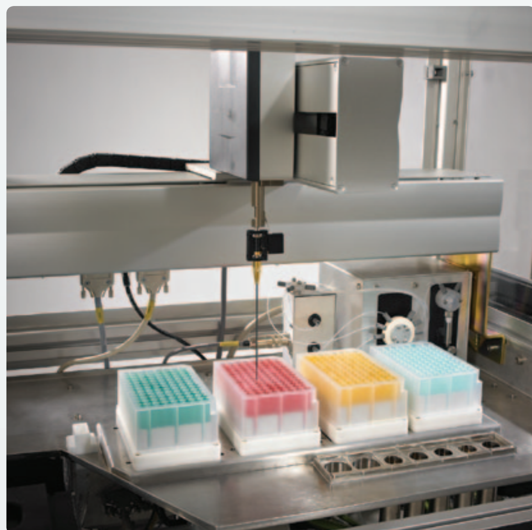
AppliedPhotophysics
Ultrasensitive Spectroscopy for the Life Sciences



Abstract *Formulation of protein drugs involves optimisation of buffer conditions to maximise the stability of the protein molecule. In this study the thermal stability of an IgG protein was determined using dynamic multimode spectroscopy (DMS) under a number of different pH conditions from pH2.2 to pH7.8.*

- *Greatest thermal stability for this IgG occurs at pH>5.0*
- *Greatest resistance to aggregation for this IgG is seen at pH<5.0*
- *Pre-transition conformation changes with pH*
- *Aggregation, when it occurs, is easily detected*
- *Quantitative results with errors are readily calculated*
- *Protein usage per denaturation is low ~60µg*
- *Unattended operation – overnight measurement*

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CHIRASCAN SERIES APPLICATION NOTE

Automated Circular Dichroism with Dynamic Multimode Spectroscopy: a powerful tool for the optimisation of protein formulation

INTRODUCTION

One of the most critical physical properties of a protein is the conformational stability of the native fold. Increased stability leads to improved storage longevity and tolerance of environmental factors like heat and mechanical stress, while reducing adverse behaviour such as aggregation (Chi et al. 2003; Wang 2005). These attributes are central to successful protein formulation and a formulation programme aims to improve conformational stability (as measured by increased resistance to temperature-induced unfolding) and to decrease the likelihood of aggregation.

Circular dichroism (CD) provides a very sensitive characterization of protein conformation (Fasman 1996; Kelly et al. 2005), and can be measured with the protein under a number of different stresses. CD is particularly useful for measuring changes in protein conformation and stability under different formulation and stress conditions.

The Chirascan™-plus Automated Circular Dichroism (ACD) system is a very powerful tool to leverage circular dichroism measurements, allowing unattended measurements of multiple samples presented in a microplate format. When the ACD is combined with the dynamic multimode spectroscopy (DMS) method of measuring protein conformation stability, it becomes possible to perform

KEYWORDS

▸ Chirascan	▸ Unfolding
▸ Circular Dichroism	▸ IgG
▸ Automated	▸ Quantitative results
▸ Thermal Stability	▸ Dynamic Multimode Spectroscopy

sophisticated measurements of thermal stability on a wide variety of proteins, or on one protein under many different conditions.

The utility of ACD-DMS in the context of protein formulation is demonstrated in this note. Data are presented from a series of DMS thermal denaturation experiments carried out on a polyclonal immunoglobulin G (Sigma-Aldrich) in various pH buffers. The data show that the structure of the protein changes at low pH to a less thermally stable form that does not aggregate upon unfolding. At neutral pH, a different pre-transition conformation exists that has a higher temperature transition mid-point (T_m) but aggregates after heat denaturation.

EXPERIMENTAL

Samples from a 10mg/mL stock in 10mM sodium phosphate buffer (pH 7.0), 150mM sodium chloride, were diluted to 1mg/mL in a 96-well microplate, by first pipetting the protein solution into the microplate, and then overlaying with the appropriate buffer conditions from a prepared master-plate. Each sample was paired with its buffer and after each denaturation experiment, a measurement of the buffer was made that could be used to baseline-correct the sample spectra, and check for potential carry-over.

