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In Situ Spectroscopic Quantification of Protein–Ice Interactions

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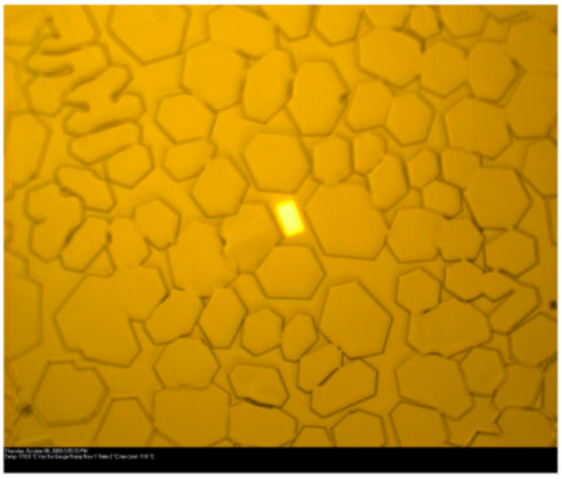
Abstract

FTIR and confocal Raman microspectroscopy were used to measure interactions between albumin and ice *in situ* during quasi-equilibrium freezing in dimethyl sulfoxide (DMSO) solutions. At temperatures of −4 and −6 °C, albumin was found to be preferentially excluded from the ice phase during near-equilibrium freezing. This behavior reversed at lower temperatures. Instead, DMSO was preferentially excluded from the ice phase, resulting in an albumin concentration in the freeze-concentrated liquid phase that was lower than predicted. It is hypothesized that this was caused by the albumin in the freeze-concentrated liquid getting adsorbed onto the ice surface or becoming entrapped in the ice phase. It was observed that, under certain freezing protocols, as much as 20% of the albumin in solutions with starting concentrations of 32–53 mg/mL may be adsorbed onto the ice interface or entrapped in the ice phase.



INTRODUCTION

Freezing plays a significant role in the stabilization and long-term preservation of biological and pharmaceutical specimens and compounds. In biobanking, fluid biospecimens such as

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serum, plasma, bronchial lavage fluid, and urine are frequently frozen and stored for future proteomics research, while protein solutions for therapeutic use are generally freeze-dried.[1–3] Freezing and freeze-drying processes impose very harsh chemical and physical stresses on proteins,[3–10] altering their characteristics (structure and activity) often irreversibly.[11–18] These stresses affect the quality of the stored biospecimens and may result in the loss of biomarker information or clinical/ diagnostic utility. Subsequent proteomics studies on frozen and thawed samples can therefore be biased.[19] The low quality of the biospecimens currently stored in biobanks has been identified as one of the major issues inhibiting scientific progress.[20] Similarly, therapeutic proteins can be damaged during freeze-processing, causing an economic loss and reducing their therapeutic impact.[21] Understanding the interactions between an ice surface and the macromolecules in the solution may therefore help to reduce product losses and to improve therapeutic efficacy.

The ice phase is expected to incorporate few or no solutes when an aqueous solution is frozen.[22] As the ice phase forms and grows, the solutes excluded from the ice phase are concentrated in the remaining liquid phase, termed the freeze-concentrated liquid (FCL) phase. When an aqueous solution containing proteins is frozen, either (1) the proteins may end up homogenously distributed within the FCL phase, or (2) the proteins may end up heterogeneously distributed in the FCL and may aggregate at the surface of or get entrapped within the ice phase. In the latter case, the protein concentration in the FCL phase would decrease. This is known to occur with antifreeze proteins,[23] which are known to adsorb to the surface of the ice[23] through the formation of hydrogen bonds between the ice surface and their ordered hydration layers.[24] With other proteins and enzymes, a handful of studies were conducted and these have reported significant interactions mainly through centrifugation and filtration of a partially frozen solution, followed by measurement the protein content in the FCL.[23,25] Other studies have used molecular techniques such as confocal Raman microspectroscopy by measuring the changes in protein conformation at the ice interface[26] and the resultant protein aggregation, which may even persist post-thaw.[4,27,28]

In this study, we show the feasibility of using FTIR spectroscopy and confocal Raman microspectroscopy (CRM) in a nondestructive fashion to quantify the adsorption of albumin onto the ice surface in partially frozen aqueous solutions of dimethyl sulfoxide (DMSO). While FTIR has been used to investigate changes in secondary structure of proteins in frozen solutions[29] and freeze-dried formulations,[30] to our knowledge, it has not been used to study protein interaction with ice in the freeze-concentrated liquid in a quantitative manner. Albumin is the most abundant protein found in the plasma. It facilitates transport for thyroid and steroid hormones[31,32] and fatty acids,[33] regulates oncotic pressure in the capillaries,[34] and is also used as a biomarker to detect and monitor renal and cardiovascular diseases.[35] The primary reason for using DMSO is that it is used very widely as a cryostabilizing agent for preservation of cells, biofluids, and tissues.[36–42] An additional advantage of using DMSO to study interactions between proteins and ice is its substantial ability to depress the melting point of binary solutions of water and DMSO.[43] This enables investigation of protein–ice interactions in the freeze-concentrated liquid during freezing over a wide range of temperatures. Nonetheless, concern has been expressed about the clinical side effects of DMSO, particularly in autologous stem cell transplants.[36,44,45] At very high concentration, DMSO has also been found to denature proteins,[46] but these concentrations are outside the concentration range used in this study.

