AppliedPhotophysics
Ultrasensitive Spectroscopy for the Life Sciences

CHIRASCAN SERIES APPLICATION NOTE

Testing the stability of antibody biotherapeutics in different formulations using dynamic multimode spectroscopy (DMS)

Abstract *This application note describes the use of a Chirascan™-plus CD spectrometer employing dynamic multimode spectroscopy (DMS) to test the stability of a monoclonal antibody in two different formulation buffers: B1 (acetate) and B2 (lactate). The technique was used to calculate the melting temperatures (T_m), and van't Hoff enthalpies($\Delta H_{van't\ Hoff}$) of the antibody in each buffer. Alongside these thermodynamic parameters, aggregation of the antibody in each of the formulations was monitored over a period of weeks by analysing their absorption spectra which are recorded simultaneously with Circular Dichroism measurements. Significant differences in absorption temperature profiles over this period of time showed that aggregation was occurring at decreasing temperatures in B1, whereas no aggregation was observed in B2. The results indicate that the antibody is more stable in B2 than B1 despite a lower T_m originally being observed in B2.*

INTRODUCTION

For a potential biotherapeutic to be a feasible candidate for marketing, it needs to be formulated in a buffer system in which it is stable. A stable protein formulation requires that minimal change occurs in the protein structure over time so that it remains in its active state. Circular Dichroism is a highly sensitive probe of protein conformation and when combined with Dynamic Multimode Spectroscopy (DMS) can be used to follow protein denaturation as a function of temperature. One assessment of protein stability is its melting temperature (T_m) which can be measured directly using this technique. However, as this study shows, this parameter on its own is not always a reliable measure of stability. This is because proteins can have a tendency to irreversibly aggregate; which, as shown here, can be dependent on the composition of its buffer. The Chirascan™-plus CD spectrometer measures absorbance at the same time as CD and can be used to measure protein aggregation due to the scattering of light that the aggregate causes.

This study determines the thermodynamic stability (T_m's, ΔH's) of a monoclonal antibody in two formulation buffers; B1 (acetate) and B2 (lactate). Also, the stability of the protein as determined by extent of aggregation is measured in each formulation over a period of 12 weeks. Prior to

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KEYWORDS

▸ Chirascan	▸ Melt temperature
▸ Circular Dichroism	▸ Formulation
▸ Reproducibility	▸ Stability
▸ Biotherapeutic	▸ Dynamic Multimode

this experiment, an interest had been raised based on differential scanning calorimetry results that showed a difference in T_m for the two formulations. Here we establish that B2 is a much more robust formulation than B1 because it prevents the antibody from aggregating over the time period measured, and therefore increases the lifetime of the active protein.

MATERIALS AND METHODS

The monoclonal antibody was supplied in two different formulations. DMS measurements were made under the conditions summarised in Table 1 using a Chirascan™-plus CD spectrometer and subsequent analyses of the data were carried out using APL's proprietary global analysis software program, Global 3.

	mAb in B1 buffer	mAb in B2 buffer
T-ramp rate	1°C/minute	1°C/minute
T-ramp range	4°C < T < 100°C	4°C < T < 100°C
New scan every	1°C	1°C
Step-size	1nm	1nm
Scan range	195nm < λ < 260nm	195nm < λ < 260nm
Time per point	0.5s < t < 0.8s	0.5s < t < 0.8s
Bandwidth	0.9nm	0.9nm
Concentration	0.5mg/mL	0.5mg/mL
Buffer	B1	B2
Pathlength	0.5mm	0.5mm
Sample volume used	130µl	130µl
Mass of protein used	65µg	65µg

Table 1. Experimental parameters for DMS measurements.

The samples were stored in a refrigerator at 4ºC when not in use and taken from the refrigerator for brief periods when measurements were carried out. The samples were degassed under partial vacuum before being transferred to a spectroscopic cuvette.

