AppliedPhotophysics

Ultrasensitive Spectroscopy for the Life Sciences

The thermal denaturation of an oligonucleotide:

a study to validate dynamic multimode spectroscopy

Abstract *Dynamic multimode spectroscopy (DMS) is a new technique from Applied Photophysics (APL) that uses the Chirascan™-plus CD spectrometer equipped with multiple spectroscopic probes to study changes in the secondary structure of proteins and other biomolecules as a function of both wavelength and temperature (see appendix).*

In this study, an oligonucleotide is used to demonstrate that the technique is reliable, reproducible and accurate. The data show that:

▶ *For a completely reversible system, the circular dichroism spectra, which are indicative of secondary structure, are identical before and after heating*

▶ *The thermodynamic parameters T_m and $\Delta H_{van't\ Hoff}$ calculated from independently measured datasets are reproducible and the same within experimental error*

INTRODUCTION

Proteins are large and complicated molecules whose behaviour is in general non-ideal and thermally-induced phase changes, even low-temperature ones that do not lead to aggregation, are rarely fully reversible. This frequently precludes the use of even simple proteins as reliable 'standards' to validate DMS (or other techniques): typically, the original secondary structure is never fully recovered after a heating cycle and at higher temperatures a tendency to self-associate and aggregate is seen. However, the oligonucleotide that is at the heart of this study was found to be reversible and show no tendency to self-associate on unfolding, making it an ideal compound with which to validate the DMS technique.

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KEYWORDS

▶ Chirascan	▶ D.N.A.
▶ Circular Dichroism	▶ Standard
▶ Thermal Denaturation	▶ Melt temperature
▶ Oligonucleotide	▶ Spectroscopy

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EXPERIMENTAL

All spectroscopic experiments were carried out using a Chirascan-plus CD spectrometer configured for DMS. A sample of a 17-base oligonucleotide (Eurogentec S.A.) at a concentration of 0.5mg/mL was prepared in phosphate-buffered saline (PBS) and degassed under partial vacuum at room temperature. 130µl aliquots of the degassed solution were pipetted into a 0.5mm cuvette and were heated continuously from 4ºC to 75ºC at a rate of 1ºC per minute or cooled from 75ºC to 4ºC at the same rate. CD and absorbance spectra in the range 200nm≤λ≤320nm were measured simultaneously during the heating / cooling cycles at a rate of 1 spectrum per minute. In this way, spectroscopic surfaces (CD and absorbance) as a function of both temperature and wavelength, for both heating and cooling cycles, were created. Ten independent measurements were made over a period of five days. The experimental parameters are shown in Table 1.

Sample	17-base oligonucleotide
Sequence (5' to 3')	TAT-AAG-AAA-ATC-TTA-TA
Buffer	PBS pH7.0
Volume	130µlitres
DNA conc.	0.5 mg/mL
Sample used	65µg
T range	4ºC – 75ºC
T rate	1ºC / minute
λ range	320nm – 200nm
Step-size	1.5nm
Time per point	0.5s
Scan rate	~1scan / minute
Cell pathlength	0.5mm
Optical probes	CD and absorbance
Experiment duration	~70 minutes

Table 1. Summary of experimental parameters.

