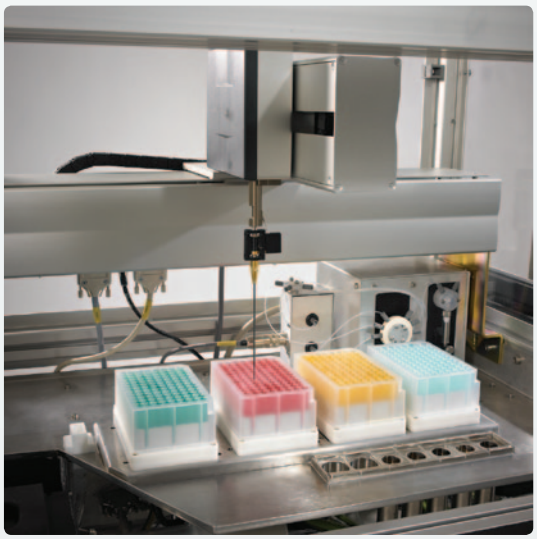
AppliedPhotophysics
Ultrasensitive Spectroscopy for the Life Sciences



CHIRASCAN SERIES APPLICATION NOTE

Mapping protein stability using Chirascan™-plus Automated CD (ACD)

Abstract *This study describes a new automated technique for measuring protein conformation by CD spectroscopy. We describe how CD changes can be followed in different pH environments. The study confirms the robustness of the technique and superb sample to sample reproducibility. With up to 200 samples per day, the improvements in productivity over conventional CD measurements enable experiments to be undertaken that were previously thought to be too time consuming or laborious.*

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INTRODUCTION

Correct protein function is critically dependent on conformation since different conformational states can exhibit considerable differences in their biochemical properties; a fact has been used extensively throughout life such as in the case of allosteric regulation. Misfolded protein conformations generally exhibit inactivity and in some cases can be highly toxic e.g. Prion diseases.

Circular dichroism (CD) spectroscopy is an exquisitely sensitive probe of protein conformation though is often perceived as a technique limited to more specialised applications. This is to some extent because, up until now, CD spectrometers have remained resolutely manual in operation which has now been overcome by new automation technology. Automation of CD spectroscopy offers a dramatic improvement in productivity over conventional CD methods, allowing up to 200 samples to be analysed per day. This substantial advancement in the field of CD spectroscopy allows experiments to be conducted that were previously considered not viable due the length of time and effort that would be required.

In this study, a Chirascan™-plus automated CD spectrometer (ACD) is used to automate the process of measuring CD and the technique has been applied to study the stability of protein conformation as a function of pH.

KEYWORDS

▸ Chirascan	▸ Stability
▸ Circular Dichroism signatures	▸ pH
▸ Protein	▸ Titration
▸ Conformation	▸ Sample to sample reproducibility

Samples were presented in a 96-well micro-titre plate and transferred to the spectrometer using a fixed-probe x, y, z robot. In a typical measurement, including the automated cleaning cycle, a rate of eight samples per hour can be comfortably achieved and the instrument can be left unattended for 24 hours. Protein usage per measurement in the far-UV is in the range 10µg-40µg and in the near-UV 20µg-100µg.

This work highlights the possibility not only of studying changes in protein conformation in response to pH but also of looking at changes in relation to ionic strength, ligand binding and identifying differences in mutant proteins in biopharmaceutical clone selection.

A series of buffered 1mg/mL bovine serum albumin (BSA) samples in a 96-well plate was prepared automatically by the fixed-probe robot of the Chirascan™-plus ACD. The phosphate-citrate buffer system ranged from pH2.2 to pH8.0 at 0.2 intervals, giving a total of 30 solutions. The samples were presented automatically to the spectrometer and their CD and absorption spectra measured. Simple treatment of the CD spectra enables the stability of the conformation as a function of pH to be established.

METHOD

Stock solutions of 4mg/mL BSA, 200mM sodium phosphate and 100mM sodium citrate were used to prepare a series of buffered samples from pH2.2 (2% phosphate-98% citrate) to pH8.0 (100% phosphate). The concentration of the buffered BSA samples was 1mg/mL; the samples were prepared directly in a 96-well plate and each protein solution was paired with its buffer. Buffers and samples were presented to the Chirascan™-plus ACD with their CD and absorption spectra measured in duplicate for each. The experimental parameters are summarized in Table 1.

