Protein structure and interactions in the solid state studied by small-angle neutron scattering

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Received 17th February 2012, Accepted 5th April 2012
DOI: 10.1039/c2fd20027a

Small-angle neutron scattering (SANS) is uniquely qualified to study the structure of proteins in liquid and solid phases that are relevant to food science and biotechnological applications. We have used SANS to study a model protein, lysozyme, in both the liquid and water ice phases to determine its gross-structure, interparticle interactions and other properties. These properties have been examined under a variety of solution conditions before, during, and after freezing. Results for lysozyme at concentrations of 50 mg mL^{-1} and 100 mg mL^{-1}, with NaCl concentrations of 0.4 M and 0 M, respectively, both in the liquid and frozen states, are presented and implications for food science are discussed.

Introduction

The role of freezing in food preservation is to prevent the growth of microorganisms and to slow chemical reactions to preserve the quality, nutrient content, texture, flavor and color of foods. The food industry must change continuously in response to consumer demands for a greater variety of high quality, convenient products at economic prices. As such, traditional methods of food preservation such as freezing need continual improvements to keep abreast of advances in technology. Thus, the freezing process and its affects on water, proteins, lipids, carbohydrates, vitamins and minerals in food must be well understood. The state diagram of foods[1] is complex because foods are complex. However, there are clear lines representing the freezing curve and glass transition that are similar to the state diagrams of simpler systems such as proteins in aqueous solutions. Thus, an understanding of the freezing process in more complex systems such as foods can be obtained by studying these simpler model systems.

During the freezing of a typical protein solution, only a fraction of the water molecules form the crystalline ice phase, whereas the remaining water molecules and other solutes present remain in the amorphous state, forming a freeze-concentrated solution, with a water concentration of around 30 percent by weight.[2] This water, confined in small areas, cannot overcome the large activation barrier that would allow it to diffuse into the ice crystal lattice. The higher the initial concentration of solute, the greater the mass fraction of water that remains unfrozen.[3] This freeze-concentration process is not new to the food science industry and, in fact, is a method used to concentrate liquid foods by allowing ice to form in the solution and then extracting the water, as ice, from the freeze-concentrated liquid phase.[4]

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Many proteins are known to suffer inactivation upon freezing, which can occur due to dissociation, aggregation or other chemical mechanisms.[3] Proteins can also become denatured, or unfolded, as a result of freezing. The extent of denaturation of proteins upon freezing depends on several factors including the initial pH, the protein concentration, the temperature of the frozen part of the solution and the presence of other substances, such as salt or sugar, in the solution.[3] NaCl can inhibit protein denaturation down to −30 °C, at which point the salt likely precipitates and, eventually, the freeze-concentrated liquid reaches the same composition as it would have in the absence of salt.[3] While structural changes in cold-denatured proteins have been studied using phosphorescence emission[5] and NMR,[6] structural studies of proteins in water ice are limited.

In this work, small-angle neutron scattering (SANS) was used to directly probe the structure and interactions of the model protein, lysozyme, in aqueous solution as a function of temperature and salt (NaCl) content. SANS probes structure and interactions on length scales of 10 Å to more than 1000 Å, making it a well-suited technique for the study of proteins in aqueous solution. Unlike X-rays, neutrons are particularly sensitive to hydrogen and the ability to substitute deuterium for hydrogen in the protein or aqueous solution makes it possible to determine the origin of features in the scattering curves. The SANS results, combined with those obtained from other techniques, were used to construct a picture of the frozen state of the lysozyme solution, including the location and aggregation state of the protein.

Materials and methods

Proteins and solutions

Hen egg lysozyme was purchased from Sigma† in powder form and used without further purification. Lysozyme solutions for SANS experiments were prepared in 99.9% D_2O (Cambridge Isotope Labs, Inc.) at protein concentrations of 50 mg mL^{-1} and 100 mg mL^{-1}. The 100 mg mL^{-1} solution was prepared with 0 mol L^{-1} (M) NaCl, whereas the 50 mg mL^{-1} solution was prepared with 0.4 M NaCl. The measured pD was near 7 for all samples.

Small-angle neutron scattering

SANS measurements were performed on the 30-meter SANS instruments[7] at the NIST Center for Neutron Research (NCNR) in Gaithersburg, MD. The neutron wavelength, λ, was 6 Å, with a wavelength spread, $\Delta\lambda/\lambda$, of 0.15. Scattered neutrons were detected with a 64 cm × 64 cm two-dimensional position-sensitive detector with 128 × 128 pixels at a resolution of 0.5 cm/pixel. The data were reduced using the IGOR program with SANS macro routines developed at the NCNR.[8] Raw counts were normalized to a common monitor count and corrected for empty cell counts, ambient room background counts and non-uniform detector response.

Data from the samples in the liquid and frozen states were placed on an absolute scale by normalizing the scattered intensity to the incident beam flux. Finally, the data were radially averaged to produce scattered intensity, $I(q)$, *versus* q curves, where $q = 4\pi\sin(\theta)/\lambda$ and 2θ is the scattering angle. A sample-to-detector distance of 1.3 m was used for measurements of lysozyme in D_2O to cover the range 0.03 $Å^{-1} \leq q \leq 0.4$ $Å^{-1}$. The scattered intensities from the samples in the liquid state were then further corrected for buffer scattering and incoherent scattering from hydrogen in the samples. The buffer scattering could not be directly subtracted from the sample scattering in the frozen state due to the presence of additional

† Certain commercial equipment, instruments, materials, suppliers, or software are identified in this paper to foster understanding. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

scattering at low q. In this case, the scattering from the samples in the frozen state were approximately corrected for background scattering by subtracting the scattering from the corresponding buffers at higher q values, where the buffer scattering was flat, and then subtracting a constant of the same magnitude from the scattering at lower q values.