The Approach

The technique developed in this study is intended to aid in understanding the interactions between proteins and ice during freezing, as well as serve as a methodology to identify

freezing parameters that minimize the harmful interactions between proteins and ice that may occur during the preservation and storage of biospecimens and pharmaceuticals.

When freezing a solution of DMSO and water under equilibrium conditions, a 35 wt % DMSO solution will follow the equilibrium freezing curve of the binary phase diagram as it is cooled to −20 °C (Figure 1). According to the phase diagram, the FCL region should contain about 37 wt % DMSO in equilibrium with ice.[43,47] To obtain the same concentration of DMSO in liquid water at −20 °C, an alternative would be to dilute the initial solution to 15 or 25 wt % DMSO and cool these under equilibrium conditions to −20 °C. Due to the freeze-concentration of the DMSO as it is excluded from the ice phase, the composition of the FCL of these diluted solutions at equilibrium at −20 °C should also be 37 wt % DMSO. The only difference among the different solutions at equilibrium at the same temperature would be the relative sizes of the FCL and ice phases. In Figure 2, aqueous solutions of 6–12 wt % DMSO with a small amount of added albumin are all at equilibrium at −6 °C. While the 12 wt % DMSO solution has just a few small ice crystals and a large FCL phase, the more dilute 6 wt % DMSO solution has an FCL phase and an ice phase that are almost equal in size.

No ternary phase diagram exists in the literature for aqueous solutions of DMSO and albumin. Even so, the addition of a small amount of albumin to aqueous solutions of DMSO should result in an additional slight depression of the equilibrium freezing point by the colligative effect.[47,48] During freezing of such a ternary solution, the mass (and molar) ratio of albumin/DMSO in the FCL should be constant if the albumin and the DMSO are excluded equally from the ice phase, even as their absolute concentrations increase due to freeze-concentration. By measuring the mass ratio of albumin/ DMSO in the FCL, it is possible to detect if either albumin or DMSO is preferentially excluded from the ice phase, since this would result in a change in the mass ratio of albumin/DMSO in the FCL. If there is preferential exclusion of albumin or DMSO from the ice phase, this effect should be greatest in solutions with greater amounts of ice. If the observed effect were due to uniform entrapment within the ice phase, it should be most significant with the greatest volume of ice. If the effect were due to accumulation of albumin or DMSO at the interface between the ice and the FCL phases, then it should be most significant with the greatest interfacial area.

Similar to during the freezing of binary DMSO solutions, dilute ternary solutions of DMSO and albumin will undergo more freeze-concentration than do more concentrated solutions to reach a common temperature, and will have a larger ice phase and smaller FCL phase. Provided that the mass ratio of albumin/DMSO is the same in both the dilute and concentrated solutions, as is the case when a concentrated solution is diluted with water, it is possible to vary the amount of ice in partially frozen systems at a given temperature and then measure the effect on the composition of the FCL. As a reference point for comparison, it is advantageous to use the composition of the FCL in which the effects of ice are minimized. This reference point is called here the minimum ice solution (MIS) and is the composition of the FCL of the partially frozen system with the least amount of ice at a given temperature. For example, the MIS on the binary DMSO and water phase diagram at −20 °C is the 35 wt % solution, which would be 2 wt % ice according to the lever rule (Figure 1). By comparing the FCL of the MIS with the FCL of the more dilute 15 and 25 wt % solutions as shown in the figure, preferential exclusion from the ice phase of either albumin or DMSO can be detected by a change in the mass ratio of albumin/DMSO. With this method, we used IR and Raman spectroscopy in a nondestructive fashion to investigate the interactions of albumin with the ice interface *in situ* in aqueous DMSO solutions in a temperature range of −4 to −40 °C.

MATERIALS AND METHODS

Experiments were conducted using Fourier transform infrared (FTIR) spectroscopy and confocal raman microspectroscopy (CRM) with solutions over a range of DMSO concentrations (6–35 wt %) and albumin concentrations (20–63 mg/mL) at temperatures of −4 to 40 °C. FTIR was chosen for its speed and lower signal-to-noise ratio in data acquisition, as well as its ability to measure freeze-concentrated liquid *in situ* without heating the sample. The disadvantage of using FTIR was its relatively poor spatial resolution of 100 μm × 100 μm. CRM can reach a spatial resolution of less than 1 μm × 1 μm; however, the weak intensity of the albumin Raman spectrum resulted in a low signal-to-noise ratio. Spatial averaging of each 4 μm × 83 μm Raman scan was required to get a sufficiently strong signal-to-noise ratio, reducing one of the benefits of CRM. The remaining benefit of CRM was that it enabled studying much thicker samples (50 μm) in which ice crystal growth was less constrained than in the very thin geometry of the FTIR experiments.

FTIR Spectroscopy

Experimental solutions were prepared gravimetrically with DMSO (99.9% purity, Sigma-Aldrich, St. Louis, MO), bovine serum albumin (MW 66.5 kDa, ≥99% Purity, Sigma-Aldrich, St. Louis, MO), and in ultrapure water (UPW). UPW was prepared by filtering deionized water through a Milli-Q water purification system (Millipore, Billerica, MA) to a final electrical resistance higher than 18.2 MΩ. All DMSO concentrations reported as wt % correspond to the mass of DMSO as a percentage of the total mass of solvent (DMSO and water). Albumin concentrations are given as (mass of albumin)/(volume of DMSO and water).