Wavelength range	260nm ≥ λ ≥ 175nm
Step-size / Bandwidth	1nm / 1nm
Time-per-point / Time-per-scan	1s / 90s
Cell path-length	0.1mm
Buffer	Phosphate-citrate, pH2.2 to 8.0
Volume per sample	40µl (50µl in well)
Protein per measurement / total	40µg / 1.2mg
Time per sample (full cycle)	7.5 minutes

Table 1. Experimental parameters.

Each buffer or sample was subject to the same regime. The fixed-probe robot transferred 40µl of solution from the well to the 0.1mm pathlength flow-cell via an injection port. The CD and absorption spectra were measured simultaneously and in duplicate. The sample was then removed from the cell. The cell was then washed and dried, ready for the next solution. Each full measurement / wash / dry cycle took 7.5 minutes.

RESULTS

All CD and absorption spectra were measured down to 175nm to establish the practical limit of measurement, which will be determined by high absorption at shorter wavelengths. As a rule of thumb, those data whose total absorption (buffer plus protein) exceeds 2AU are excluded from subsequent analysis. The highest total absorption occurs at approximately pH5.0, reaching 2AU at 188nm. Therefore all data at shorter wavelengths than this have been excluded. Figure 1 shows the buffer-subtracted CD and absorption spectra (260nm ≥ λ ≥ 188nm) and how they change from pH2.2 to pH8.0.

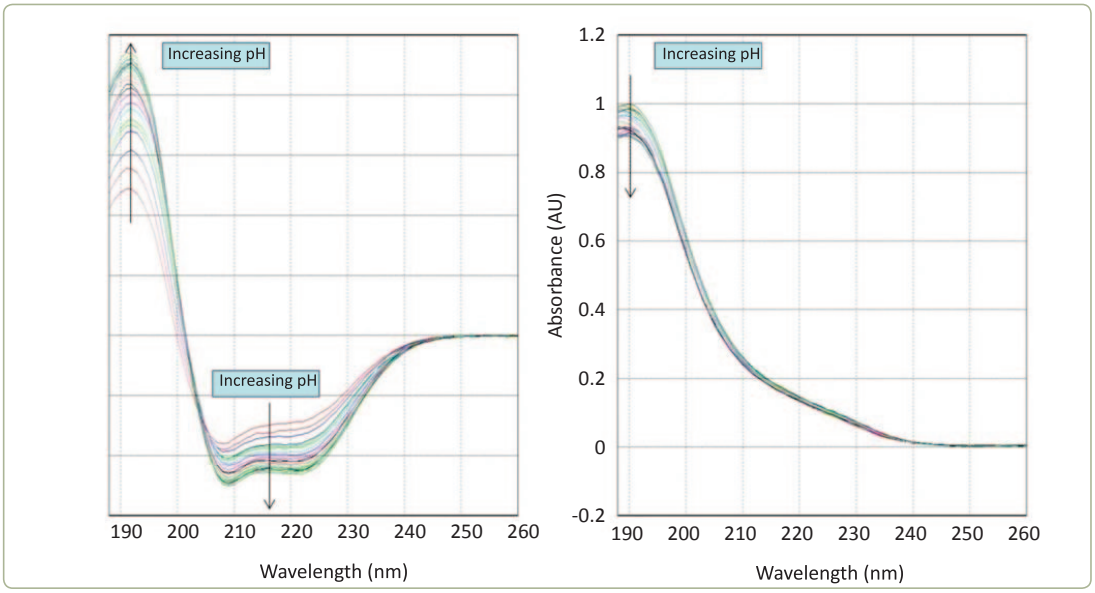

Figure 1. Background-subtracted CD and absorption spectra of BSA showing change with pH.

A closer look at the CD spectra in Figure 1 shows that there are two isosbestic points, one occurring at 206nm in the range pH2.2 to pH3.8 and the second occurring at 201nm in the range pH4.0 to pH8.0 (Figure 2). This agrees with findings reported in the literature[1] which suggest that BSA adopts three different conformations in the range of pH covered in this study, these being the N-form which occurs at physiological pH, the F-form which may also be physiologically active and the extended or E-form, which dominates at lower pH values.