Prior to performing the DMS measurement, the light intensity was adjusted to ensure that photo-bleaching did not interfere with the measurements. The maximum safe light intensity was established by reducing the spectral bandwidth until no decay of the CD signal was seen during a dummy DMS run with the temperature of the sample held constant at 35ºC (Figure 1). Once the maximum safe light intensity had been established, a multi-wavelength thermal denaturation measurement was carried out four times for each formulation.

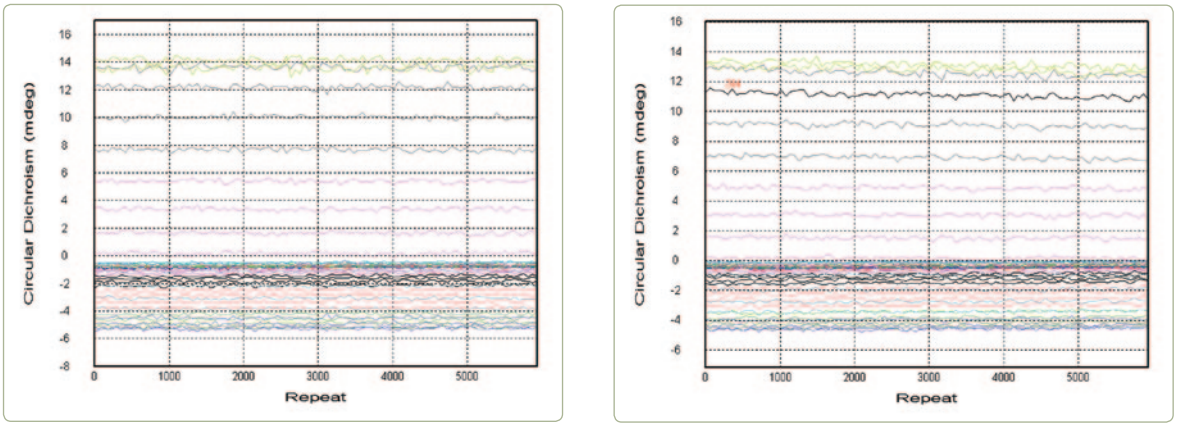


Figure 1. B1 (left) and B2 (right). 100 scans (6000s) at 35ºC and 0.9nm BW. No photo-bleaching.

RESULTS AND DISCUSSION – THERMODYNAMIC PARAMETERS

Tables 2 and 3 show the results of the Global 3 analysis of the 4 replicates of B1 and B2 respectively. Each dataset was analysed twice to establish the effect on the result of different starting values for the refinement.

The B1 datasets were analysed using 4-transition, no-baseline models. The fourth transition was used to account for the aggregation phase which occurred at higher temperatures. Attempts at excluding data affected by aggregation and using a 3-transition model meant it was difficult to identify the phase transitions in the derivative plots The 4-transition model was therefore preferred.

The B2 datasets were analysed using 3-transition, no-baseline models. A fourth transition was not required because there was no evidence of aggregation and therefore no significant distortion of the third transition.

	T_m1 °C	T_m2 °C	T_m3 °C	ΔH1 kJ/mol	ΔH2 kJ/mol	ΔH3 kJ/mol
B1 1.1	68.4 (1)	75.8 (1)	83.3 (1)	373 (22)	788 (31)	716 (12)
B1 1.2	67.1 (1)	75.0 (1)	83.1 (1)	422 (26)	649 (28)	665 (10)
B1 2.1	50.0 (2)	81.8 (2)	85.1 (1)	118 (10)	189 (3)	658 (25)
B1 2.2	49.6 (2)	80.9 (1)	85.1 (1)	207 (43)	199 (48)	640 (15)
B1 3.1	68.9 (2)	77.7 (1)	84.3 (1)	154 (9)	496 (37)	837 (14)
B1 3.2	68.0 (2)	77.2 (1)	84.1 (1)	147 (12)	523 (44)	776 (12)
B1 4.1	67.5 (1)	77.6 (1)	84.5 (1)	208 (21)	390 (26)	689 (16)
B1 4.2	68.4 (2)	77.9 (1)	84.5 (1)	194 (14)	380 (28)	719 (16)
Average*	68.1	76.9	84.0	250	495	711
Std Dev*	0.7	1.2	0.6	117	185	70
*omitting 2.1 and 2.2						

Table 2. B1-buffered antibody, global analysis results.

