AppliedPhotophysics

Ultrasensitive Spectroscopy for the Life Sciences

Screening optimal buffer conditions for a therapeutic antibody using Chirascan™-plus Automated Circular Dichroism

Abstract *The early stages in the development of a novel therapeutic drug involve indentifying the conditions in which a protein is natively folded, including pH and ionic strength. These will form the basis of the formulation buffer. Circular dichroism (CD) spectroscopy is able to identify very small changes in protein conformation and can therefore be used to screen for these conditions. Conducted manually, this can be a time-consuming and tedious task. Chirascan-plus ACD can be used to speed up the process and eliminate human errors.*

This paper demonstrates the process using a currently marketed therapeutic antibody (adalimumab), over the pH range (2.2 to 7.8) and the NaCl concentration range (75 to 575mM). It was found that the neutral folded form of adalimumab is stabilized in the pH range at all the ionic strength conditions used. There is also a small trend upwards in pKa due to ionic strength.

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INTRODUCTION

The first step in the formulation process of a therapeutic protein drug is to determine the conditions of pH and ionic strength in which the protein is natively folded. These will then form the basis of the formulation buffer.

Circular Dichroism (CD) spectroscopy probes the protein conformation, and so is a particularly powerful way of monitoring the changes in protein structure induced by changes in buffer conditions. Applied Photophysics' Automated Circular Dichroism (ACD) system, in which up to 200 samples per day can be analysed with unattended operation, can easily leverage the value of this probe by allowing a much larger range of conditions to be explored.

In this study, a currently marketed therapeutic antibody (adalimumab) was diluted into a number of buffer conditions with varying pH (2.2 to 7.8) and ionic strength (75 to 575 mM) conditions in two 96 well microplates, and then automatically sampled using an ACD autosampler on a Chirascan™-plus CD instrument.

The antibody had already undergone an analogy of the formulation process, in which CD was used to determine the optimal pH range of the natively folded protein. This study was carried out manually, and simply looked at the pH stability range. Using Automated Circular Dichroism a similar study was carried out with minimal user intervention, and the screen was extended to the effect of ionic strength.

KEYWORDS

▶ Circular Dichroism	▶ Antibody
▶ Chirascan	▶ Optimal Conditions
▶ Adalimumab	▶ Automated
▶ Therapeutical	▶ Protein

MATERIALS AND METHODS

Adalimumab was purchased as a single preloaded automatically injecting syringe at a formulated concentration of 50mg/mL in the adalimumab formulation buffer [Abbott labs]. The solution was transferred to a microcentrifuge tube and was stored in this way at 4°C until used. It was noted that some evidence of protein precipitation, which was cleared by centrifugation, was present after storage.

The samples were prepared in two 96-well microplates, first by pipetting protein solution into the microplate, and then overlaying with the appropriate buffer conditions from a prepared masterplate. This provided a nominal protein concentration of ~1 mg/mL. The concentration was not known inaccurately, due to the precipitation losses.

The conditions screened were pH from 2.2 to 7.8 in 0.4 pH steps, of a 75 mM Phosphate/Citrate buffer. Additionally, 6 complete pH screens were performed in NaCl concentrations from 0 to 500 mM in 100 mM steps. The total number of sample and buffer pairs was 90 (180 individual samplings), and the acquisition took ~24 hours. The total volume for each sample well was 100 μl (65 μL required for sampling, and 35 μl as excess volume in the sample well).

Buffers and samples were presented to the Chirascan™-plus sequentially using the ACD autosampling system, and CD and absorption spectra were measured simultaneously for all samples. The sample cell used was a 0.2mm pathlength cell specially designed for the ACD. Spectra were collected from 190 nm to 260 nm, 1 nm step-size, with 1 second per point sampling time. All reported spectra are an average of 4 repeat spectra after buffer baseline subtraction.

Due to absorbance of constituents of the adalimumab formulation buffer, the cutoff of the buffer absorbance (>2.0 AU) was determined to be 203 nm under the most absorbing buffer conditions. All spectra were therefore restricted to the range 203 to 260 nm. The collected spectral files were automatically averaged, and buffer spectra subtracted to provide corrected raw CD spectra. These were plotted against the pH at each salt concentration and the multidimensional dataset fitted to a single set of species spectra for all ionic conditions, with different pKa's, using the solver plugin for Microsoft® Excel® based on the method of Kemmer and Keller (2010). The pKa was calculated from the standard Henderson–Hasselbalch equation.