Each experiment involved placing 100 nL of the experimental solution between two CaF_2 windows. The windows were sealed with vacuum grease to eliminate evaporation and to generate a thin film approximately 1 μm thick. The sealed sample was then transferred to an infrared microscope attached to an FTIR spectrometer (Thermo-Nicolet Continuum equipped with a Mercury Cadmium Telluride detector, Thermo Electron, Waltham, MA) equipped with an FDCS 196 (Linkam Scientific Instruments Ltd., UK) freeze-drying cryostage. The FTIR sampling resolution was 4 cm^{-1}, and 128 IR scans were averaged per spectrum in the 4000–930 cm^{-1} wavenumber range. The IR spectra were analyzed using OMNIC (Thermo-Nicolet) software.

Equilibrium Freezing Experiments

The sample solutions were cooled at 30 °C/min until frozen (formation of ice crystals was observed visually and spectroscopically). The samples were then warmed back up slowly to close to the melting temperature until a few small crystals remained in the solution. The samples were then recooled down to a common final temperature at the cooling rate described in Table 1. The cooling rates were chosen so that ice growth occurred slowly over 20–40 min in order to minimize any concentration gradients in the FCL resulting from diffiusion limitations. The small crystals acted as nucleation points for crystal growth and ensured that equilibrium-freezing experiments were conducted in a repeatable manner. At the final temperature, the most concentrated starting solution was made up primarily of FCL and only a few small ice crystals. The effect of ice growth on the FCL was minimized in this case, which allowed the FCL of this solution to serve as a control and a basis for comparison. See Figure 1.

Bulk Freezing Experiments at −20 °C

Pharmaceutical formulations are sometimes stored at length in freezers at −20 °C.[49–51] Once a sample has reached equilibrium under these conditions, there should be no additional ice

growth. To evaluate the interactions that may occur between ice and albumin over time in the absence of ice growth, similar to what would occur during long-term storage, as well as to clarify any interactions observed during near-equilibrium freezing experiments, solutions of DMSO and albumin were added to ice at −20 °C. The amount of ice was controlled by freezing known quantities of water (0.1–1.5 mL) in 15 mL polypropylene centrifuge tubes in a temperature and humidity-controlled walk-in freezer maintained at −20 °C. A 35 wt % DMSO solution with 30 mg/mL albumin was allowed to thermally equilibrate overnight in the freezer, and then was added in varying quantities (0.25–1.0 mL) to the centrifuge tubes containing ice. The concentration of DMSO was chosen to minimize any ice growth or melting of the ice at −20 °C. After 30 h, the liquid phase from all samples was extracted with a 25 gauge needle and syringe, removed from the freezer, and then stored at 4 °C until use. FTIR was then used to determine the composition of the extracted liquid in order to detect any changes in the mass ratio of albumin/DMSO that resulted from the 30 h of contact with ice. Samples were cooled at 30 °C/min until frozen, then warmed until a few ice crystals remained, and then cooled at 1 °C/min to −35 °C, after which spectra were collected in the resulting FCL.

Confocal Raman Microspectroscopy

Experimental solutions were prepared gravimetrically with DMSO (≥99.7% purity, Sigma-Aldrich, St. Louis, MO), bovine serum albumin (≥98% purity, Sigma-Aldrich, St. Louis, MO), and deionized (DI) water. Each experiment involved placing 1 μL of the experimental solution in a microchannel machined in a 50 μm thick silicone film, which was sandwiched between two quartz windows. The sample was then transferred to a confocal Raman microscope (Nanophoton, Osaka, Japan) equipped with an FDCS 196 (Linkam Scientific Instruments Ltd., UK) freeze-drying cryostage. The microscope had a 100× Nikon air objective (NA 0.90) and a 532 nm AR-ion laser operated at 10 mW for excitation and a CCD detector electrically cooled to −70 °C. Spatial resolution was 350 nm in the x-direction and 800 nm in the vertical direction. Scans were conducted over a 4 μm × 83 μm region of the sample with a spectral resolution of 6 cm^{-1} in the 4662–4670 cm^{-1} wavenumber range at an exposure time of 0.025 s per pixel. The spectra collected from all pixels were averaged for each scan (7980 spectra per scan) and were analyzed using CytoSpec software (Boston, MA) and Matlab (MathWorks, Natick, MA).

In confocal raman microspectroscopy experiments, the sample solutions were frozen and then warmed in the same fashion as the FTIR spectroscopy experiments. The samples were then cooled at 0.5 °C/min to −17.5 °C after nucleation at the freezing temperature following the same procedures described in the FTIR experiments above. The small crystals remaining in the liquid after freeze and thawing back to freezing temperature acted as nucleation points for crystal growth and ensured that equilibrium-freezing experiments were conducted in a repeatable manner.

