Towards a unified biophysical characterization platform: Combining dynamic light scattering and Raman spectroscopy to determine protein structure and stability

Malvern Instruments Bioscience Development Initiative

Executive summary

The combination of dynamic light scattering (DLS) with Raman spectroscopy has the capability to characterize a wealth of chemical, structural, and physical parameters of therapeutic proteins. Raman spectroscopy simultaneously derives protein secondary structure markers (amide I and III) and tertiary structure markers (aromatic side chains, disulfide bond, hydrogen bonding, local hydrophobicity). These markers can be monitored under controlled conditions by the determination of spectral peak position, shape, and/or intensity. Raman is able to make these structural determinations at formulation concentrations, 50 mg/mL or greater, rather than at the diluted concentrations required by conventional methods, i.e. typically less than a few mg/mL for circular dichroism (CD).

DLS using backscatter detection is capable of measuring the hydrodynamic radius of proteins at high concentrations (over 50 mg/mL). As the technique is based on light scattering, and scattering intensity scales with r^6, it is exquisitely sensitive to the formation of aggregates. Therefore, changes in size distribution and polydispersity as a function of a variety of perturbations (temperature, pH, salt concentration, etc.) can be monitored, enabling the derivation of protein interaction and kinetic information, i.e. k_D, relaxation times.

Combining these two techniques into a single system allows for the measurement of size and structure from a single small volume sample under identical conditions. This combined approach is unique in that it can indicate, for example, if a protein has changed its structure and then aggregated, aggregated without structural change, or changed structure and remained un-aggregated. It is therefore able to provide useful insights into the mechanism of aggregate formation.

Introduction

Aggregation and native-state unfolding in protein formulations is of critical importance to manufacturers and patients alike. Product quality and safety concerns require both an understanding of aggregation, and also careful monitoring for long-term problems related to formulation stability. The combination of Raman spectroscopy and DLS

is perfectly suited to exploring the aggregation of proteins. Raman spectroscopy derives information about protein unfolding by monitoring the changes in molecular vibrations which occur as the result of changes in the protein's secondary and tertiary structure. DLS, on the other hand, is a technique which can be used to determine the hydrodynamic radius of a therapeutic protein in solution, the sample's polydispersity, and also sample interactions. By combining these two analytical approaches into a single system, a wealth of chemical, structural and physical parameters can be determined. Protein secondary and tertiary structure, melting temperature (T_m), onset temperature of aggregation (T_{onset}), and transition enthalpy values can all be derived, as well as aggregation propensity, protein solubility, and the potential for high viscosity at formulated concentrations. The complementary nature of the results obtained from DLS and Raman spectroscopy on the same sample may provide unique insights into the mechanisms of aggregation and unfolding.

To achieve concurrent DLS and Raman measurements, Malvern Instruments' Zetasizer Helix (ZS Helix) integrates a fiber-coupled Raman spectrometer with a Zetasizer Nano ZSP to provide DLS (colloidal stability) and Raman (conformational stability) data sequentially on a single sample. The Zetasizer Nano system integrates proprietary non-invasive backscatter (NIBS) detector technology with dynamic (DLS), static (SLS), and electrophoretic (ELS) light scattering to measure the hydrodynamic radius of proteins from 0.3 nm to 10 µm, at concentrations ranging from 0.1 mg/mL to 100 mg/mL, or more. Raman spectra are collected using 785 nm excitation (~280 mW) from 150 cm^{-1} to 1925 cm^{-1} at 4 cm^{-1} resolution. Sample aliquots (~120 µL) are introduced into the temperature-controlled sample compartment using a quartz cuvette with a 3 mm pathlength. The sample compartment temperature can be set from 0°C to 90°C ± 0.1°C. Thermal ramp studies are conducted by collecting Raman and DLS data at a series of predefined temperature increments, while isothermal incubation experiments are performed by collecting DLS and Raman data at a low temperature, quickly increasing the temperature, and collecting numerous Raman and DLS data over an extended period of time at the higher temperature.

