Application Note

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Characterization of Protein Thermal Stability Using UV-Vis Spectroscopy

Benefits of the Agilent Cary 3500 UV-Vis spectrophotometer with a Peltier temperature-controlled multicell in protein therapeutic research

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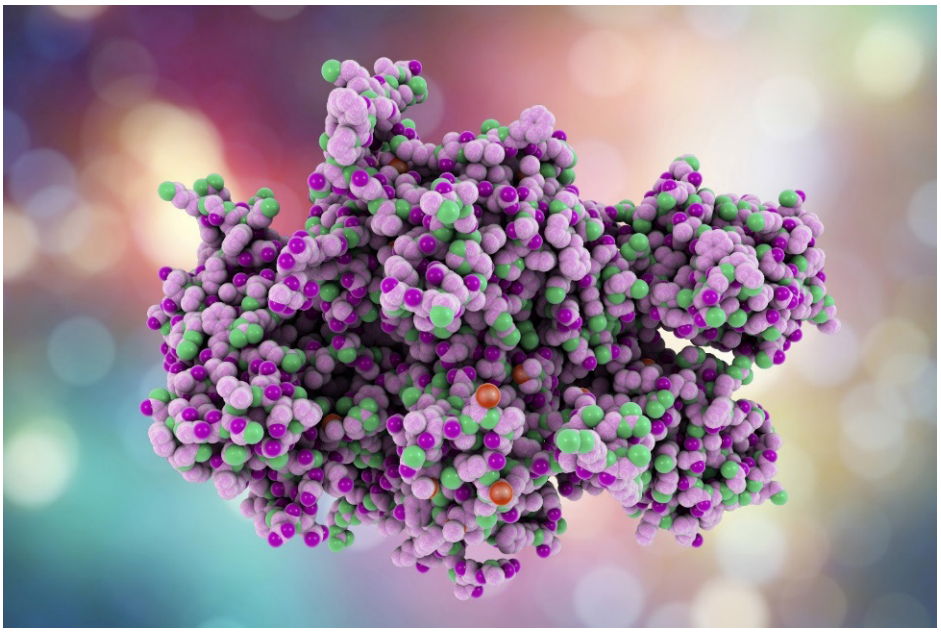

Abstract

The thermal stability of proteins is an important characteristic of biotherapeutics, as the data provide an insight into the physical and chemical properties that influence the stability and structure of proteins. This study demonstrates the fast and simple characterization of protein thermal stability and melting temperature of a protein using the Agilent Cary 3500 UV-Vis spectrophotometer fitted with a Peltier temperature-controlled multicell module.

Introduction

Drug discovery is a multifaceted process in which potential new medicines and their therapeutic effects must be demonstrated. Proper identification and characterization of drug targets are important steps in the process of drug discovery that can facilitate lead-therapeutic compound identification.[1] For protein therapeutics, biochemical characterization includes molecular weight, homogeneity and heterogeneity, solubility, thermal stability, amino acid sequence composition, secondary structure, three-dimensional structure, and protein modification.[2,3]

Thermal stability data provide an insight into the physical and chemical properties that influence protein stability and structure. Melting temperatures can be determined by monitoring a change in the sample's properties as the temperature gradually increases using a variety of techniques (Table 1). When proteins are subjected to elevated temperatures, the secondary and tertiary structure changes. These structural changes expose the hydrophobic core and lead to protein aggregation.[4] The temperature at which half of the protein is still in its soluble form is known as the apparent melting temperature (T_m), as shown in Figure 1. This temperature can be increased by the presence of a bound ligand.[5,6] Thermal stability studies contribute to the understanding of ligand-binding, drug stability, shelf life, and optimal storage conditions – useful information for future production and commercialization of the drug.

Thermal stability analysis of proteins using UV-Vis spectroscopy

Table 1 summarizes the advantages and disadvantages of the various techniques that are used to measure the thermal stability of proteins. UV-Vis spectroscopy overcomes many of the challenges of the analysis. UV-Vis provides direct absorbance measurements without the need for fluorescent labels, measurements can be performed using small amounts of sample, and the results are quick and easy to interpret and report.