	T_m1 °C	T_m2 °C	T_m3 °C	ΔH1 kJ/mol	ΔH2 kJ/mol	ΔH3 kJ/mol
B2 1.1	66.8 (2)	80.5 (2)	86.3 (1)	166 (25)	258 (15)	867 (11)
B2 1.2	67.3 (2)	79.4 (1)	86.1 (1)	139 (18)	314 (19)	821 (10)
B2 2.1	64.4 (1)	77.0 (1)	85.7 (1)	191 (16)	465 (18)	695 (8)
B2 2.2	63.0 (1)	76.5 (1)	85.7 (1)	322 (24)	430 (18)	669 (8)
B2 3.1	64.6 (1)	76.5 (1)	85.4 (1)	408 (25)	391 (17)	734 (10)
B2 3.2	65.2 (1)	76.8 (1)	85.5 (1)	370 (22)	408 (17)	756 (10)
B2 4.1	64.2 (1)	76.6 (1)	85.5 (1)	436 (31)	493 (21)	678 (8)
B2 4.2	65.8 (1)	77.2 (1)	85.6 (1)	376 (26)	564 (23)	707 (9)
Average	65.2	77.6	85.7	301	415	752
Std Dev	1.4	1.5	0.3	118	97	67

Table 3. B2-buffered antibody, global analysis results.

Key: B1 / B2 n.x = B1 / B2 measurement n, analysis x
Figure in brackets = the calculated error in the last significant figure or figures
Average = the average of the measurements
Std Dev = the population standard deviation

For both B1 and B2 datasets, there is significant variation in the individually calculated enthalpies, especially for ΔH1 and ΔH2, even for a single dataset. This is because the transitions overlap extensively and a range of results will account for the observations equally well. The third and best defined of the transitions gives a calculated enthalpy that does not show quite such variation. The averages of the results for the enthalpies indicate that they are the same within experimental error.

The calculated transition mid-points are much more robust than the calculated enthalpies, because a transition mid-point is fairly insensitive to the width of the transition. Nonetheless, there is a surprising result for one of the B1 datasets (B1 measurement 2), where the mid-points are anomalous. Repeat analysis of the dataset gives much the same result each time and at the time of writing, there is no explanation for this anomaly. It has been excluded from the calculation of the average and standard deviation.

The results suggest that the difference in T_m1 for B1 and B2 is greater than can be accounted for by experimental error and that it is higher for the B1 formulation; T_m2 for both formulations is the same within experimental error. The difference in T_m3 is significant with that of B2 being higher than B1.

RESULTS AND DISCUSSION – DENATURATION PROFILES

During the course of making the above measurements, it was noted that the B1 formulation was showing a tendency to aggregate at lower temperatures than had previously been observed. Figure 2 shows the denaturation signature for the B1 formulation at an interval of about 10 weeks and Figure 3 shows a comparison of the B2 formulation at an interval of about 12 weeks. The difference is striking.