Raman spectroscopy

Raman spectroscopy is an effective approach to determine protein secondary and tertiary structural characteristics. A variety of secondary structural markers, including amide I

(1600 cm^{-1} - 1700 cm^{-1}), amide III (1200 cm^{-1} - 1350 cm^{-1}), and α-helix backbone (930 cm^{-1} - 950 cm^{-1}) can be monitored. Any changes in these regions indicate a secondary structural change in the protein. Additionally, Raman spectroscopy is highly sensitive to the symmetrical vibration modes of the aromatic side chains, i.e. phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp), and as such, has been widely used as a probe of protein tertiary structure. There are several well-established and frequently used Raman markers that provide insights into the environment of these aromatic side chains, including Trp at 1550 cm^{-1} and Tyr at 850 cm^{-1}. Variations in these areas suggest a change in the local hydrophilic/hydrophobic environment of the side chain, which is characteristic of unfolding.

With its ability to elucidate protein structural changes, Raman spectroscopy is ideal for studying unfolding proteins. One situation in which proteins are known to unfold and aggregate is when exposed to thermal stress. Included below are Raman spectra from a lysozyme sample that was exposed to thermal stress using a thermal ramping experiment. Figure 1 shows spectra from the lysozyme sample at 20°C (blue line) and 80°C (red line).

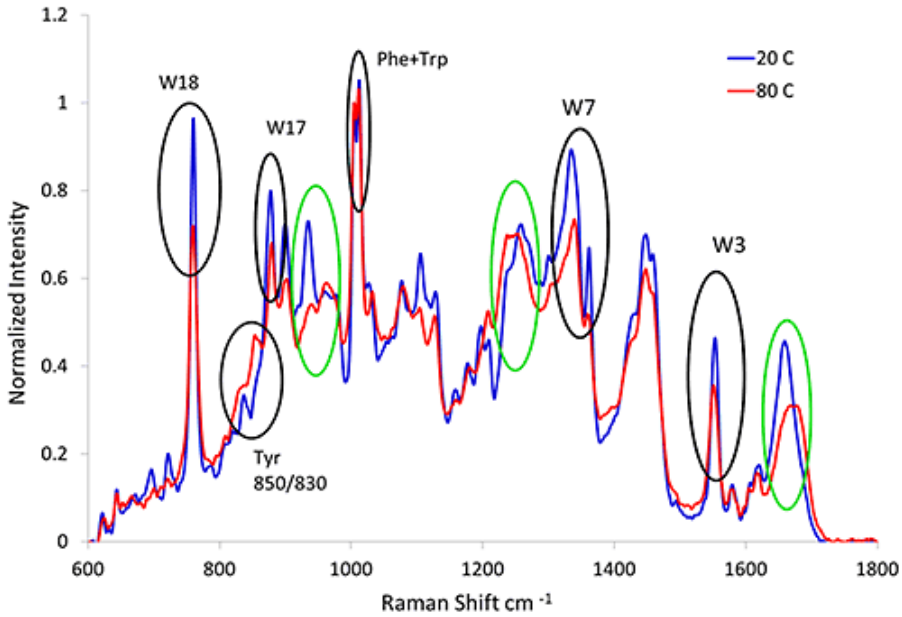

Figure 1: Raman spectra of lysozyme (pH 7.4) before (blue line) and after (red line) heat treatment. Changes in the secondary (green circles) and tertiary (black circles) structural markers are observed as a result of thermal stress

Figure 1 clearly demonstrates that as the temperature increases, the secondary structure (green circles) of the lysozyme changes. These changes are illustrated with peak shifts and/or intensity changes. Concurrent with changes in secondary structure are changes in the aromatic side chains (black circles). These tertiary structural markers indicate changes in the local aqueous environments of the side chains, changes in the hydrogen bonding of the side chains, and changes in the dihedral bonding angles. Based on the secondary and tertiary structural changes observed from the Raman spectra, it is evident that lysozyme is undergoing structural changes while exposed to thermal stress.

Dynamic light scattering

Traditionally, DLS was only thought to be useful for obtaining size information from low concentration samples, and it was widely applied for determining the hydrodynamic radius of proteins in solution. However, with the inclusion of backscattering detection, the size of proteins in solution (50 mg/mL or greater) can be determined. In addition to size measurements, DLS supplies information regarding the size distribution, or polydispersity (PDI), of the sample and can be used to measure T_{onset}. And due to its sensitivity to size, DLS is ideal for detecting small amounts of large aggregates in solution. Figure 2 (below) includes results from a bovine serum albumin (BSA) thermal ramping experiment.