This application note demonstrates the benefits of the Cary 3500 UV-Vis spectrophotometer (Figure 2) for characterizing thermal stability and reproducibility of proteins. The Cary 3500 UV-Vis spectrophotometer uses an integrated in-cuvette temperature probe that provides feedback to the Peltier block, enabling control of the temperature of the solutions during measurements. An eight-position multicell holder is built into the instrument and uses water-free, air-cooled Peltier devices to control the temperature of samples between 0 and 110 °C. For increased experimental capability, each cuvette position of the multicell is measured simultaneously, providing a faster analysis compared to other techniques.

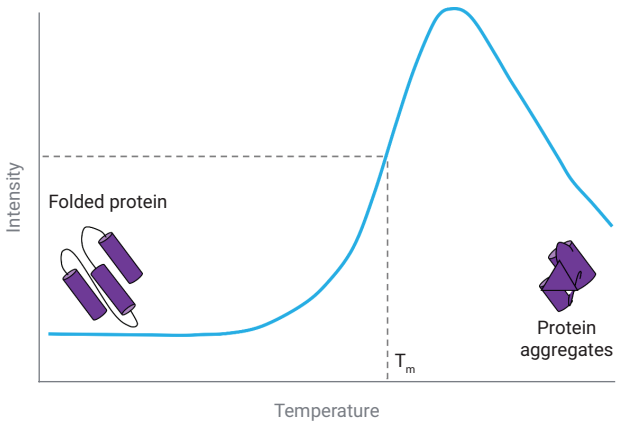

Figure 1. Typical response of protein intensity (or absorbance) as temperature increases.

Table 1. Techniques used to measure the thermal stability of proteins.

Technique	Principle	Advantages	Disadvantages
Circular Dichroism Spectroscopy[7,8]	Measure absorbance in terms of ellipticity using circular polarized light.	– Small amount of material needed – High sensitivity – High resolution	– Time consuming – Some buffers cause interference in the far UV
Differential Scanning Calorimetry[9,10]	Measure change in heat capacity with respect to a reference material.	– Small amount of material needed – High resolution	– Interpretation of results can be difficult
Thermal Shift Assay[11,12]	Measure changes in fluorescence upon binding of a fluorophore dye to hydrophobic molecules.	– Can run more than one sample in RT-PCR machine – High sensitivity – Small amount of material needed	– Need for fluorescent probes – Fluorescent probes may cause conformational changes – Proteins should have a pocket to accommodate fluorescent probes

Figure 2. The Agilent Cary 3500 UV-Vis multicell spectrophotometer can be used for up to four temperature experiments, across eight cuvette positions, simultaneously.

Figure 3. Agilent ultra-microcell used in this study. The extremely small, focused beam of the Agilent Cary 3500 UV-Vis spectrophotometer passes through small, permanently aligned apertures that require no adjustment.

Experimental

Samples

A sample of *Escherichia coli* disulfide bond isomerase A (*Ec*DsbA, native state) was provided by La Trobe Institute for Molecular Science (LIMS), La Trobe University, Australia. A solution of ≈0.6 mg/mL of *Ec*DsbA was prepared in a buffer solution containing 50 mM NaCl, 1 mM EDTA, and 100 mM sodium phosphate, adjusted to pH 7.0. Agilent ultra-microcell, 50 µL volume, 10 mm optical pathlength quartz cuvettes were used (part number 5062-2496, Figure 3). Neat phosphate buffer was used as the reference (blank) solution.

Instrumentation and method

A Cary 3500 UV-Vis multicell Peltier spectrophotometer was controlled using Agilent Cary UV Workstation software. The method parameters used to measure protein absorbance and thermal stability are shown in Tables 2 and 3, respectively. For the thermal stability analysis study, data were collected every 0.1 °C using a ramp rate of 0.1 °C/min.

Table 2. Wavelength range scan parameters.

Parameter	Value
Instrument	Agilent Cary 3500 UV-Vis spectrophotometer
Wavelength range	200 to 400 nm
Signal averaging time	0.1 s
Spectral bandwidth	2 nm
Sample volume	80 µL, additional volume was added to accommodate for evaporation

Table 3. Thermal melt experimental parameters.