Samples were transferred from the plate to a 0.2mm spectroscopic flow-cell via an injection port, using a fixed-probe x, y, z robot. Each sample was heated continuously at 1°C per minute from 20°C to 95°C while measuring far-UV spectra from 250nm to 200nm in 1nm steps with a sampling time of 0.8s per point. Each spectrum took approximately 1 minute to measure, giving a spectrum for every 1°C rise in temperature and thus a total of 75 far-UV spectra per denaturation. The conditions screened were from pH2.2 to pH7.8 in 0.8pH steps, controlling the pH by varying the relative concentrations of citrate and phosphate in a 75mM phosphate-citrate / 150mM sodium chloride buffer.

Parameter	Value
Protein concentration	1mg/mL
Buffer (pH2.2 – pH7.8)	75mM citrate-phosphate / 150mM NaCl
Volume used per denaturation	60µl
Protein used	~60µg
Cell path-length	0.2mm
T-ramp range and rate	20°C – 95°C @ 1°C/minute
Duration per denaturation	75 minutes
Optical properties measured	CD and absorption
Wavelength range measured	250nm – 200nm
Bandwidth	1nm
Step size / time per point	1nm / 0.8s

Table 1. Summary of experimental parameters.

At the end of a thermal ramp to 95°C, the sample holder temperature was returned to the start temperature in preparation for the next sample. In the time required to equilibrate the cell, an extensive cleaning routine was performed to ensure there was no carry-over of denaturated or aggregated protein.

All thermal data analysis was carried out by the Applied Photophysics' Global 3 analysis package.

RESULTS

The denaturation data for this polyclonal IgG do not show the separate CH2, Fab, CH3 transitions that might be expected from a monoclonal IgG because of overlap of the contributions from the various IgG components of the sample. When the unfolding is not accompanied by aggregation (at low pH), it is adequately described using a single transition model with linear pre- and post-transition baselines. At higher pH, the unfolding is accompanied by aggregation but a significant loss of conformation has occurred by the time of onset of aggregation (see Figure 2). Although it is impossible to distinguish the individual transitions in the data, the fact that there is separation of the first unfolding transition and aggregation indicates that the aggregation step is likely to be associated with the second or third transitions, rather than the first. The datasets above pH4.0 are fitted to a two-transition model with a single pre-transition baseline, with the second transition used in place of the post-transition baseline.

Figure 2 shows some representative raw data from the larger dataset, with low pH samples showing a change in profiles and spectra compared to those at neutral pH. This change in stability at low pH is concomitant with a significant shift in the spectrum of the folded protein. The folded and unfolded spectra (Figure 1) as derived from the fit to the data, show significant differences, indicating that there has been a destabilising change in the folded structure at low pH.

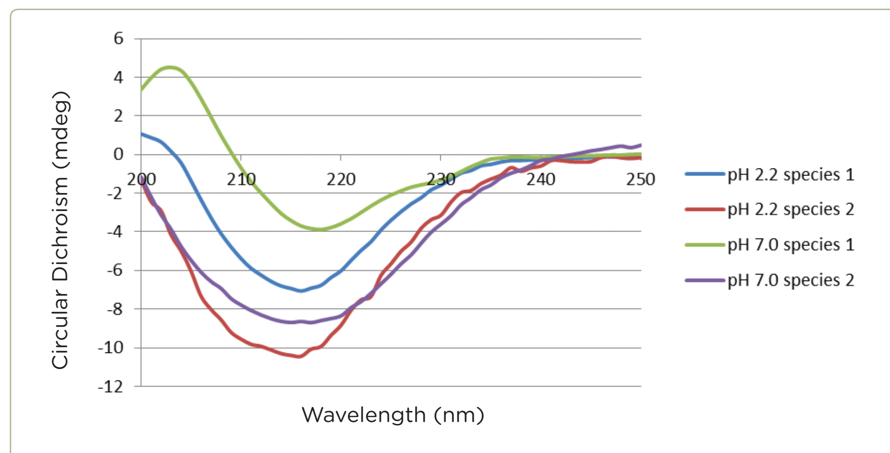

Figure 1. Fitted folded (species 1) and unfolded (species 2) at neutral and low pH. The spectra have been derived from the global model fit.

