Freezing-induced Perturbation of Tertiary Structure of Monoclonal Antibody

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Introduction

- Recombinant DNA technology has made it possible to produce novel therapeutic proteins. Monoclonal antibodies comprise the majority of recombinant proteins currently in the clinic.[1]
- Most proteins are marginally stable and subject to numerous degradative processes in aqueous solution. These degradative processes can often be retarded or eliminated by storage of proteins at subzero temperatures (i.e.,cryopreservation).
- However, freeze-thawing cycle usually results protein partially unfolding, and consequently forming aggregates, which may induce immunogenic response.
- Fluorescence spectroscopy is commonly used to study perturbations of protein tertiary structure induced by chemical denaturants, pH changes, pressure and temperature. However, its use in freezing studies has been limited.
- Strambini and coworkers reported that binding of ANS to proteins in ice can provide a general monitor of ice-induced alterations of their tertiary structure.[2-3]
- In this study, both intrinsic and extrinsic fluorescence spectroscopy were employed to document real-time freezing-induced perturbations of tertiary structure of a monoclonal antibody (IgG).

Materials and methods

Model protein: Monoclonal antibody (IgG) 0.5mg/ml

Buffers: 10 mM Potassium Phosphate, pH 3,8
10 mM Sodium Acetate, pH 4
Salt: 0, 150mM Potassium Chloride

1-anilino-8-naphthalene sulfonate (ANS): 75μM

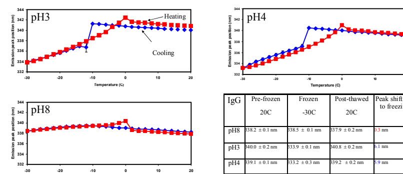
Instruments:
QuantaMaster spectrofluorometer equipped with a thermostated cuvette holder

Cuvettes (disposable cuvettes, Light transmittance range: 275-800nm)

The effects of pH and salt (150 mM KCl) were studied during cooling and heating between 20ºC and -30ºC

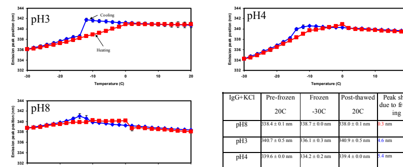
Fluorescence spectra were fitted to Extreme peak function (Origin 7,OriginLab) to obtain the associated λmax values.

Results and discussion



IgG	Pre-frozen 20C	Frozen -30C	Post-thawed 20C	Peak shift due to freezing
pH8	338.2 ± 0.1 nm	338.5 ± 0.1 nm	337.9 ± 0.2 nm	0.3 nm
pH3	340.0 ± 0.2 nm	333.9 ± 0.1 nm	340.8 ± 0.2 nm	6.1 nm
pH4	339.1 ± 0.1 nm	333.2 ± 0.3 nm	339.2 ± 0.2 nm	5.9 nm

Figure 1 IgG intrinsic (Trp) fluorescence emission peak position as a function of temperature in pH3, 4 and 8 buffer. Table 1 list fluorescence emission peak positions and peak shift due to freezing. Data represent average ± standard deviation of triplicate samples.



IgG+KCl	Pre-frozen 20C	Frozen -30C	Post-thawed 20C	Peak shift due to freezing
pH8	338.4 ± 0.1 nm	338.7 ± 0.0 nm	338.0 ± 0.1 nm	0.3 nm
pH3	340.7 ± 0.5 nm	336.1 ± 0.3 nm	340.9 ± 0.5 nm	4.6 nm
pH4	339.6 ± 0.0 nm	334.2 ± 0.2 nm	339.4 ± 0.0 nm	5.4 nm

Figure 2 IgG+150mM KCl intrinsic (Trp) fluorescence emission peak position as a function of temperature in pH3, 4 and 8 buffer. Table 2 list fluorescence emission peak positions and peak shift due to freezing. Data represent average ± standard deviation of triplicate samples.

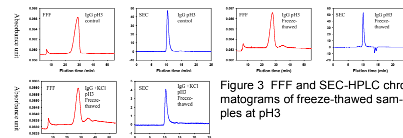


Figure 3 FFF and SEC-HPLC chromatograms of freeze-thawed samples at pH3

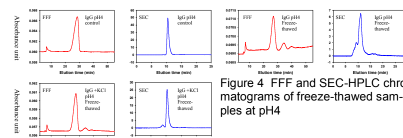


Figure 4 FFF and SEC-HPLC chromatograms of freeze-thawed samples at pH4

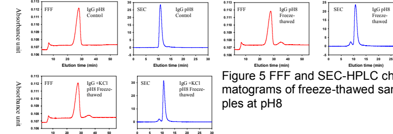
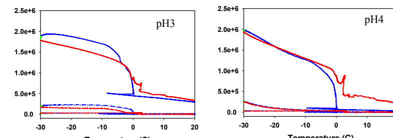