RESULTS

The overlayed spectra at all wavelengths show a transition underway at the lowest pH condition measured (2.2) and finishing at approximately pH 4.0. It should be noted that at the lowest concentration of NaCl the transition is almost completed at pH 2.2 while higher NaCl concentrations show significantly more of the transition (Figure 1).
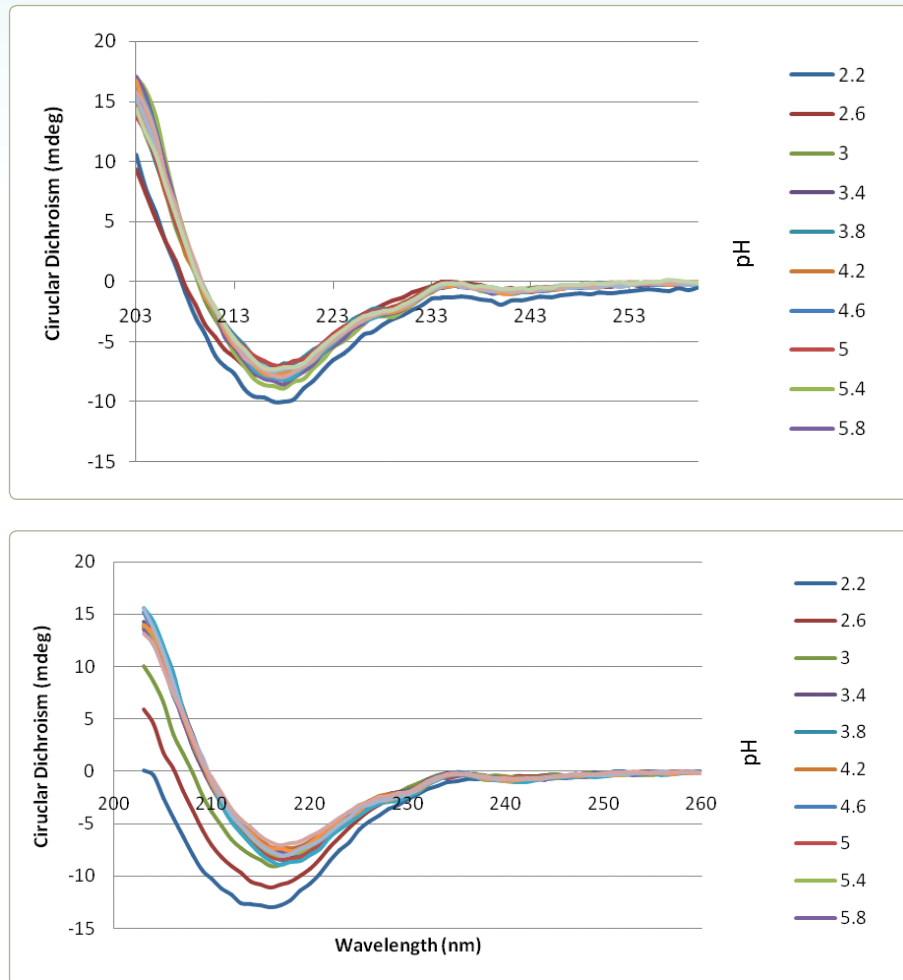

Figure 1. Overlayed experimental data from pH titration of Humira at 0 mM (top) and 200 mM NaCl (bottom). Note the difference in size of the observed pH transition.
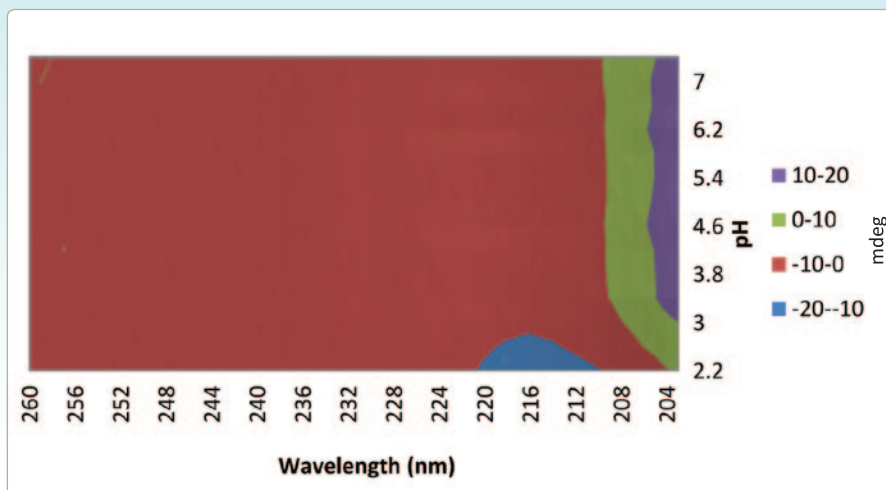
Figure 2. As an example, the 3D contour plot of the change in CD with pH with 200mM NaCl. Note the transition evident at pH 2.2-3.8.

