region (Figure 14), which suggests secondary structure was not significantly altered. Similarly, Raman spectra in the regions of Amide I, III and the backbone skeleton bands indicated the treated HSA did not have observable alterations in secondary structure (data not shown). A recent study also evaluated the effects of H$_2$O$_2$ on the structure and aggregation of HSA employing infrared spectroscopy, Far-UV CD spectroscopy and light scattering. It was also found the secondary structure of HSA was not affected significantly by H$_2$O$_2$ treatment.$^{114}$

However, differences in the Raman spectra for control and H$_2$O$_2$-treated HSA were observed in the 800 cm$^{-1}$ to 1300 cm$^{-1}$ region (Figure 15A). In both the raw spectrum for H$_2$O$_2$-treated HSA and the difference spectrum, a new peak around 1045 cm$^{-1}$ was apparent. This peak has been assigned to SO$_2$ stretching and was probably due to methionine and/or cystine oxidation.$^{114-117}$ However, the oxidation stress employed in our experiment was not as harsh as that described in earlier studies$^{115,116}$ in which the peracid oxidation of disulfide bridges of the cystine was induced by much higher concentrations of H$_2$O$_2$. Therefore, in the spectrum for H$_2$O$_2$-treated HSA from our studies the SO$_2$ stretching band was more likely due to methionine oxidation, which does not require as harsh treatment conditions as those required for cystine oxidation.$^{114,115}$

Before evaluation of the thermal stability of H$_2$O$_2$-treated HSA, the aggregates formed during the 5-day incubation were removed by preparative ultracentrifugation. The control sample was also processed by ultracentrifugation. The protein concentrations in the supernatants of both samples were adjusted to 18 mg/mL, and thermal stability was studied
with Raman spectroscopy and DLS (Figure 15B, C). As observed in our earlier experiment (Figure 8A) the Amide I peak position for control, untreated HSA started from 1654 cm\(^{-1}\) at 20\(^\circ\)C and shifted up to 1662 cm\(^{-1}\) at 90\(^\circ\)C. With the H\(_2\)O\(_2\)-treated sample, the peak center was \(~\)1655 cm\(^{-1}\) at 20\(^\circ\)C, and the final peak position was 1662 cm\(^{-1}\) at 90\(^\circ\)C. With H\(_2\)O\(_2\)-treated HSA there was a gradual transition during heating rather than the sharp one noted for temperature-dependent peak position for the untreated HSA sample. H\(_2\)O\(_2\)-treated samples had lower T\(_{\text{onset}}\) and T\(_{\text{m}}\) values than did the untreated protein (Table 1), indicating that oxidation reduced thermal stability. Similarly, by DLS, H\(_2\)O\(_2\)-treated HSA showed a lower T\(_{\text{agg}}\) value as compared to that for untreated HSA (Table 1).

![Raman spectra of 12mg/mL HSA at pH 7 after 5 days isothermal incubation at 37\(^\circ\)C with or without H\(_2\)O\(_2\) treatment measured at 20\(^\circ\)C. H\(_2\)O\(_2\) treatment resulted into only slight intensity loss and peak broadening at Amide I region 1654.5 cm\(^{-1}\) peak.](image)

Figure 14. The Raman spectra of 12mg/mL HSA at pH 7 after 5 days isothermal incubation at 37\(^\circ\)C with or without H\(_2\)O\(_2\) treatment measured at 20\(^\circ\)C. H\(_2\)O\(_2\) treatment resulted into only slight intensity loss and peak broadening at Amide I region 1654.5 cm\(^{-1}\) peak.
Figure 15. A. Raman spectra acquired at 20°C (from ~800 cm\(^{-1}\) to ~1300 cm\(^{-1}\)) of 18 mg/mL HSA with or without H\(_2\)O\(_2\) treatment. The scaled difference spectrum is also shown. The SO\(_2\) stretching reflected by the increase of intensity at 1045 cm\(^{-1}\) was labelled in the figures respectively. The spectra were the average of triplicate samples. B. The shifting of Amide I peak centers of HSA with or without H\(_2\)O\(_2\) treatment during thermal ramping. The solid lines were sigmoidal fitting of the data points. C. The z-averaged hydrodynamic size of HSA with or without H\(_2\)O\(_2\) treatment during thermal ramping. Error bars stand for standard deviation of 3 independent samples.

It should be noted that, even after ultracentrifugation to remove the aggregates of HSA formed during H\(_2\)O\(_2\) treatment, the hydrodynamic diameter determined from DLS was ~14 nm; compared to 7 nm for the control sample of HSA. The presence of residual aggregates, as well as oxidized HSA molecules, in the treated sample might have contributed to the greater hydrodynamic diameter and lower thermal stability. In an earlier study\(^{114}\),
however, based on Rayleigh light scattering intensity, oxidized HSA had a lower aggregation propensity during heating than untreated HSA. The discrepancy between our results and those from the earlier study might be because in our experiment the HSA was stressed with H$_2$O$_2$ longer and at higher temperature (37°C for 5 days versus 25°C for 1 day). In addition, the protein concentration in our experiment was 46 mg/mL versus 2 mg/mL in the earlier study. These differences in results between the two studies further illustrate that one cannot assume similar effects will be observed during heating studies unless all conditions are exactly matched between experiments.

**Thermal stability of IVIG**

Monoclonal antibodies have become one of the most important categories of therapeutic proteins, and evaluation of thermal stability is crucial for research and development of antibody therapeutic products$^{118,119}$. Because these products are often used at relatively high doses when delivered by subcutaneous administration, the protein concentration in monoclonal antibody products can be 50 to 100 mg/mL, or maybe even higher. And for thermal stability assessment, it is important to study protein samples at the high concentrations used for the actual dosage form.

To mimic such studies, we examined the thermal stability of an ultracentrifuged IVIG sample at 51 mg/mL by Raman spectroscopy and DLS. The protein solution was prepared in the commercial product formulation of 250 mM Glycine buffer at pH 5. The Raman spectra at 20°C, 60°C and 90°C were selected for initial comparison (Figure 16). In contrast to results with HSA, which has mostly native alpha helix structure, there were minimal temperature-induced shifts in the Amide I peak position for the spectra of IVIG at 20°C to 90°C.
protein aggregation was not expected to result in as much increase in overall beta sheet native secondary structure. Therefore, formation of intermolecular beta sheet during protein aggregation was not expected to result in as much increase in overall beta sheet content measured with Raman spectroscopy as that observed with HSA. Antibodies have mostly beta sheet native secondary structure. Therefore, formation of intermolecular beta sheet during protein aggregation was not expected to result in as much increase in overall beta sheet content measured with Raman spectroscopy as that observed with HSA. Also, with Raman spectroscopy we were not able to distinguish intermolecular beta sheet in aggregated IVIG from intramolecular beta sheet in the native protein.

Figure 16. A comprehensive analysis of Raman spectra of 51 mg/mL IVIG during thermal ramping from 20°C to 90°C. Spectra at 20, 60 and 90°C were selectively shown for comparison. The vertical dash lines were drawn to highlight the changes of characteristic Raman bands along with the change of temperature. The peak shifting of Tyr 850 cm⁻¹ and 830 cm⁻¹ were shown in the upper-left inset and the peak shifting of the Trp 1550 cm⁻¹ was shown in the upper-right inset. Error bars stand for standard deviation of 3 independent samples.
Considering the limited resolution with individual spectra for detecting the secondary structural transition of IVIG during heating, we determined difference spectra by subtracting the spectrum obtained at 20°C from those for the protein at different temperatures during heating (Figure 17B). With this approach we were able to resolve increased intensities in bands at 1668 and 1686 cm\(^{-1}\) with increased temperature (Figure 17C). Difference spectra provided a sensitive means by which to detect the small increases in beta sheet content in the IVIG sample during heating studies, and might be particularly useful for studying monoclonal antibody therapeutic products.

![Figure 17. A. The Amide I region in the Raman spectra for IVIG acquired at 20°C and 90°C during heating. B. Difference spectra in the Amide I region obtaining by subtracting the Raman spectrum at 20°C from those acquired during heating at the designated temperatures. C. Intensities of bands at 1668 cm\(^{-1}\) and 1686 cm\(^{-1}\) in the difference spectra as a function of temperature.](image-url)
Assessment of tertiary structure during heating also improved the resolution of structural changes detected with Raman spectroscopy. During heating of IVIG the Raman spectra showed substantial peak shifts for the tyrosine Fermi doublet at 850 cm\(^{-1}\) and 830 cm\(^{-1}\) and tryptophan at 1550 cm\(^{-1}\) (Figure 16 inset). There was about a 2 cm\(^{-1}\) reduction in peak positions for the Tyr 850 and 830 cm\(^{-1}\) peaks. The Trp 1550 cm\(^{-1}\) peak downshifted around 3 cm\(^{-1}\). Those peak shifts were caused by changes of the local environment of Tyr and Trp, consistent with tertiary structural alterations of IVIG during heating\(^{40}\). The \(T_{\text{onset}}\) and \(T_m\) values were determined for Tyr 830 cm\(^{-1}\), Tyr 850 cm\(^{-1}\) and Trp 1550 cm\(^{-1}\) as shown in Table 1. In addition, the intensity ratio of the Fermi doublet of Trp at 1360 cm\(^{-1}\) and 1340 cm\(^{-1}\), another tertiary structure marker for proteins\(^{40}\), went from 0.3 to 0.05 (data not shown), with the \(T_{\text{onset}}\) at 62.7±1.2°C and \(T_m\) at 68.1±0.9°C (Table 1).

In an earlier study, second derivative UV spectra and intrinsic fluorescence spectroscopy of mAb samples also indicated that Trp had increased solvent exposure with increasing temperature\(^{120}\). As shown in our work and the earlier study, changes in tertiary structure could be especially valuable to understand mAb structure and thermal stability under circumstances where minimal secondary structure changes occur\(^{121}\).

Based on the concomitant DLS analysis in our study of IVIG, aggregation was initiated at around 56°C and preceded further as the temperature increased to form large assemblies of over 100 nm at 90°C (Figure 18A and B). The initial apparent hydrodynamic diameter was 3.5 nm at 20°C as compared to typical mAb size of ~10 nm\(^{122}\), which is most likely due to the solution conditions employed and the relatively high concentration of IVIG in the sample. The macromolecule concentration effect on molecular diffusion has been developed as\(^{123}\):
\[ D_m = D_o [1 + K_D C + K_2 C^2 + \ldots] \]  

Equation 3

where \( D_m \) is mutual diffusion coefficient or the apparent diffusion coefficient, \( D_o \) is the value of \( D_m \) at infinite dilution, \( K_D \) is the first order interaction parameter, \( K_2 \) is the second order interaction parameter, and \( C \) is protein concentration. At relative low protein concentration the higher order concentration effect, \( K_2 \), and above are negligible, and the first order interaction parameter \( K_D \) could be measured by the correlation between diffusion coefficient and protein concentration to reflect extent of intermolecular interaction.\(^{124}\) For example, the apparent diffusion coefficient of a monoclonal antibody (pI = 8.5) measured with DLS increased almost linearly with an increase in protein concentration in the range of 2 to 12 mg/mL at pH 4 and 6.\(^{125}\) At pH 4 and 6, the net positive charge on the mAb molecule dominates intermolecular interactions, and the repulsive interaction causes the positive correlation of \( D_m \) to protein concentration (or positive \( K_D \)).\(^{125}\) At higher protein concentration not only electrostatic interactions but also factors such as Van der Waals interactions and excluded volume effects, should be considered.\(^{126}\) In our study, with the pI of IgG molecules in IVIG ranging from about pH 4.7 to 7.5,\(^{88}\) the majority of the IgG population should be positively charged at pH 5. Therefore, repulsive interactions between the positively charged molecules and an increased diffusion coefficient is expected with increasing protein concentration. In fact, an increase in the apparent diffusion coefficient was seen when the IVIG protein concentration was increased from 1 mg/mL to 50 mg/mL (data not shown), and the apparent size of IVIG decreased from 9 to 3.4 nm. However, this underestimation of molecular size should not affect aggregation onset point determinations because these values depend on the relative size change rather than the absolute size.
with infrared spectroscopy combined a series of slightly modified biophysical methods.

