



Abstract A stable, correctly-folded protein is an absolute requirement for a successful biotherapeutic and the stability of a desired conformation is determined by suitable formulation. Chirascan™ dynamic multimode spectroscopy (DMS) is a new technology from Applied Photophysics that uses multiple spectroscopic probes to monitor changes in secondary structure as a function of temperature and to determine the thermodynamics of unfolding. Chirascan™ DMS will:

- ▶ Prove that a protein is initially correctly folded which is a requirement for biological activity
- ▶ Distinguish between unfolding and aggregation steps, which is of potential relevance to future immunogenicity
- ▶ Identify the structural components that change and the order in which they denature, giving valuable information on conformation
- ▶ Determine quantitatively the T_m and enthalpy values as indicators of stability

In this study, DMS is applied to the denaturation of a monoclonal antibody under different pH conditions to show the potential of the technology in biotherapeutic development.

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CHIRASCAN SERIES APPLICATION NOTE

A new approach for optimising biotherapeutic development using Chirascan™ Dynamic Multimode Spectroscopy (DMS)

INTRODUCTION

The stability of a protein therapeutic is conventionally monitored by calorimetric techniques, particularly differential scanning calorimetry (DSC), yielding thermodynamic parameters such as the enthalpy and the mid-point of a conformational transition. The thermodynamic parameters are indicative of the relative stabilities of proteins in different conditions but calorimetric techniques cannot tell you how the conformation changes, whether or not a conformational change leads to aggregation, or if the protein is in its desired conformation prior to heating.

Knowledge of secondary structure will confirm whether or not the protein is correctly folded initially, and observing the secondary structure as a function of temperature will tell you how its conformation changes on heating. However, the only common method to determine protein secondary structure in solution is circular dichroism (CD) and monitoring CD as a function of temperature can be a time-consuming business^[1].

Chirascan™ dynamic multimode spectroscopy combines the benefits of spectroscopic and calorimetric measurements into a single, rapid, information-rich measurement that generates results from a complete experiment in about an hour.

KEYWORDS

▶ Chirascan	▶ Optimising
▶ Spectroscopy	▶ Stability
▶ Dynamic Multimode	▶ Protein
▶ Biotherapeutic	▶ Circular Dichroism

EXPERIMENTAL CONDITIONS AND INSTRUMENT SETUP

Chirascan™ DMS was used for all the experiments. Samples of a monoclonal antibody (mouse anti-human IgG-Fc sub-class IgG2a (Abazyme, MA, USA)) at concentrations of 0.25mg/ml and 0.5mg/ml were buffered at pH7.0 and pH4.2 respectively. Protein/buffer solution in a 1mm cuvette was heated from 4°C to 76°C at a rate of 1°C per minute; the sample temperature was recorded using an in-sample thermocouple. CD and absorbance spectra were measured during heating such that new scans commenced at intervals of 1°C. The duration of each of the two experiments was less than 75 minutes.

Sample	mouse anti-human IgG	mouse anti-human IgG
Buffer	pH7.0 (45mM phosphate)	pH4.2 (45mM phosphate 25mM ascorbate)
Volume	300µL	300µL
Protein conc.	0.25 mg/mL	0.5mg/mL
Protein used	75µg	150µg
T range	4°C - 76°C	4°C - 76°C
T rate	1°C / minute	1°C / minute
l range	260nm - 198nm	260nm - 205nm
Step-size	1nm	1nm
Duration	<75 minutes	<75 minutes
Cell pathlength	1mm	1mm
Optical probes	CD and absorbance	CD and absorbance

Table 1. Summary of experimental parameters.

RESULTS AND DISCUSSION - PH7.0

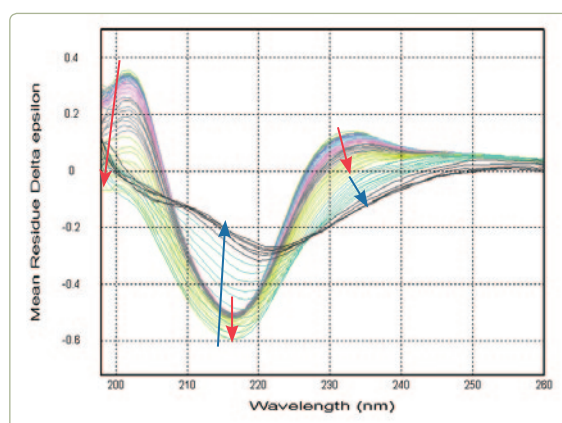


Figure 1. CD Spectra of mAb at multiple temperatures.