Lysozyme solutions were loaded into demountable 2 mm path length titanium cells with titanium windows. Samples were measured at temperatures between 20 °C and −80 °C. The 0 M NaCl sample was cooled by placing it in a closed cycle refrigerator that was at 20 °C and slowly lowering the temperature, in increments, to 10 °C, 5 °C, 0 °C, −5 °C, −10 °C, −20 °C, −40 °C and −80 °C. The sample was allowed to remain at each temperature for 30 min before making a measurement and then moving to the next temperature. The actual measurement time at temperature was 5 min. The 0.4 M NaCl sample was cooled in the same way, except it was measured at −25 °C and −30 °C in addition to the temperatures above, since its freezing temperature is near −25 °C.

Data analysis

Data from samples in both the liquid and frozen states were fit using an empirical functional form for SANS data characterized by a broad scattering peak. This model function is part of the NCNR IGOR SANS data analysis package and curve fitting was accomplished using the non-linear curve fitting routine within IGOR. The scattered intensity, $I(q)$ is calculated using the relation:

$$I(q) = \frac{A}{q^n} + \frac{C}{1 + (|q - q_o|\xi)^m} + B \quad (1)$$

where A, B, and C are constants, n is the Porod exponent, m is the Lorentzian exponent and ξ is the Lorentzian screening length. The Porod exponent results mainly from the scattering at lower q values and is applicable when there is scattering in this region froFm particle aggregates. The aggregate can be a dimer, trimer or higher oligomer of the particle. Porod scattering can also result due to scattering from interfaces between co-existing phases. The Lorentzian parameters are derived mainly from the higher q region and are applicable when a broad scattering peak due to interactions between particles is present in the data. The average center-to-center distance between neighboring particles, d, also known as the d-spacing, is related to the peak position, q_o, as $d = 2\pi/q_o$. While many factors such as solution conditions can play a role in determining the actual distance between neighboring particles in solution, this approximation allows the change in particle concentration upon crowding to be followed in a simple manner. The constant, B, is a background term.

Results and discussion

SANS from 0 M NaCl lysozyme upon freezing

The SANS data from lysozyme at 100 mg mL^{-1} in D_2O with 0 M NaCl are plotted on a log(I) *vs.* log(q) scale in Fig. 1. At 20 °C, clear signs of interparticle interference, *i.e.*, interaction between the lysozyme particles, are seen in that the data show a downturn at the lowest q values. This downturn in the data at low q allows for the observation of a broad peak in the data that is centered at approximately $q =$ 0.1 $Å^{-1}$. The presence of such a peak, and the absence of significant scattering at lower q values, suggests that the solution is fairly monodisperse and consists mainly of interacting lysozyme monomers. Such interactions can be due to the fact that proteins at higher concentration are more closely packed such that the solution has a higher degree of order. Thus, a d-spacing exists between neighboring proteins as defined above. Long-ranged, repulsive electrostatic interactions between proteins, especially in this case where there is no salt in the solution to screen them, can cause

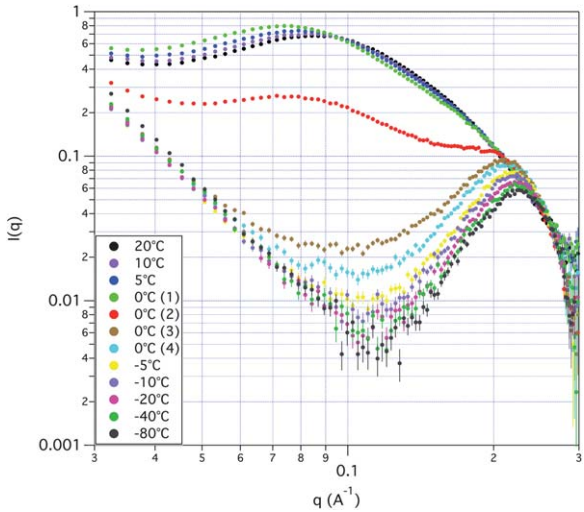

Fig. 1 SANS from lysozyme in 0 M NaCl D_2O solution as a function of temperature. Error bars represent plus and minus the combined standard uncertainty of the data collection.

a similar effect. As the temperature is slowly reduced from 20 °C to 0 °C, the data show a clear shift of the broad peak in the data to lower q values. Thus, the d-spacing becomes slightly greater as the solution cools to 0 °C. This is perhaps due to the expansion of water as it freezes, thus allowing for a greater distance between protein molecules at a given concentration. Alternatively, it could be due to temporal clustering of the protein molecules as the temperature is reduced.[9]

As the solution is equilibrated at 0 °C, the data show a reduction of the scattered intensity as a whole, as well as a shift of the broad scattering peak back to a higher q value and the appearance of an additional peak near $q = 0.2$ Å^{-1}, indicating that a subset of proteins are now much closer together in the solution. At this point, the solution is in a mixed state, with the scattering showing evidence of lysozyme with the same d-spacing as in the liquid state, as well as with a significantly smaller d-spacing. While it is assumed that this peak is correlated with the onset of freezing, this cannot be confirmed by the SANS data alone.

As the sample continues to freeze at 0 °C, the scattering shows only a well-defined peak near $q = 0.2$ Å^{-1}, along with sharply increasing scattering at lower q values. The peak near $q = 0.2$ Å^{-1} is a result of interacting lysozyme monomers that are closer together than in the liquid state, due to the freeze-concentration effect as ice forms in the solution. As the sample continues to freeze down to −80 °C, the peak continues to sharpen and shift to higher q values.