Parameter	Value
Instrument	Agilent Cary 3500 UV-Vis spectrophotometer
Wavelength	280 nm
Signal Averaging Time	2 s
Spectral Bandwidth	2 nm
Data Interval	0.1 °C
Ramp Rate	0.1 °C/min
Temperature Control	Peltier block
Temperature Range	25 to 90 °C
Smoothing And Derivative Filter Size	25
Smoothing And Derivative Data Interval	0.1 °C
Analysis Lower Temperature	30 °C
Analysis Upper Temperature	90 °C
Cuvette	Ultra-microcell quartz cuvette
Sample Volume	70 µL

The signal was averaged for 2 seconds before each data point was recorded. The ramp rate of 0.1 °C/min was used due to the sample susceptibility to aggregation at higher ramp rates. The experimental temperature was controlled through the Peltier block of the Cary 3500.

Results and discussion

To determine the maximum absorption peak of the *Ec*DsbA protein, a wavelength scan was performed by the Cary 3500 UV-Vis spectrophotometer using the parameters given in Table 2. The Cary 3500 fitted with the multicell module allows seven samples and one reference solution to be measured simultaneously. This feature increases confidence in the results, as measurements are collected at the same time under identical conditions. If a small sample volume is required, e.g., 70 µL, measurements can be performed using an ultra-microcell (Figure 3). As the Cary 3500 system has a small and focused beam, it allows accurate measurements to be performed using ultra-microcells without any pre-alignment. The maximum absorption peak for *Ec*DsbA was found at 280 nm, as shown in Figure 4.

For the thermal stability analysis of the *Ec*DsbA protein, the absorbance at 280 nm was monitored as the temperature increased. Sample temperature was controlled using the Peltier block temperature with a ramp rate of 0.1 °C/min, as specified in Table 3. As the temperature increased, a peak was observed, indicating a change in the absorbance of the protein caused by a change in the protein structure. The thermal melt plot (Figure 5) shows a transition occurring at high temperature (above 65 °C) for the *Ec*DsbA protein.

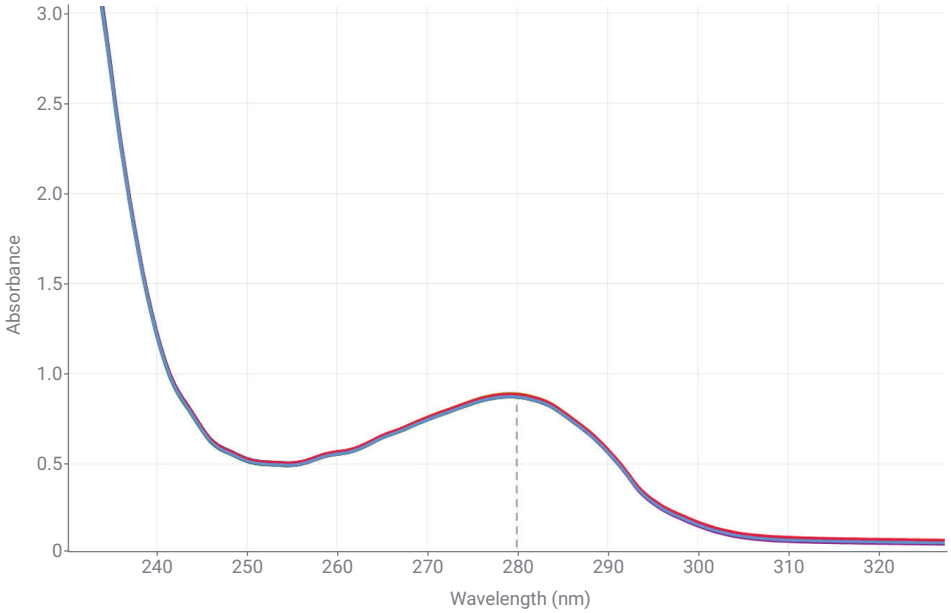

Figure 4. Wavelength range scans of seven separate samples of the *Ec*DsbA protein (native state), showing the *Ec*DsbA protein peak at 280 nm. The seven samples were measured simultaneously using ultra-microcells.

