Measuring Aggregation Propensity of a Protein using SUPR-CM

Summary

- Aggregation is a major challenge in biologics development, and can be assessed using isothermal chemical denaturation.
- Gibbs Free Energy values determined for different concentrations of protein using SUPR-CM.
- High-throughput capabilities of SUPR-CM allowed for rapid processing of samples.
- Aggregation propensity measured for the same protein in two different buffers (phosphate and acetate).
- Evidence showing phosphate significantly increases propensity for the unfolded protein to aggregate.
- Acetate buffer showed no statistically significant change in ΔG° values, with over 4x reduction in aggregation propensity when compared to the phosphate samples.

Applied Innovations in Protein Characterisation

Introduction

Protein aggregation is one of the biggest problems to tackle that's linked to protein instability.[1] Aggregation can occur throughout the development, formulation, manufacture and distribution of a new biologic, and can result from temperature, agitation, freeze/thawing, and formulation conditions.[2,3] Aggregates can be immunogenic, causing immune reactions and tolerance issues with prolonged use of a biotherapeutic. In order to maintain the effectiveness of the therapeutic, the protein construct and formulation conditions need to be optimized to prevent or mitigate aggregation.

Along with measuring conformational stability, isothermal chemical denaturation (ICD) can be used to determine a protein's propensity to aggregate.[4] This is determined by measuring the Gibbs Free Energy (ΔG°) via ICD at different protein concentrations.

Depending on the change of the ΔG° values with protein concentration, it can be determined whether the protein's folded or unfolded state is likely to result in aggregation. An increase in ΔG° indicates that the folded state is prone to aggregation, while a decrease in ΔG° is evidence that the unfolded state is prone to aggregation. If there is no change in ΔG°, then there are no intermolecular interactions, and it is unlikely that aggregation will occur.[5]

In this application note, the SUPR-CM fluorescence plate reader was used to determine the aggregation propensity of the model protein lysozyme in two different buffer solutions in order to illustrate how ICD can be used to assess which buffer condition would result in fewer aggregates.

[1] Wang W. Protein aggregation and its inhibition in biopharmaceutics. International Journal of Pharmaceutics. 2005; 289(1–2): 1–30.

[2] Wang W, Roberts CJ. Protein aggregation – Mechanisms, detection, and control. International Journal of Pharmaceutics. 2018; 550(1–2): 251–268.

[3] Mahler H-C, Friess W, Grauschopf U, Kiese S. Protein aggregation: Pathways, induction factors and analysis. Journal of Pharmaceutical Sciences. 2009; 98(9): 2909–2934.

[4] Schön A, Clarkson BR, Siles R, Ross P, Brown RK, Freire E. Denatured state aggregation parameters derived from concentration dependence of protein stability. Analytical Biochemistry. 2015; 488: 45–50.

[5] Rizzo JM, Shi S, Li Y, Semple A, Esposito JJ, Yu S, et al. Application of a High-Throughput Relative Chemical Stability Assay to Screen Therapeutic Protein Formulations by Assessment of Conformational Stability and Correlation to Aggregation Propensity. Journal of Pharmaceutical Sciences. 2015; 104(5): 1632–1640.

