

Comparing pH and Buffer Solutions for Stabilising a Monoclonal Antibody using the SUPR-CM High-Performance Plate Reader

Introduction

Advances in recombinant DNA technologies have facilitated an increase in the number of monoclonal antibodies (mAbs) that have been developed for therapeutic applications.[1], [2] The high specificity of mAbs allows for targeted therapeutics with fewer side effects as well as the development of specific drug delivery molecules.[2]

Development of mAb biologics includes optimizing the stability of the mAbs by alteration of the solution conditions. The formulation process involves screening different buffers and pH conditions before experimenting with other excipients.[2] The number of solution conditions that can be altered can increase the time and quantity of mAb needed to run all the formulation experiments.

Currently, thermal approaches like differential scanning fluorimetry (DSF) and differential scanning calorimetry (DSC) are often preferred despite drawbacks like non-equilibrium measurements and thermal alteration of solvent conditions.[3] Chemical denaturation has the advantage of reversible, equilibrated measurement of protein stability. Chemical denaturation uses chaotropic agents (like guanidine hydrochloride or urea) to unfold the protein,[4] and by measuring the fraction of unfolded protein at different denaturant concentrations, the stability of the protein can be determined.

Utilizing chemical denaturation, the stability of a novel mAb was quickly determined for different buffer solutions at different pH values, using the SUPR-CM fluorescence plate reader. Equilibrium conditions were achieved by letting the prepared microplates incubate for 24 hours, as previously determined for this mAb (data not shown).

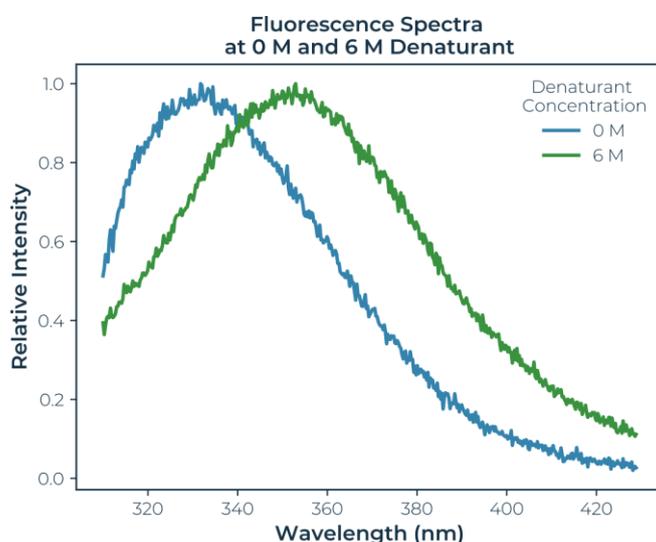


Figure 1 – Fluorescence spectra of IgG1 mAb at 0 M and 6 M GuHCl. The redshift in the spectrum can be used to measure the unfolding of the protein.