No more than one transition was evident at any of the ionic conditions studied. The data were therefore fitted to a single pKa transition. A global fit was used, minimised to the same species' spectra for all ionic strength conditions, while allowing the pKa to vary between ionic strength conditions. The species' spectra fit resulting from the minimisation is shown in Figure 3.

The spectrum of species 2 is the neutral pH species, and therefore is well represented in the dataset, the species 1 spectrum is determined by extrapolation from the lowest pH condition.
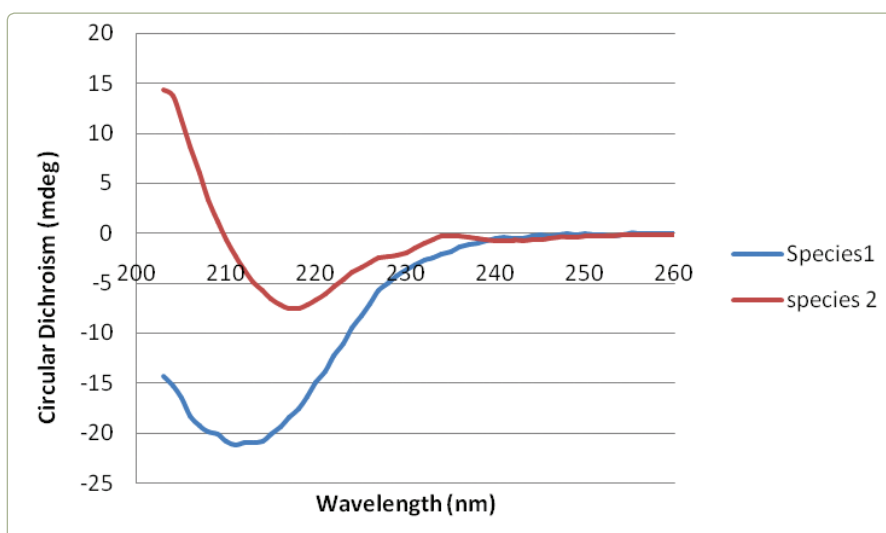

Figure 3. Derived Spectrum of the Low pH Species (1) and High pH Species (2).

The fitted pKa's are shown in Figure 4. These demonstrate a rise in pKa with NaCl at sub 200mM NaCL concentrations. Obviously the accuracy of this fit is limited by the range of pH conditions used, with significant proportion of the spectrum change outside the pH range of 2.2 to 7.8.
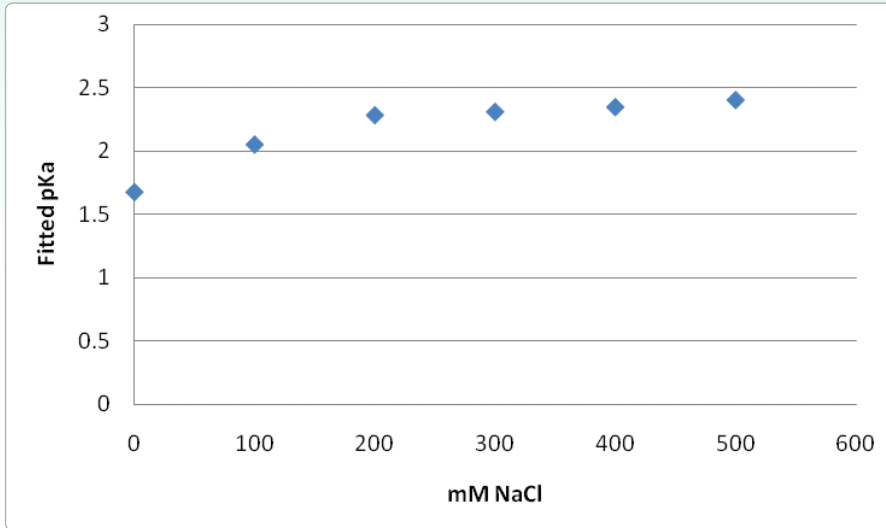

Figure 4. Fitted pKa with NaCl concentration. Note the trend at low salt concentrations.