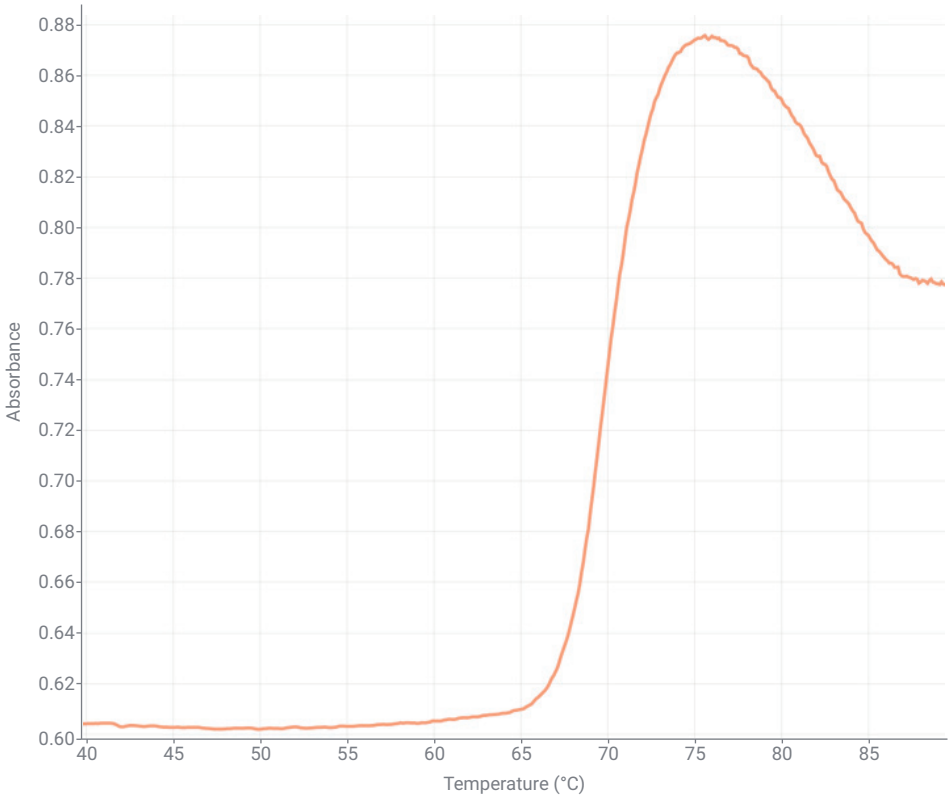

Figure 5. Thermal melt plot of the *Ec*DsbA protein. Changes in absorbance with increasing temperature indicate structural changes to the protein.

The Cary UV Workstation software calculates the T_m of biological samples using the first derivative of the measured absorbance data. T_m can be determined by selecting the appropriate smoothing and derivative filter sizes along with the upper and lower temperature for analysis. The T_m of *Ec*DsbA was found to be 69.86 °C (n = 6; Figure 6) using the analysis parameters given in Table 3.

The calculated T_m for the *Ec*DsbA protein obtained using the Cary 3500 UV-Vis spectrophotometer agreed with the previously reported results obtained using circular dichroism, as shown in Table 4.14, 15

The precision of the measurement of the T_m of *Ec*DsbA by the Cary 3500 was evaluated by running a repeatability study. The protein sample solution was split into six portions and each portion was analyzed separately using the 70 µL ultra-microcell. The repeatability of the results was assessed by calculating the average and the standard deviation of the T_m values of *Ec*DsbA (Table 4). The Cary 3500 UV-Vis system showed excellent precision, with a standard deviation of only 0.16 °C over six measurements.