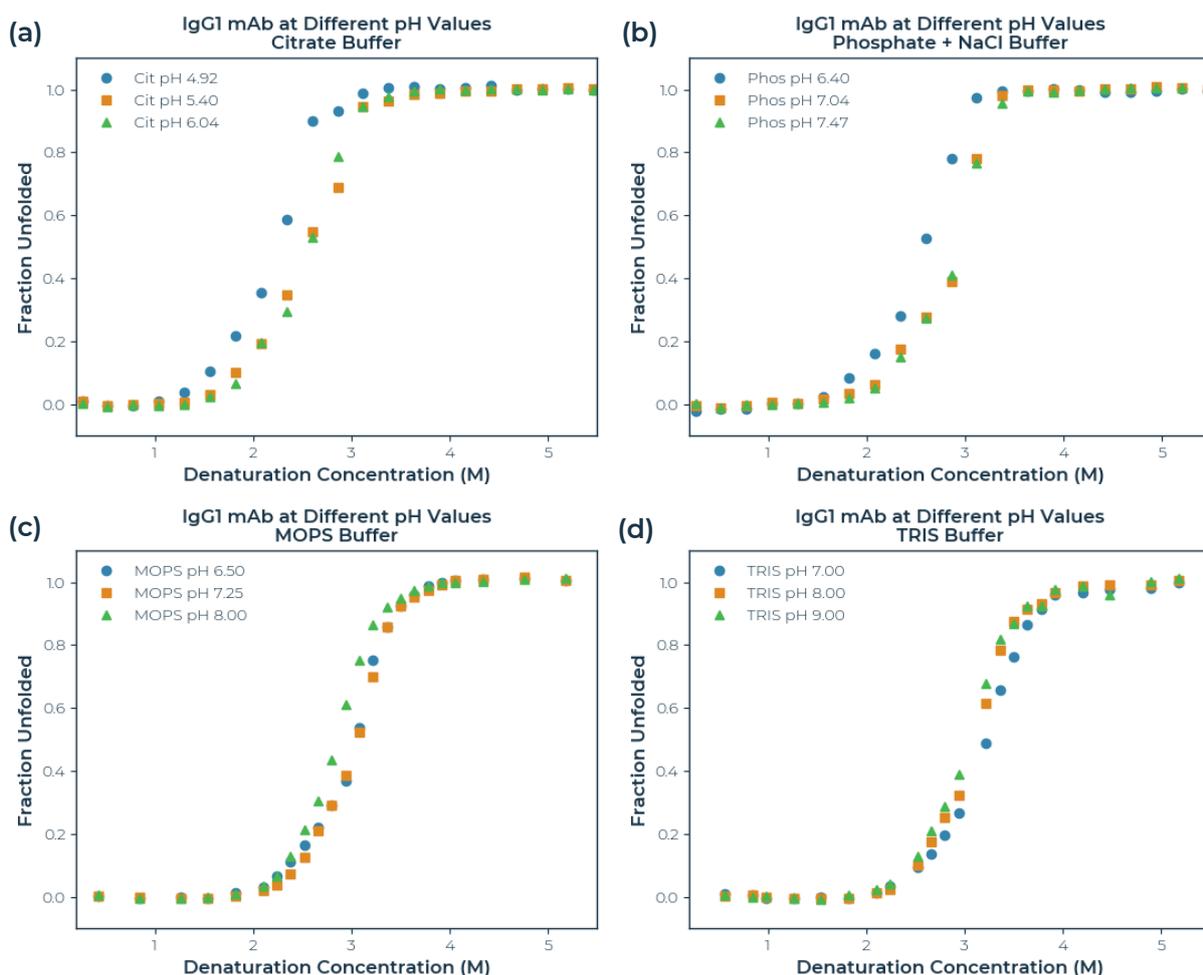


Figure 2 – Fraction unfolded values against denaturant concentration for each pH value measured. The plots are grouped by buffer, with (a) for citrate, (b) for phosphate, (c) for MOPS and (d) for TRIS.

Results

Increasing the concentration of the denaturant causes the mAb to unfold. The unfolding of the protein alters the spectral properties of the tryptophan molecules as the local environment changes.^[5] This transition can be seen in **Figure 1** as a redshift in the spectral peak going from 0 M to 6 M guanidine hydrochloride (GuHCl).

The ratio of intensity values was used to quantify the unfolding process. The fluorescence maximum for each peak in **Figure 1** is at 330 nm (0 M) and 355 nm (6 M); thus, the ratio of intensity values at these wavelengths is an indication of the degree of unfolding and can be used to calculate a denaturation curve from which key thermodynamic parameters can be determined.

The ratio data exhibits three characteristic features: The lower plateau where the majority of antibodies are in the folded state. The upper plateau where the majority of antibodies are in the unfolded state. Finally, there is the region between where the antibodies transition from the folded to the unfolded state.

The fraction unfolded was calculated for each dataset and plotted in **Figure 2**. Looking at the data for the citrate buffer (**Figure 2(a)**) shows that the lowest pH value (pH 4.92) has reduced the stability of the antibody since the transition region has shifted to lower denaturant concentrations when compared to the pH 5.40 and 6.04 samples. This is a similar case for the phosphate buffer (**Figure 2(b)**), where the pH 6.40 sample has not performed as well as the pH 7.04 and 7.47 samples.

Table 1 – The pH values of each buffer solution used to assess the stability of the mAb.

Buffer	pH Values		
Citrate	4.92	5.40	6.04
Phosphate	6.40	7.04	7.47
MOPS	6.50	7.25	8.00
TRIS	7.00	8.00	9.00

For the MOPS and TRIS buffers, a difference in pH did not show as large a change. There is an observable difference between the MOPS pH 8.00 sample and the other MOPS samples in **Figure 2(c)**. The TRIS buffer (**Figure 2(d)**) also shows a slight difference between pH 7.00 and the other two pH values. However, these changes are not as pronounced as with the citrate or phosphate samples, implying that the mAb has greater stability across a wider range of pH values for the MOPS and TRIS buffers than the citrate and phosphate buffers.

The data was fitted to both a two-state and a three-state function to establish an appropriate model for this antibody. A comparison between the residuals (not shown) of the two-state and three-state model confirmed the three-state model to be more appropriate.