As there is no significant difference in the derived pKa at 200mM NaCl or higher, we can use this particular concentration to demonstrate what conclusions can be made about the suitable pH range for a formulation. Figure 5 demonstrates the relative ratios of species 1 (acidic) and species 2 (neutral) present. As can be observed, there is >98% species 2 present at pH 4 and higher.
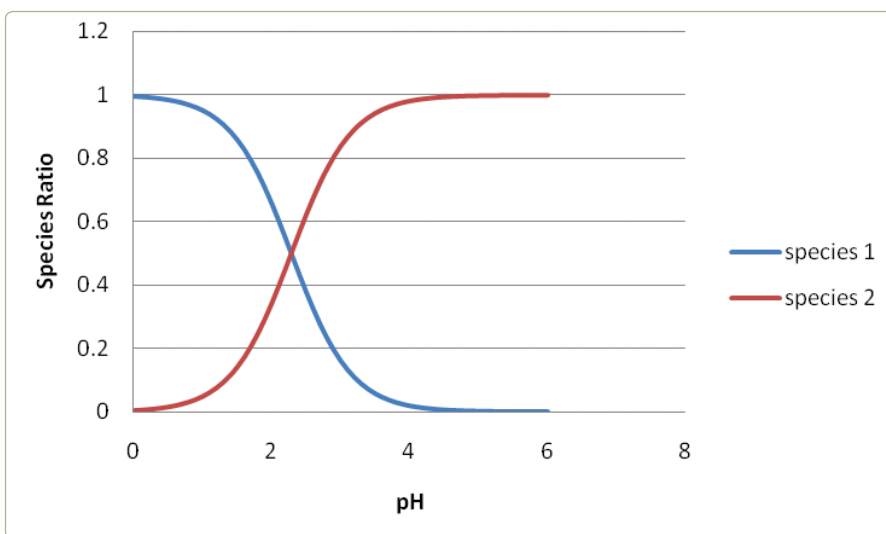

Figure 5. Demonstration of the relative ratio of the 2 species spectrum of the pKa fit at 200 mM NaCl. Note the crossover pKa point at pH 2.4.
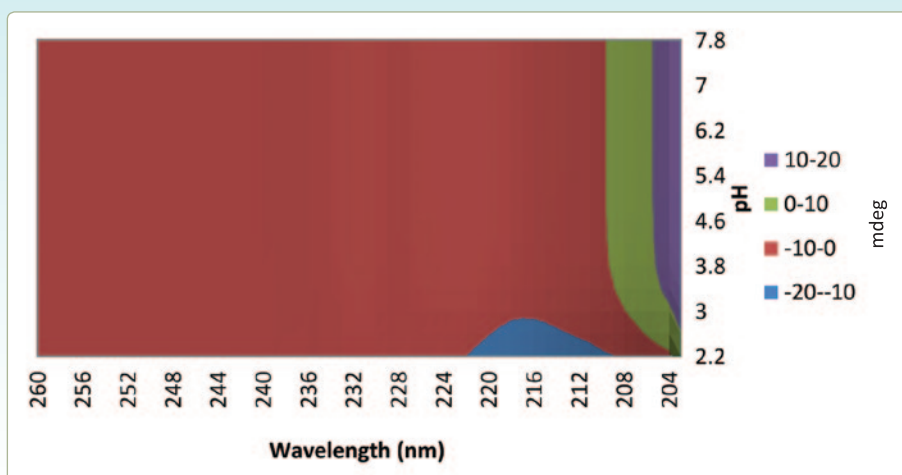
Figure 6. Fitted 3D spectra contour plot, indicating the region of change (ph 2.2-4.0) and the region of stability (4-7.8).

Figure 6 is the fitted spectra pH contour plot, visually indicating insignificant change at higher than pH 4.0.

CONCLUSION

This study describes a new automated method for the optimisation of buffer conditions for a therapeutic protein drug, using circular dichroism as a probe of the protein conformation.

The ability of the new ACD to sample upwards of 100 separate buffer conditions within a 24 hour period allows a significantly larger number of formulation buffer conditions to be compared using CD as a probe.

The neutral folded form of adalimumab is stabilised in the pH range 4.0 to 7.8 at all ionic strength conditions measured. There is also a small trend upwards in pKa due to ionic strength.

Although only ionic strength and pH were studied in this case, the same experimental setup can be used to probe the effect on protein conformation of any number of solution conditions, including the effect of excipients and detergents.

REFERENCES

G. Kemmer & Sandro Keller, *Nature Protocols* 2010,**5**, pp. 267 – 281.

Abbott laboratories, Humira Prescribing Information, Ref:03-5236, Revised: January, 2003.

C Martin, presentation at the PDA conference, Oct 25th 2005, "Humira®- from Development to Commercial Scale Production".

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.

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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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