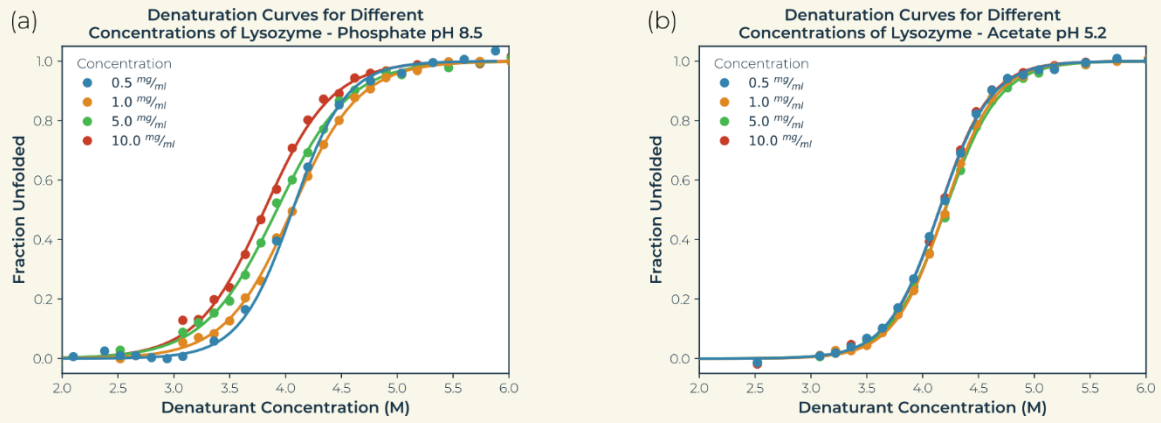

Figure 1 – Denaturation curves showing the change in the fraction of unfolded protein at different concentrations of denaturant. The plots show the change in denaturation curve for different concentrations of lysozyme in (a) pH 8.5 phosphate and pH 5.2 acetate buffer. The dots represent the measured data while the solid line is the trendline from fitting a two-state function to the data.

Method

STOCK SOLUTIONS

Stock solutions of phosphate and acetate buffer were made at 0.1 M concentration and had pH values of 8.5 and 5.2, respectfully. Denaturant stock solutions of guanidine hydrochloride were prepared in each buffer at 7 M. Solutions of lysozyme were prepared at concentrations of 0.5 $^{mg}/_{ml}$, 1 $^{mg}/_{ml}$, 5 $^{mg}/_{ml}$, and 10 $^{mg}/_{ml}$ for each buffer. Protein samples were incubated at 20°C for 24 hours to allow stock solutions to equilibrate.

DISPENSING REAGENTS

Samples for chemical denaturation were prepared in the wells of a 384-well microplate (black, Greiner). A Mantis® liquid handler (Formulatrix®) was used to dispense reagents into the wells of the microplate. 24 denaturant concentrations were prepared from 0 M to 6 M. The volume of protein dispensed per well was 7 µl and the total well volume was kept at 50 µl. Prepared plates were incubated for 24 hours at 20°C to allow the samples to reach equilibrium.

FLUORESCENCE MEASUREMENTS

The incubated microplates were measured with the SUPR-CM fluorescence plate reader. The well measurement time was adjusted as the sample concentration increased. The 0.5 $^{mg}/_{ml}$ samples used a 500 ms well measurement time. The well measurement time was decreased to 250 ms for the 1 $^{mg}/_{ml}$ sample, while the 5 $^{mg}/_{ml}$ and 10 $^{mg}/_{ml}$ samples used a 100 ms well measurement time. Changing the well measurement time ensured that substantial fluorescence signals were measured without saturating the array detector.

The fluorescence data were converted to denaturation curve data by plotting the ratio of intensities (350 nm & 330 nm) against denaturant concentrations. Gibbs Free Energy values were calculated from fitting a two-state function to denaturation curve data.

Results

The profiles of the denaturation curves of **Figure 1** shows a two-state behaviour that is to be expected for this globular protein.[1, 2] Comparison of the data profiles in **Figure 1** shows a larger change for the phosphate samples than the acetate samples. The profiles of the phosphate sample of **Figure 1(a)** show an initial change in transition region gradient (from 0.5 $^{mg}/_{ml}$ to 1 $^{mg}/_{ml}$) before the profiles shift to lower denaturant concentrations. By comparison, the acetate sample profiles show only minor changes.

[1] Ahmad F, Bigelow CC. Estimation of the Free Energy of Stabilization of Ribonuclease A, Lysozyme, a-Lactalbumin, and Myoglobin. The Journal of Biological Chemistry. 1982; 257(21): 12935–12938.

[2] Ahmad F, Contaxis CC, Bigelow CC. Free Energy Changes in Lysozyme Denaturation. Journal of Biological Chemistry. 1983; 258(13): 7960–7963.

Table 1 – Values of Gibbs Free Energy for different concentrations of lysozyme in phosphate and acetate buffers.