The observed peaks in the scattering occur due to the crowding of protein as the solution freezes. As ice forms, the remaining unfrozen water becomes freeze-concentrated in a separate phase. The structure of a solution of phosphate buffer saline (PBS) at a temperature of −26 °C has been described, using confocal Raman microscopy, as consisting of ice crystals surrounded by narrow channels and more rounded domains that contain unfrozen water.[10] This phase separation induces segregation of the protein, forming a region consisting of freeze-concentrated protein. While protein was observed in the ice phase of PBS containing both lysozyme and trehalose, the concentration of both protein and trehalose in the unfrozen water phase was about two orders of magnitude greater. Similarly, NMR experiments[11] have shown that a solute-rich liquid phase persists in a solution of bovine serum albumin, potassium fluoride and water down to temperatures as low as −100 °C. Fig. 1 shows that the formation of the freeze-concentrated protein regions can clearly be detected in the SANS data as the solution is frozen.

The increased scattering at lower q values arises from larger structures in the system. Such scattering was observed even in the absence of protein,[12] indicating that it is arising, at least partially, from the ice structure itself. A contrast variation series of experiments performed on −40 °C frozen water solutions of several mixtures of D_2O : H_2O (by volume) in the absence of protein and salt showed an absence of scattering at a solution composition of 8% D_2O : 92% H_2O.[12] Under these conditions, the neutron scattering length density matches that of air. Thus, the scattering at low q values is due to cracks in the ice that create a contrast in neutron scattering length density between air and ice. The cracks are large features, explaining why the scattering occurs mainly at lower q values. However, a second contrast variation series performed on the same frozen water solutions with 100 mg mL^{-1} lysozyme and 0 M NaCl showed that the low q scattering was still present at a solution composition of 8% D_2O : 92% H_2O.[12] This means that there also must be scattering from large-scale protein structures in the frozen samples, in addition to the scattering from the cracks in the ice itself. It is not possible to determine from the SANS data alone whether these protein aggregates are in the ice or unfrozen water phase. Perhaps the protein is aggregating at the boundaries of the water and ice phases in addition to being forced into tight clusters due to the small space available in the water phase. If there is some protein in the ice phase, it could propagate to the edges of the cracks formed during the freezing process, where it could form large aggregates.

SANS from 0.4 M NaCl lysozyme upon freezing

The SANS data from lysozyme at 50 mg mL^{-1} in D_2O with 0.4 M NaCl are plotted on a log(I) *vs.* log(q) scale in Fig. 2. Unlike the 0 M NaCl data at 20 °C, there is no indication of a peak due to interparticle interference. While the concentration of this sample is one-half of the 0 M NaCl sample, a peak in the scattering should be easily visible if the solution consists of interacting lysozyme monomers.[12] Rather, the 0.4 M NaCl data show an upward slope at lower q values, which is usually an indication of the presence of higher order aggregates in the sample. Aggregation could be due to the fact that the salt is screening the long-ranged repulsive electrostatic interaction between proteins, allowing them to come closer together and form larger aggregates. The presence of aggregates in the solution means that the solution is no longer

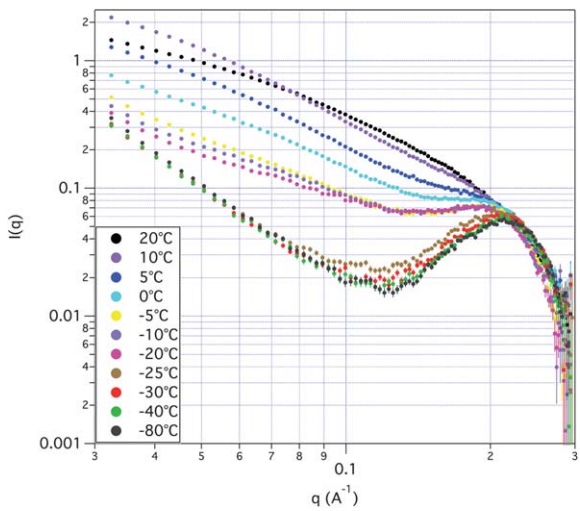

Fig. 2 SANS from lysozyme in 0.4 M NaCl D_2O solution as a function of temperature. Error bars represent plus and minus the combined standard uncertainty of the data collection.

monodisperse. This would also explain the absence of an interaction peak in the data, as there is no longer a well-defined average *d*-spacing between particles. As the temperature is slowly reduced from 20 °C to 5 °C, the data show a decrease in the scattering at lower q values, indicating that the population of large aggregates is decreasing, and the appearance of a peak near $q = 0.2$ Å^{-1}, indicating the existence of close-packed protein at temperatures above the freezing point of the water in the solution. The temperature at which this liquid–liquid phase separation occurs is consistent with that found in previous studies of lysozyme in 0.4 M NaCl solution.[13] However, it is uncertain what effect the existence of protein aggregates in the liquid state has on the packing of the proteins as the solution cools. The fact that a peak exists implies that there is a population of proteins in the solution that is more monodisperse than before. This correlates with the decrease in scattering at lower q values, suggesting that some of the larger aggregates originally in the solution are breaking up into smaller particles that are becoming more concentrated as the solution cools.

The low q scattering continues to decrease as the temperature is reduced to −20 °C. The shape of the scattering curve then changes dramatically below −20 °C, as the salt begins to crystallize out of solution (at −21 °C).[3] As the sample continues to freeze, by lowering the temperature down to −80 °C, the peak near $q = 0.2$ Å^{-1} continues to sharpen and shift to higher q values, indicating that the distance between proteins in the freeze-concentrated phase continues to decrease with decreasing temperature.