Figure 18. A. Z-averaged hydrodynamic size of IVIG during heating. B. DLS intensity distributions of IVIG at 20, 40, 50, 60, 70, 80°C. In panel A, error bars denote standard deviation of values obtained for 3 independent samples.

Usually, high concentration mAb formulations pose challenges for analytical characterization and formulation development. Attempts have been made to obtain analytical data of highly concentrated solutions of mAb’s, either solely by infrared spectroscopy\textsuperscript{127}, or with infrared spectroscopy combined a series of slightly modified biophysical methods\textsuperscript{120,128}. For the former, the thermal stability of bovine IgG was tested up to 200 mg/mL at varied pH values. For the latter, fluorescence, UV, CD and infrared spectroscopies, and DSC were combined to test the effects of pH on the thermal stability of two mAbs across a concentration range 0.1 to 190 mg/mL. Interestingly, at pH 6, the bovine IgG was found to have increasing $T_m$ values from 71 to 73°C when the protein concentration increased from 25 to 100 mg/mL, but the value decreased slightly at 200 mg/mL. However, for the study of the two mAbs, spectroscopic techniques probing tertiary structure demonstrated a decrease in the apparent thermal melting temperature of $\sim$ 5-20°C with increasing protein concentration. In these two studies, the apparent melting temperature decrement was attributed to increased aggregation at higher protein concentration. However, no direct size measurements were
performed. Conversely in another recent study, only light scattering was applied to characterize protein aggregation at concentration up to 190 mg/mL, but no structure information was supplied. In contrast, with the combined DLS-Raman system both protein conformational and aggregation can be studied at the same time.

Conclusions

The combination of Raman spectroscopy and DLS offers unique advantages such as low sample volume requirement, easy background subtraction, compatibility with high protein concentration, and simultaneous analysis for both size and conformation including protein secondary and tertiary structure information. The robustness of a combined Raman-DLS system for protein thermal stability evaluations has been documented by the current study, and further applications of this system for other protein systems will be forthcoming.
CHAPTER III
CHARACTERIZATION OF SIZES OF AGGREGATES OF INSULIN ANALOGS AND THE CONFORMATIONS OF THE CONSTITUENT PROTEIN MOLECULES: A CONCOMITANT DYNAMIC LIGHT SCATTERING AND RAMAN SPECTROSCOPY STUDY

Introduction

Currently, the fast-acting insulin analogs, lispro, aspart, and glulisine, are the most widely used insulins in diabetic patients. In contrast to native human insulin, the analogs have weaker monomer-monomer interactions, which upon administration allow for quicker dissociation from the oligomeric state to the biologically active monomer. As a result, in patients the analogs have a quicker onset of activity than does human insulin. For each of the analogs a specific mutation is responsible for the weaker intermolecular interactions. Lispro has the penultimate two amino acids in the B-chain reversed compared with insulin (from Pro28-Lys29 to Lys28-Pro29). In the B-chain, aspart has one substitution, Pro28 to Asp; and glulisine has two substitutions, Asn3 to Lys and Lys29 to Glu.

In commercial formulations of lispro and aspart, meta-cresol and/or phenol and zinc ions promote native state assembly of the insulin molecules, which in turn increases protein storage stability. In addition, meta-cresol/phenol also serve as antimicrobials. In the commercial formulation of glulisine, meta-cresol is included as a stabilizer and antimicrobial preservative, but the formulation does not include zinc ions.

There can be a gradual loss of meta-cresol and phenol from the formulations during administration of the analogs into patients with pump and catheter systems. The

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reduction in the concentration of these stabilizers can lead to aggregation and even precipitation of insulins in the pump reservoir and tubing \textsuperscript{54,56,57}. Published laboratory studies have characterized the aggregation of insulin analogs resulting from preservative depletion\textsuperscript{48,58}. In one study, it was found that heating and agitation of lispro, aspart and glulisine -- in the absence of meta-cresol or phenol -- caused all of the analogs to precipitate via fibril formation\textsuperscript{48}. Although the structural changes resulting from fibril formation were not characterized, it has commonly been observed that the secondary structure of insulin molecules is converted from native alpha-helix to non-native intermolecular beta sheet during fibril formation\textsuperscript{59-63}. In another study, this structural transition was observed for lispro when solutions were seeded with preformed aggregates to induce fibril formation\textsuperscript{64}. In more recent work, it was observed that quiescent incubation of lispro or aspart samples, which had been depleted of meta-cresol and phenol, at 37°C caused formation of soluble aggregates without observable fibrils\textsuperscript{58}. However, the conformations of insulin analog molecules in the soluble aggregates were not studied. Therefore, in contrast to the wealth of published information on structural changes in insulin molecules upon conversion to insoluble fibrils, there are not similar published insights into the structural changes arising when insulin analogs are converted into soluble aggregates.

In the current study, we addressed this issue by analyzing samples of the lispro, aspart and glulisine with concomitant Raman spectroscopy and dynamic light scattering (DLS)\textsuperscript{132}. After removal of preservatives, 20 mg/mL solutions of each of the three insulin analogs were incubated quiescently at 37°C for 30 days. At certain time points during the incubation, samples were removed and characterized for protein secondary and tertiary structure with Raman spectroscopy. Concomitantly in the same sample cuvette, protein and aggregate sizes
were determined with DLS$^{132,133}$. Also, size exclusion chromatography (SEC) was used to quantify the levels of native insulin and aggregates in the incubated samples. Finally, the relative thermal stabilities of the insulin analogs at 20 mg/mL were evaluated during heating studies with concomitant Raman spectroscopy and DLS.

Materials and Methods

Materials

Insulin analogs lispro (Humalog®-Eli Lilly and Company), aspart (Novolog®-Novo Nordisk) and glulisine (Apridra®-Sanofi-Aventis) were purchased from the University of Colorado at Boulder’s Wardenburg pharmacy. Lispro is formulated in 1.88 mg/mL sodium phosphate buffer with 16 mg/mL glycerol, 3.15 mg/mL meta-cresol and 0.0197 mg/mL zinc (pH 7.0-7.8). Aspart is formulated in 1.25 mg/mL phosphate buffer with 16 mg/mL glycerol, 1.5 mg/mL phenol, 1.72 mg/mL meta-cresol, 0.58 mg/mL sodium chloride and 0.0196 mg/mL zinc (pH 7.2-7.6). Glulisine is formulated in 6 mg/mL Tris buffer, 3.15 mg/mL meta-cresol, 5 mg/mL sodium chloride and 0.01 mg/mL Tween 20 (pH 7.0-7.8). All other chemicals were purchased from Fisher Scientific (Hampton, NH) and were of reagent grade or higher quality.

Preparation of insulin analog solutions

Preservatives (meta-cresol and/or phenol) in the insulin formulations were removed with Zeba desalting columns (Thermo Scientific, Rockford, IL), as described previously$^{58}$. As a result of this process, each insulin analog was in a solution with the respective original formulation composition, but without preservative. The protein was concentrated to 20
mg/mL with Amicon Ultra 3000 MWCO centrifugal filters (Millipore, Cork, Ireland). Insulin concentration was determined by UV absorbance at 277.5 nm with an extinction coefficient of 0.9521 cm⁻¹•mL•mg⁻¹, assuming the same extinction coefficient for insulin monomer, dimer, tetramer and hexamer. Aliquots (200 µL) of each insulin solution were incubated quiescently at 37°C in 1.3 mL glass vials (West Pharmaceuticals, Exton, PA), and analyses were performed at day 0, 4, 10, 20 and 30 by UPLC-SEC and concomitant Raman spectroscopy/DLS. For each insulin analog solution, three sample vials were prepared for each time point. Thus, for each time point, independent triplicate samples were analyzed.

**Size-exclusion chromatography**

Sample aliquots were removed from the vials, placed into Eppendorf tubes and centrifuged at 14,100 x g for 10 minutes. Aliquots of the “supernatants” were analyzed. It should be noted, however, that no pellet was observed for any of the centrifuged samples.

Native insulin and soluble aggregate levels were quantified with SEC using Waters Acquity UPLC BEH200 (Waters Corp., Milford, MA) SEC column (4.6×150 mm²) on an Agilent 1100 HPLC (Santa Clara, CA). UV detection was at 280 nm. The mobile phase consisted of 100 mM sodium phosphate (pH 7.0), 100 mM sodium sulfate and 0.05% w/v NaN₃; with a flow rate of 0.208 mL/min and run time of 37 mins. For the purpose of quantification, the total peak area before incubation was considered as 100%. The peak areas for native species and soluble aggregates in samples taken at various incubation time points were calculated based on the initial total peak area.
Concomitant Raman spectroscopy and DLS

Raman spectroscopy-DLS measurements were performed on a Zetasizer Helix system (Malvern Instruments, Malvern, UK)\textsuperscript{132}. For a typical experiment, a ~ 120 µL sample was loaded into a 3×3 mm quartz cuvette which was placed into a Peltier temperature-controlled sample compartment. Raman and DLS data were acquired sequentially\textsuperscript{21}. More detailed information on the instrument can be found in previous study\textsuperscript{132}. Raman scattering was excited by a 785 nm laser with approximately 280 mW power. Raman spectra of corresponding buffers for the protein samples were acquired under the identical conditions and subtracted from the spectra for the protein solutions. Unless otherwise noted, Raman spectra were collected with 10 co-additions of a 20 s exposure. DLS data were collected at a 173-degree backscattering angle from a 632 nm laser.

Insulin analog conformational stability during heating

Solutions (20 mg/mL) of each insulin analog were heated from 20 to 90°C with data acquisitions at a 1°C increment. During heating, Raman spectra were collected at each temperature, as described previously\textsuperscript{1}. For each analog, the heating study was repeated three times, with a fresh solution used each time.

Results and Discussion

Quantitation of levels of native insulins and aggregate by size-exclusion chromatography

Figure 19A shows the SEC chromatograms of insulin lispro samples that were analyzed during the time course of the incubation. Native insulin eluted at around 12 min
whereas the high molecular weight species (HMWS) eluted from 4 min to 10 min. The gradual loss of the peak for native insulin and gain of the HMWS peak indicated the conversion of native insulin species into higher order oligomers as a function of incubation time.

Figure 19. A. Size exclusion chromatography characterization of lispro during isothermal incubation. Peak integration of main peak and HMWS peak and unrecovered protein percent for SEC chromatography of B. lispro, C. aspart and D. glulisine. Error bar denotes standard deviation for values obtained for 3 independent samples.

Furthermore, there was a progressive reduction in total peak area in the chromatograms for lispro samples, reflective of a reduction in recovery of protein during sample processing and/or chromatography. When there is such a reduction in protein recovery, it may be ascribed to pelleting of so-called “insoluble aggregates” during the pre-chromatography centrifugation step. Also, there can be loss of protein during an SEC run; e.g., due to adsorption to the column matrix\textsuperscript{134}. We did not observe a pellet for any of the
samples; even those that had more than 30% reduction in total peak area on the SEC chromatograms. If, for example, 20% of the protein in a sample (0.2 x 10 mg/ml x 0.15 ml = 0.3 mg) had centrifuged down as precipitated protein, we would have expected to have seen an obvious pellet at the bottom of the centrifuge tube. Because we could not determine the cause of the loss of this material, we refer to it in the graphs as “unrecovered protein percent”.

For lispro, the main peak decreased to 74% at day 4 and further decreased to 26% by day 10 (Figure 19B). The main peak continued to decrease, and at the end of the 30-day incubation the remaining main peak was only 5%. The HMWS peak area increased from 0% at day 0 to 63% at day 30. In addition the amount of unrecovered protein gradually increased from 0% to 33%. In contrast to the fibrillation of insulin/insulin analogs observed previously under conditions such as extremely low pH or agitation, lispro under the commercial formulation conditions (except for depletion of preservatives) formed mostly soluble aggregates and no obvious insoluble aggregates or fibrils.