Figure 5 FFF and SEC-HPLC chromatograms of freeze-thawed samples at pH8

- At pH 8, freezing and thawing caused minimal change in the wavelength of maximum emission for tryptophan fluorescence, with or without KCl, indicating the polarity of the microenvironment of tryptophan residues in IgG did not change substantially. (Figure 1-2)
- At pH 3 and 4, the intrinsic fluorescence emission spectra were blue shifted in the frozen state, implying that tryptophans were exposed to a more apolar microenvironment. (Figure 1-2)
- Table 1-2 list fluorescence emission peak positions and peak shift due to freezing.
- Size-exclusion high performance liquid chromatography (SEC-HPLC) and asymmetrical flow field-flow fractionation(AFFF) studies of the freeze-thawed samples showed that IgG aggregates formed during freeze-thawing at pH 3, 4 and 8 either with or without 150 mM KCl. KCl facilitated aggregation at pH 3, but reduce it at pH 4. These findings are consistent with the results of fluorescence spectroscopy. (Figure 3-5)



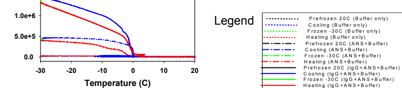


Figure 6 IgG ANS fluorescence intensity as a function of temperature. Excitation: 350nm; Emission: 480nm

Legend
- Prefrozen 20C (Buffer only)
- Cooling (Buffer only)
- Frozen -30C (Buffer only)
- Heating (Buffer only)
- Prefrozen 20C (ANS+Buffer)
- Cooling (ANS+Buffer)
- Frozen -30C (ANS+Buffer)
- Heating (ANS+Buffer)
- Prefrozen 20C (IgG+ANS+Buffer)
- Cooling (IgG+ANS+Buffer)
- Frozen -30C (IgG+ANS+Buffer)
- Heating (IgG+ANS+Buffer)

- At pH8, fluorescence intensity of the external probe ANS increased dramatically when antibody was frozen. These results suggest an increased accessibility to ANS of the protein's hydrophobic sites, which is commonly associated with formation of partially unfolded protein molecules. This effect was nearly completely reversible upon thawing. (Figure 6)
- The presence of 150 mM KCl reduced ANS fluorescence intensity in both frozen and thawed samples. (Figure 7)

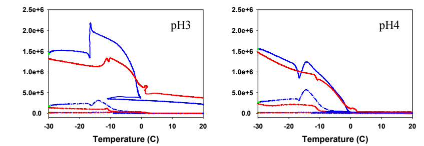


Figure 7 IgG + 150mM KCl ANS fluorescence intensity as a function of temperature. Excitation: 350nm; Emission: 480nm

Legend
- Prefrozen 20C (Buffer +KCl)
- Cooling (Buffer +KCl)
- Frozen -30C (Buffer +KCl)
- Heating (Buffer +KCl)
- Prefrozen 20C (ANS+KCl+Buffer)
- Cooling (ANS+KCl+Buffer)
- Frozen -30C (ANS+KCl+Buffer)
- Heating (ANS+KCl+Buffer)
- Prefrozen 20C (IgG+KCl+ANS+Buffer)
- Cooling (IgG+KCl+ANS+Buffer)
- Frozen -30C (IgG+KCl+ANS+Buffer)
- Heating (IgG+KCl+ANS+Buffer)

- Compared with pH8, ANS fluorescence intensity showed a distinct increase in pre-frozen samples at pH3, implying that the protein partially unfolded at such a low pH. The increase of ANS fluorescence intensity was larger during freezing at pH 3 and 4 than that at pH8. The effect was attenuated in the presence of 150 mM KCl in frozen samples at pH3 and 4. (Figure 6-7)
- In contrast, however, the presence of 150mM KCl reduced ANS fluorescence intensity in thawed samples at pH4 while KCl fostered ANS fluorescence intensity in thawed samples at pH3.

Conclusions

- Freezing-induced perturbations of tertiary structure of IgG were documented by intrinsic fluorescence spectroscopy and the extrinsic probe ANS.
- Fluorescence spectroscopy is a simple and effective technique, and should be useful in general to study in real-time the effects of freezing on therapeutic proteins.

Future directions

- we will study the effects of stabilizers such as sugars, surfactants, amino acids and salts on the perturbation of protein tertiary structure upon freezing and thawing.
- Next, we will use IR spectroscopy to study the perturbation of secondary structure of monoclonal antibody during freezing and thawing.

Reference

[1] Reichert, J.M., et al., *Monoclonal antibody successes in the clinic.* Nat Biotechnol, 2005. **23**(9): p. 1073-8.
[2] Gabellieri, E. and G.B. Strambini, *Perturbation of protein tertiary structure in frozen solutions revealed by 1-anilino-8-naphthalene sulfonate fluorescence.* Biophys J, 2003. **85**(5): p. 3214-20.
[3] Gabellieri, E. and G.B. Strambini, *ANS fluorescence detects widespread perturbations of protein tertiary structure in ice.* Biophys J, 2006. **90**(9): p. 3239-45.