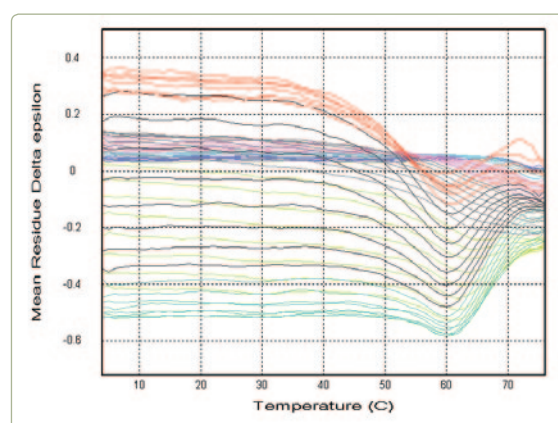


Figure 2. CD-temperature profiles at multiple wavelengths

The CD data for the mAb at pH7.0 are displayed in figures 1 and 2 as a function of wavelength and temperature. There are 73 individual CD spectra in figure 1 with a new scan started every 1°C, and 64 CD-temperature profiles in figure 2, one for each integer wavelength from 197nm to 260nm. No significant change in the CD signature occurs below about 35°C. In the range 35°C<T<60°C a rapid change accompanied by loss of amplitude of the positive peaks at 202nm and 233nm takes place (indicated by the red arrows in figure 1)

with a concomitant increase in the amplitude of the negative peak at 216nm. A second transition, indicated by the blue arrows, occurs above 60°C, where the most significant change is the loss of amplitude of the peak at 216nm.

Figure 3 shows the absorbance as a function of temperature – note that at approximately 60°C there is an abrupt change in the apparent absorbance of the sample even for those traces that represent wavelengths at which there is no absorbing species ($\lambda > 250\text{nm}$). In fact, such a change in the absence of a chromophore is due to light scattering caused by the onset of aggregation and therefore the second transition seen in the CD data is accompanied by aggregation.

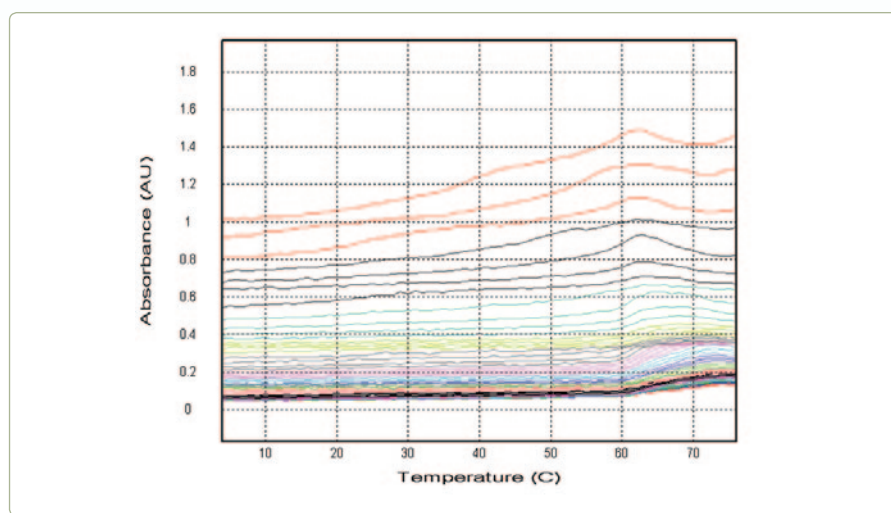


Figure 3. Absorbance-temperature profiles at multiple wavelengths.