Modeling of protein–protein interactions

The SANS data for the 0 M NaCl and 0.4 M NaCl lysozyme solutions cooled from 20 °C to −80 °C were fit with the broad peak functional form defined in eqn (1). For the 0 M NaCl samples in the liquid state, the Porod exponent was fixed at 0.0001, since there is no scattering from large scale structures in the system. The average *d*-spacing between the lysozyme particles cannot be determined from the fit unless the scattering curve exhibits an interaction peak. Thus, q_o was fixed at 0 when no interaction peak was present, as was the case for the 0.4 M NaCl solution at temperatures above 5 °C. Fig. 3 shows an example of the best-fit broad peak curve along with the data for both solutions in the liquid and frozen states.

The resulting best-fit parameters for both the 0 M NaCl and 0.4 M NaCl lysozyme solutions at all temperatures are listed in Table 1. It can be seen from this information that the *d*-spacing decreases with decreasing temperature, as expected since the proteins are being forced closer together as the temperature is decreased. This is further illustrated in Fig. 4, which shows a plot of the *d*-spacing as a function of temperature for both solutions. There is an increase in *d*-spacing of the 0 M NaCl sample as it approaches the freezing point and a sharp decrease in *d*-spacing as the sample freezes at 0 °C. The *d*-spacing continues to decrease with decreasing temperature all the way down to −80 °C. A population of close-packed particles is not apparent in the 0.4 M NaCl solution until the temperature is lowered to 5 °C. The *d*-spacing has a large error above −25 °C because the interaction peak in the data is not sharp. It becomes sharper below the freezing point of the salt solution (−21 °C). Also, the *d*-spacing at the lowest temperature is slightly larger for the 0.4 M NaCl sample as compared to that for the 0 M NaCl sample.

The final concentration of protein in the freeze-concentrated phase is independent of the original protein concentration in the solution.[3,12] Yet, the spacing between lysozyme particles is slightly larger in the frozen 0.4 M NaCl solution all the way down to −80 °C, well below the freezing point of the salt solution. Thus, the packing of proteins in the freeze-concentrated phase is different in the two cases, perhaps due to a difference in protein shape. Either the shape of the monomer is different or perhaps there are both monomers and dimers or other small oligomers present in this phase, which may certainly be the case since there were aggregates in the system before freezing.

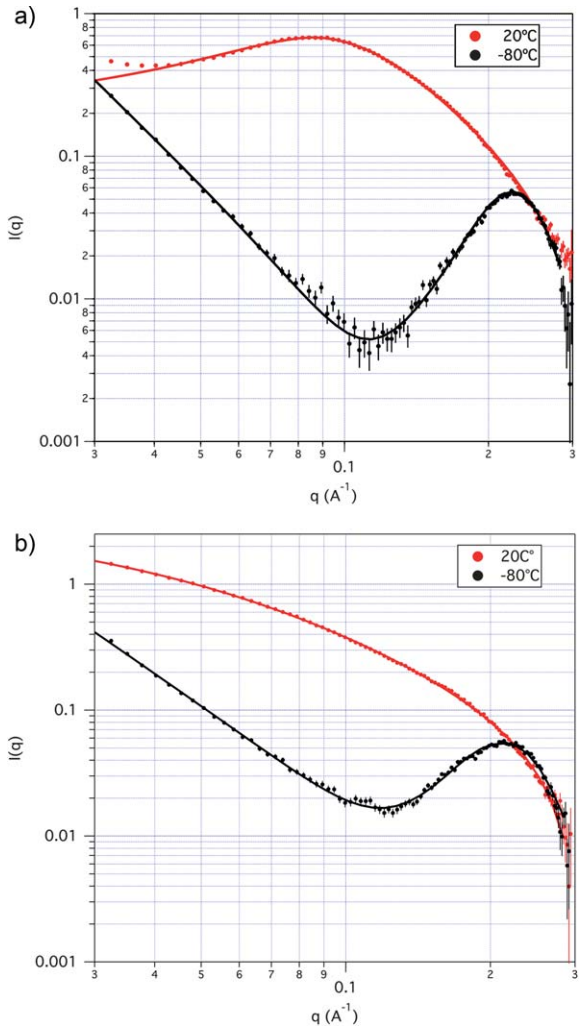

Fig. 3 SANS data from (a) 100 mg mL^{-1} lysozyme in 0 M NaCl D_2O solution and (b) 50 mg mL^{-1} lysozyme in 0.4 M NaCl D_2O solution in the liquid and frozen states. Solid lines in (a) and (b) represent best-fit curves to eqn (1). Error bars represent plus and minus the combined standard uncertainty of the data collection.

It can also be seen from Table 1 that the Porod exponent behaves differently upon freezing of the 0 M NaCl and 0.4 M NaCl solutions. This is further illustrated in Fig. 5, which shows a plot of the Porod exponent as a function of temperature for the two solutions. In this case, the Porod exponent arises due to scattering from a population of large particles in the solution and its value gives an idea of the morphology of these aggregates.[14,15] Lysozyme in 0 M NaCl solution does not significantly aggregate in the liquid state, as evidenced by the lack of significant scattering at low q values. However, lysozyme in 0.4 M NaCl solution in the liquid state has a Porod exponent around 1, which can be indicative of rod-like particles with lengths much longer than their widths. Given the lack of an interaction peak in these data, it is likely that the rod-like particles are polydisperse in length and/or width. However,

Table 1 Broad peak fitting parameters[a]

