The potential of UVRR for characterizing the structure, conformation and stability of biopharmaceutical protein formulations

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Introduction

Protein based drugs are attracting increasing interest and many products are marketed [1]. However, in contrast to conventional small molecule drugs which are designed to have a stable active form (typically a single well-defined polymorph) and where formulation routes are well-established, protein-based drugs are much less stable and present very significant formulation challenges. For example, it is well known that biological malfunctions, and hence disease states, can be induced by the failure of proteins to fold correctly, or to remain correctly folded. Similarly, in several in vitro experiments, protein aggregates have been found to be particularly cytotoxic. This means that for a protein drug it is important to determine that the drug is correctly folded and that it remains correctly folded for the shelf-life, which for an economically viable protein drug is 18-24 months. Typically, the stability of protein drugs must be maximised through carefully designed formulation which keeps the drug in its active form and with minimized degradation. A key problem, which we address here, is to find a method to determine the structure of the drugs after they have been stabilised within such formulations.

The use of Raman spectroscopy for protein studies is already well described in the literature [2-4], particularly the sensitivity of the spectra to conformational changes. However, we have found that attempting to carry out non-enhanced Raman studies of even relatively concentrated (1% by mass) aqueous protein solutions gives spectra in which the protein bands are weak. This makes them susceptible to interference from other components in the solution, including the solvent itself, as well as buffer components, signals from the sample holder etc.

It is not possible to increase the concentration of the proteins because the measurements need to be carried out on the therapeutic dosage forms, which are typically <1% by mass, so some form of enhancement is required. One possibility is the use of low refractive index hollow waveguides, which have previously been used for dilute protein solutions [5] but this approach is not compatible with our ultimate goal, which is to establish a methodology for high throughput screening of such samples. SERS is not appropriate because of the likelihood of degradation/conformational perturbation which will be induced by the enhancing medium. This leaves UV resonance Raman (UVRR) as the obvious choice, [2,6]

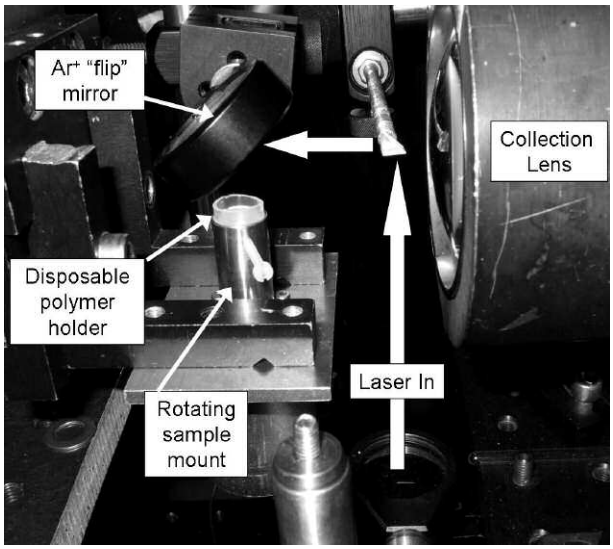

Figure 1. Photo of the sample holder and set-up.

particularly since it is non-contact and potentially can be used in high throughput screening mode. Moreover, it might be expected these types of experiments would be technically less demanding than temperature-jump induced time-dependent protein folding experiments which have already been demonstrated. [7]

The primary goal of these investigations was to determine the extent to which other experimental factors, such as the introduction of vaccine adjuvants or some turbidity would have on the ability of UVRR to detect key changes in the drug structure, as well as to explore the feasibility of using the technique for high throughput screening studies.

Experimental

Raman spectra were recorded using 244 nm laser excitation generated by frequency doubling an Innova FRED 300c Ar^+ laser. The power of the fundamental was locked at 120 mW which resulted in 26 mW of 244 nm at the sample.

To allow rapid interchange between samples but minimize both cross contamination and interfering Raman scattering signals from the container, a new sampling system was developed. This system allowed disposable