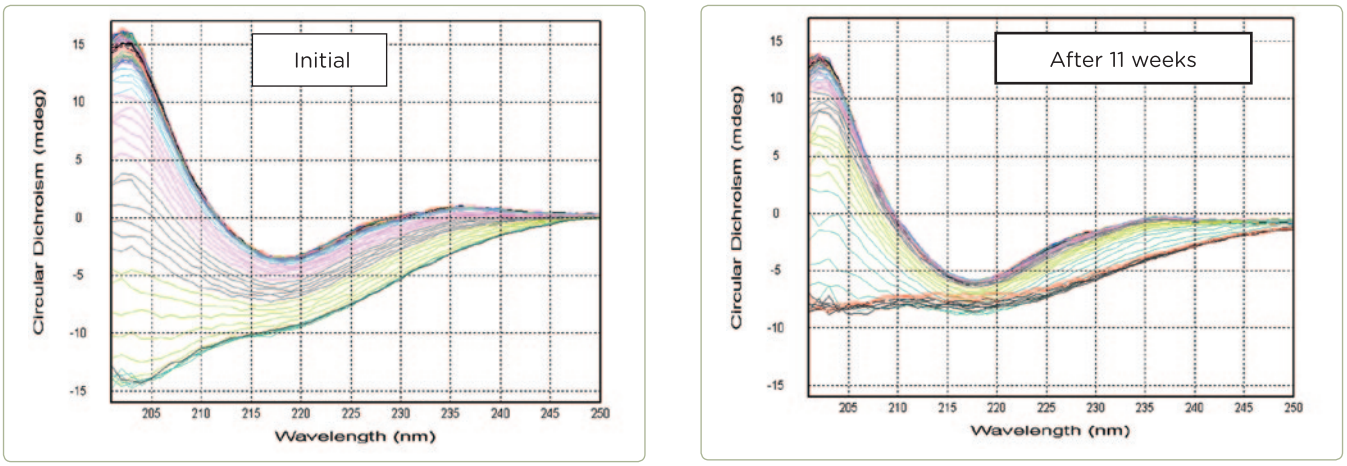


Figure 2. Long-term stability of antibody in acetate (B1).

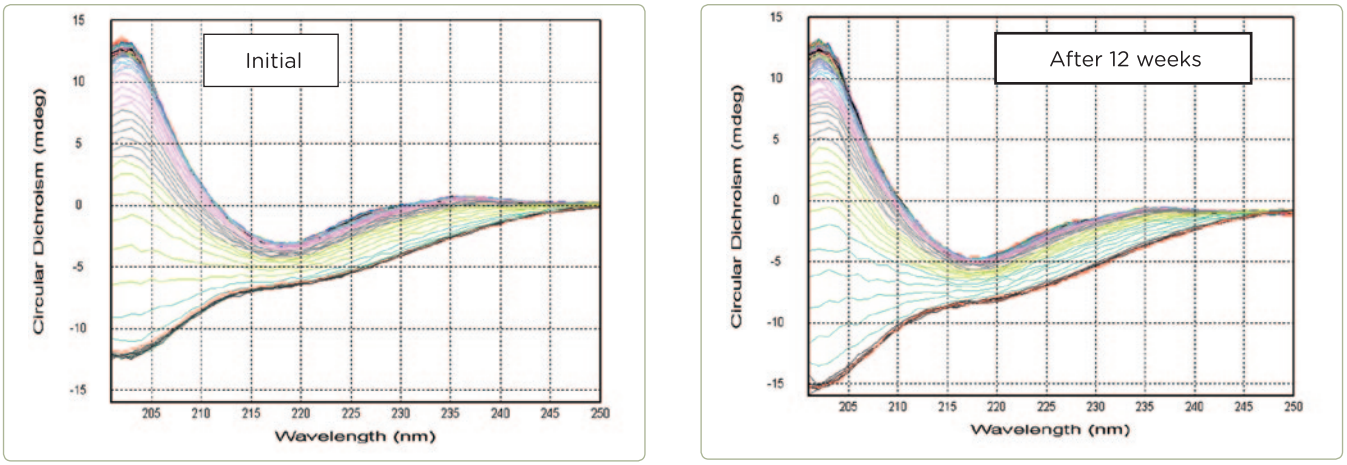


Figure 3. Long-term stability of antibody in lactate (B2).

Examination of the absorption profiles of the two formulations over the period that the samples had been in our possession showed quite clearly that the aggregation onset temperature for the B1 formulation was decreasing with passing time, whereas the B2 was not. Indeed, the B1 data, even when initial experiments were carried out, showed a tendency to aggregate at very high temperatures (>95°C) where the B2 did not.