For aspart, at day 20 the main peak was still about 81% of the total, the HMWS peak was 15% and there was only minimal amount of unrecoverable protein (Figure 19C). For glulisine, the main peak did not decrease until day 10, when 99% native species still existed (Figure 19D). At day 30, the main peak was 83% of the total while the HMWS peak 15%, and the percentage of unrecoverable protein was minimal. Thus, glulisine was much more resistant to aggregation than aspart or lispro, and lispro had a much faster aggregation rate than the other two analogs. In a previous study, although the protein concentration used was 3.5 mg/mL, similar degradation profiles in the absence of preservatives were found, i.e. after
the 10-day incubation the majority of the lispro degraded, while the majority of the aspart was still native species, and minimal aggregation occurred for glulisine\textsuperscript{58}.

However, in another study it was found that when incubated at 37°C with agitation in the absence of preservatives, aspart completely aggregated within 1 hr, and lispro and glulisine completely aggregated by 4 hr\textsuperscript{48}. Such accelerated aggregation rates compared to those observed in studies with quiescent incubations could be due to the greater stresses arising during agitation, including protein adsorption and aggregation to air-water interface, as well as protein desorption due to changes in interfacial surface area.

**Analysis of incubated insulin analog samples with concomitant DLS and Raman spectroscopy**

We took advantage of the unique capabilities of the Zetasizer Helix instrument to obtain size and protein conformational data for the incubated insulin analog samples. It is important to note that the samples were placed directly into the cuvette without any prior processing such as centrifugation. Thus the results are reflective of all protein species in the samples. And in the cases (i.e., lispro samples after 20 or more days of incubation) where the native protein had been almost completely converted into aggregates, the data provide valuable information on conformation of the constituent insulin molecules within the aggregates, as well on aggregate size.

In the DLS characterization, results were analyzed as both the intensity percent versus size and the volume percent versus size (Figures 20). The intensity distribution is helpful for detecting small amounts of large aggregates because the scattering intensity is proportional to the sixth power of the particle diameter. Thus, DLS can be very sensitive to even trace
amounts of relatively large particles\textsuperscript{132}. When converted to volume distribution based on Mie theory, the intensity bias can be minimized, and relative volume proportion of each size species can be estimated\textsuperscript{135}. However, the assumptions associated with Mie theory during the conversion bring in error in particle sizes especially for aggregated protein samples in which the aggregated species are usually not homogeneous and/or spherical\textsuperscript{135}. Even without exact sizing, the volume distributions obtained still provide useful insight into relative levels of different sized populations of species.

In the intensity distribution for lispro at day 0, there was a major peak at 7 nm for the native protein and minor peaks around 30 and 80 nm (Figure 20A). The polydispersity index (PDI) for the lispro sample was 0.23 at day 0, indicating a relative heterogeneous particle population. At day 4, a peak around 20 nm with almost equal intensity to the native insulin peak was apparent. As incubation time increased, the peaks progressively shifted to positions for larger sized species. By day 30, there were two peaks with equal intensity at 17 and 40 nm. When the light scattering data were converted into volume distribution (Figure 20D) the initial size at day 0 for native lispro was around 5.8 nm. With time of incubation the single peak was observed at positions for larger sizes to about 12 nm at day 30, reflecting the extensive aggregation of the protein. During incubation the PDI increased slightly to 0.25 at day 4 and remained unchanged until day 30, indicating the consistency of the heterogeneity of the particle population.

The DLS intensity distribution for aspart showed that at day 0 there was only one peak at 4 nm with polydispersity (PDI) at 0.06, indicating a relatively homogeneous size distribution. From day 4, the peak intensity at 4 nm started to decrease while multiple peaks at several hundred nanometers appeared. And the PDI increased to approximately 0.7 since
by day 4 due to the appearance of large aggregates in a range of sizes. But the volume distribution indicated only slight size increases from 4 nm to 5 nm from day 0 to day 30 (Figure 20E), meaning the aggregates represented only a small fraction of the total sample mass.

Figure 20. DLS characterization of insulin analogues during isothermal incubation. A.-C. Intensity size distributions of lispro, aspart and glulisine respectively. D.-F. Volume size distribution of lispro, aspart and glulisine respectively. Error bar denotes standard deviation for values obtained for 3 independent samples.

In the case of glulisine, the intensity distribution also had a single peak centered at 5 nm at day 0 with PDI at 0.13. Large peaks at 200 nm appeared for samples as early as day 4 of incubation (Figure 20C). And subsequently the PDI increased to 1, which indicated a
greatly heterogeneous size distribution. However, the volume distribution showed only slight increases in size (from 5 nm to 6 nm) from day 0 to day 30 (Figure 20F).

Overall, these results demonstrated that for aspart and glulisine the levels of aggregates formed during incubation were low although the sizes of the aggregates were relatively large. And the results from DLS analysis of the incubated samples were consistent with those from SEC and documented much lower levels of aggregates for aspart and glulisine compared to lispro.

The monomer and dimer of insulin have reported hydrodynamic diameters of ~2-3 nm, and the value for the hexamer is about 5.6 nm\(^{136,137}\). Earlier studies with sedimentation velocity ultracentrifugation showed that in the absence of preservatives and the presence of zinc, lispro existed mostly as monomer with a small polydispersed population of aggregated species\(^{58,138}\). In contrast, under the same conditions, with insulin aspart the largest fraction was of assembled molecules. Insulin glulisine consisted mostly of species with sizes between monomer and hexamer\(^{58}\). To compare our results to those from the earlier ultracentrifugation study, it is important to consider experimental and instrument capability differences. The capability of DLS to resolve different size species is limited, and our samples were at much higher protein concentration (20 mg/mL) than used in the previous studies\(^{58,138,139}\). The DLS peaks at 7 nm and 5.8 nm in the intensity distribution and volume distributions, respectively, of lispro at day 0 might represent hexamers and maybe some even higher-order oligomers. For aspart and glulisine, the sizes measured at day 0 were in between the size of monomer/dimer and hexamer, i.e. peaks at 4 nm and 5 nm in the intensity and volume distributions, and no larger size species were detected. Therefore, although the protein concentrations in the earlier studies were lower than we used, our DLS data showed that the
assembly states of insulin analogues in the absence of preservatives were consistent with results of previous reports\textsuperscript{58}.

Concomitant with DLS analysis, to gain insight into the conformation of the insulin molecules, Raman spectra were acquired for the incubated insulin analog samples. In Figure 21, the Raman spectra for lispro in the range of 400 to 1750 cm\(^{-1}\) are shown as a function of time of incubation. The amide I band from \(~1600\) to \(1670\) cm\(^{-1}\), originates principally from the protein backbone’s C=O stretching coupled with minor contributions from C-N stretching and N-H in-plane bending\textsuperscript{140,141}, and is sensitive to the secondary structure change. It is clear

![Figure 21](image.png)

Figure 21. Raman spectra of lispro in the process of incubation. The conformation markers of Amide I, Amide III \(\alpha\) helix, skeleton stretching \(\nu(c-c)\), tyrosine Fermi doublets and disulfide bond vibration of \(\nu(s-s)\), \(\nu(c-s)\) were labelled with dotted line. Each spectrum denotes the mean of 3 independent samples.
that from spectra of lispro samples at day 0 and day 30 that the amide I peak gradually shifted from 1658 to 1668 cm\(^{-1}\), which is an indication of loss of native alpha helix structure and gain of non-native beta sheet structure\(^{141}\).

In Figure 22, the Raman spectra for the samples at incubation time points of day 0 and day 30 for lispro, aspart and glulisine were compared. In contrast to results observed with lispro during incubation, the changes in the spectra of aspart and glulisine were barely detectable, consistent with the minimal aggregation observed during incubation of these analogs.

Second derivative spectra of the amide I region of Raman spectra of lispro also indicated the native alpha helix structure was converted to non-native beta sheet during incubation. For aspart and glulisine this conversion was minimal (Figures 22 and 23). Another secondary structure marker is amide III in the region of 1200 to 1300 cm\(^{-1}\), attributed to the in-plane bending of N-H and stretching of C-N\(^{140}\). The peak at 1270 cm\(^{-1}\) was assigned to amide III alpha helix\(^{141}\). For the spectra of lispro samples, this peak gradually decreased in intensity as a function of time of incubation. Furthermore, the Raman peaks at 892 cm\(^{-1}\) and 943 cm\(^{-1}\), assigned to the skeleton stretching \(\nu(C-C)\)\(^{141}\), had remarkable loss of peak intensity, which also confirms the significant rearrangement of the polypeptide chain orientation. In contrast, spectra of samples of aspart and glulisine had minimal intensity changes in either amide III 1270 cm\(^{-1}\) or the skeleton stretching \(\nu(C-C)\) at 892 cm\(^{-1}\) or 943 cm\(^{-1}\).
Figure 22. Raman spectra of lispro, aspart and glulisine at day 0 and day 30. The conformation markers of Amide I, Amide III α helix, skeleton stretching $\nu(c\cdot c)$, tyrosine Fermi doublets and disulfide bond vibration of $\nu(s\cdot s)$, $\nu(c\cdot s)$ were labelled with dotted line. Each spectrum represents the mean of 3 independent samples.

The disulfide bond region in Raman spectra also provides important structural information, especially the S-S stretching ($\nu(s\cdot s)$) at $\sim$513 cm$^{-1}$ and the C-S stretching ($\nu(c\cdot s)$) of C-S-S-C group at $\sim$664 cm$^{-1}$. Insulin analogs contain one intra-chain disulfide bond in A chain and two inter-chain disulfide bonds between the A and B chains$^{142,143}$. During incubation of lispro, the samples’ spectra had shifting of $\nu(s\cdot s)$ stretching to lower frequencies. This change and the decrease of intensity for $\nu(c\cdot s)$ stretching are related to the distortion in the orientation of the C-S-S-C moiety$^{141}$. But no such changes were observed in the spectra for either aspart or glulisine.
Figure 23. Second derivative of amide I region for insulin analogues A. lipsro, B. aspart, and C. glulisine during isothermal incubation.

To quantify the changes in conformation, amide I peak shifting, intensity changes at peak 892 cm\(^{-1}\) and the peak shifting of the ν(s-s) stretching of insulin analogs were plotted and compared in Figure 24. For spectra of lispro, the amide I peak center in frequency increased during incubation (Figure 24A). For aspart and glulisine spectra there were only slight increases. The peak intensity of skeleton bands at 892 cm\(^{-1}\) in lispro spectra dropped as much as 61% from day 0 to day 10 and then stayed unchanged until day 30. For glulisine spectra, the intensity stayed unchanged until day 10 and only decreased slightly by 13% at day 30. For aspart spectra, the intensity kept decreasing but only 37% of the original intensity was lost at day 30 (Figure 24B). The ν(s-s) stretching in spectra of lispro shifted from 513.1 cm\(^{-1}\) to 505.6 cm\(^{-1}\) from day 0 to day 30. In contrast, in spectra for aspart the peak shifted
from 512.4 cm$^{-1}$ to 510.9 cm$^{-1}$ and glulisine spectra had the peak shifting from 512.7 cm$^{-1}$ to 511.2 cm$^{-1}$ (Figure 24C).

To quantitatively assess the secondary structure changes during incubation, the Raman spectra were analyzed as described in the Materials and Methods. At day 0, lispro contained 53.3% alpha helix and 8.8% beta sheet, aspart contained 52.4% alpha helix and 11.2% beta sheet, and glulisine contained 51.1% alpha helix and 12.3% beta sheet. These values reasonably match those obtained by x-ray crystallography of insulin (45% alpha helix and 12% beta sheet)\(^{144}\).

Spectra for samples analyzed during incubation, show that values for lispro had progressive decrease of alpha helix content from 53.3% to 35.9% and increase of beta sheet content from 8.8% to 26.0% by day 30 (Figure 24D). This indicated that the aggregated species at day 30 still retained considerable amount of native alpha helix structure while part of the structure had been converted into nonnative beta sheet structure. At day 30, aspart and glulisine had no significant increase in beta sheet content.