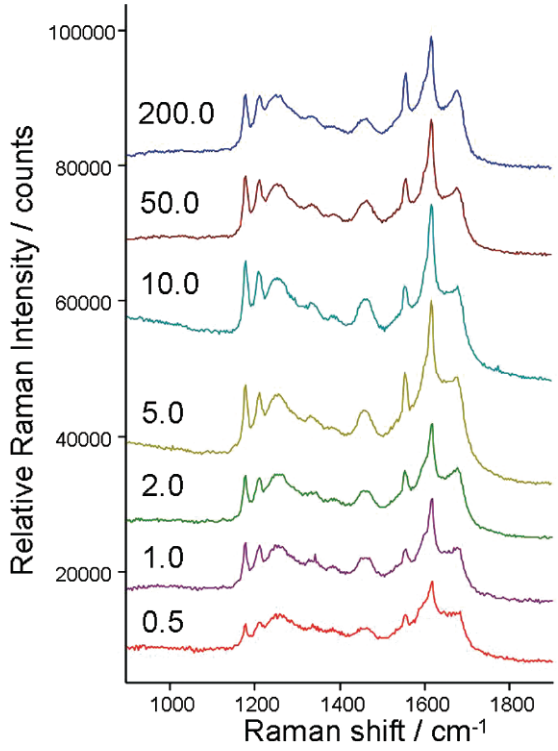

Figure 2. Demonstration of the wide range of concentrations that could be probed by UVRR. The values are in mg/ml of salmon calcitonin.

polymer containers to be used but minimized degradation by rotating the samples at a speed of 58 revs/min (figure 1). The sample containers were filled with the same volume of sample each time to ensure a constant focal position. Samples could be changed within 20 seconds by flipping the Ar^+ turning mirror mount out of the beam path.

A Spex Triplemate spectrograph (filter stage: 1200 grooves/mm, spectrograph: 3600 grooves/mm) was used along with an Andor DU420A CCD camera (1024 × 255) at -80°C as the detector. The measured spectral range was 325-2230 cm^{-1}, with a resolution of 2 cm^{-1}. Accumulations were carried out in kinetic mode with 5 or 10 minute total integration but readouts every minute. Spectra reported here are the sums of the first five minutes of the kinetic runs, except the antibody signals which are the first three minutes.

Samples investigated included various formulations of therapeutic antibodies, peptides (e.g. salmon calcitonin) and proteins (e.g. growth factors). Problems related to: changes during storage, conformational change with pH, buffer type and concentration, chemical stability, protein aggregation, depot formulations and gel formation, ability to measure in turbid systems and stability of proteins adsorbed onto adjuvants were all investigated during the course of this work.

Results and discussion

Due to the very large range of samples that it was possible to study with our high throughput sampling set-up and the relative brevity of this report, only some representative results are reported here.

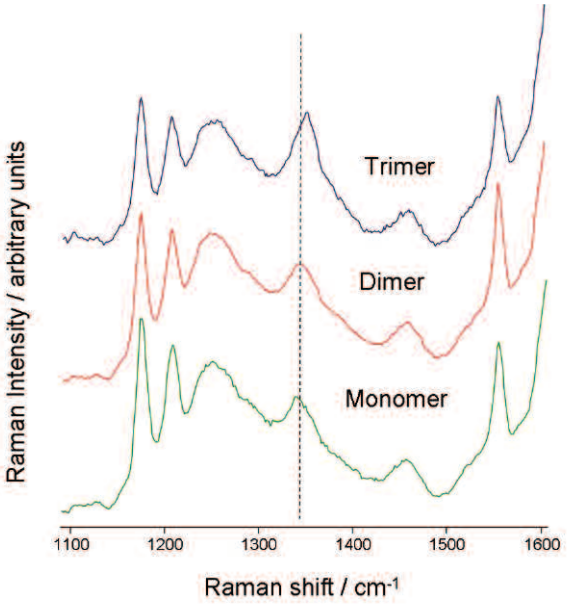

Figure 3. Spectra from the different fractions of BSA. The dashed line marks the middle of the tryptophan W7 doublet.

Figure 2 demonstrates the wide range of concentrations that can be probed by UVRR. Concentration of the peptide salmon calcitonin (sCT) was varied between 200 mg/ml and 0.5 mg/ml. Although the water signal had to be subtracted out of the spectra at ≤2.0 mg/ml the sCT spectra are clearly visible over this very broad range. This is of particular interest since many of the standard techniques currently used are limited to a relatively small concentration range [1].

Preliminary experiments were also carried out to determine the extent to which experiments at this excitation wavelength can be used to probe protein structure. Figure 3 shows the spectra (5 min accumulation) of 3 different fractions of the bovine serum albumin (BSA), for ease of interpretation they are labeled monomer, dimer and higher molecular weight species. As the fraction length increases there is a shift in the tryptophan W7 doublet peak to higher wavenumber. This indicates that the tryptophan in the BSA is in a more hydrophobic environment [2]. This result gives some indication of the amount of folding that is occurring within the BSA in solution and may provide a standard marker in future investigations for BSA.