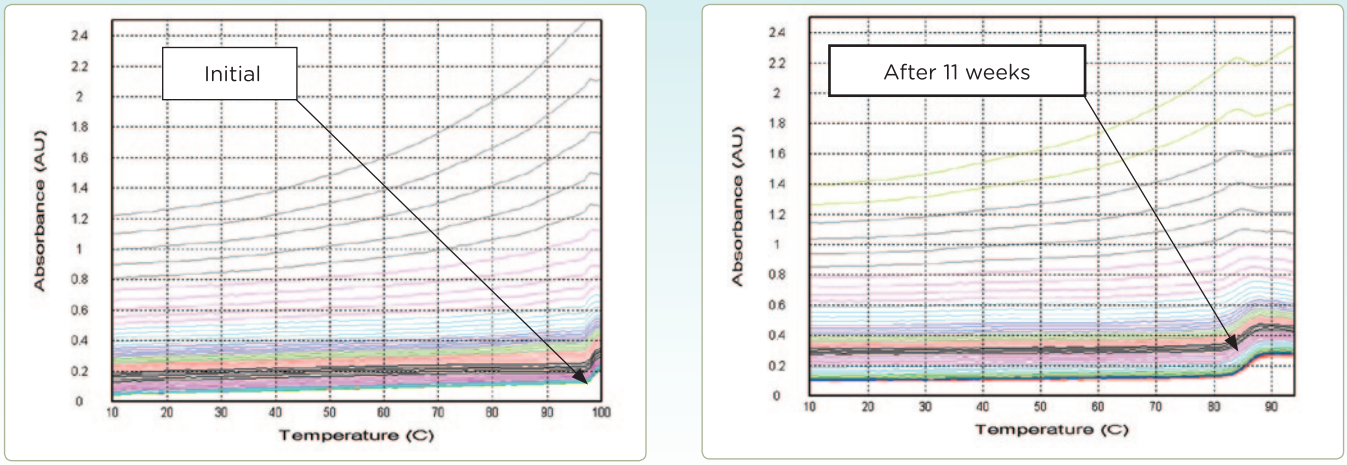


Figure 4. Acetate (B1) absorption temperature profiles: aggregation onset at 97ºC and 82ºC.

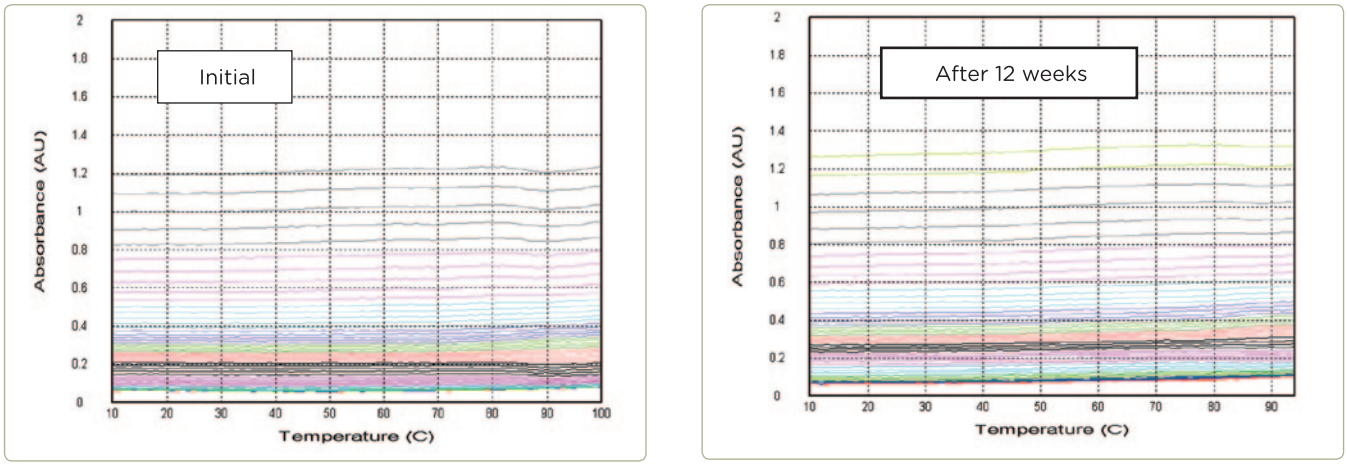


Figure 5. Lactate (B2) absorption temperature profiles: no evidence of aggregation.