Concentration (mg mL^{-1})	NaCl (M)	T/°C	Porod Exponent	q_o (Å^{-1})	d-spacing (Å)	χ^2
100	0	20.00 ± 0.05	0.0001	0.0859 ± 0.0001	73.14 ± 0.08	2.3
100	0	10.00 ± 0.05	0.0001	0.0830 ± 0.0002	75.7 ± 0.2	1.8
100	0	5.00 ± 0.05	0.0001	0.0793 ± 0.0001	79.2 ± 0.1	1.7
100	0	0.00 ± 0.05	0.0001	0.0738 ± 0.0001	85.1 ± 0.1	2.0
100	0	0.00 ± 0.05	0.0001	0.0736 ± 0.0002	85.4 ± 0.3	1.0
100	0	0.00 ± 0.05[b]	3	0.20 ± 0.03	31 ± 4	0.8
100	0	0.00 ± 0.05	3.13 ± 0.06	0.2062 ± 0.0002	30.47 ± 0.03	2.2
100	0	0.00 ± 0.05	3.27 ± 0.06	0.2113 ± 0.0002	29.74 ± 0.03	1.4
100	0	−5.00 ± 0.05	3.22 ± 0.06	0.2161 ± 0.0002	29.08 ± 0.03	1.3
100	0	−10.00 ± 0.05	3.11 ± 0.06	0.2181 ± 0.0002	28.81 ± 0.03	1.4
100	0	−20.00 ± 0.05	3.13 ± 0.05	0.2209 ± 0.0002	28.44 ± 0.02	1.1
100	0	−40.00 ± 0.05	3.05 ± 0.05	0.2237 ± 0.0003	28.09 ± 0.04	1.4
100	0	−80.00 ± 0.05	3.23 ± 0.05	0.2245 ± 0.0003	27.99 ± 0.04	1.5
50	0.4	20.00 ± 0.05	1.1 ± 0.2	0	—	1.3
50	0.4	10.00 ± 0.05	1.38 ± 0.02	0	—	2.5
50	0.4	5.00 ± 0.05	1.54 ± 0.01	0.2 ± 0.05	31 ± 6	3.8
50	0.4	0.00 ± 0.05	1.35 ± 0.01	0.2 ± 0.05	31 ± 6	2.0
50	0.4	−5.00 ± 0.05	1.27 ± 0.01	0.2 ± 0.05	31 ± 6	1.5
50	0.4	−10.00 ± 0.05	1.06 ± 0.01	0.2 ± 0.05	31 ± 6	1.9
50	0.4	−20.00 ± 0.05	0.83 ± 0.02	0.2 ± 0.05	31 ± 6	1.6
50	0.4	−20.00 ± 0.05[b]	2	—	—	—
50	0.4	−25.00 ± 0.05	2.88 ± 0.03	0.2042 ± 0.0003	30.77 ± 0.05	1.4
50	0.4	−30.00 ± 0.05	2.81 ± 0.03	0.2086 ± 0.0003	30.12 ± 0.04	1.3
50	0.4	−40.00 ± 0.05	2.43 ± 0.06	0.2109 ± 0.0004	29.79 ± 0.05	1.2
50	0.4	−80.00 ± 0.05	2.59 ± 0.06	0.2127 ± 0.0004	29.54 ± 0.06	1.1

[a] Errors represent one standard deviation. [b] Indicates second population.

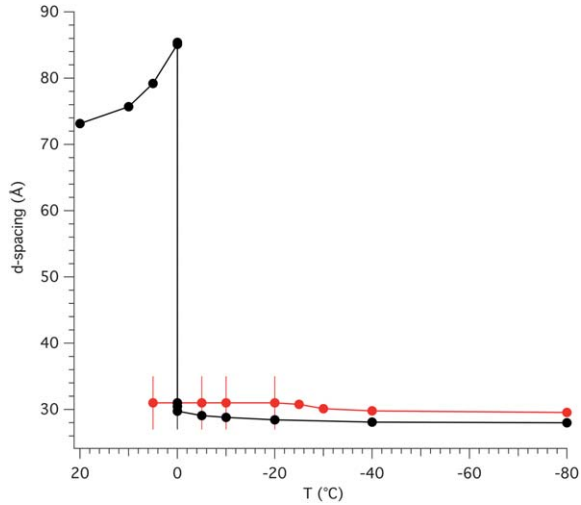

Fig. 4 Average *d*-spacing between lysozyme molecules as a function of temperature determined from fitting the data to eqn (1) for the 0 M NaCl (black) and the 0.4 M NaCl (red) samples. Error bars represent one standard deviation. The lines between points are included for clarity.

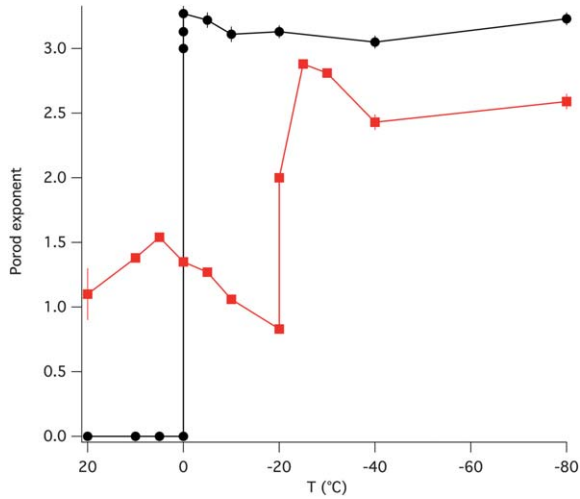

Fig. 5 Porod exponent for lysozyme as a function of temperature determined from fitting the data to eqn (1) for the 0 M NaCl (black) and the 0.4 M NaCl (red) samples. Error bars represent one standard deviation. The lines between points are included for clarity.

the fitted Porod region is based on the model assumed in eqn (1). The Porod exponent determined assuming a different fitting function could be different. Thus, a physical interpretation beyond the fact that there is scattering from large structures may not be warranted.