Importantly, the plot of the amide I peak center versus the mean size measured by DLS showed that there were good correlation between the structure features represented by the amide I peak center and the volume mean size in the sample (Figure 25). These results document that structural changes observed were due to formation of aggregates. Accordingly, there were large changes noted in the structure of samples lispro molecules during incubation because of the high degree of aggregation. In contrast, with aspart and glulisine, there were minimal aggregation and conformation alterations.
Figure 24. Quantitative analysis of Raman spectra makers in A. Raman shift at Amide I, B. Intensity of skeleton bands at 892 cm\(^{-1}\) and C. Raman shift at \(\nu(s-s)\) stretching for insulin analogues lispro (blue circle), aspart (red square), and glulisine (green triangle) during incubation. D. Quantitative analysis of \(\beta\) sheet components of insulin analogues during isothermal incubation. Error bar denotes standard deviation for values obtained for 3 independent samples.

Formation of lispro soluble aggregates during incubation resulted in conversion of a substantial fraction of the secondary structure from native alpha helix into non-native beta sheet. However, there was still considerable amount of alpha helix remaining after the protein molecules were converted into the soluble aggregates. This behavior is different from what has observed during insulin fibrillation when almost complete conversion to beta sheet structure occurs. Although there are not Raman spectroscopy studies on fibrils of insulin analogs, there are several publications in which are fibrillation human or bovine insulins was studied by Raman spectroscopy, as well as by infrared spectroscopy\(^{59,61,62,141,145-147}\). For
example, when bovine insulin was heated at 80°C to form fibrils, the majority of secondary structure was converted into beta sheet with ~10% alpha helix left. In another study, after dissolving bovine insulin in acidic buffer and heating at 50°C, infrared spectra showed that the native alpha helix structure was almost completely diminished while various beta sheet structures dominated the secondary structure components.

Figure 25. Correlation between Amide I peak center and DLS measured volume mean size during isothermal incubation for lispro (blue circle), aspart (red square), and glulisine (green triangle). Error bar denotes standard deviation for values obtained for 3 independent samples. Inset shows the zoom-in of the amide I region for lispro at day 0 (blue) and day 30 (red).

Finally, heating-induced aggregation and structural changes of the insulin analogs were investigated whether the aggregate rates observed during isothermal incubation correlated with the relative thermal stabilities of the molecules. The three insulin analogs at 20 mg/mL were heated from 20 to 90°C. As an indicator of the secondary structure, the peak
center of amide I was plotted against temperature (Figure 26). Whereas the spectra for all insulin analogs had a gradual increase in the amide I peak center with the increase of temperature, the onset temperature ($T_{\text{onset}}$) and midpoint temperature ($T_m$) for the shifting were substantially different. The $T_{\text{onset}}$ was 69, 79 and 81°C and the $T_m$ was 78, 85 and 85°C for lispro, aspart and glulisine respectively. The thermal stability of lispro was thus lower than aspart and glulisine, which correlated well with the relative aggregates rates noted during isothermal incubation. For aspart and glulisine, although they had similar thermal stabilities, the differences in assembly state and/or formulation compositions might have contributed to their different aggregation rates.

Figure 26. Shifting of amide I peak center during thermal ramping from 20 to 90°C for insulin analogues lispro (blue circle), aspart (red square), and glulisine (green triangle) at 20 mg/mL. Error bar denotes standard deviation for values obtained for 3 independent samples.
Conclusions

This study demonstrated that during isothermal of incubation insulin analogs after depletion of preservatives, the aggregation rates of lispro > aspart ~ glulisine; with aspart having an earlier aggregation onset time than glulisine. More importantly, with the concomitant measurements of structural transitions and size changes we were able to characterize the conformation of lispro after it was converted to soluble aggregates. The dominant structural alterations were a partial conversion of native alpha helix into non-native beta sheet and distortion of disulfide. These studies not only provide insights into structural behavior of insulin analogs during aggregation but also demonstrate the uniquely valuable data that can be acquired with concomitant DLS and Raman spectroscopy.
CHAPTER IV

CHARACTERIZATION OF NANOPARTICLE TRACKING ANALYSIS FOR QUANTIFICATION AND SIZING OF SUBMICRON PARTICLES OF THERAPEUTIC PROTEINS

Introduction

Protein aggregation is ubiquitous during the development, production and clinical use of therapeutic protein products\textsuperscript{148,149}. Conditions during manufacturing, shipping, storage, and delivery to patients that can induce aggregation include exposures to light, elevated temperatures, freeze-thawing, agitation, particles of foreign materials and various interfaces\textsuperscript{5,16}. A major concern is that protein aggregates, including submicron and micron-sized particles, may lead to adverse and unintended immunogenicity in patients\textsuperscript{13,150}. Accordingly, regulatory agencies are requesting that manufacturers provide more rigorous characterization and quantification of subvisible particles in their therapeutic protein products\textsuperscript{15,151}.

One difficulty in meeting this regulatory expectation is that no single analytical method or instrument can cover the entire size range of particles from submicron to micron-sized to visible\textsuperscript{5,66,67}. Also, each approach has key limitations and is critically affected by instrument parameters and sample characteristics, which must be evaluated rigorously in order to maximize utility of the method for analysis of therapeutic proteins. For micron-sized particles, light obscuration, electric sensing zone-based detection and flow microscopy are available and have been evaluated in detail in earlier investigations\textsuperscript{5,66,67,148,152-154}. DLS and nanoparticle tracking analysis (NTA) are useful for submicron particles. DLS can provide size distributions based on scattering intensity fluctuations, but cannot determine particle

\textsuperscript{3} Published with permission from Journal of Pharmaceutical Sciences volume 104, issue 8, August 2015, pages: 2441-2450.
concentrations. NTA provides both particle counts and size distributions, but there has been limited published research on the impact of operational and sample parameters on data acquisition, analysis and robustness. In the current study we use a model therapeutic protein and synthetic nanoparticles to critically evaluate such factors with the NanoSight LM20 NTA instrument.

An NTA instrument combines a high-resolution camera with a microscope to record the motions of individual nanoparticles in solution. Particles are visible in the system because of the light scattering that occurs when they are illuminated by the integrated laser. During sample analysis, individual particles are tracked spatially in the recorded videos. The diffusion coefficients of the particles are obtained and the hydrodynamic diameter of each particle is calculated according to the Stokes-Einstein equation.

NTA’s utility and capabilities have recently been evaluated in a few research areas\textsuperscript{139,155-159}. For example, NTA has been used to characterize nanoparticles arising in therapeutic protein products such as IgG and interferon-\(\beta\)-1a under various stresses\textsuperscript{65,160}; and, along with other particle characterization techniques, to compare the particles in a series of commercial interferon-\(\beta\) products\textsuperscript{28}. Also, the particle sizing capabilities of NTA have been found to be superior to DLS\textsuperscript{139,154}. With polydispersed samples of protein aggregates and drug delivery system nanoparticles, NTA was better able to resolve different-sized populations of particles than DLS.

Although useful results have been obtained with NTA in such studies, the complexity of the method and the potential impacts of instrument settings and sample properties can lead to erroneous results. For example, a recent study by Weinbuch et. al. showed that the nanoparticulate impurities in excipients such as sugars could interfere with NTA
measurements. In addition, the scattering intensity of particle species has been shown to have a profound effect on measuring particle concentration. The particle concentrations of weak scatterers were under-counted by four orders of magnitude for DNA and around 20-fold for fibrillar protein aggregates when compared to their expected concentrations. The goal of the current study was to systematically evaluate NTA instrument settings, operating parameters, sample handling methods and sample properties (e.g., protein concentration) on the accuracy and reproducibility of nanoparticle sizing and counts with the NanoSight LM20 instrument. For this study we used human serum albumin (HSA) as a model therapeutic protein.

Because only a minute sample volume is recorded and analyzed with this instrument, a multiplication factor of approximately 15 million is used to convert the raw particle numbers measured to particles per milliliter. To increase sample volume/tracking numbers measured during an experiment, a syringe pump connected to the sample chamber may be used. We therefore investigated the effect of sample flow rate through the chamber on particle counting and sizing. We also evaluated the effect of the raw number of particles detected during each measurement (referred to as “complete tracking number” in the instrument software) on the robustness of the particle data. During image analysis with the NanoSight LM20 instrument (software NTA 2.2) the minimum expected particle size (MES) must be set by the operator. Because this parameter can greatly affect results for a given sample, we also tested the effect of MES settings on particle concentration and on the accuracy of particle size determination.

Finally, it is becoming common to formulate therapeutic proteins such as monoclonal antibodies at concentrations of 50-100 mg/mL or even higher. Samples at such high
protein concentrations can cause difficulties during particle characterization with methods such as microscopic imaging and DLS\textsuperscript{67,161}. In the current study we investigated the capabilities of NanoSight for counting and sizing particles in samples with high protein concentrations by studying different particle species (latex beads, gold nanoparticles and protein aggregates) spiked into particle-free HSA solutions at various protein concentrations.

**Materials and Methods**

**Materials**

Human serum albumin (HSA, fraction V fatty acid free) was purchased from MP Biomedical, (Carlsbad, CA) and formulated with phosphate buffer (0.1 M sodium phosphate, pH 7.09). Latex beads size standard (100 nm) was purchased from Thermo Scientific (Waltham, MA). Gold particle size standard (50 nm) was purchased from Nanocs (NYC, NY). All chemicals were purchased from Fisher Scientific (Hampton, NH) and were of reagent grade or higher quality.

**Instrument configuration and experimental methods**

The NanoSight (Model LM20, NanoSight Ltd. Amesbury, United Kingdom) instrument was equipped with a 405 nm laser light source (Model # LM12B). NTA 2.2 software was used to collect and analyze videos of particle tracks. For each experiment, unless otherwise noted, 500 µL samples were drawn into 1 mL silicone oil-free plastic syringes (National Scientific Company, TN), which were used for sample injection into the instrument sample chamber.
In our studies with the NanoSight Model LM20 instrument, data were acquired using the video capture mode, which records videos of the sample for post hoc analysis. In this mode, the user defines the length of video acquisition time from 10 to 215 seconds, depending on the observed particle concentration.

The current study was performed on the older LM20 system, which is no longer marketed but still used in many labs. Also, results obtained with the LM20 system should be directly applicable to any of the current NTA systems available, including the NanoSight LM10, NS300, and NS500 systems.

**Evaluation of complete tracking number**

The raw number of particle detected during analysis is referred to as complete tracking number. To initially investigate the importance of this parameter, a suspension was prepared by diluting the 100 nm latex beads size standard with MilliQ water to achieve a desired number of particles per video frame (>10). For complete tracking number evaluation, the sample was measured with video record mode, and analysis was stopped when desired complete tracking numbers were obtained: 50, 100, 200, 400, and 800. During analysis, the detection threshold was set at 8, minimum expected particle size was set at 100 nm.

Also, blur and minimum track length were set at auto. Blur is a smoothing function used to remove scattering noise around and within a particle. A large value may reduce the noise but also cause smaller particles to be overlooked. Minimum track length determines the minimum number of steps that a particle must take before its size value can be included in the final result. A large minimum track length value will omit small fast-moving particles and cause bias towards a larger than expected size distribution**162. In our evaluations, we used
the auto setting for those two parameters because we found it to be sufficient for the samples we studied.

Next, the effect of complete tracking number was studied with HSA particles. To generate protein particles, a HSA solution (30 mg/mL) was agitated in a 15 mL polypropylene conical tube at 300 rpm with an orbital shaker for 24 hours at room temperature. The agitated HSA solution was then diluted to a protein concentration of 1 mg/mL with phosphate buffer. The sample was evaluated with complete tracking numbers of 50, 100, 200, 400, 800, and 1200. During analysis, the detection threshold was set at 6, minimum expected particle size was set at 50 nm and blur size and minimum track length were set at auto.