CONCLUSION

DMS datasets for an antibody biotherapeutic formulated in acetate and lactate buffers were measured over a period of several weeks. Each DMS dataset was obtained in under 100 minutes and used only 65µg of protein. In spite of the complexity of the systems under investigation, with multiple and overlapping phase transitions, the CD temperature profiles of the DMS data enabled the calculation of robust values for the temperature mid-points for each phase transition in the acetate and lactate formulations. Significant differences in the mid-points of the first and third phase transitions can be seen (T_m1 and T_m3); the mid-points of the second transitions (T_m2) are the same within experimental error.

The enthalpies calculated from the CD temperature profiles show considerable variation between DMS measurements of the same sample and within a dataset when different starting values are given for the refinement. This is an inevitable consequence of the degree of overlap of transitions in these complex systems because a range of results will account for the observations equally well.

The CD spectra of the DMS data were used to identify changes in the secondary structure of the antibody. Both acetate and lactate formulations maintain the antibody in its mainly β-sheet conformation as they age at low temperature and, initially, the conformation changes on heating are very similar, with each formulation taking a virtually identical structural pathway to a recognisable unfolded state. As the samples age, the denaturation pathway of the antibody in acetate buffer changes dramatically but there is no such change for the antibody in lactate buffer, whose denaturation pathway was identical to that of the initial measurement.

The absorption spectra of the DMS data were used to identify aggregation in the acetate formulation and, with the CD spectra, to assess the long-term stability for both formulations. The temperature of the onset of aggregation of the antibody in the acetate formulation was found to decrease significantly with sample age whereas no tendency to aggregate was found for the lactate formulation irrespective of the age of the sample (up to 12 weeks). The results suggest that despite the significantly lower T_m of its first transition which, if taken in isolation might suggest the contrary, the antibody is more stable in the lactate than in the acetate formulation.

It is interesting to note that the data presented here support opinion voiced at a recent Biocalorimetry Conference[1], that T_m on its own may not be a particularly good indicator of stability and that resistance to aggregation once unfolding has occurred is likely to be a better indicator. This highlights the value of using DMS in formulation testing during biotherapeutic antibody development.

REFERENCES

[1] Applications of BioCalorimetry, Heidelberg, July 7-10 2008.

APPENDIX 1

DYNAMIC MULTIMODE SPECTROSCOPY – AN INTRODUCTION

Dynamic multimode spectroscopy (DMS) is the name given to a new experimental technique developed at Applied Photophysics for determining how a protein or other bio-macromolecule (e.g. DNA, RNA) behaves when subjected to changing, usually increasing, temperature. Conventionally, such thermal denaturation experiments have been carried out either as continuous temperature ramps at a single wavelength (which assumes that the behaviour of the protein at a single wavelength is representative of the behaviour at all wavelengths), or as a series of spectra measured after thermal equilibration at discrete temperature intervals (which even at coarse temperature intervals of 5ºC can take many hours to complete and assumes that kinetic effects do not influence the result). In the general case, neither of these assumptions is correct.