	Phosphate		Acetate	
Lysozyme Conc. ($^{mg}/_{ml}$)	$\Delta G°$ (kJ mol^{-1})	C_m (M)	$\Delta G°$ (kJ mol^{-1})	C_m (M)
0.5	41.69	4.06	43.74	4.14
1.0	32.94	4.05	45.07	4.19
5.0	31.22	3.92	42.65	4.20
10.0	30.71	3.81	44.56	4.15

These trends are reflected in the fitted values shown in **Table 1**. The C_m values for the phosphate samples 0.5 $^{mg}/_{ml}$ and 1 $^{mg}/_{ml}$ are similar meaning the change in $\Delta G°$ values stems from a change in gradient of the transition region. The phosphate sample C_m values decrease by 0.25 M while the acetate C_m values show no trend and only have a statistical range of 0.06 M.

The Gibbs Free Energy values from fitting a two-state function to the denaturation curves are plotted in **Figure 2** and the values obtained are listed in **Table 1**. The most noticeable difference is that the phosphate buffer has lower values of $\Delta G°$ than the acetate buffer. Which is mainly due to the self-association of the lysozyme.

It is also apparent that the $\Delta G°$ values for the phosphate samples decrease as the protein concentration increases. This implies that the unfolded state is more likely to form aggregates.[1] Going from 0.5 $^{mg}/_{ml}$ to 10 $^{mg}/_{ml}$, the phosphate sample's $\Delta G°$ values decreases by 10.98 kJ mol^{-1}. This is significantly more than the 2.42 kJ mol^{-1} change seen with the acetate samples. This is evidence that the acetate buffer helps mitigate the aggregation of the protein.

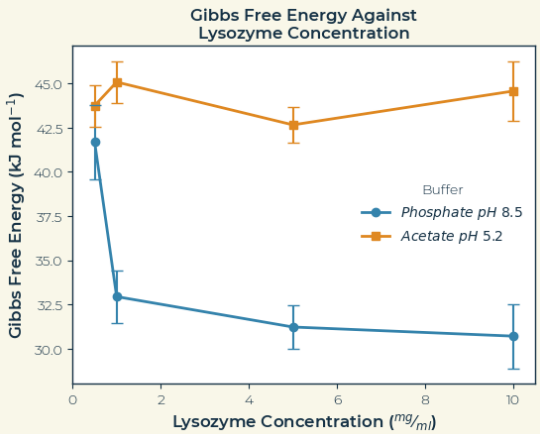

Figure 2 – The Gibbs Free Energy values for different concentrations of lysozyme in phosphate and acetate buffer. Values obtained from the two-state fitting of **Figure 1**. Error bars equate to one standard deviation.

Included in **Figure 2** are the errors associated with the $\Delta G°$ values. These errors are the standard deviations from fitting the two-state function to the denaturation curves and were consistently below 5%.

Conclusion

Given the impact aggregation can have on the safety and efficacy of a biologic and the regulatory interest in controlling it,[2] there is a need for rapid determination of aggregation propensity. The SUPR-CM provides rapid, high throughput measurement of protein aggregation propensity.

The aggregation propensity for lysozyme was measured in two different buffers using the SUPR-CM fluorescence microplate reader. Measuring the Gibbs Free Energy values for different protein concentrations showed that phosphate buffer increased the likelihood of the unfolded state to aggregate. This was evidenced by the 10.98 kJ mol^{-1} decrease in $\Delta G°$ as the protein concentration was increased. The acetate buffer showed no statistically relevant change in $\Delta G°$

[1] Rizzo JM, Shi S, Li Y, Semple A, Esposito JJ, Yu S, et al. Application of a High-Throughput Relative Chemical Stability Assay to Screen Therapeutic Protein Formulations by Assessment of Conformational Stability and Correlation to Aggregation Propensity. Journal of Pharmaceutical Sciences. 2015; 104(5): 1632–1640.

[2] Cordoba-Rodriguez R. V. Aggregates in MAbs and Recombinant Therapeutic Proteins: A Regulatory Perspective. BioPharm International. 2008; 21(11): 1–8.

suggesting no intermolecular interaction and no aggregation propensity. Combined with the small change in acetate sample C_m values, the data of **Figure 2** illustrates acetate as being the more preferential buffer to reduce aggregation.