**Evaluation of minimum expected particle size (MES)**

The effect of choice of MES was investigated with gold nanoparticles, latex beads and HSA particles. An aliquot (3 µL) of the original stock solution of 50 nm gold particles was diluted with 1 mL MilliQ water. The 100 nm latex bead stock solution was diluted 10^5-fold with MilliQ water. To prepare HSA samples with particles, 0.1 mg/mL HSA was agitated in a 15 mL polypropylene conical tube at 300 rpm with an orbital shaker for 24 hours at room temperature. Videos were recorded and analyzed with MES settings of 30, 50, 80, 100, 150, 200, 250, 300 and 400 nm. For evaluation of all samples in the study of effects of MES, other instrument parameters settings were: detection threshold of 8; and blur size and minimum track length were set at auto.
Effect of pump flow rate

A syringe pump (Model# NE-1000, New Era Pump Systems Inc., NY) was connected to the NanoSight sample chamber. Flow rates of 0, 3, 5, 7, 10, and 15 µL/min were tested. The 100 nm latex bead standard was diluted with sufficient MilliQ water to generate particle concentration of ~10 particles/video frame. For data analysis, the detection threshold was set at 6, MES was set at 100 nm, and blur size and minimum track length were set at auto. Three independent sample replicates were measured at each of the different flow rates. To evaluate the combined effects of MES and flow rate, the 100 nm latex bead standard solution was diluted with MilliQ water and analyzed with different MES settings at different flow rates, with three independent samples measured each time.

Evaluation of effects of protein concentration

To evaluate the effects of protein concentration, HSA solutions were prepared at concentrations of 1, 2, 4, 10, and 20 mg/mL. The solutions were centrifuged at 112,000×g for 8 hours at 4ºC to remove particles (particle-free solutions). In one study, a 5 µL aliquot of the 50 nm gold particle suspension was pipetted into 1 mL MilliQ water or into 1 mL of each of the 5 different HSA solutions. Independent triplicate samples were prepared by pipetting the aliquot of gold particle suspension into three separate tubes of water or each HSA solution.

To study the effects of protein concentration on nanoparticle sizing and counting for latex beads, a stock suspension was prepared by diluting 1 µL of 100 nm latex bead standard into 1 mL of phosphate buffer (0.1 M sodium phosphate, pH 7.09). A 5 µL aliquot of this suspension was pipetted into 1 mL of the particle-free HSA solutions at the different protein concentrations. Five replicates were prepared and analyzed for each protein concentration.
To study effects of protein concentration on the characterization of protein particles, HSA protein aggregates were prepared by end-over-end agitation of 1 mg/mL HSA solution in a 15 mL polypropylene conical tube. The tube was placed on a multipurpose rotator (Model 151, Scientific Industries Inc., NY) and rotated between 40-60 rpm overnight at room temperature. Then 50 µL of agitated 1 mg/mL HSA solution was spiked into 950 µL of particle-free HSA solutions at the different protein concentration. Five replicates were prepared and analyzed for each protein concentration.

For NTA data analysis, the detection threshold was set at 6, and blur size and minimum track length were set at auto. And the MES was set at 50 nm for 50 nm gold particles, 100 nm for latex beads and 50 nm for HSA protein aggregates.

**Effect of serial dilution of aggregated HSA solutions**

HSA solutions with 1, 2, 4, 10, and 20 mg/mL protein concentration solutions were agitated end-over-end with a multipurpose rotator (Model 151, Scientific Industries Inc., NY) between 40-60 rpm overnight at room temperature. The solutions were then filtered with a 220 nm PES syringe filter (Millex-GP, Millipore Corp., MA) to remove larger particles. To prepare particle-free solutions, a non-agitated solution of HSA at the same protein concentrations noted above was centrifuged at 112,000×g for 3 hours at 20ºC and then filtered with a 220 nm PES syringe filter. Agitated solutions were serially diluted (2, 4, 8, and 16-fold) into centrifuged particle-free HSA solutions of corresponding protein concentrations. Triplicates were prepared by pipetting the protein aggregates into three separate tubes for solutions at each protein concentration. For NTA data analysis, the
detection threshold was set at 6, the MES was set at 50 nm, and blur size and minimum track length were set at auto.

**Statistical analysis**

The unpaired t-test was used for statistical evaluation, and p < 0.05 was considered statistically significant.

**Results and Discussion**

**Evaluation of complete tracking number**

During an NTA measurement, each particle’s movement is tracked in consecutive video frames. The software calculates the particle’s diffusion coefficient and size based upon the trace of the moving particle. The total count of particles depends on the complete tracking number, which in turn is determined by the number of video frames analyzed and the concentration of particles in the sample. The concentration of particles in a sample is determined by first dividing the complete tracking number by the number video frames analyzed. This value of particles per video frame is then converted into particles per ml by multiplying by approximately 15,000,000. The multiplication factor is dictated by the actual sample volume probed by the laser, which can vary slightly from sample to sample. In our preliminary studies, we determined that optimal sample particle concentrations for the NanoSight instrument were between $10^8$ and $10^9$ particles/mL.

We first investigated the effect of number of complete tracks in the analysis on particle size distribution and counts. For this study, we analyzed the particle size and
concentration with different complete tracking numbers for 100 nm latex beads and HSA protein aggregates.

With latex 100 nm beads, at complete track values of 50 and 100, there was peak splitting in the size distribution (Figure 27A). The cause of this splitting is not known. For 200 or greater number of complete tracks, as expected the size distributions obtained for 100 nm latex beads were single peaks and centered around 100 nm. Neither the mean particle size measured nor the particle concentrations varied significantly as a function of the complete tracking numbers investigated (Figure 27C,D).

The size distribution of HSA protein particles obtained with complete tracking numbers of less than 400 were very noisy with several peaks below 50 nm (Figure 27B). The instrument is not capable of measuring protein particles smaller than about 50 nm. Thus, the peaks below 50 nm were artifacts. With higher tracking numbers the size distributions were substantially smoother and large peaks below 50 nm were not present. Neither the mean particle size measured nor the particle concentrations varied significantly as a function of the complete tracking numbers investigated (Figure 27C,D).

Thus, for both latex beads and HSA particles the value of complete tracking number only minimally affected the mean size measured or the particle concentrations, but a higher complete tracking number resulted in a smoother particle size distribution. Also, for the protein particles, higher tracking numbers resulted in a decrease in the artifact peaks below 50 nm. We assume that the smoother, less noisy distributions are more representative of the actual particle size distribution and thus recommend that complete tracking numbers of greater than 400 be used for analysis of nanoparticles in protein samples.
Figure 27. A. Size distributions of 100 nm latex beads. B. Size distributions of protein particles in 1 mg/mL HSA with different complete tracking numbers in video-capture mode. C. Particle sizes of 100 nm latex beads and protein particles in 1 mg/mL HSA measured by NTA with different complete tracking numbers. D. Particle concentrations of 100 nm latex beads and protein particles in 1 mg/mL HSA measured by NTA with different complete tracking numbers. Video analysis was stopped at different complete tracking numbers as shown in the figure.

**Evaluation of minimum expected particle size (MES)**

NTA uses the Brownian motion of individual particles to calculate a size distribution of nanoparticles the sample. The size of the search area where the NanoSight instrument will expect a particle in the subsequent video frame is set using the MES parameter (NanoSight
LM20 instrument manual). As the MES increases, the search area becomes smaller since larger particles have shorter diffusion distances in a given time period.

We first investigated the effects of the MES parameter with 50 nm gold particles and 100 nm latex beads. For the 50 nm gold particles, the mean size measured was about 50 nm when the MES was set at 30 nm to 100 nm, but when the MES was set at 150 nm and greater, the mean size increased progressively increased (Figure 28A). Similarly, with 100 nm latex beads, when the MES was set at 150 nm or smaller, the size distribution was centered around 100 nm, and at higher values of MES the size distribution progressively increased (Figure 28B).

With 50 nm gold particles, the particle concentrations obtained had largest values when the MES was set near the particles’ expected size of 50 nm. With the 100 nm latex beads the effects of MES on particle concentration were similar, but the greatest concentration was noted with the 100 nm MES setting. Although sample replicates were not studied in these experiments, it should be noted that these same trends were observed in multiple repeats of the experiments.

Next, we analyzed the size distribution and particle counts of 0.1 mg/mL agitated HSA solutions (Figure 28 C and D). For the HSA aggregates, a higher MES setting also made the size distribution shift up, and the particle counts also changed with the MES values.

These effects of changing MES can be explained by the principle on which the particle tracking is based. When the NanoSight software tracks an individual particle, it creates a search area around the particle where the software will search for its location in the next video frame. However, if the search areas of two particles overlap, both particles will be excluded from the final analysis. For small MES values (larger search area), particle search
areas have a greater chance to overlap resulting in exclusion of both particles from the analysis. This effect will be more prevalent when particle concentration is high. Conversely, when MES is set at a value larger than the actual particle size, the search area is reduced. Particles can diffuse out of the search area between video frames, resulting in fewer tracked particles and a decrease in the observed particle concentration. These observations are in agreement with a prior assessment\textsuperscript{139}, in that an overestimation of MES results in reduction in analysis of small particles whereas too low a MES setting causes overall reduction in particles analyzed.

Newer versions of software (NTA 2.3 and newer) than the version that we had available on our instrument provide for an automatic MES setting. With this approach during analysis of a given sample, the software determines the sizes of particles in the first 100 video frames during and uses a proprietary algorithm to establish the MES setting. With this additional feature in the software, the inaccuracy in nanoparticle characterizations caused by incorrect MES settings might be mitigated. Considering the sizes of protein particles in a given protein sample are generally unknown, an automatic MES setting is recommended for measuring protein samples. For the current study, the auto function was not yet available. So we used a MES value of 50 for further characterizations of protein samples, because 50 nm was the minimum size at which particles could be reliably characterized.
Figure 28. A. Size distributions of 50 nm gold particles using different MES settings during analysis. 3 μL original stock solution was diluted with 1 mL Millipore water. The same video was analyzed with different MES settings. B. Size distributions of 100 nm latex beads with different MES settings used during analysis. 100 nm latex beads were diluted 10^2-fold with Millipore water. The same video was analyzed with different MES settings. C. Size distributions of 0.1 mg/mL agitated HSA solution with different MES settings used during analysis. D. Determined particle concentrations of 50 nm gold particles, 100 nm latex beads and HSA aggregates from above using different MES settings for the analysis of the same video.

**Evaluation of pump flow rate**

Because of the small sample volume analyzed, we explored the use of a syringe pump to increase this volume and investigated the effects of flow rate. When a flow is applied to the sample, for the individual particle size calculations the NTA software will accommodate the collective drift. For the initial study, the size distribution and particle concentrations of
100 nm latex bead samples were analyzed under different flow rates, with all other instruments settings held constant. The mean particle sizes did not change significantly until a flow rate of 10 µL/min (Figure 29A-E). The mean sizes measured were 117±4, 115±2, 111±1 and 93±1 nm for flow rates of 0, 5, 7, and 10 µL/min, respectively. In addition, a decrease in particle concentration was only observed when flow rates of 10 µL/min and above were used (Figure 29F). Also, the number of particles included in the distributions generally decreased with increased flow rate, with a significant loss in tracked particles occurring above a flow rate of 7 µL/min. Total particles tracked at 10 and 15 µL/min were below the threshold described above of 200 total particles tracked.

Figure 29. A.-E. Size distributions of 100 nm latex beads at different flow rates. 100 nm latex beads were diluted in Millipore water to a concentration of about 10 particles/frame. Error bars are the standard deviation of three measurements of the sample. F. Resulting concentrations of 100 nm latex beads at the different flow rates from above. Error bar stands for standard deviation of the three measurements.
Overall, it can be concluded that the lower flow rates (≤ 7 µL/min) did not significantly affect the size distributions, particle concentrations or complete tracking numbers. Because of the small sample view area, a low flow rate is recommended for increasing sampling volume, and, in turn, to obtain a more accurate representation of the total particles within a sample, especially when the particles per video frame are fewer than 10. At high flow rates (above 7 µL/min) the particle characterization can be significantly altered. Particles might be pushed out of the search area and excluded from the analysis. As a consequence the particles would not be tracked accurately, resulting in decreases in particle counts and complete tracking numbers, and inaccurate particle sizes.