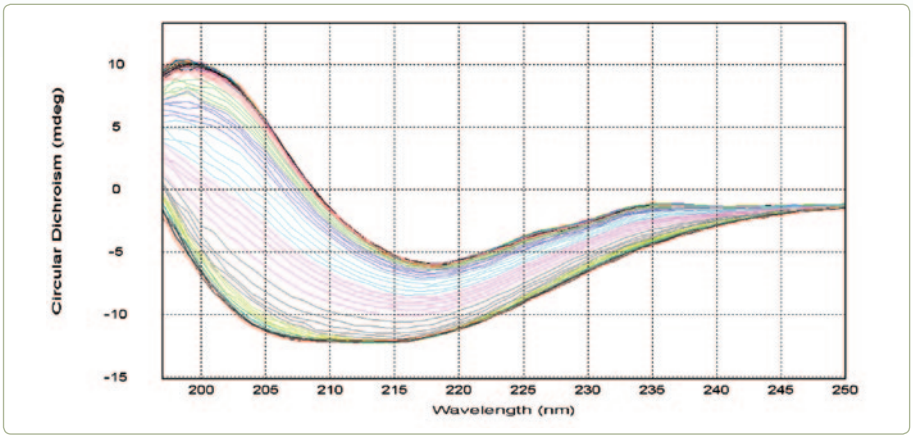
Dynamic multimode spectroscopy combines the two conventional methods into a single, complete experiment which is quick and easy to perform. To a very good approximation, this method eliminates kinetic effects, and enables the measurement of CD at all chosen wavelengths therefore eliminating assumptions about the behaviour of the protein at different wavelengths.

A DMS experiment will always record the circular dichroism and absorption spectra simultaneously and, where cuvette geometry allows, e.g. in the near-UV, the fluorescence excitation spectrum is also recorded.

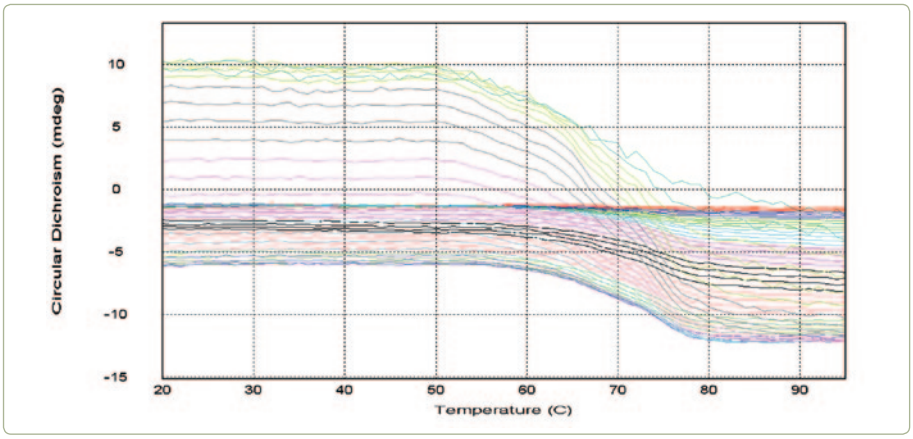
- **Dynamic** refers to the continuous change in an independent parameter other than wavelength, in this case temperature.
- **Multimode** refers to the simultaneous measurement of more than one spectroscopic probe.
- **Spectroscopy** refers to the fact that these measurements are full spectroscopic, not single wavelength, measurements.

In the course of a DMS experiment, a three-dimensional picture is developed in which CD and absorption measurements are made at every user-defined point in the wavelength-temperature plane. For example, a typical experiment in the far-UV (say 195nm-260nm, 20ºC-95ºC) will be set up such that a measurement is made at integral wavelength intervals and at temperature intervals of approximately 1ºC, which means that the CD and absorption surfaces generated will each contain approximately 66 x 75 = 4950 spectroscopic data-points.

