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Concomitant Raman spectroscopy and dynamic light scattering for characterization of therapeutic proteins at high concentrations

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ABSTRACT

A Raman spectrometer and dynamic light scattering system were combined in a single platform (Raman–DLS) to provide concomitant higher order structural and hydrodynamic size data for therapeutic proteins at high concentration. As model therapeutic proteins, we studied human serum albumin (HSA) and intravenous immunoglobulin (IVIG). HSA concentration and temperature interval during heating did not affect the onset temperatures for conformation perturbation or aggregation. The impact of pH on thermal stability of HSA was tested at pHs 3, 5, and 8. Stability was the greatest at pH 8, but distinct unfolding and aggregation behaviors were observed at the different pHs. HSA structural transitions and aggregation kinetics were also studied in real time during isothermal incubations at pH 7. In a forced oxidation study, it was found that hydrogen peroxide (H_2O_2) treatment reduced the thermal stability of HSA. Finally, the structure and thermal stability of IVIG were studied, and a comprehensive characterization of heating-induced structural perturbations and aggregation was obtained. In conclusion, by providing comprehensive data on protein tertiary and secondary structures and hydrodynamic size during real-time heating or isothermal incubation experiments, the Raman–DLS system offers unique physical insights into the properties of high-concentration protein samples.

There are several points during the development history of a therapeutic protein product where there is a need to rapidly assess protein structure, aggregation, and thermal stability. For example, during early development of a therapeutic monoclonal antibody (mAb)[1], relative physical stability (e.g., during heating) and aggregation propensity are often compared among several candidate variants of the antibody [1]. 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 [2,3]. 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 [4–6]. 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.

Traditionally for such studies, multiple techniques have been used separately to characterize protein conformation, aggregation, and thermal stability, with the results from different instruments being combined to provide an overall assessment of the protein's physicochemical properties [7]. 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 [8,9]. Other techniques such as field flow fractionation (FFF) and analytical ultracentrifugation (AUC) are used for orthogonal confirmation of SEC results [10–12]. 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, including intrinsic/extrinsic fluorescence, ultravi-

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[1] *Abbreviations used:* mAb, monoclonal antibody; SEC, size exclusion chromatography; DLS, dynamic light scattering; SLS, static light scattering; UV, ultraviolet; CD, circular dichroism; IR, infrared; HSA, human serum albumin; IVIG, intravenous immunoglobulin; Tyr, tyrosine; Trp, tryptophan; H_2O_2, hydrogen peroxide; PLS, partial least squares; DSC, differential scanning calorimetry; IgG, immunoglobulin G.

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olet (UV) absorbance, far- and near-UV circular dichroism (CD), and infrared (IR) [10,12].

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 mAb product. Obtaining more than one data type from a given sample (e.g., UV absorbance spectra and turbidity measurements, fluorescent spectra and SLS measurements) helps to conserve precious protein. In addition, making simultaneous measurements on a sample ensures 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 mAb therapeutics and some older products such as human serum albumin (HSA) and intravenous immunoglobulin (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 might 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 nanometers (nm) to a few micrometers (μm). The 173° backscattering detector minimizes interference from multiple scatterings and enables the collection of size distribution data of high-concentration protein samples [10]. 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) [13–17]. Compared with 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 [18]. Raman spectroscopy is also ideally suited to studying proteins at high concentration [19].

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. In addition, by acquiring Raman spectra and light scattering data during real-time heating studies and during isothermal incubations at various temperatures, we were able to directly compare the effects of temperature or time of incubation on protein secondary and tertiary structure as well as aggregation.

In our experiments, thermal stability of 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 (pHs 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 hydrogen peroxide (H_2O_2) and the effects on structure, aggregation, and thermal stability were compared with 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) and IVIG (Gammagard Liquid, Baxter HealthCare) were purchased from the University of Colorado at Boulder's Wardenburg Pharmacy. All other chemicals were purchased from Fisher Scientific (Hampton, NH, USA) 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, Malvern, UK) was combined with a Kaiser Raman RxN1 spectrometer (Kaiser Optical Systems, Ann Arbor, MI, USA). The instrument is a prototype system developed by the Malvern Bioscience Development Initiative (Columbia, MD, USA). A 785-nm laser with an approximately 280-mW laser power source was used for Raman spectroscopy. DLS data were collected at 632 nm with a 173° backscattering detector that minimizes interference from multiple scatterings and enables the collection of size distribution data of high-concentration protein samples [10]. As shown

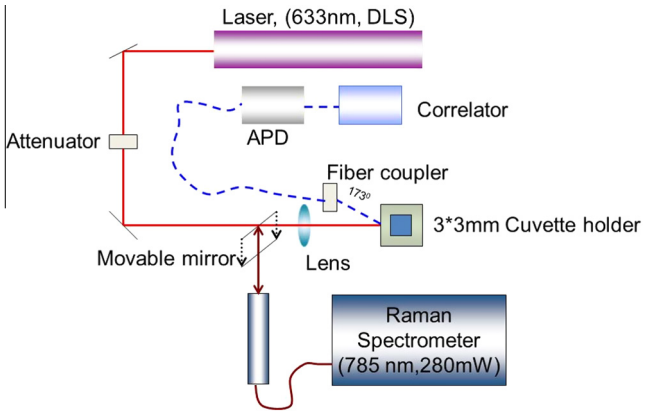

Fig.1. Diagram of the instrument with Raman spectroscopy combined with DLS. APD, avalanche photodiode.