The combined effects of flow rate and MES setting on particle characterization were next investigated. For flow rates greater than 7 µL/min and at suboptimal MES settings (> 100 nm) there were substantial reductions in the measured concentrations of 100 nm latex beads (Figure 30). In addition, the measured mean particle sizes increased with increased MES setting for flow rates ≤ 7 µL/min (data not shown). For flow rates ≥ 10 µL/min, the majority of particles could not be tracked accurately. At higher MES settings (≥ 200 nm), the search area around a given particle in a frame decreased, causing particles under high flow rates to more easily leave the search area. Overall, it was found that slower flow rates had a higher tolerance to suboptimal MES settings. It is therefore advised to run a sample with lower flow rate (≤7 µL/min) in order to mitigate potential errors associated with an incorrect MES setting.
Figure 30. Combined effects of flow rate and MES settings on particle concentrations of 100 nm latex beads detected by NTA. Error bars are the standard deviation of three measurements.

**Evaluation of high concentration protein formulation**

Scattering of the laser light by high concentration protein samples may hinder accuracy in determining size and concentration of nanoparticles with NTA\textsuperscript{69,161}. To characterize the effects of high concentration protein formulations, we examined the particle size distributions and counts of various particle species added to solutions of increasing protein concentration. Identical quantities of gold particles, latex beads or protein aggregates were spiked into particle-free HSA solutions, which had been centrifuged to remove pre-existing particles. As shown in Figure 31, a reduction in the contrast was seen between 50 nm gold particles and the 10 mg/mL HSA background solution compared with the contrast observed with the H\textsubscript{2}O background. This reduction in contrast was also observed with 100 nm latex beads and HSA protein aggregates when placed into a 20 mg/mL background HSA solutions versus a 1 mg/mL background HSA solution (Figure 31). This loss of contrast caused problems for particle characterization. For example, the height of the peak
Figure 31. Screenshots of recorded videos demonstrate the contrast between particles and their background. The left panels show 50 nm gold particles in H2O and 10 mg/mL clean HSA solutions. The middle panels show 100 nm latex beads in 1 mg/mL and 20 mg/mL clean HSA solutions. The right panels show the HSA protein aggregates in 1 mg/mL and 20 mg/mL clean HSA solutions. Centrifugation was used to remove aggregates from HSA solutions prior to spiking by the indicated particles.

corresponding to the 50 nm gold particles decreased as HSA solution concentration increased, resulting in decreased particle counts (Figure 32A-F). This effect was also observed with 100 nm latex beads (Figure 33) and HSA aggregates (Figure 34). Notably, for HSA aggregates, a significant decrease in particle counts was detected at background protein concentrations as low as 4 mg/mL.

The high protein concentration background affected characterization of protein aggregates more significantly than the gold or latex particles, likely due to the lower refractive index of the protein particles compared to that for the gold or latex particles. As demonstrated in a recent NTA evaluation, a weak scattering intensity of some protein aggregates likely contributed to the under-estimation of counts for fibrillar protein aggregates.
as well as for other protein aggregates with extended, non-spherical morphologies. Thus, light scattered by high concentration protein samples might limit accurate quantification of nanoparticles in a sample when using NTA.

However, even at high background protein concentrations, particle sizing accuracy was not significantly affected (Figures 32-34). Because NanoSight calculates diffusion coefficients directly from the real time Brownian movement of individual particles, as long as there is sufficient contrast between particles and their background, they can be accurately tracked and their size measured by the NanoSight detector and software.

For high concentration protein formulations, dilution may be an option to reduce the background light scattering. However, dilution could result into disassembly of particles and other aggregates. To test the effects of dilution on nanoparticle counts, first aliquots of particle-containing, agitated HSA solutions, prepared from HSA solutions at a range of protein concentrations, were mixed with particle-free HSA solutions of the same protein concentrations. As can be seen in Figure 35, at all protein concentrations tested, there was a linear correlation between particle counts obtained from NTA and the reciprocal of the dilution factor. These data indicate that NTA can provide reliable relative nanoparticle counts for samples of a given protein with the same protein concentration and solution conditions. For such a case, the video capture and analysis settings must remain identical among all samples to provide reliable particle counts.
Figure 32. A.-E. Size distributions of 50 nm gold particles spiked in clean HSA solutions. Error bars are the standard deviation of 3 independent samples. F. Determined concentrations of 50nm gold particles spiked in different clean HSA solutions. Error bars are the standard deviation of 3 independent samples.

Figure 33. A.-E. Size distributions of 100 nm latex beads spiked in clean HSA solutions. Error bars are the standard deviation of 5 independent samples. F. Determined concentrations of 100 nm latex beads spiked in different clean HSA solutions. Error bars are the standard deviation of 5 independent samples.
Figure 34. A.-E. Size distributions of HSA aggregates spiked in clean HSA solutions of increasing concentrations. Error bars are the standard deviation of 5 independent samples. F. Determined concentrations of HSA aggregates spiked in different clean HSA solutions. Error bars are the standard deviation of 5 independent samples.

Figure 35. Correlation of detected particle concentration to the dilution factors. Error bars are the standard deviation of 3 sample measurements. Samples were serially diluted two-fold from the initial samples.
In contrast to the results obtained with nanoparticle-containing solutions of HSA were diluted into particle-free solutions at the same protein concentration, when a particle-containing HSA was diluted into protein-free buffer, there was not a linear correlation between expected particle concentrations and actual particle counts (data shown). Overall, there was not any clear relationship between the particles counts obtained with NTA and the dilution factor. This result suggests that dilution in buffer resulted in particle disassembly, but that the magnitude of this effect was not proportional to extent of dilution.

We therefore suggest that for a given protein and solution conditions (e.g., protein concentration, pH, ionic strength, excipients) relative nanoparticle concentrations be compared reliably between samples. However, prior to making these comparisons, to assure that relative counts are linear with particle concentration, counts should be measured after serial dilution of a particle-containing sample into particle-free solutions at the same protein concentration. Also, for these measurements the instrument setting should be the same for each sample analyzed. Such assessments of relative levels of nanoparticles may include comparing nanoparticle concentrations in different lots of a therapeutic protein product and determination of effects of accelerated degradation conditions (e.g., agitation or heating) on the rate of nanoparticle formation in a given protein formulation.

**Conclusion**

It has been established that NTA is an effective method to analyze nanoparticles and protein aggregates. In this work, we systematically explored how changes in the NTA user settings affected measured particle size distributions and concentrations. Settings such as the complete tracking numbers, minimum expected particle size, and pump flow rate can have significant effects on the resulting particle counts and size distributions. Complete track
numbers of at least 400 are suggested for protein particle analysis. A correct setting for the MES is crucial for correct size distributions of particles, but use of the automatic setting feature in the newer software versions may mitigate this concern. Caution must be taken when applying a flow to a sample as flow rate can affect both the observed particle distributions and the determined concentrations. Furthermore, with high protein concentration formulations for therapeutic proteins, high protein background concentrations significantly affect particle counts, but particle are sized correctly. In this situation dilution into particle-free protein solution at the same concentration may allow for relative comparisons to be made in particle concentrations. Overall, with appropriate insight into effects of sample and instrument parameters, NTA provides an effective means of obtaining valuable nanoparticle concentration and size distribution data for therapeutic protein samples.
CHAPTER V
REDUCED SUBVISIBLE PARTICLE FORMATION IN LYOPHILIZED INTRAVENOUS IMMUNOGLOBULIN FORMULATIONS CONTAINING POLYSORBATE 20

Introduction

Traditionally, both aqueous and solid dosage forms have been employed for therapeutic protein formulations. Although development of lyophilized formulations tends to be more time-consuming and expensive than the development of liquid formulations, lyophilized formulations are often chosen for proteins whose instability in aqueous formulations restricts their shelf life\textsuperscript{163,164}. Out of over 140 therapeutic protein drugs in the market in 2012, 64 were marketed as lyophilized products\textsuperscript{74}.

Instabilities may still arise in lyophilized protein formulations. Protein degradation may derive from various stresses that protein molecules are exposed to during lyophilization\textsuperscript{75,165-170}. During freezing, proteins may be subject to cold denaturation\textsuperscript{171}, ice-water interfaces\textsuperscript{172}, high salt concentrations\textsuperscript{75} and freezing-induced pH shifts\textsuperscript{173}. Drying can cause unfolding of protein molecules as water is removed from their surfaces\textsuperscript{75,174}. During long-term storage, in spite of the low translational mobility of protein molecules in glassy solids, local movement or fast dynamics may still result in chemical and physical degradation\textsuperscript{175}. If unfolded or conformationally-perturbed protein molecules are formed during lyophilization or storage in the dried state, aggregation may occur upon reconstitution\textsuperscript{176}.

In addition, protein molecules could adsorb to, or be kinetically trapped at, the ice-water interface\textsuperscript{169,172,177-179}. After sublimation of ice crystals during primary drying, these
protein molecules would be found at the remaining glassy solid-air interface\textsuperscript{76,77,169}. In two recent studies, the extent of damage (oxidation, deamidation and aggregation) to two therapeutic proteins observed after long-term storage correlated directly with the amount of protein exposed on the glassy solid-air interface\textsuperscript{76,77}. Annealing the frozen formulations prior to drying reduced the specific surface areas of the glassy solid and the fraction of protein exposed at the solid-air interface, which in turn significantly reduced degradation of the protein\textsuperscript{76}.

The first goal of the current study was to test the hypothesis that conditions that result in lower amounts of a model antibody therapeutic on the surface of glassy solids would in turn reduce aggregation and particle formation rates during storage. Strategies can be employed on both lyophilization cycle and formulation components to vary the amount of protein on the surface. For example, a pre-drying annealing step during lyophilization can promote the growth of large ice crystals at the expense of the smaller ones and subsequently reduce the total ice-water interfacial surface area\textsuperscript{174,180}. Non-ionic surfactants such as polysorbate 20 (ps20) could potentially compete with protein molecules for ice-water interface adsorption\textsuperscript{169} and thus reduce the amount of protein per unit surface area.

A second goal of the study was to characterize the subvisible particle levels in reconstituted lyophilized formulations. Subvisible particle characterization is particularly valuable in detecting trace amount of protein aggregates\textsuperscript{70-72}. Particle counting methods provide a much more sensitive means by which to detect and quantify protein aggregation than traditional methods such as size exclusion chromatography (SEC). In two recent examples, particle formation was observed after mAbs were subjected to freeze-thawing\textsuperscript{70} or pumping\textsuperscript{71}, but no loss of monomer or formation of soluble aggregates could be detected.
with SEC. Limited studies have been performed on particle levels in reconstituted
lyophilized protein formulations. A single published study found that the addition of sucrose
and sorbitol significantly reduced the amount of subvisible particles observed in reconstituted
lyophilized IgG1 formulations\textsuperscript{73}.

In this study, aggregation and particle formation of intravenous immunoglobulin
(IVIG) was compared in formulations with buffer alone, ps 20, trehalose or trehalose plus
ps20. The specific surface areas, the surface protein fraction and the secondary structure of
the lyophilized formulations were measured after lyophilization. To evaluate the effect of
ice-water interfacial surface area, a post-freezing, pre-drying annealing step was included in
the lyophilization cycle, and results were compared to those obtained when a cycle without
an annealing step was used. After reconstitution, protein aggregation was monitored by SEC
and subvisible particle analysis with the flow imaging microscopy instrument and the
resonant mass measurement (RMM) instrument. Protein aggregation was also measured after
reconstitution of dried formulations that had been stored at 50°C. Finally, the potential
protective effect of ps20 during reconstitution was studied by analyzing aggregation after
reconstituting of lyophilized IVIG formulations with water or with ps20-containing solutions.