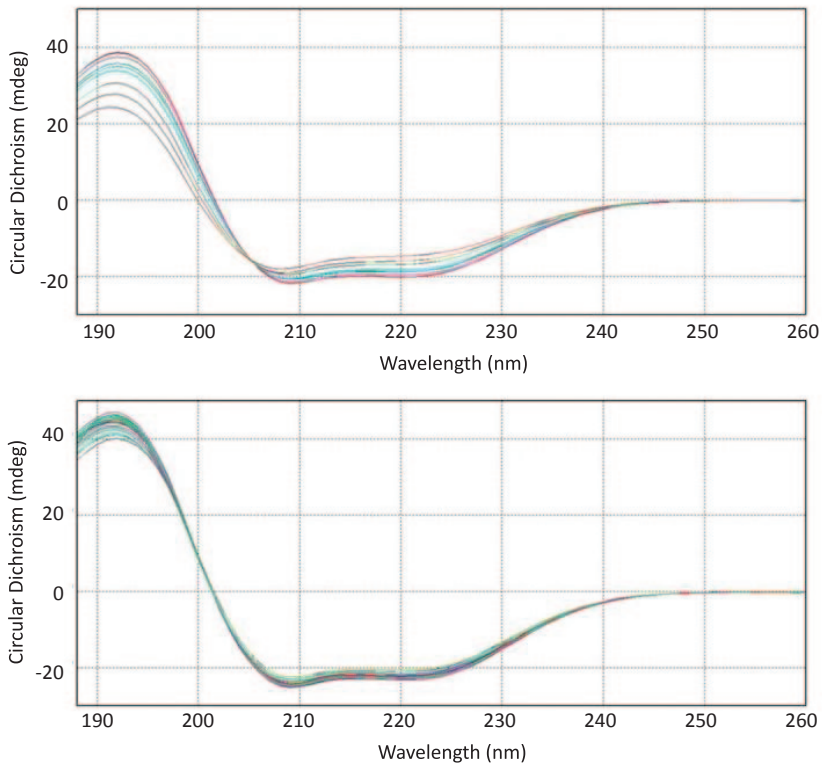

Figure 2. CD spectra showing isosbestic points at 206nm (top) and 201nm (bottom).

Each sample was paired with its respective buffer. The buffer is non-chiral and its changing composition should have no effect on the CD spectra but it serves as a convenient internal drift standard for the CD measurements and a control to detect contamination. Its absorption, however, will change with composition and for this reason it is important to match buffer and sample.

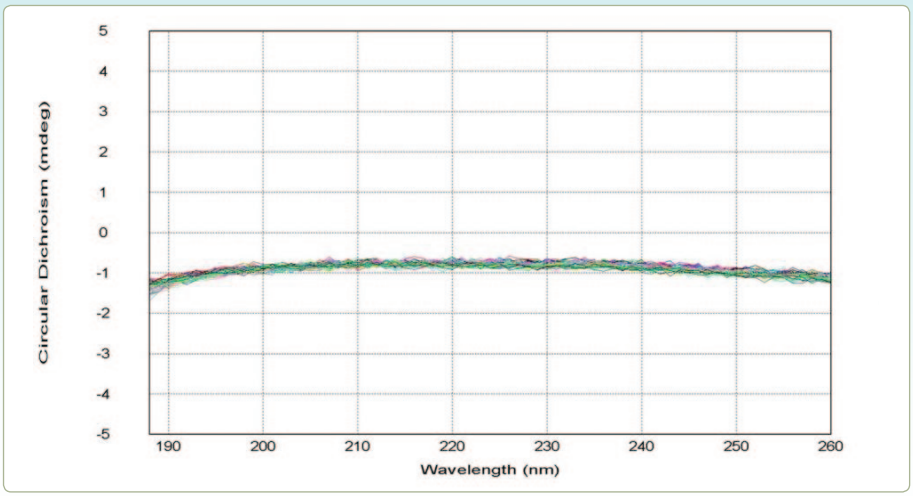

Figure 3. Overlay of CD baseline spectra of phosphate-citrate system from pH2.2 to pH8.0.

Figure 3 shows the sixty buffer CD baselines from the duplicate measurements of the thirty different buffer solutions. There is no significant drift during the experiment and the peak-to-peak spread is about 0.3 millidegrees. Nor is there any evidence of cross-contamination, indicating that the cleaning regime is very effective.

The data were used to generate a conformational stability map as a function of pH. Simply plotting a crude contour map of the raw data will give an immediate qualitative feel for the stability of the protein– see Figure 4.