RESULTS AND DISCUSSION - PH4.2

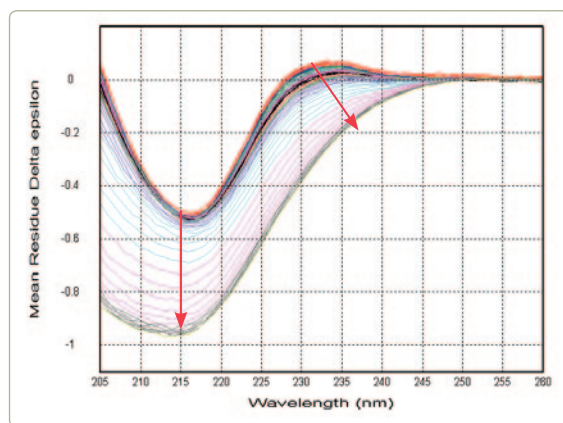


Figure 4. CD Spectra at multiple temperatures.

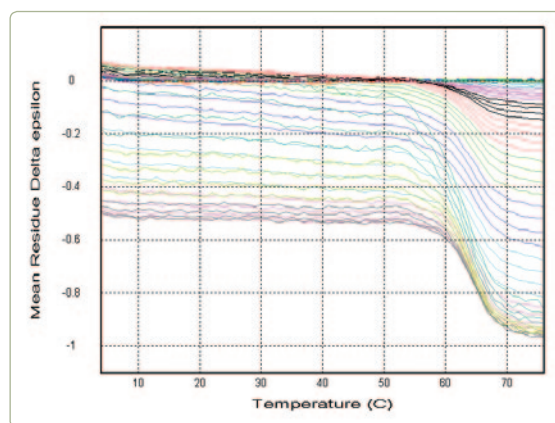


Figure 5. CD-temperature profiles at multiple wavelengths.

The CD data for the mAb at pH4.2 are displayed in figures 4 and 5 as a function of wavelength and temperature. There are 73 individual CD spectra in figure 4 with a new scan started every 1°C, and 56 CD-temperature profiles in figure 5, one for each integer wavelength from 205nm to 260nm inclusive. No significant change in CD occurs below 50°C and the transition appears to be biphasic, indicated by the red arrows.

Inspection of figure 6 shows no increase in apparent absorbance at wavelengths where there is no chromophore suggesting that there is no aggregation associated with the unfolding event, in contrast to the same sample at pH7.0.

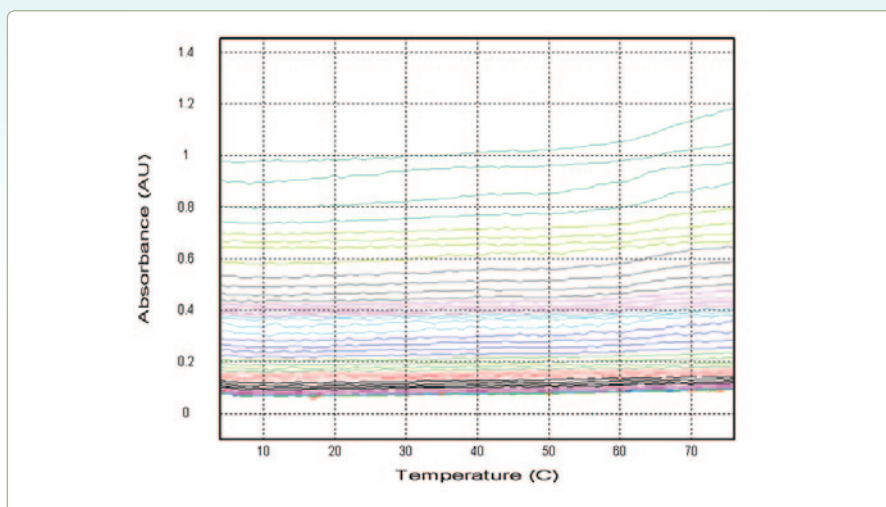


Figure 6. Absorbance-temperature profiles at multiple wavelengths.

RESULTS AND DISCUSSION - ANALYSIS OF THE DATA

A data reduction technique known as singular-value decomposition was used to identify the number of independent species represented in the CD data: there were three for both pH7.0 and pH4.2, although as noted above, the CD-temperature profiles at pH4.2 do not obviously show this. Triphasic denaturation models were therefore used in fitting the data.

The data across all wavelengths were used in non-linear least-squares refinement of the models. Constraints were applied to ensure that the calculated values and profiles remain physically meaningful. The results with errors in brackets are given in the table below.