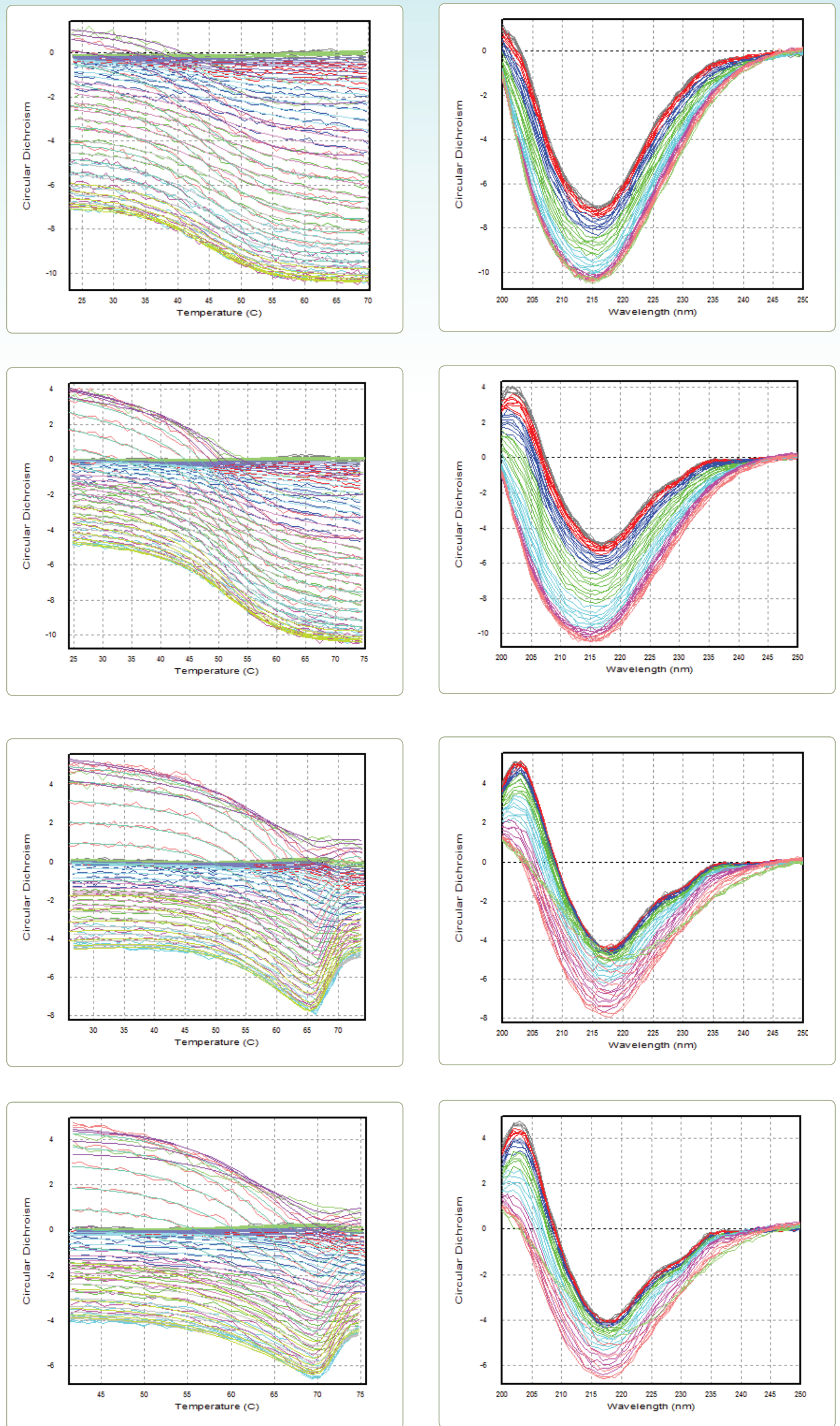

Figure 2. DMS CD thermographs of IgG in selected pH buffers. Left are temperature vs. CD for all wavelengths and the fitted model in blue. Right are the spectra at each temperature.

Plotting the measured T_m vs. the pH of the buffer shows a distinct trend (Figure 3), with samples in pH buffers lower than 4.8 demonstrating a significant decrease in thermal stability. Extending the study below pH 2.0 could possibly show the complete pH transition to the low pH, low thermal stability form of the protein, with a T_m below 45 °C.