RESULTS AND DISCUSSION

The oligonucleotide adopts a predominantly hairpin conformation at or below room temperature and a predominantly single strand conformation at temperatures above about 50ºC. A set of CD spectra and temperature profiles from a single DMS dataset are shown in Figure 1.
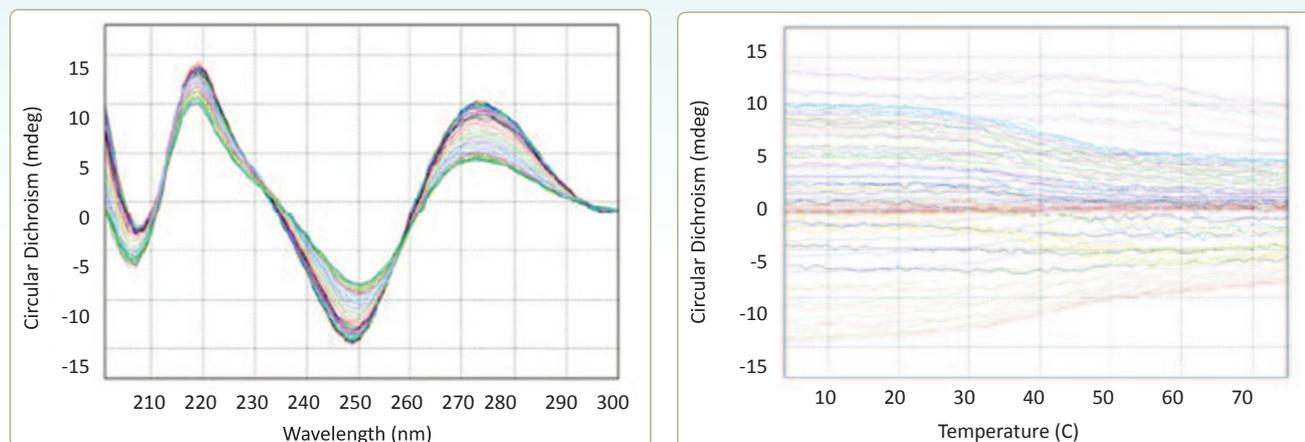
Figure 1.   A DMS dataset showing CD as a function of wavelength and temperature.

Ten independent measurements were made over a period of five days. Each dataset was analysed with APL's proprietary Global3 program using a single transition, double baseline (1T2B) model and the results are reported in Table 3. There is slight variation in the parameters T_m and $\Delta H_{van't\ Hoff}$ calculated for independent measurements but the standard deviations in the averages of the independent measurements were small and the range of calculated values fell within ±1.7 standard deviations for both transition mid-points and van't Hoff enthalpies.

Sample name	T_m ºC	ΔH_{vH} KJ/mol
Sample1 Day1 Up	38.1	128
Sample1 Day2 Up	38.0	126
Sample1 Day3 Up	38.4	130
Sample2 Day3 Up	37.6	127
Sample2 Day4 Up	38.1	129
Sample2 Day4 Down	38.2	136
Sample2 Day4 Up2	38.3	128
Sample2 Day4 Down2	37.9	130
Sample3 Day5 Up	37.9	128
Sample3 Day5 Down	38.1	136
Average of all 10 measurements	38.1	130
Standard deviation	0.3	4
Average of 7 measurements (up)	38.1	128
Standard deviation (up)	0.3	2
Average of 3 measurements (down)	38.2	134
Standard deviation (down)	0.2	3

Table 3. Results of global analysis of spectroscopic data.

It is worth noting that the transition mid-point temperatures are identical within experimental error for the 'up' cycles and the 'down' cycles. This is because the temperature probe is immersed in the sample itself and the true sample temperature is measured, rather than using the heating-block temperature as an approximation to the sample temperature. If the latter method were employed, as is often done for spectroscopic measurements, there would be a lag between block and sample temperature that would lead to a systematic error in the results. The T_m on the 'up' cycle would be too low and on the 'down' cycle, too high.