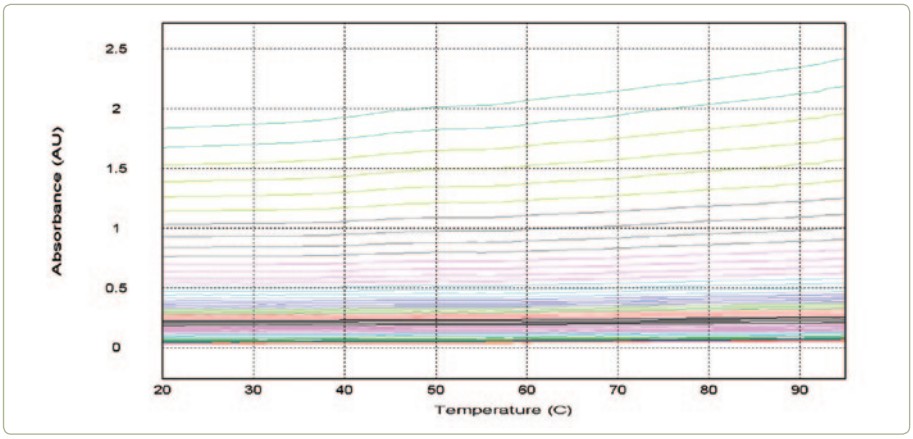
The duration of a DMS experiment is determined by the rate and range of the continuous temperature ramp. A typical protein denaturation would run from 20ºC to 95ºC at 1ºC per minute, giving a total of 75 minutes. The wavelength scanning parameters are set such that a full wavelength scan at a resolution of 1nm is measured for every 1ºC rise in temperature. Thus, a full spectrum is measured approximately every one minute. The unique technology incorporated in Chirascan™-plus ensures that the signal-to-noise of CD spectra measured under these conditions is more than good enough for subsequent interpretation and quantitative analysis.



CD spectra of a monoclonal antibody (250nm-197nm, 20ºC-95ºC) measured in a DMS experiment…



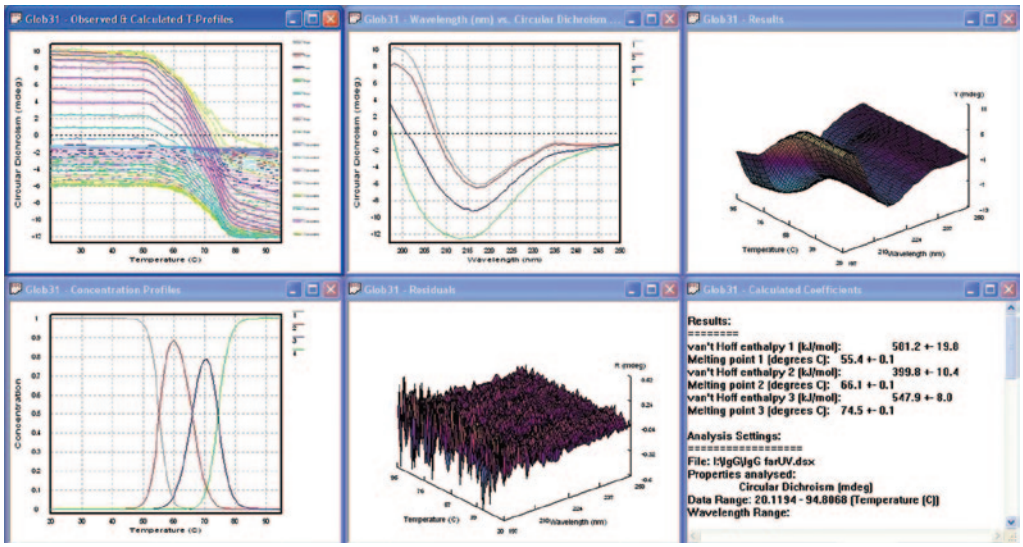
…and the corresponding CD temperature profiles for each of the wavelengths…



…and the simultaneously-measured absorption temperature profiles for each of the wavelengths.

Note that the absorption profiles at those wavelengths where there is very little or no absorption remain constant as a function of temperature, indicating that no insoluble aggregates are formed. If aggregation had occurred, there would have been a sharp, apparent change in absorption due to light scattering by particulates.

DMS is not simply about information-rich measurements: it is also about analysis. In order to take full advantage of the richness of the experimental data, Applied Photophysics has developed a global analysis software product, **Global3**, that takes into account all the data to calculate the spectra and concentration profiles of all the contributing species as well as the mid-points and van't Hoff enthalpies of the transitions between them. Global3 is a comprehensive package that generates both structural and thermodynamic results for the system under study and is unique to Applied Photophysics.



Results of a Global3 analysis of monoclonal antibody far-UV data using a three-transition model.

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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Applied Photophysics was established in 1971 by The Royal Institution of Great Britain