

Circular Dichroism for Assessing the Solid-State Structure of Immobilised Proteins or Antigens

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Introduction

Determination of the effect of immobilisation on the secondary and tertiary structure of antigens or therapeutic proteins is generally difficult because conventional circular dichroism (CD) analysis cannot be applied to particulates. This study aimed to develop a method for obtaining good quality CD spectra of antigen bound to adjuvant and protein bound to excipient microcrystals.

CD Spectroscopy

CD is a powerful spectroscopic tool for elucidating structural aspects of proteins. Far and near UV CD spectra provide information about secondary and tertiary structure respectively and are used to detect changes in conformation as a protein environment is changed. CD has primarily been used to study proteins in solution and membrane proteins in vesicles and sheets. In heterogeneous systems artefacts can arise from the effects of differential light scattering and absorption flattening^{1,2}. This has restricted the use of CD with particulate systems

Aims of Study

- Develop a rotating CD cell suitable for making spectroscopic measurements on suspensions
- Carry out measurements of model proteins bound to alhydrogel, silica and salts in aqueous and solvent
- Optimise optics and spectral analysis in order to obtain useful information about the conformation of immobilised proteins

Development of Rotating CD Cell Holder

CD measurements of particulate suspensions are prone to artefacts arising from

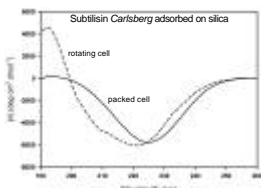
- sample sedimentation during the measurement process
- optical effects involving light scattering and absorption flattening^{1,2}

To avoid sedimentation of the sample a motor driven rotating cylindrical sample cell holder was constructed and incorporated into the sample compartment of a conventional Jasco J-810 spectropolarimeter.



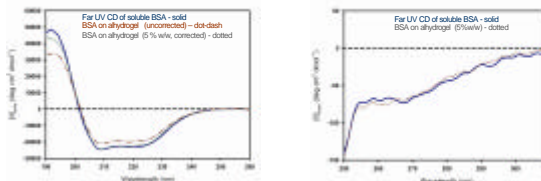
A correct combination of rotation speed (40-100 rpm) and cell path length (generally 0.02 cm for far UV and 0.5 cm for near UV) is necessary to avoid uneven distribution of particles in the light beam.

A comparison was made between the far UV CD spectra of proteins adsorbed onto 60 µm silica particles and packed tightly into a 0.01 cm path length cell and spectra obtained with the rotating system. Spectra obtained with the rotating cell were clearly superior as illustrated right.



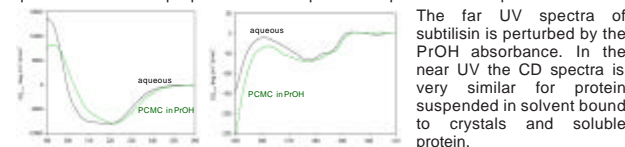
CD of proteins bound to alhydrogel

Bovine serum albumin (BSA) was adsorbed onto the adjuvant alhydrogel to provide a highly scattering suspension typical of parenteral vaccines. Far UV and near UV CD spectra were measured in buffer and compared to the spectra of soluble BSA. Spinning down the suspension gave a flat baseline demonstrating complete binding of protein to the alhydrogel.



CD of Protein Coated Microcrystals (PCMC) in propanol

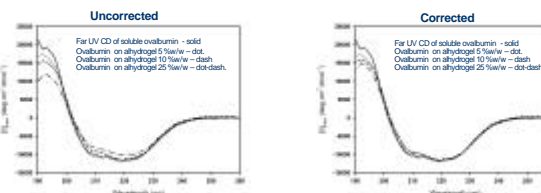
Subtilisin Carlsberg was coated onto potassium sulphate microcrystals and CD spectra measured in propanol were compared to aqueous soluble protein.



This indicates subtilisin must bind to PCMC in a near native conformation.

Correcting for differential scattering and absorption flattening

Effects of light scattering on CD spectral shapes are minimal in the presence of achiral particles but significant for chiral particles due to differential scattering of right and left circularly polarised components. Binding of protein to alhydrogel generates chiral particles. Decreasing the distance between the sample and the detector window to 2 mm was used to minimise this problem.



For stable protein suspensions, absorption flattening is the most significant factor contributing to CD spectral distortion. It occurs because of close packing of the chromophores (protein molecules) on immobilisation, resulting in a smaller total cross-sectional area than if they were uniformly dispersed. The degree of flattening at any given wavelength is proportional to the total absorbance at that wavelength. An empirical approach was adopted to correct CD spectrum for absorption flattening based on using the measured high tension voltage (HTV) to derive the total absorbance (A) of the sample, via the in-built Jasco software.

Discussion

CD spectra were successfully obtained for suspensions of immobilised protein in both aqueous and organic solvents. The rotating cell allowed particles of >50 µm to be kept in homogeneous suspension throughout the measurement process while optimisation of optics and sample concentration was found to lead to significant increases in spectral intensity. By taking into account differential scattering absorption flattening effects could be compensated for. The resultant corrected spectra could be used to gain insights into the protein secondary structure by application of standard analysis tools such as Selcon3 and K2D provided by DICHROWEB. An analysis for BSA bound to alhydrogel is shown below.

Secondary structure analysis of BSA and BSA-alhydrogel suspension in PBS

Protein	Protein loading	Q correction	Helix	Sheet	Turns	Unordered
X-ray (HSA)	-	-	70	0	15	15
BSA (sol)	-	-	73	5	7	15
BSA 1	5	No	62	8	11	19
BSA 1	5	Yes	70	8	9	14

The analysis shows there are only small changes to secondary structure on binding of BSA to alhydrogel. Interestingly, this is contrary to a recent published study where infrared was used to deduce the protein became more disordered³. From the near UV spectra it is clear that subtle changes in BSA tertiary structure do take place on binding. This is consistent with published fluorescence measurements showing a blue shift upon adsorption to alhydrogel³.

This type of analysis can clearly also be applied to antigen bound to adjuvant to give valuable insights into changes in antigenicity observed between different formulations. It may also be used to monitor the structure of therapeutic proteins within solid-state formulations following preparation or storage.

Details of CD Measurements

CD spectra were recorded using a Jasco J-810 spectropolarimeter at 20 °C. Spectra were obtained by averaging 6 scans measured at scan rate of 50 nm min⁻¹, time constant of 0.5 s, bandwidth of 1 nm for far UV and 2 nm for near UV. Blank spectra of aqueous buffer or suspension were used to correct observed spectra. Spectra were smoothed by the means-movement method using Jasco Spectra Analysis software and subjected to secondary structure analysis using Selcon3 and K2D provided by DICHROWEB. Quality of fit was judged from normalised root mean square deviation with value ± 0.25 considered as a good fit. For K2D maximum error values indicate a poor data analysis. Reproducibility of the spectra was found to be within $\pm 5\%$ of the spectral amplitude from three measurements of separate suspensions of the same sample.

References

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Conclusions

Circular dichroism can be used to directly assess the conformation of proteins immobilised on a range of support materials. By optimisation of the cell and optical configuration to minimise the effects of scattering, and the use of a simple empirical correction process to account for absorption flattening, high quality spectra can be obtained and used to probe both secondary and tertiary structure.