From the fitting, the key descriptive parameters were obtained for each denaturation curve at each pH value.[6] The parameters of interest are the ΔG° and the C_m values. The ΔG°_1 and C_{m1} values are the Gibbs Free Energy and the midpoint denaturant concentration for the folded/intermediate transition. In contrast, the ΔG°_2 and the C_{m2} values are the Gibbs Free Energy, and the midpoint denaturant concentration for the intermediate /unfolded transition.[6]

The pH values with the highest ΔG° and C_m values (for each buffer) were plotted together in **Figure 3**, along with the fitted trendline. The ΔG° and C_m values for the data in **Figure 3** are listed in **Table 2**. Comparing the C_m values show that the citrate and phosphate samples have lower values than MOPS and TRIS. This can also be seen in **Figure 3** as the transition regions occur at lower denaturant concentrations.

The phosphate sample produced the highest ΔG°_2 value at 84.85 kJ mol⁻¹. However, the first transition region has the lowest ΔG° value at 27.25 kJ mol⁻¹. This large variability between transition regions, coupled with the lower C_m values, ranks citrate and phosphate below MOPS and TRIS as optimal buffers for this mAb. Comparing the data for MOPS and TRIS in **Figure 3** would suggest that TRIS has improved the mAb stability the most since the data is shifted to higher denaturant concentrations. Using simplified fittings and C_m rankings showed TRIS to have a 0.14 M increase over MOPS (data not shown). However, comparing the fitted values of **Table 2** reveals that the MOPS sample has higher ΔG° values and a higher C_{m1} value.

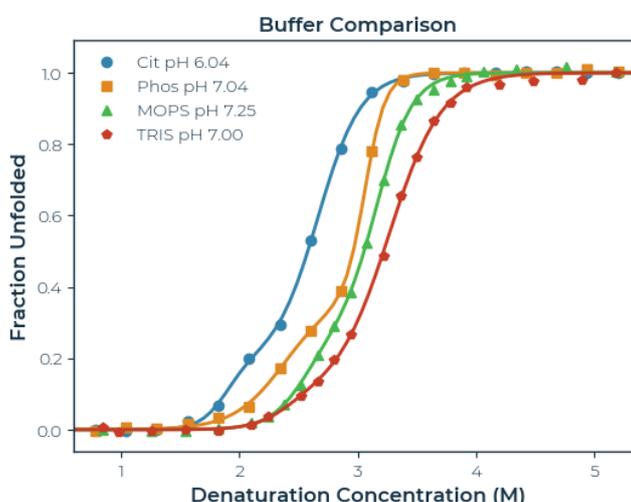


Figure 3 – Fraction unfolded plots for the best performing pH value, for each buffer tested along with the trendline from fitting a three-state function.

Table 2 – The ΔG° and C_m values from fitting a three-state function to the data of **Figure 3**.

Sample	ΔG°_1	C_{m1}	ΔG°_2	C_{m2}
Citrate	40.63	1.89	35.82	2.66
Phosphate	27.25	2.39	84.85	3.06
MOPS	39.66	2.55	50.86	3.17
TRIS	37.44	2.40	36.37	3.27

Conclusion

Chemical denaturation experiments were performed on an IgG1 mAb to establish which buffer and pH improved the stability of the mAb the most. Equilibrated samples were prepared in 384-well microplates and rapidly measured with the SUPR-CM. Both MOPS and TRIS improved the C_m values over phosphate and citrate. Quantification of more detailed denaturation data, obtained with SUPR-CM, revealed MOPS to have the higher C_{m1} value and higher ΔG° values. The high quality, detailed denaturation data produced with the SUPR-CM highlights the overall improvement in stability of the mAb when using a MOPS buffer at pH 7.25.

Methodology

Stock solutions of citrate, phosphate, TRIS, and MOPS were prepared at 0.1 M. The IgG1 mAb stock concentration was 1.25 mg/mL, and a 7 M guanidine hydrochloride stock solution was prepared. Buffer solutions had their pH values adjusted to the values shown in **Table 1**. The denaturant was dissolved in the appropriate buffer, and the solution pH was altered to match the buffer solution. Reagents were dispensed into the 384-well microplates (Greiner) by a Mantis[®] Liquid Handler (Formulatrix[®]). Samples were prepared with 24 denaturant concentrations from 0 M to 6 M. Samples were dispensed in triplicate and had a final protein concentration of 50 µg/mL per well. The total well volume was kept consistent at 50 µL.

The microplates were incubated for 24 hours (20°C), ensuring the samples reached equilibrium. After the incubation period, fluorescence spectrum data were acquired with the SUPR CM microplate reader. The mAb was excited at 280 nm and fluorescence recorded from 310 nm to 435 nm. Each microplate was measured in 2.5 mins, which produced an excellent signal-to-noise ratio while not compromising on the speed of data acquisition. Denaturation curves were generated by plotting the ratio of intensities at 355 nm and 330 nm against denaturant concentration. Denaturation curves were fitted to a three-state function to determine ΔG° and C_m values.

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