Materials and Methods

Materials

IVIG (Gammagard\textsuperscript{®} Liquid, Baxter HealthCare Corp) was purchased from the
University of Colorado at Boulder’s Wardenburg Pharmacy and used before the expiration
date. High purity $\alpha,\alpha$-trehalose dihydrate was purchased from Pfanstiehl (Waukegan, IL).
All other chemicals were purchased from Fisher Scientific (Hampton, NH) and were of
reagent grade or higher quality. Lyophilization glass vials (5 mL, catalog # 68000318) and butyl rubber stoppers (catalog # 19560042) were purchased from West Pharmaceutical Services (Linville, PA).

**Sample preparation**

100 mg/mL IVIG in the original formulation buffer (250 mM glycine, pH 5) was dialyzed at 4°C against 2 mM sodium citrate solution at pH 4.6, with three changes of the external solution; at ~1000 times exchange volume each time. The dialyzed IVIG was then prepared at 1 mg/mL concentration in four different formulations: 2 mM sodium citrate; 2 mM sodium citrate and 0.03% ps20 (w/v); 2 mM sodium citrate and 5% (w/v) trehalose; 2 mM sodium citrate, 5% (w/v) trehalose and 0.03% ps20 (w/v). All formulations had a pH value of 4.6. To remove protein particles prior to experiments, the samples were centrifuged at 12,000 × g for 3 hours with a sw28 rotor in a Beckman Optima LE-80k ultracentrifuge and then filtered through a 220 nm PES low protein-binding syringe filter (Millipore, Billerica, MA). All formulations were prepared in at least 3 replicate vials for each sampling time point.

**Protein lyophilization**

Samples (1mL) were pipetted into 5 mL glass vials, and then the vials were loaded onto the shelves of a FTS Lyostar 3 lyophilizer at room temperature. During the standard lyophilization cycle, the shelf temperature was initially set at 10°C, and samples were allowed for equilibration for 1 h. Then shelf temperature was reduced to -5°C at 1°C/min and held for 20 min. A second ramp of shelf temperature to -45°C at 1.3°C/min was then started.
After holding samples at -45°C for 400 min, primary drying was performed by ramping the shelf temperature to -20°C at 2.5°C/min, setting chamber pressure at 70 mTorr and holding samples under this condition for 1400 min. Then the shelf temperature was elevated to 33°C at 0.3°C/min to initiate secondary drying, and samples were maintained at 33°C and 70 mTorr for 4 h. After freeze-drying, all vials were back filled with dry nitrogen and sealed with stoppers in the chamber.

To evaluate the effect of pre-drying annealing, an annealing step was added before the primary drying of the standard lyophilization cycle. After holding the samples at -45°C for 400 min, the shelf temperature was increased to -5°C over 30 min and maintained at -5°C for 6 h. Then the shelf temperature was cooled down to -45°C at 1.3°C/min and held at this temperature for 6 h. Then the primary drying was initiated as described in the standard lyophilization cycle.

**Long-term storage**

After lyophilization, one set of samples was analyzed immediately. Other samples were stored at 50°C incubator and removed from for analysis at weeks 2, 4, 8 and 16.

**Reconstitution of lyophilized formulation**

MilliQ water was used to rehydrate the lyophilized cake. Aliqouts of 950 µL and 1 mL milliQ water were pipetted into vials with or without trehalose, respectively. Then samples were allowed to sit on the lab bench for at least 30 min before analysis.

To evaluate the effect of ps20 upon reconstitution, 5% trehalose formulation was reconstituted with 950 µL MilliQ water containing 0.03% ps20.
Infrared spectroscopic (IR) analysis of IVIG secondary structure

In a nitrogen dry box, 500 mg KBr powder was mixed thoroughly with ~ 13 mg lyophilized cake (containing ~0.25 mg protein), ground with a pestle and mortar and then transferred to a stainless steel die (13 mm internal diameter). The die was pressed with hydraulic press (Carver Model “C”, Wabash, IN) to form a pellet. The pellet was then analyzed with a Proto-3DS infrared spectrometer (Biotools, FL). For comparison, the spectrum of IVIG (20 mg/ml) in aqueous solution was also analyzed. Under single-beam transmission mode, 200 interferograms from 4000-1000 cm⁻¹ were collected and averaged. The spectra were processed with GRAMS® software for water vapor subtraction, second-derivative calculation, baseline correction and area normalization, as previously described.  

Size exclusion chromatography (SEC)

Reconstituted samples were placed into Eppendorf tubes and centrifuged at 14,100 x g for 10 minutes. The supernatants were then analyzed by SEC. A Tosoh (San Francisco, CA) TSK G3000SWXL column (7.8×300 mm²) and an Agilent 1100 (Santa Clara, CA) system were used for quantifying monomer and protein aggregate levels. The mobile phase was 100 mM sodium phosphate, 100 mM sodium sulfate and 0.05% w/v NaN₃ at pH 7, and the flow rate was 1 mL/min.

Flow imaging microscopy

A FlowCAM® instrument was used to measure particle size and concentration in the size range of ≥ 1 µm. A 0.5 mL sample was loaded into the sample holder and 0.3 mL was analyzed at flow rate of 0.08 mL/min, after priming. Both dark and white pixel thresholds were
set at 15. A customized optical filter was used to remove air bubbles and the remaining particles were considered as protein particles. The total mass of protein in particles with size \( \geq 2 \) µm was estimated using the ellipsoid-volume method described by Kalonia et al\(^{72}\).

**Resonant mass measurement (RMM)**

Resonant mass measurement (RMM) was used to determine size and concentration for particles in the size range of \( \sim 0.2 \) to \( 5 \) µm. Densities of air bubbles and protein particles were set at \( 0.00123 \) g/cm\(^3\) and \( 1.4 \) g/cm\(^3\) respectively. Samples (~150 µL) were loaded into the instrument, and samples were analyzed either for total particle number of 100 or 10 mins, whichever came first. The total mass of protein particles in each sample was also determined.

**Surface area measurement**

The specific surface area (SSA) was measured with a Quantachrome Autosorb-1 (Boynton Beach, Florida) instrument. Sample preparation and experimental procedure were the same as described previously\(^{76}\).

**Electron spectroscopy for chemical analysis (ESCA)**

ESCA was used to analyze the surface elemental composition of the freeze-dried cake as previously described\(^{21}\).

**Calculation of surface fraction of IVIG**

IVIG includes human IgG1, 2, 3, 4 and each species is polyclonal, and thus there is no defined sequence for each of the protein molecule. To estimate the theoretical
composition of the protein, we used the amino acid sequence of a known intact human IgG1, which on a C-molar basis has an atomic composition of CH\textsubscript{1.546}N\textsubscript{0.273}O\textsubscript{0.308}S\textsubscript{0.0064}. Within the formulations studied here, the total mass of cake is around 52 mg and the N\% in the bulk is 0.33\%. Assuming that the ESCA technique reports atomic composition in a surface layer that is approximately 100 Å deep and the density of the cake is around 1.1 g/cm\textsuperscript{3}, the mass of protein on the surface can be calculated as previously described as below:\textsuperscript{76}

\[
m\textsubscript{surface}/m\textsubscript{total} = SSA \times l/\rho \times N\% / 17.0\% \quad \text{Equation 4}
\]

where \(m\textsubscript{surface}\) is the mass of protein on the surface, the \(m\textsubscript{total}\) (52 mg) is total mass of the lyophilized cake per vial, SSA is specific surface area per gram cake (m\textsuperscript{2}/g), \(l\) (10 nm) is the surface thickness measured by ESCA, and \(\rho\) (1.1 g/cm\textsuperscript{3}) is the density of the cake. N\% is the surface N percentage and the 17\% is the theoretical overall N content of IVIG.

**Results and Discussion**

**Protein structure after lyophilization**

Second-derivative spectra of samples were obtained immediately after lyophilization. For all of the formulations and lyophilization conditions that were examined, the protein spectra in the amide-1 region were essentially identical, but bands were shifted somewhat compared to those in the spectrum for the native protein in aqueous solution (Figure 36). Furthermore, after 16-week storage at 50°C, no spectral differences were found for the protein in any of the formulations (data not shown).
SSA, surface N percentage and amount of protein on the surface

BET krypton adsorption was used to measure the SSA of the lyophilized formulations (Table 2). Annealing reduced the SSA to half compared with that obtained with the standard lyophilization procedure. This effect can be ascribed to the increased in ice crystal sizes caused by Ostwald ripening during annealing\textsuperscript{174,180,183}. In contrast, with either lyophilization procedure the SSA was unaffected by the inclusion of ps20 in the formulation.

Figure 36. Secondary structure of IVIG with different formulations and lyophilization procedures after lyophilization characterized by FTIR. A. Comparison between IVIG aqueous sample and IVIG lyophilized samples by standard lyophilization procedure. B. Comparison between IVIG lyophilized samples by standard lyophilization and annealing procedures. Each spectrum is the average of 3 independent samples.
In addition, ESCA measurement were performed to obtain the N% at the surface of lyophilized formulation (Table 2). If the IgG molecules within the sample were homogeneously distributed throughout the solid cake, N% was expected to be approximately 0.33%. In all the formulations studied, however, the N% measured for the outmost 100 Å of the dried cake were 2- or 3-fold higher, which suggested that a higher amount of protein per surface area were accumulated at the surface than in the bulk. Surprisingly, annealing was found to increase the surface N% of IVIG, whereas addition of ps20 had no significant effect (Table 2). Overall, the total amount of protein adsorbed onto the surface of the dried cake was about the same for both formulations and was not affected substantially by the lyophilization process.

Table 2. SSA and surface fraction measurement of IVIG with different formulations and lyophilization procedures after lyophilization. Each value represents the mean and standard deviation of 3 independent samples.

<table>
<thead>
<tr>
<th>formulation</th>
<th>Standard Lyo</th>
<th>Annealing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>trehalose</td>
<td>trehalose+ps20</td>
</tr>
<tr>
<td>SSA(m^2/g)</td>
<td>1.34±0.10</td>
<td>0.69±0.04</td>
</tr>
<tr>
<td>N(%)</td>
<td>0.63±0.15</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>Surface Fraction of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVIG(%)</td>
<td>2.9±0.69</td>
<td>2.8±0.46</td>
</tr>
</tbody>
</table>

It has been suggested that proteins might adsorb to the ice-water interface as a result of the amphiphilic nature of protein molecules and the relative hydrophobicity of the interface. If this mechanism were dominant, then PS20 would be expected to compete with protein molecules for adsorption, thus reducing the amount of protein found at the interface. However, this effect was not observed in our studies. Another mechanism by
which protein molecules might be accumulated in the surface layer is due to the diffusion-limited transport of protein molecules away from the freezing front of growing ice crystals. At the sub-zero temperatures and high viscosities found within the frozen formulation, the extension of ice crystal surfaces could be much faster than the diffusion rate of protein molecules. As a result, protein molecules may be concentrated in the immediate region of the ice front and entrapped there as ice growth progresses\(^\text{185}\). During annealing, additional growth of ice crystals due to Ostwald ripening might have accentuated this protein accumulation behavior.

In an earlier study that examined the effects of annealing on frozen formulations of human growth hormone during lyophilization, annealing decreased the SSA by \(~50\%) but did not affect the surface N\(^%\)\(^\text{76}\). The greater size and resulting reduced diffusivity of IVIG molecules (ca. 150 kD) as compared to growth hormone molecules (ca. 22 kD) might account for the different levels of surface accumulation during annealing.