Sample 2 was used to complete 2 full thermal cycles on day 4. The CD spectra at the end of the first and second full cycles were compared. For each set, the 3 CD spectra taken at the lowest temperatures at the end of the cycle (4ºC, 5ºC and 6ºC) were averaged. The amplitudes of the CD peaks and troughs at the end of the second cycle were very slightly larger than those at the end of the first cycle (see Figure 2) and the differences were attributed to evaporative loss causing a slight increase in concentration of the oligonucleotide.
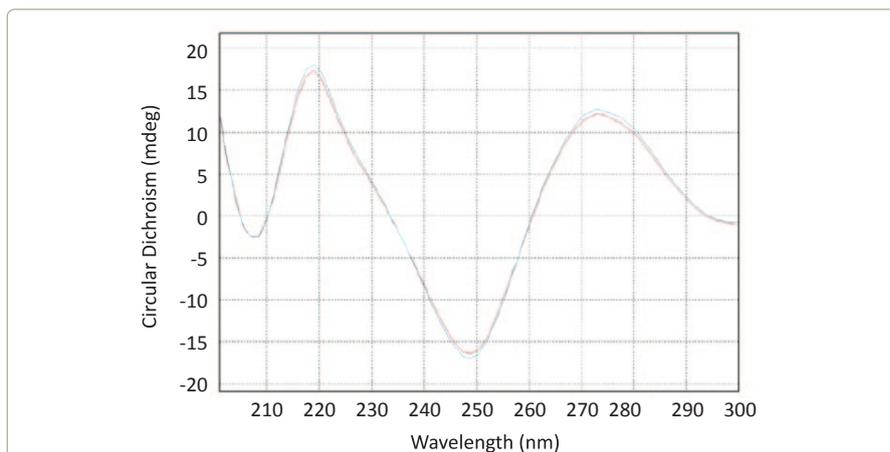
Figure 2. Average of 3 spectra (4ºC-6ºC) at end of 1st (red) and 2nd (turquoise) cycles.

This can be readily corrected because the absorbance spectra, which are recorded simultaneously with the CD spectra, are proportional to concentration. The ratio of the differences in amplitude in the corresponding absorbance spectra at three different wavelengths were averaged (Table 4) and the result was used to correct for the small amplitude difference in the CD spectra. Following this correction, the CD spectra overlaid perfectly (Figure 3).

Wavelength	Abs 1	Abs 2	Ratio
261nm	0.8779	0.9146	1.0418
232.5nm	0.3519	0.3693	1.0494
210nm	1.1228	1.1638	1.0365
		Average:	1.0426

Table 4. Correction factor for CD spectra calculated from absorbance spectra.
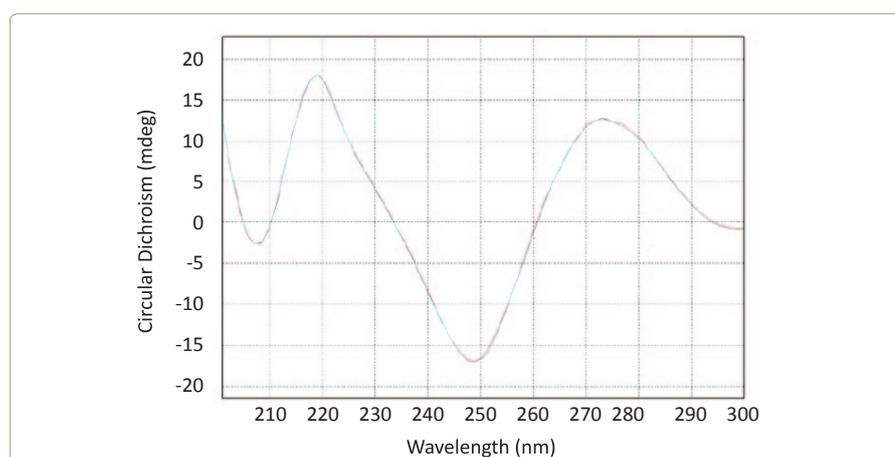
Figure 3. Overlay of CD spectra after one and two full heating and cooling cycles, corrected for evaporative loss using absorbance spectra.

CONCLUSION

The experiment was undertaken to demonstrate that when sample variability is taken out of the equation, dynamic multimode spectroscopy produces reliable results. The high degree of reproducibility in the DMS results underlines the precision of the technique. The experimentally determined T_m agrees with the theoretical one calculated using the Wallace-Ikatura formula and suggests that DMS is accurate as well as precise, although DSC experiments are planned to confirm this.

REFERENCES

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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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