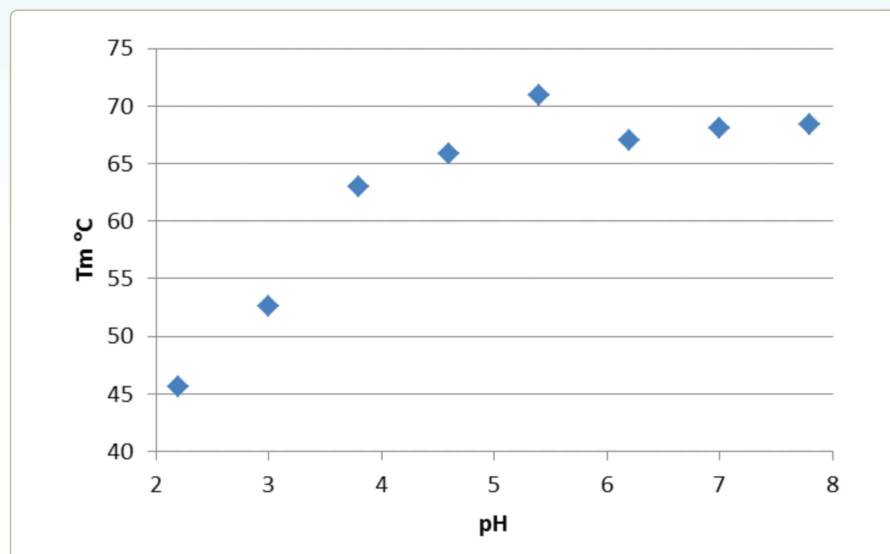

Figure 3. Effect of buffer pH on the T_m of FIII9-10: note the >20 °C drop in T_m at low pH.

Looking at the absorbance signal obtained simultaneously with the CD measurement at wavelengths where there is little absorbance (Figure 4), it is possible to highlight optical density increases due to scattering of light by aggregated particles. In buffer with a pH less than 4.0, there is no observable aggregation for the IgG molecule. At neutral pH there is significant aggregation occurring which is easily observed in the absorbance signal.

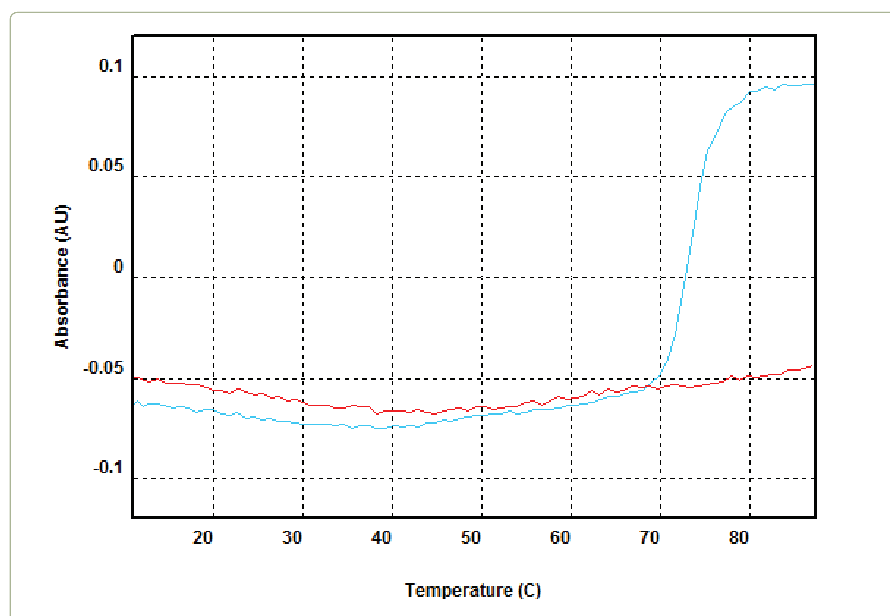

Figure 4. Absorbance thermograms at 250nm of IgG in pH7.0 (blue) and pH2.2 (red) buffers.

SUMMARY

The results from one overnight Chirascan™-plus ACD DMS experiment have provided a number of significant insights into the effect of pH on the stability of the protein. These are that the polyclonal antibody is more thermally stable at neutral pH, from 4.8 to 7.8, than it is at lower pH; that the loss of thermal stability at lower pH is concomitant with a change in the pre-transition folded structure of the protein; and once the proteins are thermally unfolded, the neutral-pH unfolded form aggregates, while the low-pH unfolded form does not.

DMS is an information-rich, label-free technique that gives access to thermodynamic, structural and aggregation data in a single experiment. The combination of DMS and automation gives rise to a very powerful tool for understanding the thermal denaturation pathway of proteins, generating quantitative results from small samples with little effort.

The automation of DMS experiments using Chirascan™-plus ACD dramatically reduces the work required to collect these data. The experiment described here took less than 30 minutes to set up and was run overnight without further user intervention.

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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.

AppliedPhotophysics

Ultrasensitive Spectroscopy for the Life Sciences

NEW APPLICATIONS FOR CD SPECTROSCOPY continued

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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