Figure 4 shows BSA mixed with two different adjuvants, the aim of this study was to analyze any interference from the turbid adjuvant. These mixtures are used as models for studying protein – aluminum adjuvant vaccines. However, as clearly shown in figure 4, once the adjuvant has been subtracted from the spectra the signal from BSA is visible. This bodes well for use of UVRR as a technique to investigate protein pharmaceuticals within their final commercial formulations.

Figure 5 shows several spectra of an antibody that is in a close-to-final formulation with several other constituents within the mixture. The combined spectra of other constituents are shown in the data taken from a placebo

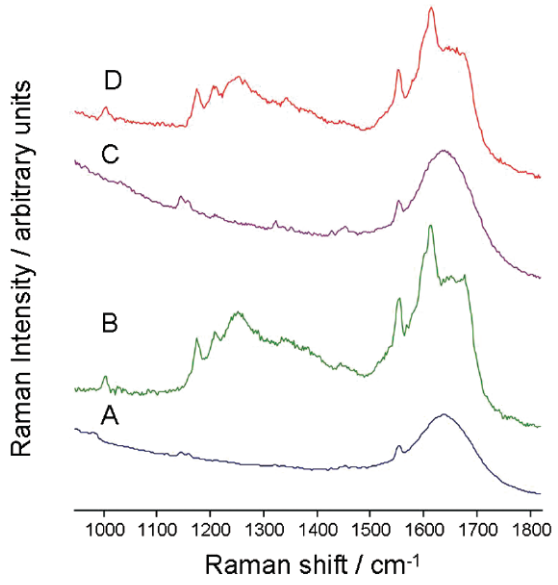

Figure 4. Influence of adjuvants. (a) 3.4 mg/ml $Al(OH)_3$, (b) 1.7 mg/ml BSA mixed with $Al(OH)_3$ which has been subtracted out. (c) 3.4 mg/ml $AlPO_4$, (d) 1.7 mg/ml BSA mixed with $AlPO_4$ which has been subtracted out.

spectrum from which only the antibody was omitted. Comparison of the spectra clearly shows that the other constituents do not interfere with the resulting UVRR spectra of the antibody. It is important to note that no subtraction of the placebo spectra has been carried out on the antibody spectra. It was also possible to selectively subtract placebo spectra to obtain the spectra of the individual components within the formulations, e.g. sucrose or Tween 80 etc.

Conclusions

We have found that UVRR is well suited to investigate biopharmaceutical protein formulations. The relatively short accumulation times required meant that the method is potentially capable of high throughput measurements. Indeed, even using the entirely manual sampling system that was built as part of this initial investigation allowed us to record data on fifty samples within one day which is already 'high throughput' in this context. Moreover, we have found that in many cases the UVRR spectra of the constituents of interest, the proteins and antibodies, are not affected by other components in the mixture, such as adjuvants, buffers, sugars and surfactants. Of particular importance is the ability of UVRR to provide high quality spectra over a very wide concentration range and to signal changes in protein structure.

This preliminary study has clearly demonstrated that UVRR has considerable potential as a tool for high throughput characterization of biopharmaceutical protein formulations. Such tools are important because they are vital in underpinning the development of clinically viable dosage forms of these valuable new drugs.

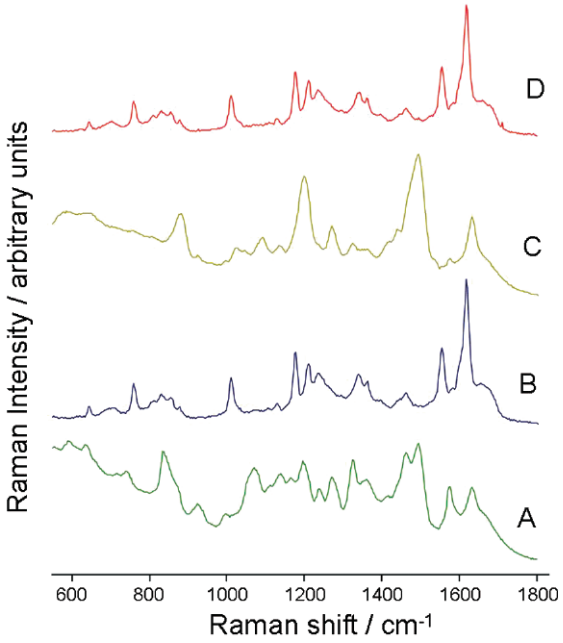

Figure 5. (a) Placebo formulation g – pH 6.0 buffer, sucrose and Tween 80. (b) Antibody formulation. (c) Placebo formulation h – pH 5.2 buffer, mannitol and Tween 80. (d) Antibody formulation.

Acknowledgements

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