IR Spectral Analysis

The composition of the FCL at a given temperature was measured by the ratio of the baseline-corrected areas of the albumin amide II band (1525–1570 cm^{-1})[52] and the DMSO ν-HCH band (1367–1485 cm^{-1}),[53,54] $\gamma \equiv A_{\text{Amide II}}/A_{\nu\text{-HCH}}$, as shown in Figure 3A. To demonstrate the accuracy of this approach, at −6 °C, a linear calibration curve was constructed by varying the amount of albumin added to a 12 wt % DMSO solution. For the highest albumin/DMSO ratio (1:2), an 11 wt % DMSO solution was substituted due to the depression of the melting point to just below −6 °C. The solutions were frozen at 30 °C/min and then warmed until a few small ice crystals remained (typically between −5.3 and −5.8 °C), and then recooled at 0.1 °C/min to −6 °C to ensure that the system was in thermodynamic equilibrium, at which point spectra were collected. The variable γ was then

plotted with respect to the known ratio of albumin to DMSO (mg/mg) (Figure 3B). The direct proportionality between γ and the albumin to DMSO ratio of the prepared solutions indicated that the chosen variable γ could be used to quantify protein concentration with high fidelity. Since the baseline of the amide II band is influenced by the δ-OH band of water, having a small amount of ice present was important to reduce variability in the water content. The varying protein amounts would shift the equilibrium freezing curve slightly, but this effect was deemed to be less significant than potential variability in water content due to evaporation during preparation of the sample.

As γ could be directly calculated from the IR spectra, it could then be used to calculate the protein content changes in the FCL *in situ*. Changes in FCL composition in the presence of varying amounts of ice were then calculated as a percentage change from the FCL of the minimum ice solution (MIS) described previously.

Raman Spectral Analysis

The same experimental protocol followed for the equilibrium freezing FTIR experiments was used to evaluate 15–25 wt % DMSO solutions with CRM. A 30 wt % DMSO solution that contained 63 mg/mL of albumin was frozen at 30 °C/min and then warmed until a few small ice crystals remained at −17.5 °C. Solutions of 25, 20, and 15 wt % DMSO were then tested with the same albumin/ DMSO ratio.

The albumin concentration in the FCL was measured by a ratio of the baseline-corrected integrated intensity of the strong amide I band and the weak tyrosine residue of the albumin (1575–1735 cm^{-1})[55] to the baseline-corrected integrated intensity of the band that included strong contributions from the antisymmetric and symmetric S–C stretching bands of the DMSO and a weak contribution from the ν-S–C of the albumin (635–750 cm^{-1}).[54,55] This ratio is denoted as $\zeta \equiv A_{\text{Albumin}}/A_{\text{DMSO+Albumin}}$ (Figure 3C). The amide I band used to quantify albumin also overlaps with the δ-OH band of water, but this band is very weak in the Raman spectra, and its effect was minimized by the constant water content at equilibrium at −17.5 °C.[56] A calibration curve was constructed by varying the amount of albumin added to a 30 wt % DMSO solution and then plotting ζ with respect to the albumin to DMSO ratio (mg/mg) (Figure 3D). A second degree polynomial was required due to the overlap of the albumin and DMSO peaks used.

RESULTS

In order to measure the exact amount of protein missing from the freeze-concentrated liquid phase due to the presence of ice, we have developed a technique to utilize *in situ* IR and Raman spectroscopy. Aqueous solutions containing 20–63 mg/mL of albumin and 6–35 wt % DMSO were studied. Temperatures at which data were collected were chosen to maximize the amount of the FCL phase in the frozen state (to maximize the signal-to-noise ratio of the spectra). Therefore, we conducted experiments with frozen solutions at −4 to −6 °C for 6–12 wt % DMSO solutions, at −8.5 °C for 12–18 wt % DMSO solutions, at −13 °C for 10–20 wt % DMSO solutions, at −17.5 °C for 15–30 wt % DMSO solutions, and at −20 °C to −40 °C for 15–35 wt % solutions.

FTIR Spectroscopy Experiments at −4 and −6 °C

For low DMSO concentration experiments with equilibrium freezing temperatures in the range of −2 and −6 °C, two ratios of albumin/DMSO, 1:3 and 1:2, were used in solutions of 6, 8, 10, 11 (for 1:2), and 12 wt % (for 1:3) DMSO, such that the FCL at equilibrium at −6 °C would contain approximately 40 or 56 mg/mL albumin (for the 1:3 and 1:2 albumin/ DMSO ratio, respectively). At the temperature where data were collected, the albumin/

DMSO ratio decreased slightly with respect to the minimum ice solution (MIS) in two conditions, but these were statistically insignificant ($p = 0.33$ and $p = 0.46$, $n = 15$) (Figure 4A, B). In all other conditions at −4 and −6 °C, there was a net increase in the albumin/ DMSO ratio that was correlated with greater ice content. The most significant increase in the albumin/DMSO ratio was observed in the FCL of the 6 wt % DMSO solutions, where ice comprised approximately 50% of the volume of the partially frozen system. This increase was statistically significant in the 6 wt % DMSO solution at both protein concentrations and temperatures ($p < 0.001$, $n = 15$ for each protein concentration and temperature). Since a net increase in the albumin/DMSO ratio would only be possible if more DMSO than albumin were missing from the FCL, either by accumulation at the ice surface or entrapment in the ice phase, this suggests that the albumin was preferentially excluded from the ice phase during freezing. This effect was greatest in the warmer temperature (−4 °C) with the most amount of ice (6 wt % DMSO). To evaluate the influence of temperature, additional FTIR experiments were run at −8.5, −13, −17.5, and −20 °C.