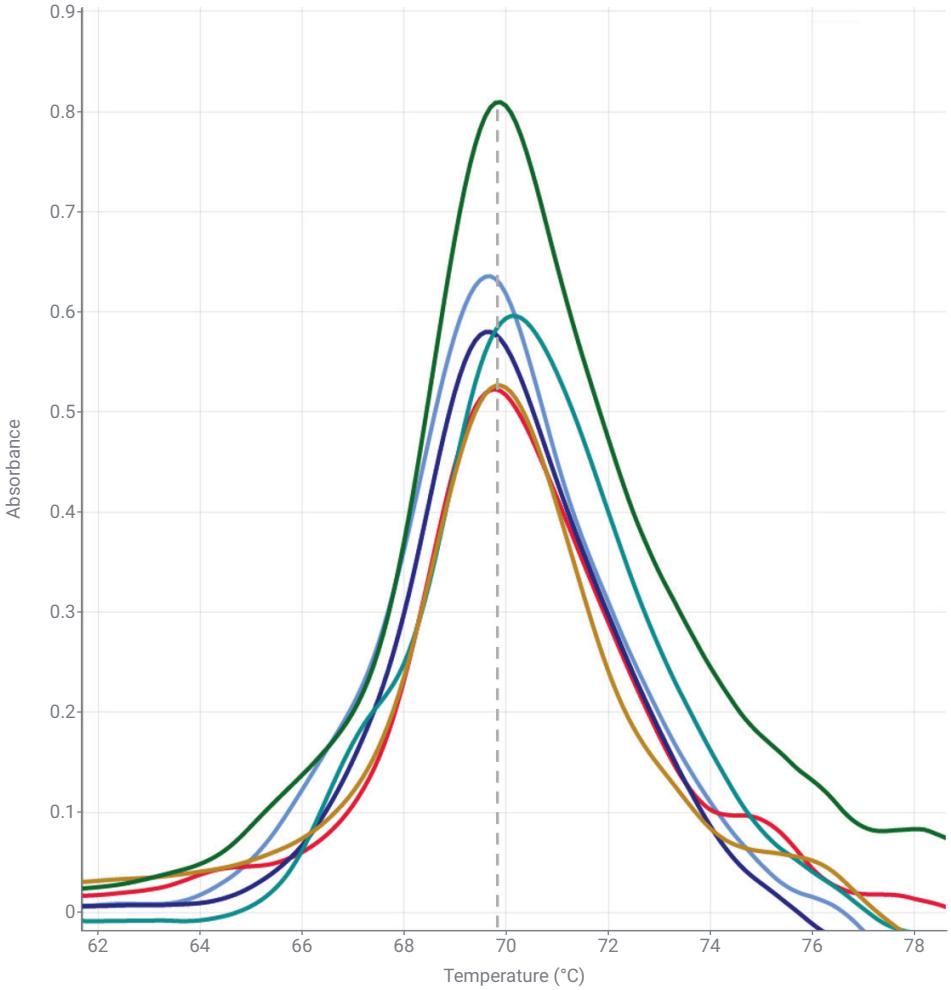

Figure 6. First derivative plots of six separate samples of the *Ec*DsbA protein obtained using the Agilent Cary UV Workstation software (T_m = 69.86 °C, n = 6).

Table 4. *Ec*DsbA T_m values measured using the Agilent Cary 3500 UV-Vis spectrophotometer and reported in the literature.

Technique	T_m of *Ec*DsbA (°C)
Cary 3500 UV-Vis Spectrophotometer Replicate 1	69.70
Cary 3500 UV-Vis Spectrophotometer Replicate 2	69.80
Cary 3500 UV-Vis Spectrophotometer Replicate 3	69.70
Cary 3500 UV-Vis Spectrophotometer Replicate 4	70.20
Cary 3500 UV-Vis Spectrophotometer Replicate 5	69.90
Cary 3500 UV-Vis Spectrophotometer Replicate 6	69.90
Average	69.86
Standard Deviation	0.16
Literature Value 1[14]	68.55
Literature Value 2[15]	69.25

Conclusion

The Agilent Cary 3500 UV-Vis spectrophotometer with a Peltier temperature-controlled multicell was used to determine the melting temperature (T_m) of six samples of *Ec*DsbA protein simultaneously. The results were reproducible, as shown by the standard deviation of 0.16 °C achieved for six portions of one sample of the *Ec*DsbA protein. Also, the average T_m of 69.86 °C calculated using the Agilent Cary UV Workstation software agreed with literature values obtained using circular dichroism spectroscopy. The results demonstrate the accuracy, precision, and speed of the Cary 3500 UV-Vis spectrophotometer for protein thermal stability analyses.

To further maximize productivity of the method, the Cary 3500 UV-Vis can measure seven samples at the same time without compromising result accuracy. The capability to simultaneously measure at least three replicates of the sample through the instrument design represents valuable time saving for laboratories that require replicate measurements.

The Cary UV Workstation software has built in smoothing and derivative calculations for ease of data analysis. Also, the software is available with optional technical controls to securely acquire, process, report, and store data. These controls are vital for laboratories that must follow the compliance guidelines of FDA 21 CFR Part 11, EU Annex 11, GAMP5, as well as ISO/IEC 17025 and EPA 40 CFR Part 160.

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