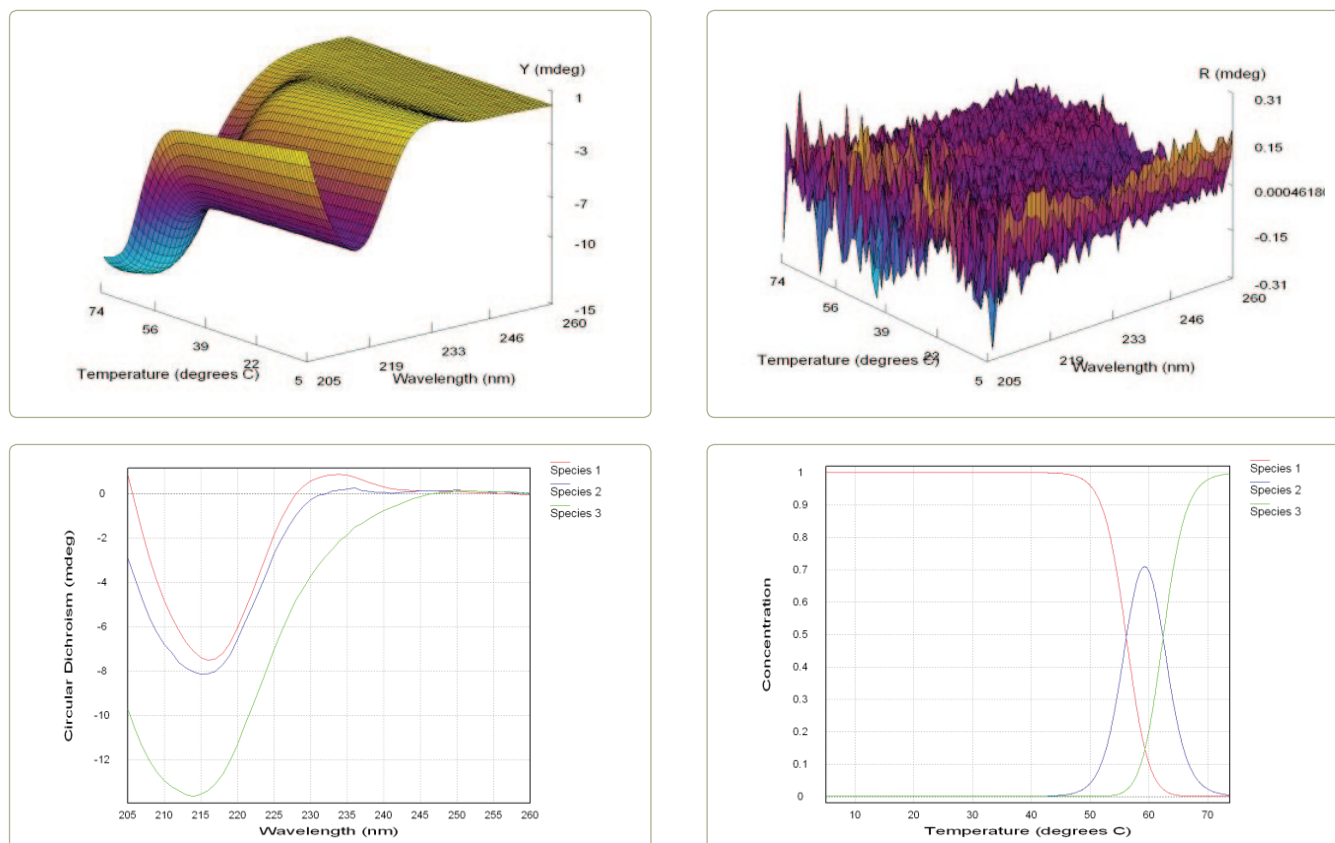
	mAb pH7.0	mAb pH4.2
T _{m1}	51.6(2) °C	56.2(1) °C
ΔH ₁	210(5) kJ/mol	453(10) kJ/mol
T _{m2}	64.3(1) °C	62.4(1) °C
ΔH ₂	446(9) kJ/mol	477(4) kJ/mol

Table 2. Transition mid-points and enthalpies.