FTIR Spectroscopy Experiments at −8.5, −13, −17.5, and −20 °C

To evaluate changes in FCL composition at lower temperatures, more concentrated DMSO solutions were necessary to ensure that there would be a sufficient volume of FCL in which to collect spectra. To evaluate partially frozen solutions at −8.5 °C, an 18 wt % DMSO solution with 30 mg/ mL albumin was diluted to 15 wt % DMSO, 12 wt %, and 9 wt %. It was not feasible to collect spectra in the FCL of the 9 wt % DMSO solution due to an insufficient volume of FCL. In the 12 wt % DMSO solution, there was a small but significant decrease (−1.7 ± 1.3%, $p = 0.017$, $n = 15$) in the albumin/ DMSO ratio in the FCL with respect to the FCL of the MIS (18 wt % DSMO). In the 15 wt % DMSO solution, which had only a small amount of ice at this temperature, only an insignificant decrease in the albumin/DMSO ratio of the FCL was observed (−1.1 ± 2.2%, $p = 0.17$, $n = 15$). See Figure 4C. To evaluate partially frozen solutions at −13 °C, a 20 wt % DMSO solution with 25 mg/mL albumin was diluted to 10 wt % DMSO. There was no significant difference in the albumin/ DMSO ratio ($p = 0.43$, $n = 15$) in the FCL of these solutions, although the standard deviation was much higher in the 10 wt % solutions (with more ice), suggesting the presence of heterogeneity in the FCL. Additional experiments at −17.5 °C were conducted with a 30 wt % DMSO solution with 30 mg/ mL albumin that was diluted to 20 wt % DMSO. At this temperature, a small decrease in the albumin/DMSO ratio in the FCL of the 20 wt % DMSO solution with respect to the 30 wt % DMSO solution was observed, but it did not reach statistical significance (−1.5 ± 3.1%, $p = 0.18$, $n = 15$). At −20 °C, a significant decrease was observed in the albumin/DMSO ratio in a diluted 15 wt % DSMO solution with respect to the original 30 wt % DMSO with 25 mg/mL albumin solution (−5.3 ± 2.7%, $p < 0.0001$, $n = 10$).

These results, when considered with those of the 6 wt % DMSO solutions at −4 and −6 °C, suggest that the presence of ice has an effect on the composition of the FCL that changes over the −4 to −20 °C temperature range. At temperatures of −4 and −6 °C, the presence of ice is associated with an increase in the albumin/DMSO ratio in the FCL of partially frozen solutions. At lower temperatures, the observed effect of ice changes and is now associated with a decrease in the albumin/ DMSO ratio. This loss of the albumin from the FCL at lower temperatures is also supported by the results of the CRM experiments (Figure 4D).

Confocal Raman Spectroscopy Experiments at −17.5 °C

Solutions of 15, 20, and 25 wt % DMSO with an albumin/ DMSO ratio of 1:4.9, such that the FCL at −17.5 °C would contain 63 mg/mL albumin, all showed significant decreases in the albumin/DMSO with respect to the MIS (30 wt % DMSO), suggesting a loss of the albumin from the FCL ($p < 0.001$, $n = 15$) (Figure 4D). The magnitude of the decrease was

greater than that observed in FTIR experiments, which may be the result of the different geometry of the experiment. In the very thin geometry of the FTIR experiments, ice crystal growth is constrained to the X–Y plane of the microscope window and the majority of the ice surface area is in contact with the top and bottom windows and not the FCL. The thicker geometry of the CRM experiments means that more of the ice surface area is in contact with the FCL instead of the top and bottom windows, which may be why the ice has a larger effect.

Additional FTIR Spectroscopy Experiments between −20 and −35 °C

To investigate temperatures below −20 °C, a 40 wt % DMSO with 25 mg/mL albumin solution was prepared and successively diluted to 35, 30, 25, and 20 wt % DMSO. The highest concentration solution of 40 wt % DMSO did not freeze consistently, so the next most concentrated solution, the 35 wt % DMSO, was used as a basis for comparison at −30 °C. At this temperature, there was no statistically significant difference between solutions. Due to unexpectedly large variations in equilibrium melt temperatures, as well as a worse signal/noise ratio with the lower protein concentration, a new 35 wt % DMSO solution with 30 mg/mL albumin was prepared and successively diluted. FCL comparison with 35 wt % DMSO solutions was possible at −30 °C, a temperature at which minimal ice content was present in the 35 wt % solution, and with the 30 wt % DMSO solution at −25 and −20 °C. The most diluted solution, the 20 wt % DMSO, showed slight increases in protein concentration with respect to the more concentrated 30 and 35 wt % DMSO solutions, whereas the 25 wt % DMSO showed slight decreases. The significant standard deviations and the absence of a consistent trend with respect to concentration make it difficult to draw any conclusions from these results alone.

Bulk Freezing Experiments at −20 °C

FTIR analysis revealed two interesting findings. There was a net increase in protein content with respect to DMSO in the extracted FCL with respect to the bulk solution. This increase was most significant in the sample with the smallest amount of ice ($p < 0.0001$, $n = 20$), such that, with respect to the FCL of this sample, there was a decreasing trend in protein content with additional ice. In an attempt to elucidate this relationship, the protein content was normalized by the composition of the extracted FCL with the smallest amount of ice, and then plotted with respect to the ratio of the minimum surface area of the ice to the volume of liquid added to the ice (Figure 5). The actual surface area was unknown, because just prior to extracting the FCL at 30 h, it was visually observed that the ice was surrounded by solution and therefore no longer had the same exposed surface area as at the start. The minimum surface area was calculated by assuming the most thermodynamically favored geometry of a sphere, using the volume of the frozen water that was initially added to the centrifuge tubes.