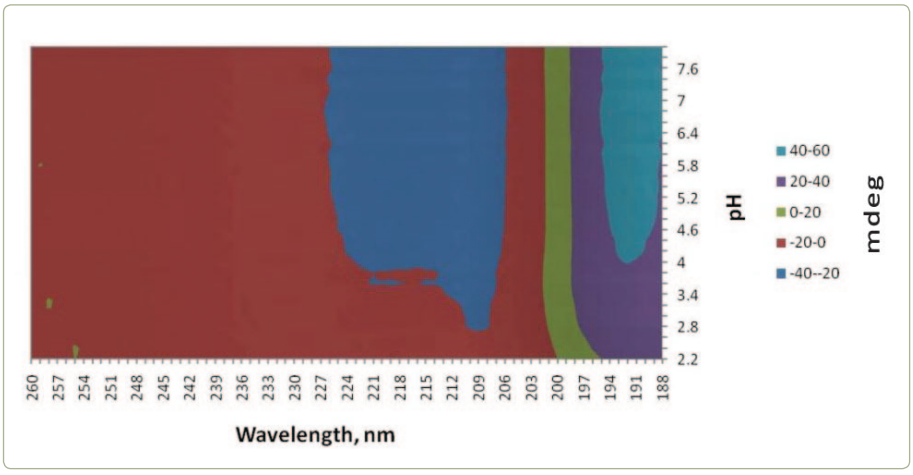

Figure 4. Contour map of raw data showing stable region at pH~6 and higher.

Another simple comparative device for assessing conformational stability is to plot the ratio of features in the raw data, for example the sum of all positive peaks to the sum of all negative peaks – see Figure 5. This technique would be useful in eliminating concentration-dependent amplitude differences for those cases where establishing a constant sample concentration is difficult.

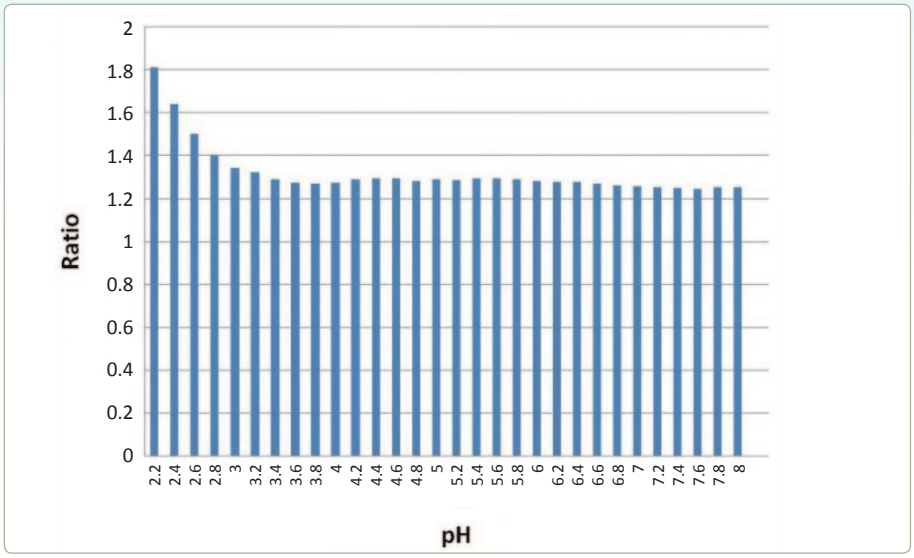

Figure 5. Plot of Σ positive to Σ negative peaks, raw data, showing gross conformational change at pH<3.4.

In addition to these simple semi-quantitative methods, global fitting of the data is recommended if the generic behaviour of the material is understood, for example if the protein has been studied before, or different members of a related family are being studied to identify different tolerances to the imposed conditions.

As discussed earlier, a three-state model is appropriate to describe the data in the current experiment. Figure 6 shows the end-point spectra after global fitting and the concentration profiles of the three species. Modelling enables us to calculate the pKa values, which were 2.34 for the E and F form and 4.44 for the F and N form equilibria respectively. Further, the concentration profiles can be used to determine the level of contamination of one conformer by another. For example, if the onset of conformational change of the N-form to the F-form were to be defined as the pH at which a 95% to 5% equilibrium were established, then we could state unequivocally that it occurs at pH5.8.