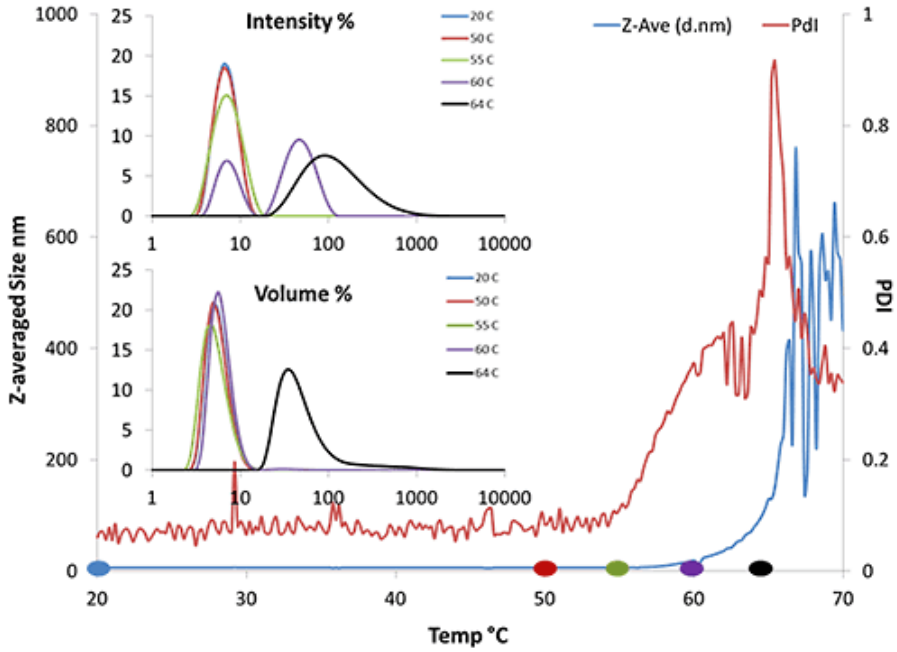

Figure 2: DLS thermal ramping data of 50 mg/mL BSA (pH 7.4). Results include Z-average size, PDI, and volume and intensity distributions

In Figure 2, the Z-averaged size trend (blue line) indicates that T_{onset} is ~62°C. The volume and intensity distributions (inset) show that as the temperature increases, there is an increase in the amount of larger particles detected by the instrument; specifically, there is an increase in larger particles at around 60°C (purple line, inset). These increases all suggest that aggregates are beginning to form. Additionally, the PDI begins to increase as early as 55°C, indicating that aggregation is beginning to occur at that temperature.

Combined DLS/Raman

Combining Raman, and its ability to monitor protein structural changes, with DLS, and its ability to monitor protein size changes, leads to a technique well suited to monitoring protein unfolding/aggregation pathways. As an example system, formulations of BSA in citrate buffer at various pHs have been used to study the link between protein unfolding and aggregation. Samples were created at 50 mg/mL concentration at pH = 4.7, 5.4, and 7.1, and the transition temperature (T_m) was determined for each by conducting ramping experiments. Samples were then incubated slightly below their T_m where unfolding should be the preferred state, but take place kinetically slowly. Representative results are shown in Figure 3 (below) for the isothermal incubation study.