DISCUSSION

The hypothesis that motivated this investigation was that albumin was not simply distributed homogeneously in the FCL and that it either accumulated or aggregated at the ice interface, or became entrapped within the ice during slow, near-equilibrium freezing. The anticipated experimental results would show either a decrease in protein content in the FCL that would support the hypothesis or no change in the FCL. The results that were obtained, however, suggest that more complicated interactions—that also involve the solvent DMSO—are occurring during freezing. In the experiments with low concentration DMSO at −4 and −6 °C, the presence of ice was correlated with a net increase in protein content with respect to DMSO in the FCL. As the ice phase grew and the FCL phase became more concentrated, the protein concentration increased more than the DMSO concentration. This same

phenomenon was also observed in the bulk freezing experiments, where larger volumes of solution were added to ice and then extracted as an FCL after being in contact with ice for 30 h at −20 °C.

This would suggest that the protein became more concentrated than expected in the FCL as a result of a loss of DMSO from the FCL phase, either during ice growth or simply in the presence of ice. In addition, the results from the equilibrium freezing experiments using FTIR and CRM at −8.5, −17.5, and −20 °C, as well as the bulk freezing experiments at −20 °C, supported the original hypothesis of a loss of protein to the ice interface or ice phase. This evidence, nonetheless, appears to be complicated by what was occurring with the DMSO, which is perhaps why the results between −20 and −40 °C did not yield a consistent trend.

A possible explanation for these observations is that albumin accumulates at the ice interface. This phenomenon is either negligible at warmer temperatures approaching 0 °C or disguised by the more significant loss of DMSO from the FCL, resulting in the observed net increase in protein content in the FCL at −4 and −6 °C in the presence of ice. The decrease in the albumin/DMSO ratio as the temperature approached −20 °C suggests that the loss of albumin from the FCL becomes more significant with decreasing temperature.

The loss of DMSO from the FCL during freezing experiments at −4 and −6 °C, as well as from the FCL of bulk solutions held at −20 °C in the presence of ice, could have multiple explanations. The first possible explanation would be the formation of DMSO-rich clusters or chains of molecules in solutions of DMSO+water+albumin. DMSO has been shown to form DMSO–water complexes with 1:2[57,58] and 1:3 ratios[43] in binary solutions, but to our knowledge, it is not established what effect the presence of protein molecules has on these arrangements at subzero temperatures. If larger clusters rich in DMSO molecules were formed, these could have an affinity for ice surfaces, such that they could congregate preferentially at the interface. Or they could simply have reduced mobility, and thereby could get caught at the interface of growing ice crystals. In both cases, there would be a heterogeneous distribution of DMSO in the FCL.

The results of the bulk freezing experiments support the conclusion that the heterogeneous distribution of DMSO-rich regions during slow freezing was the result of an affinity of DMSO for the ice surface and not reduced mobility. While it is possible that reduced mobility of the DMSO caused the increased protein content relative to DMSO observed in the FCL extracted from solutions held at −20 °C for 30 h in the absence of significant ice growth, this seems unlikely, unless the clusters of DMSO-rich regions were large enough to rival the size of the albumin macromolecules.

Another possible explanation for the increase in albumin observed in the FCL of solutions held at −20 °C is that some of the DMSO crystallized out of the liquid phase. This would not need to have been triggered by the presence of ice, since it could have also occurred and gone undetected in the bulk solution held at −20 °C. Samples for FTIR analysis were only taken from this solution once it had been warmed back to 4 °C and stirred again. Subsequent X-ray diffraction experiments at −6 and −20 °C (data not shown here), however, showed only the presence of ice and did not confirm the presence of any non-ice crystals.

The most compelling evidence of a loss of albumin from the freeze-concentrated liquid comes from the experiments conducted over 30 h in a large-scale freezer, held at the common industry standard temperature of −20 °C. These experiments were designed to isolate the interactions between the ice interface and albumin in the absence of ice growth. Thus, the results, which showed a correlation between increased ice surface area and loss of

albumin from the FCL over the relatively short time scale of 30 h, have implications for the long-term storage of albumin.

SUMMARY

In this study, we have developed a novel technique using FTIR and confocal Raman microspectroscopy to measure interactions between ice and proteins *in situ* during freezing. We have shown that during freezing, as a function of temperature, dimethyl sulfoxide (DMSO) and bovine serum albumin are preferentially excluded from the ice phase, resulting in significant deviations from the theoretical composition of the freeze-concentrated liquid phase. As a result, as much as 20% of the albumin in solution may leave the freeze-concentrated liquid following freezing, and may be adsorbed onto the ice interface or entrapped in the ice phase.