**Protein aggregation characterized by SEC**

In samples of IVIG measured prior to lyophilization, 91% monomer and 9% dimer were detected. In all lyophilized formulations, including formulations containing only buffer and IVIG, monomer and dimer levels were largely unaffected by lyophilization and immediate reconstitution (Figure 37 A-D). Thus, at the resolution of degradation provided by SEC, IVIG was remarkably stable during freezing, drying and rehydration. For many other proteins, including some antibodies, lyophilization in the absence of stabilizing excipients results in measurable aggregation by SEC\(^\text{176,186,187}\).
During long-term storage of formulations without trehalose, the level of insoluble protein aggregates and high molecular weight soluble oligomers quickly increased, with a concomitant decrease in monomer level. In contrast, formulations with trehalose (either with

Figure 37. Protein aggregation of IVIG with standard lyophilization procedure characterized by SEC. The formulations are: buffer alone (blue circle), buffer plus PS20 (red square), buffer plus trehalose (green upward triangle) and buffer plus trehalose plus PS20 (purple downward triangle). The percentage of each protein species was calculated: A. Monomer peak, B. Insoluble aggregates, C. Dimer peak, and D. High molecular weight species peak. The percentages of each protein species before lyophilization are the initial data points in each panel. The vertical dash line in each panel is drawn to distinguish data points from pre-lyophilization data. Each data point and error bar denote the average and standard deviation of 3 independent replicates respectively. For some data points, error bars are not visible because they are smaller than the symbols. Lines were drawn to lead the eye.
or without ps20) stabilized IVIG through 8 weeks of storage at 50°C, and only slight
decreases in monomer and increases in insoluble aggregates levels were observed at week 16.
Similarly, with SEC characterization, no effects of pre-drying annealing on protein
aggregation levels were observed immediately after lyophilization and reconstitution, or after
long-term storage and reconstitution (Figure 38). Trehalose provided such robust
stabilization that any additional protection provided by PS20 or annealing was masked and
could not be detected based on SEC analysis.

**Protein degradation characterized by particle analysis**

Counting of subvisible particles provides a much more sensitive tool for detection and
quantification of protein aggregates than SEC. In lyophilized and reconstituted IVIG
samples, more microparticles (of size ≥ 1 µm) were observed in the formulations with
trehalose than in the formulation containing buffer alone (Figure 39A). We estimated the
total mass of particles of size ≥ 2 µm based on a recently proposed ellipsoid-volume
method (Figure 39B). The total mass of particles of size ≥ 2 µm was ~300 ng/mL,
representing ~1/3000 of the total protein mass in the sample. Almost no particles were
detected in the formulations containing PS20. The total mass of protein particles ≥ 2 µm in
the PS20-containing formulations was ~10 ng/mL.

During storage of IVIG in the lyophilized trehalose formulations (with or without
PS20), no changes in particle counts or mass were detected over the 16-week storage period.
In contrast, for formulations in buffer alone or buffer plus PS20, between 8 and 16 weeks of
storage particle counts in the rehydrate samples exceeded the upper measurement limits for
the instrument (Figure 39A and B), Similar trends in the results were seen for samples that were lyophilized following an annealing step in the frozen solid (Figure 39 C and D).

Figure 38. Protein aggregation of IVIG with annealing procedure characterized by SEC. The formulations are: buffer alone (blue circle), buffer plus PS20 (red square), buffer plus trehalose (green upward triangle) and buffer plus trehalose plus PS20 (purple downward triangle). The percentage of each protein species was calculated: A. Monomer peak, B. Insoluble aggregates, C. Dimer peak, and D. High molecular weight species peak. The percent of each protein species before lyophilization are the initial data points in each panel. The vertical dash line in each panel is drawn to distinguish data points from pre-lyophilization data. Each data point and error bar denote the average and standard deviation of 3 independent replicates respectively. For some data points, error bars are not visible because they are smaller than the symbols. Lines were drawn to lead the eye.
Particles were also analyzed by RMM, which is capable of sizing and counting particles in the size range of about 0.2 to 5 µm (depending on the sensor and the particle type). Immediately after lyophilization, the IVIG formulations with buffer alone and with trehalose had higher numbers of particles compared to formulations containing PS20 (Figure 40A). However, total particle masses were indistinguishable across formulations. During long-term storage in formulations without trehalose particle counts increased over time,
exceeding the upper limit of detection by 16 weeks. For the trehalose formulations, no significant increases in particle concentration or mass were observed during storage. In the trehalose formulation containing PS20, particle levels were initially very low and did not change during storage. Annealing did not affect the overall trends for any of the formulations tested (Figure 40C and D).

RMM can detect not only particles that have a density greater than that of the bulk fluid, but it can also detect positively buoyant particles\textsuperscript{188}. In all of the lyophilized and rehydrated IVIG samples, RMM detected positively buoyant submicron-sized particles (data not shown). These positively buoyant particles were most likely air bubbles that were formed due to the entrapment of air voids of the cake structure in liquid phase during reconstitution. If protein adsorbs to such bubbles, aggregation could potentially result. Future studies are warranted to investigate the characteristics of submicron-sized bubbles generated during reconstitution and their potential effects on protein aggregation.

**Effect of ps20 during reconstitution**

Interestingly, the presence of PS20 did not reduce IVIG concentration in the surface layers of lyophilized formulations, but did greatly reduce particle formation in formulations containing trehalose. It has also been shown that the presence of polysorbate in reconstitution solutions can reduce aggregation\textsuperscript{189}. We investigated this potential effect by
reconstituting the lyophilized trehalose formulation of IVIG with water or with aqueous solution containing 0.03% PS20. No significant differences in aggregate levels were detected by SEC (data not shown). However, particle analysis by both FlowCAM® and RMM showed that rehydration of the lyophilized IVIG formulation with 0.03% PS20 significantly reduced particle formation (Figure 41). The total concentration of particles detected by FlowCAM® was significantly lower with ps20 rehydration, besides, the total mass of protein
particles ≥ 2 µm was 279 ng/mL with water rehydration, but was reduced to 24 ng/mL with ps20 rehydration (Figure 41B). These results indicated that the reduced particle levels observed in PS20 might be predominately due to effects manifesting during rehydration.

Another mechanism by which PS20 may protect proteins from aggregation is by altering the kinetics of dissolution during reconstitution of lyophilized formulations. It has previously been shown that PS20 accumulated at the solid-air interface, with their hydrophobic tail oriented towards the air. During reconstitution, this outwardly-facing hydrophobic layer impedes wetting, thus slowing dissolution\textsuperscript{176}. The slow dissolution of the cake allows protein more time to refold during reconstitution, reducing the likelihood of aggregation\textsuperscript{176}. However, this mechanism would not be operative when PS20 is present only in the reconstitution solution. PS20 did not reduce the amount of IVIG present in surface layers of lyophilized formulations, nor is it required to be present in the lyophilized solid to reduce particle formation. Thus the mechanism by which PS20 protected IVIG from particle formation remains unclear. Speculatively, surfactants might reduce agglomeration of the smallest particles formed during rehydration, rendering the particles too small (e.g. <0.2 µm) to be detectable by methods such as RMM and FlowCAM\textsuperscript{®}.
Figure 41. Effect of ps20 during lyophilization and reconstitution with different treatment. Treatment 1 is water reconstitution of IVIG with the trehalose plus ps20 formulation, treatment 2 is ps20 reconstitution of IVIG with the trehalose formulation and treatment 3 is water reconstitution of IVIG with trehalose formulation. A. FlowCAM® measured microparticle size distribution. B. Protein particle mass in the size range of ≥2 µm calculated based E-V model. C. Protein particle mass detected by RMM.
CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

The current thesis work addresses issues closely related to the development of therapeutic protein drugs in the biotechnology industry, i.e. retaining protein stability and preventing protein aggregation. Due to the delicate nature of protein molecules and the negative effect of protein aggregates on the quality of therapeutic protein drugs, it is critical to characterize protein conformation, stability and aggregation during therapeutic protein drug development.

In this thesis work, I first evaluated emerging techniques developed to characterize protein stability and aggregation. By combining the principles of Raman spectroscopy and DLS, I was able to simultaneously characterize both size and protein conformation including secondary and tertiary structure and robustly evaluate protein thermal stability. In addition to the enriched stability profiles, the unique advantages of this system also include low sample volume requirement, easy background subtraction and compatibility with high protein concentration. Similar to FTIR spectroscopy, Raman spectroscopy has been used to characterize solid protein\textsuperscript{190}, thus it is also promising to evaluate the capability of this system in characterizing the conformation of lyophilized protein samples. With Raman spectroscopy valuable information on tertiary structure of the dried protein could be obtained, as well as results on secondary structure.

A further application of the combined Raman spectroscopy and DLS system in characterizing the incubation stability of insulin analogs demonstrated that insulin lispro underwent a partial conversion from native alpha helix to non-native beta sheet and distortion of disulfide bonds after being converted to soluble aggregates. In addition, the protein
conformation and size measurement by the combined Raman spectroscopy and DLS system in this study enabled the non-intrusive characterization and comparison of aggregation rates and structure perturbation for three different fast-acting insulin analogs. Nevertheless, Raman spectroscopy analyzes the averaged conformation of the all protein species in a sample and thus is not sensitive to perturbation of a small proportions of protein molecules. To better compare the conformational perturbation of aggregated insulin analogs, future studies need to be done to focus on separating the aggregated protein species and analyzing their conformations directly.

Another emerging technique, nanoparticle tracking analysis (NTA), was also evaluated for its application in characterizing protein aggregates in the submicron size range in therapeutic protein. I found that NTA could provide an effective means of obtaining valuable nanoparticle concentration and size distribution data for therapeutic protein samples with appropriate handling of sample (e.g. inclusion of a pump for better sampling or sample dilution in the same protein concentration background) and setting of instrument parameters (e.g. complete tracking numbers and minimum expected particle size). Due to the lack of standardized protein particles with defined size and particle concentration, the evaluation of NTA in counting and sizing still had to be performed on particle standards such as latex beads or gold nanoparticles, which have different refractive index compared with protein particles. However, it was demonstrated in this study that the refractive index of the targeted particles or the contrast between particles and the background solution significantly affected the accuracy of particle counting. Therefore, the availability of standardized protein nanoparticles in the future will benefit the robustness of evaluation of the capability of NTA
and other emerging particle characterization techniques in characterizing protein particles in therapeutic proteins.

Lastly, as an effort to improve protein stability of lyophilized dosage forms, the effect of ps20 and annealing in protecting antibody stability was evaluated. It was found that ps20 effectively reduced the generation of subvisible particles thereby decreasing protein aggregation. The protective mechanism of ps20 traditionally involves competing with protein molecules for adsorption on ice-water interface during freezing. However in my study, ps20 might play a more significant role during the reconstitution and not during freezing. The influence of annealing was also investigated in this study, but no significant effect was seen compared with standard lyophilization procedure. Annealing reduced the SSA by \( \sim 50\% \), however number of protein molecules per surface area was higher. Therefore I concluded that annealing did not significantly change the protein fraction on the air-solid surface. Compared with previous studies on KGF and rhGH, it appears that IVIG is not as sensitive to surface exposure. Thus using a surface-sensitive model protein for future studies to investigate the effect of annealing and the number of protein molecules per surface area might improve our understanding of the behavior of ps20 in protecting proteins in lyophilization process.

Overall, this thesis work critically evaluated emerging biophysical techniques that could improve characterization of protein stability and aggregation in development of therapeutic protein drugs. High concentration dosage will continue to be the mainstream formulation strategy for therapeutic protein (mostly monoclonal antibodies) due to patient compliance. The comprehensive stability characterization of protein molecules will benefit the protein drug development, especially at high concentration dosage. Yet, formulations
under development still encounter numerous difficulties that resulted from complicated protein properties due to high concentration, such as viscosity, susceptibility to aggregation etc. Dedicated efforts are required to improve stability by engineering optimal drug candidate, screening optimal formulation and exploiting powerful biophysical characterization techniques. Subvisible particle especially submicron particle will continue to be a major challenge to develop stable and safe therapeutic protein drugs. Current available techniques for submicron particle characterization are limited in the capability of size range coverage or quantification. A better understanding of protein aggregation pathway and the correlation between submicron particles and immunogenicity will require more robust techniques for submicron particle quantification. In addition, the study of the effect of ps20 on IVIG protein stability during lyophilization provided an insight into the potential mechanism by which protein molecules are protected during freeze-drying stress and reconstitution. After reconstituting lyophilized cake we found the existence of nanobubbles, which were overlooked previously. Recent decade has seen a rising interest in the characterization and application of nanobubbles. The potential involvement of nanobubbles in the effect of ps20 on protein aggregation during reconstitution will inspire future studies on the role of nanobubbles on protein stability in general.
REFERENCES