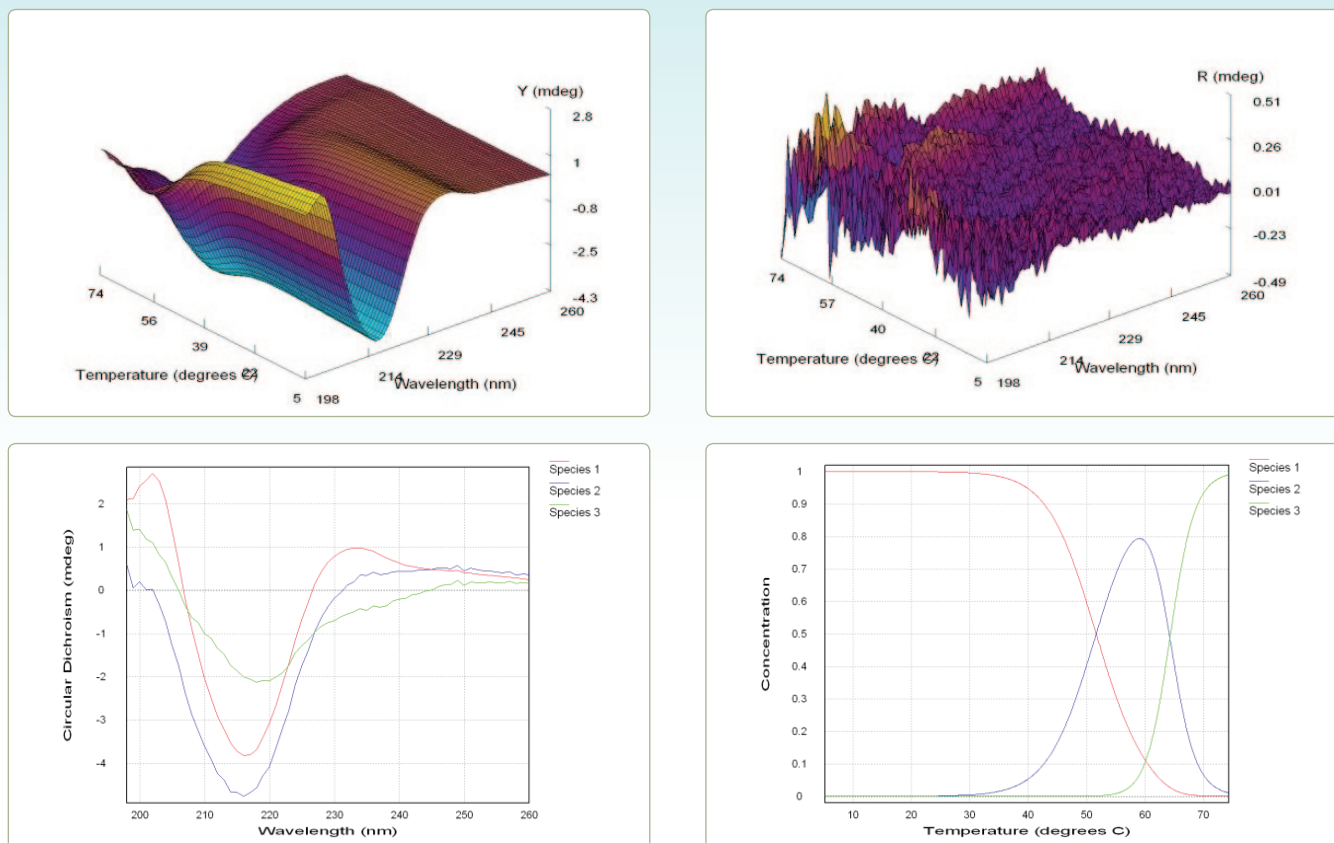
If a higher T_m is taken as an indicator of better long-term stability of the protein, then T_{m1} indicates that the protein at pH4.2 will be more stable. A larger enthalpy of transition indicates a narrower transition and therefore a later onset of loss of conformation, which is a desirable characteristic; ΔH_1 indicates that the protein at pH4.2 has the sharper transition.