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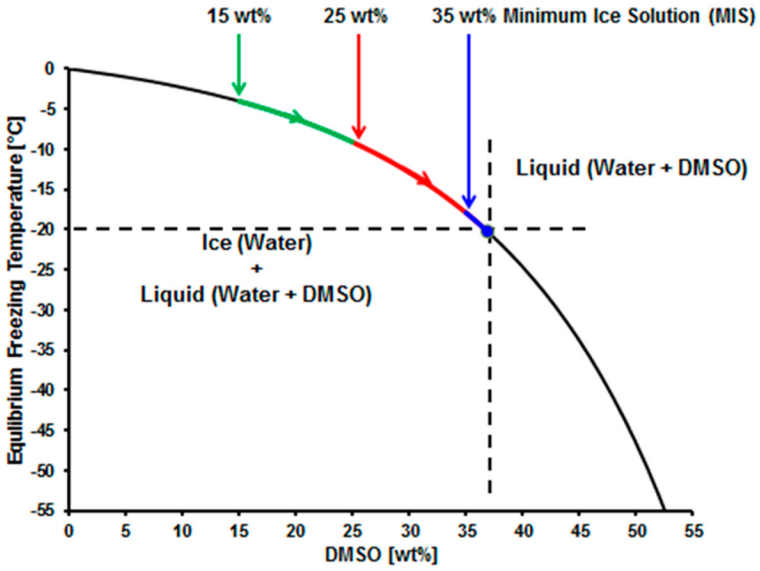

Figure 1.
Binary DMSO and water phase diagram, adapted from the polynomial curve fit created by Kleinhans and Mazur to fit Rasmussen's phase diagram.

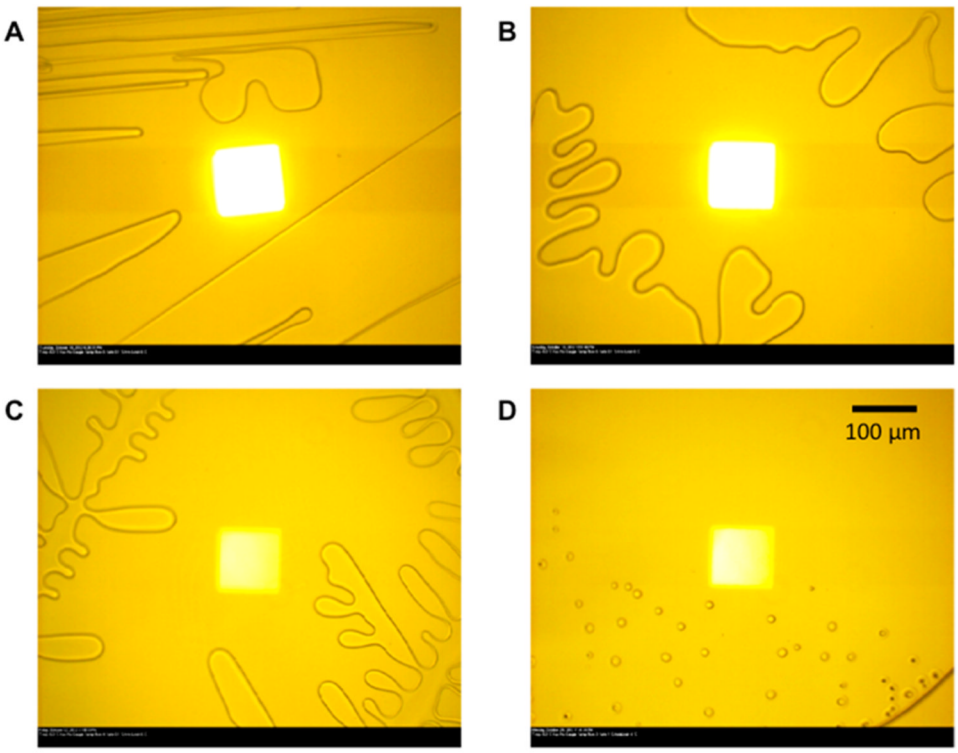

Figure 2.
Partially frozen solutions at −6 °C: (A) 6 wt % DMSO, 1:3 albumin/DMSO; (B) 8 wt % DMSO, 1:3 albumin/DMSO; (C) 10 wt % DMSO, 1:3 albumin/DMSO; (D) 12 wt % DMSO, 1:6 albumin/ DMSO.

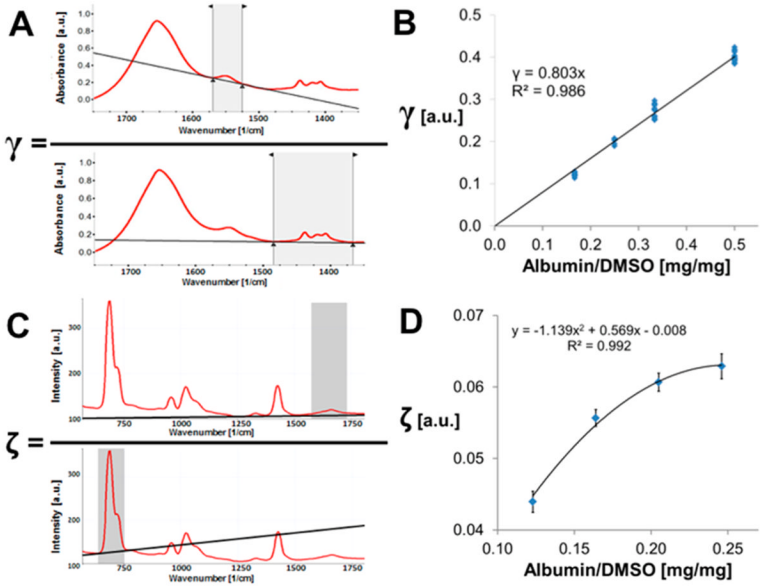

Figure 3.