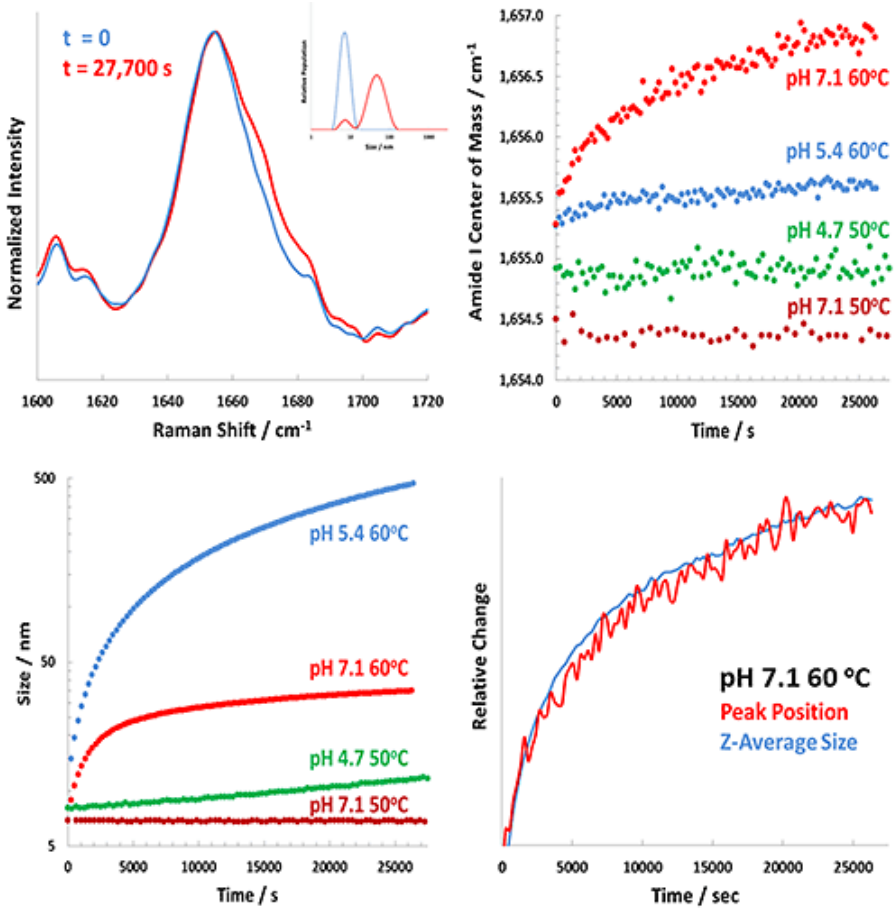

Figure 3: Representative results of for the Raman-DLS study of BSA at various pH values. The upper left panel displays the change in the Amide I band, as well as the change in Z-average size distribution (inset) for BSA at pH 7.1 for 60°C incubation. The upper right panel shows the change in the Center of Mass (CoM) of the Amide I band as a function of incubation time for each formulation measured, and the lower left panel shows the corresponding size data. The lower right panel shows the strong relationship between the structural changes (unfolding) and the Z-average size for BSA at pH 7.1

The upper left panel shows the extent of change in the pH 7.1 BSA sample at 60°C incubation between t = 0 (blue) and t = 7.8 hours (red) for both the amide I band and the DLS results (inset). Complete analysis of the Raman (amide I) and DLS data are shown in the upper right and lower left panels, respectively. The analysis reveals significant pH-dependence in the behavior of both BSA unfolding and aggregation. For example, in the lower right panel of Figure 3, the time-dependence of the amide I band and the Z-averaged size are superimposed. This plot demonstrates that the structural and size changes closely track one another. It also suggests that the structural unfolding of the protein tracked by Raman spectroscopy results in a larger BSA monomer (up to 35 nm), but that little aggregation of the BSA occurs. Observing the results at pH 5.4 reveals a different picture; that is, the structural changes in the protein are very small (~0.25 cm^{-1} change in Center of Mass after 7.8 hours), but the size changes are significantly larger (> 500 nm after 7.8 hours). The pH 4.7 results, nearest the isoelectric point (pI), show a small change in size at 50°C, and no discernible change in structure. At 60°C, above T_m for BSA at pH 4.7, the protein was seen to precipitate instantaneously. This matches expectations near pI, as charge-charge repulsion of protein monomers is not present and aggregates are free to form.

The combination of Raman spectroscopy and DLS provides a clear window into how access to both structural and size data can be used to understand processes of interest to the bioanalytical community.

Conclusions

The combination of DLS and Raman spectroscopy in a single instrument allows for elucidation of protein structure and size changes from a single sample. DLS, with its ability to measure protein size, is a good indicator of protein aggregation, and Raman spectroscopy, with its ability to probe protein secondary and tertiary structure is also a good indicator of protein unfolding and potentially aggregation. Combined, these techniques can give a unique insight into the unfolding/aggregation pathway, providing critical characterization information.

About Malvern's Bioscience Development Initiative

Malvern Instruments' Bioscience Development Initiative (BDI) was established to accelerate innovation, development and the promotion of new technologies, products and capabilities to address unmet measurement needs in the biosciences markets.

Malvern Instruments Limited
Grovewood Road, Malvern,
Worcestershire, UK. WR14
1XZ

Tel: +44 1684 892456
Fax: +44 1684 892789
www.malvern.com