The structural information inherent in the data permits further investigation of the behaviour of the protein. Reference to the results of the refinements in figures 7 and 8 illustrates the following points:

- ▶ The CD signature of the secondary structure is a very good indicator of how the protein is folded and can be used to judge if the protein is correctly folded at the outset of the measurement. The familiar CD signature of a mAb is seen at the outset of both experiments, indicating that the protein is correctly folded at both pH7.0 and pH4.2.
- ▶ The CD signature identifies the main secondary-structural components (α -helix, β -sheet etc.), which of them change under stress, how they change and the order of their changing. Monoclonal antibodies are largely β -sheet proteins; the protein at pH7.0 and pH4.2 show a similar initial change to an intermediate but at higher temperatures, the behaviour is different. At pH7.0, further unfolding leads to a collagen-like CD signature and is accompanied by aggregation (seen from the absorbance spectrum); at pH4.2, further unfolding leads to an extended conformation with no apparent associated aggregation.
- ▶ Most therapeutic antibodies will need to be active at physiological pH and body temperature. It is interesting to note that at pH7.0 the monoclonal antibody is starting to unfold at 37°C. This is best seen by reference to Species 1 (native conformation) in figure 8d overleaf.



Figures 7a-7d. mAb at pH4.2 – triphasic model – top: the model and residual surfaces, bottom: the model species and their relative concentration profiles.



Figures 8a-8d. mAb at pH7 – triphasic model – top: the model and residual surfaces, bottom: the model species and their relative concentration profiles.

CONCLUSION

Dynamic multimode spectroscopy is a powerful addition to the range of biophysical tools available in biotherapeutic development. In addition to thermodynamic parameters which are indicative of the relative stability of proteins in different conditions, detailed information about the behaviour of the secondary structure and aggregation is readily available.

The technique is not restricted to far-UV secondary structure analysis – it can be used equally well in the near-UV to study the change in the chiral environment of the aromatic chromophores of amino-acid residues, giving insights into the stability of the protein's tertiary structure.

REFERENCES

- [1] Norma J. Greenfield, Nature Protocols, Vol.1 No.6, 2006, 2527.

NEW APPLICATIONS FOR CD SPECTROSCOPY

Optimising biotherapeutic formulations

Used as a label free stability-indicating assay, Chirascan™-plus automated circular dichroism (ACD) can identify good formulation candidates earlier for further downstream processing. By culling formulations that are likely to fail early and focusing on those that are more viable for real time and accelerated stability studies, users can make savings in both time and money.

Combining the label-free and information-rich technique of dynamic multimode spectroscopy (DMS) with the productivity of automation gives a whole new approach to establishing conformational stability under different formulation conditions. The conformational integrity of biotherapeutics as a function of more than one stress condition (e.g. Temperature, pH, ionic strength) is readily determined in unattended operation.

Statistical comparison of similar proteins (Biosimilarity)

Research into biosimilar pharmaceutical products has grown exponentially over the last few years turning it into a multi-billion pound business. Structure, biological activity and stability are just a few of the complex studies required and, traditionally, these use multiple techniques which are very time consuming and labour intensive.

Automation lends itself to measuring samples repeatedly and thus to generating statistical comparisons. To answer the question: 'Are these two CD spectra the same?' is no longer a matter of guesswork – a statistical significance can be associated with the measurements and a quantitative judgment about similarity or otherwise can be made.

Drug Discovery

Fast determination of protein characteristics is key to any drug discovery department in pharmaceutical research. Applied Photophysics offers a unique solution providing simultaneous circular dichroism, absorbance and fluorescence measurements in a single, easy to use, automated experiment.

The Chirascan™-plus ACD spectrometer can provide structural, functional, thermodynamic and aggregation data. By automating our system we provide unparalleled productivity, low sample volumes and no human error reducing the pressure on analytical labs and enabling them to focus on discovery.

Protein Engineering

Monoclonal antibodies, antibody-like proteins, and other biotherapeutics represent a large and growing number of molecular entities entering human clinical trials in virtually all disease indications. The long-term stability of these potential therapeutics is of crucial importance for their development to drug products.

The Chirascan™-plus ACD spectrometer provides rapid, accurate, and easy to perform measurement of the thermal melting (T_m) points, which has proven to be an exceptionally good indicator of the relative stability of engineered proteins.

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