FTIR and Raman albumin/DMSO ratio calibration: (A, B) Ratio of baseline-corrected areas for y and linear calibration curve. (C, D) Ratio of baseline-corrected areas for ζ and quadratic calibration curve.

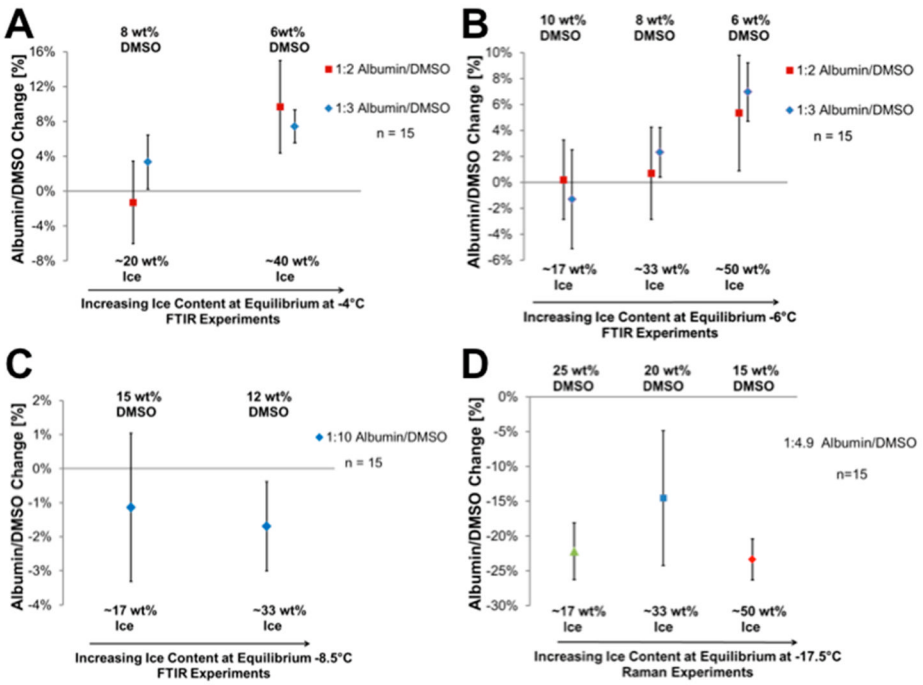

Figure 4.

(A–C) FTIR results: Change in albumin/DMSO ratio at −4, −6, and −8.5 °C. (D) Raman results: Change in albumin/DMSO at −17.5 °C.

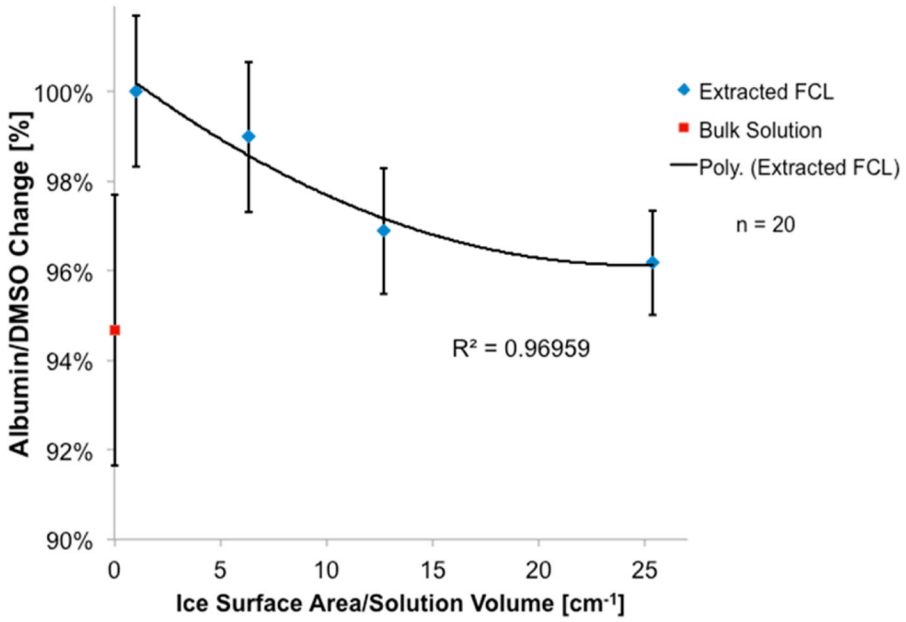

Figure 5.

Bulk freezing experiments: the albumin content in the FCL extracted from the samples after 30 h at −20 °C is plotted versus a measure of the effective surface area that could interact with the FCL. The γ values are normalized by the γ of the FCL extracted from the solution with the least amount of added ice (shown as 100%). The starting bulk solution is also included in the plot.

Table 1

Cooling Parameters of Equilibrium Freezing Experiments Using FTIR

final temperature (°C)	DMSO concentration (wt %)	cooling rate (°C/min)	temperature of spectra collection (°C)
−6.0	6, 8, 10, 12	0.1	−4.0, −6.0
−8.5	12,18	0.2	−8.5
−13.0	10, 20	0.2	−13.0
−17.5	20, 30	0.5	−17.5
−20.0	15, 30	0.5	−20.0
−40.0	20, 25, 30, 35	0.5 °C/min to −20 °C; 1.0 °C/min to −40 °C	−20.0, −25.0, −30.0, −35.0, −40.0