Upon freezing, the data from both the 0 M NaCl and 0.4 M NaCl show signs of larger aggregates in the system, in addition to the close-packed population of small particles. The Porod exponent for the 0 M solution is near 3 upon freezing. For the 0.4 M solution, the Porod exponent increases to a value between 2 and 3 upon freezing. However, as stated previously, contrast variation experiments have shown

that the low q scattering in the frozen state also arises in part due to the formation of cracks in the ice structure. The differences observed in the Porod exponent are real and indicate the different morphology of the scatterers upon freezing. But, experiments performed in 8% D_2O solution, where the scattering from the cracks in the ice is negligible, would be necessary to isolate the scattering from the protein aggregates in the system.

Slow *versus* fast cooling

For the purposes of this work, slow cooling is defined as lowering the temperature in increments, from 20 °C down to −80 °C as described above, allowing the sample to equilibrate at each temperature increment for 30 min. On the other hand, fast cooling is defined as changing the temperature, in one increment, from 20 °C down to the desired frozen-state temperature (−80 °C in this case), and allowing the sample to cool directly to that temperature without stopping at any temperatures in between. Plunging the sample into liquid nitrogen or placing it directly into a freezer from room temperature would also be defined as fast cooling procedures for the purposes of this work. When the slow cooling procedure is followed, the two-dimensional SANS scattering pattern (prior to converting to $I(q)$ *vs.* q) is symmetric. However, when a fast cooling procedure is followed, the scattering pattern contains asymmetric "flares" that are indicative of an ordered structure at very long length scales relative to SANS, *i.e.*, microns or larger.[12] Such structures may arise due to strain on the sample when subjected to a large temperature change.[16]

Regardless of the cause of the asymmetric features, their main effect on the SANS data is to increase the scattering at low q values significantly. This also results in an apparent broadening of the interaction peak near $q = 0.2$ Å^{-1}. However, the location of the interaction peak, indicating the distance between the freeze-concentrated lysozyme particles, is not affected. This is illustrated in Fig. 6, which shows a log(I) *vs.* log(q) plot of the data from two 100 mg mL^{-1} lysozyme in 0 M NaCl D_2O solutions that were cooled to −80 °C using either the slow or fast cooling methods. Following the slow cooling method results in cleaner scattering curves and sharper interaction peaks in the frozen state, making it easier to fit the data to theoretical functions.

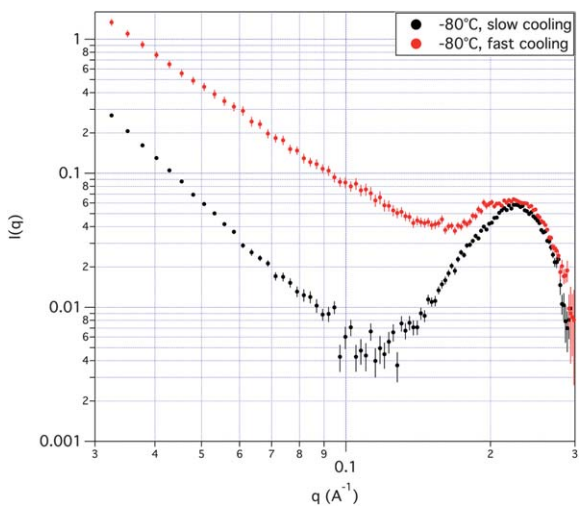

Fig. 6 SANS data from 100 mg mL^{-1} lysozyme in 0 M NaCl D_2O solution cooled to −80 °C from 20 °C by both the slow and fast cooling methods as described in the text. Error bars represent plus and minus the combined standard uncertainty of the data collection.

Multiple freezing and thawing cycles

In order to assess the effect of multiple freezing and thawing cycles on the lysozyme solutions, both solutions were cooled to −80 °C from 20 °C multiple times, using both the fast and slow cooling methods as described above. Fig. 7a shows log(*I*) *vs.* log(*q*) plots of the data from the 100 mg mL^{-1} lysozyme in 0 M NaCl D_2O solution that was cooled to −80 °C, warmed to 20 °C and then cooled again to −80 °C using the slow cooling method. It can be seen that the scattering curve is reproducible at all *q* values. This was found to be the case after several additional cooling cycles. Fig. 7b shows log(*I*) *vs.* log(*q*) plots of the data from the same system at 20 °C after several freeze–thaw cycles to final temperatures ranging from −20 °C to −80 °C using both the slow and fast cooling methods. Again, the data are essentially reproducible at all *q* values.