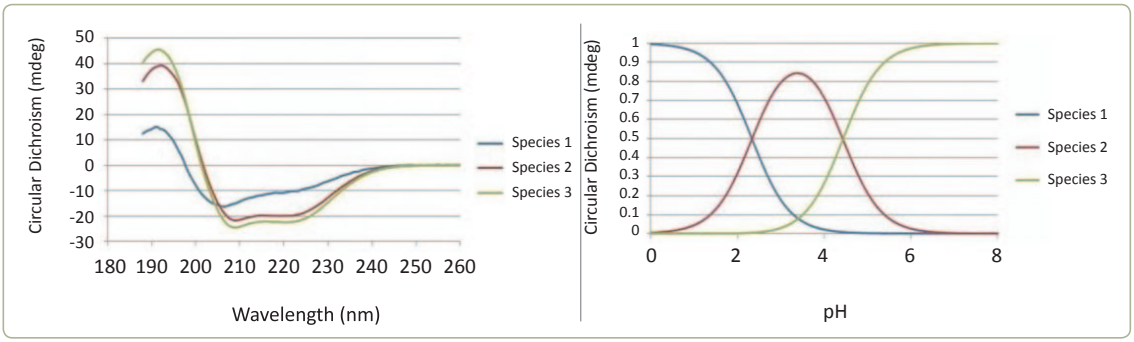

Figure 6. Three-state end-point spectra (left) and their concentration profiles (right).

Figure 7 shows the calculated contour map for the three-state model as a function of pH. The regions of conformational stability of the calculated spectra are well-defined, unambiguous and need no expert interpretation. The map shows that the conformation is stable at pH5.8 and above and that some amplitude changes begin to occur as the pH is reduced from this value until at about pH4.2, major changes commence. The calculation shows that the conformational changes to the E-form are complete by pH1.5.

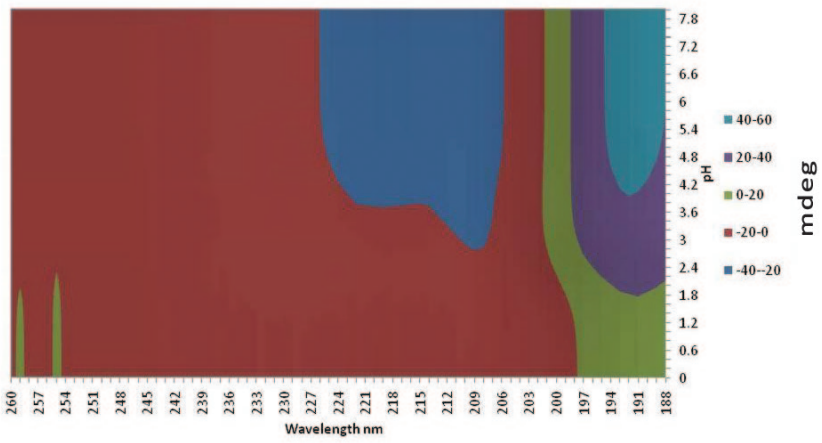

Figure 7. Contour map of calculated spectra over an extended pH range.

The pH resolution used in this study at 0.2 is very fine and in general, conformational change can be perfectly adequately monitored at intervals in pH of 0.4, which would reduce by half the quantity of protein used. Likewise the concentration could be halved without compromising the result, which would again reduce protein usage. For a typical phosphate-citrate range (pH2.2 to pH8.0), total protein usage could be as little as 300µg, making it a very economical technique for the information returned.

CONCLUSION

This study describes a new automated method for the study of conformational stability of proteins as a function of an independent variable, in this case pH. It has been shown that the automated preparation of samples and subsequent automated, simultaneous measurement of CD and absorption spectra is now feasible.

The new Chirascan™-plus ACD delivers excellent sample-to-sample reproducibility, efficient and effective inter-sample *in situ* cleaning and superb instrument stability. It is economical in sample consumption and can analyse up to 200 samples per day in unattended operation, representing a very considerable increase in the productivity of CD spectroscopy.

The methods for data analysis and interpretation provide at-a-glance, semi-quantitative stability information using the raw data alone and quantitative results can be obtained with minimum effort. The latter is particularly useful if the generic behaviour of a family of proteins is understood and relative conformational stabilities are desired.

The model protein BSA has been used in this study but automated CD spectroscopy could be equally well applied to the study of the relative conformational stabilities of bio-therapeutic candidates in response to changing pH, ionic strength, ligand binding and other variables. The improvement in reproducibility, productivity and sample economy brought to CD by automation give it the qualities needed to help identify promising bio-therapeutic candidates at the pre-formulation phase of product development.

REFERENCES

[1] Carter D.C. and Ho J.X. Structure of Serum Albumin pp153-203, Advances in Protein Chemistry, Vol.45, *Lipoproteins, Apolipoproteins and Lipases*, Academic Press 1994, Ed. Verne Schumaker. ISBN: 0-12-034245-6.

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