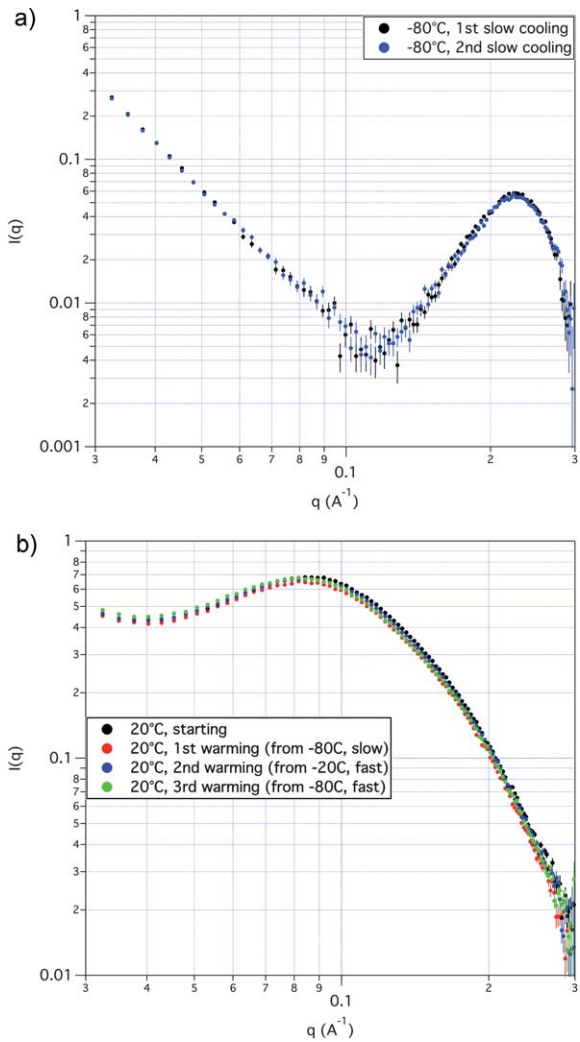

Fig. 7 (a) SANS data from 100 mg mL^{-1} lysozyme in 0 M NaCl D_2O solution cooled to −80 °C from 20 °C by the slow cooling method as described in the text. (b) SANS data from 100 mg mL^{-1} lysozyme in 0 M NaCl D_2O solution warmed to 20 °C from −80 °C by both the slow and fast methods as described in the text. Error bars in (a) and (b) represent plus and minus the combined standard uncertainty of the data collection.

On the other hand, the scattering data from the 50 mg mL^{-1} lysozyme in 0.4 M NaCl solution was not reproducible upon re-freezing. This is evident from Fig. 8a, which shows log(I) *vs.* log(q) plots of the data after the first and second cooling cycles using the slow cooling method. Fig. 8b shows log(I) *vs.* log(q) plots of the data from the same system at 20 °C after thawing the sample from −80 °C using the slow cooling method. It is clear that the original SANS curve was not reproduced after even one freeze–thaw cycle.

The SANS data obtained from the lysozyme solutions after several freeze–thaw cycles are reproducible at 20 °C and −80 °C for the 0 M NaCl solution, but not for the 0.4 M NaCl solution. This indicates that the aggregates formed during freezing of the 0 M NaCl solution are reversible upon thawing. However, the 0.4 M NaCl solution already contains aggregates before the first freeze–thaw cycle

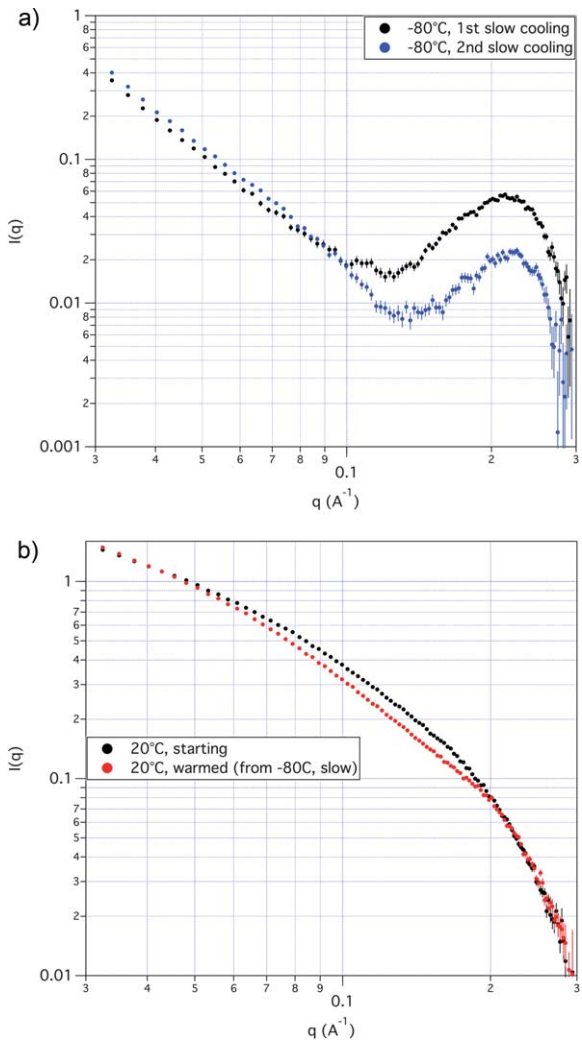

Fig. 8 (a) SANS data from 50 mg mL^{-1} lysozyme in 0.4 M NaCl D_2O solution cooled to −80 °C from 20 °C by the slow cooling method as described in the text. (b) SANS data from 50 mg mL^{-1} lysozyme in 0 M NaCl D_2O solution warmed to 20 °C from −80 °C by the slow cooling method as described in the text. Error bars in (a) and (b) represent plus and minus the combined standard uncertainty of the data collection.

and the solution is polydisperse. The nature of the polydispersity changes during the freeze–thaw cycles such that the original population of aggregates is not reproduced during subsequent freezing and thawing. However, in both cases, the aggregates formed are weakly associated with each other, as they either break up completely upon thawing, as in the 0 M NaCl solution, or they break up and reform differently upon thawing, as in the 0.4 M NaCl solution. This is consistent with size exclusion chromatography data that showed no significant aggregates in either solution before and after freeze–thawing, indicating that the aggregation is reversible.[12]

Proteins that have a larger equilibrium binding constant than lysozyme are more highly associative and may respond very differently upon freezing and thawing. Such proteins would tend to form more stable aggregates when subjected to freeze-concentration. The rates of chemical modifications would also tend to increase in such proteins, irrespective of the aggregation state. Thus, undesirable effects such as permanent aggregates, harmful chemical modifications and protein denaturation may exist upon thawing of such proteins.

Conclusions

Based on the information obtained from the SANS data of the 100 mg mL^{-1} lysozyme solution with 0 M NaCl, along with information obtained by others using Raman scattering[10] and NMR,[11] as well as a basic knowledge of the behavior of water upon freezing,[3] a cartoon of the morphology of the sample in the frozen state can be constructed. As drawn in Fig. 9, the frozen state consists mainly of ice crystals, with regions of amorphous water containing freeze-concentrated protein. The SANS data clearly show that large structures are present in the system. While some of this scattering is due to the scattering from large cracks in the ice matrix, SANS contrast variation experiments[12] showed that there is a contribution from protein aggregates. It is speculated that these aggregates form at either the ice–water or ice–air interfaces. The Raman results showed that the majority of the protein exists in the amorphous water phase.[10] Thus, while some of the protein aggregates

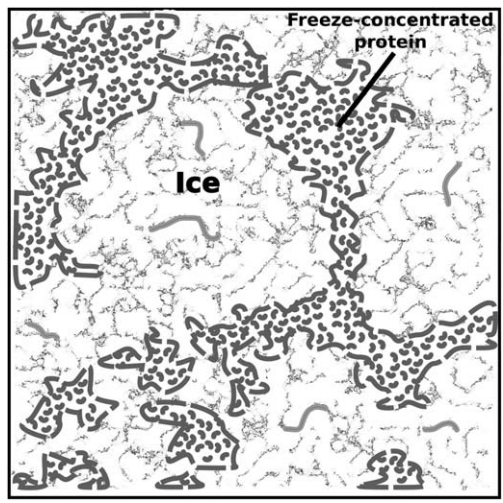

Fig. 9 Cartoon of the morphology of 100 mg mL^{-1} lysozyme in 0 M NaCl D_2O solution at −80 °C (based on Dong *et al.*[10]). The ice and freeze-concentrated protein phases are labeled. The dark grey lines represent large protein aggregates at the boundary between the ice and freeze-concentrated protein regions. The lighter grey lines in the ice phase represent possible protein aggregates trapped at the ice–air interface. Dimensions of the freeze-concentrated proteins and protein aggregates are enlarged for clarity.

may be trapped in the ice phase at the ice–air interface, the majority of the protein is drawn to be at the ice–water interface in Fig. 9. The remaining protein is shown to exist as close-packed monomers in the unfrozen amorphous water region.

The morphology of the 50 mg mL^{-1} lysozyme solution with 0.4 M NaCl is more complicated. The SANS data show that lysozyme doesn't exist as a monomer in solution. Rather, a distribution of sizes of aggregates is likely present. However, the data also show a liquid–liquid phase separation at 5 °C, well above the freezing temperature of the water component in the solution. The SANS data further indicate that the *d*-spacing is similar to that obtained for the 0 M NaCl solution at 0 °C and only decreases slightly below −20 °C, when the salt is frozen out of solution. This begs the question as to the mechanism of the "freeze-concentration" effect in both the 0 M NaCl and the 0.4 M NaCl samples. Is it mainly driven by a liquid–liquid phase separation as the temperature is lowered and then "frozen" into place as ice forms in the system and traps the protein in the amorphous water phase? Does this explain the slightly larger *d*-spacing observed for the 0.4 M NaCl sample from the onset of the phase separation all the way down to −80 °C? Further studies on the freezing of several different proteins in several different salt solutions should shed additional light on this issue.

Future directions

Neutron techniques are currently being explored for their potential contributions to food science, as evidenced by two recent meetings[17,18] and a recent paper on the subject.[19] SANS is being used to study the nanoscale structure of systems of interest to the food science community, such as food proteins, starch granules, polymers and emulsions.[19] The vast majority of these studies are performed on systems in the liquid state. However, SANS is also well-suited to the study of proteins in other biotechnologically-relevant phases. This work shows that SANS can potentially answer some important questions regarding the structure and interactions of food constituents, such as proteins, in the solid state. SANS can be used to ascertain the nature of interactions between proteins in both the liquid and solid states and under a variety of different solvent conditions. Moreover, SANS can be used to determine whether the cooling rate or multiple freeze–thaw cycles affect these interactions.

It is more difficult to ascertain the shape of the protein under conditions where it is interacting with other proteins in the solution. This hampers the ability to determine whether there is a shape change in the protein upon freezing, such as that which may occur due to denaturation, for instance. Some limited information can be obtained using alternative fitting functions that take the shape of the particle into account. However, such fitting methods are limited to those shapes that can be described in a functional form.

Work is currently underway to use real-space, all-atom computational modeling techniques to model the SANS data from close-packed protein systems. In this case, the actual structure of the protein is being used, as defined from X-ray crystallography or NMR spectroscopy. Typically, these structures are verified or refined based on SANS data from the protein in dilute solution. The solid state can then be modeled *in silico* and compared to SANS data, allowing the shape of the protein to be considered as the proteins become more concentrated and pack together more closely. To this end, it would be useful to repeat the current studies on proteins that are more asymmetric than lysozyme to determine the limits to this approach.

Contrast variation is a powerful method that can be useful for obtaining additional information about the nature of the low *q* scattering in the SANS data. By adjusting the $D_2O : H_2O$ ratio in the solvent, measurements can be made under conditions where either the scattering from large protein structures or from cracks in the ice are negligible. This allows the separation of the scattering from those two components to allow a better understanding of the nature of each. Contrast variation can also be used in a similar manner to eliminate or enhance the scattering

contribution from many salts and sugars. Thus, further work can be done on the 0.4 M NaCl lysozyme solution to determine if the contribution to the scattering from the salt component can be separated from that of the protein and ice components.

Finally, SANS can be used to study proteins in other solid states. The scattering from freeze-dried protein samples can be measured to determine the nature of the protein interactions in the system. It is more difficult to use contrast variation in this case since there is no bulk water in the system. However, if the protein is freeze-dried in the presence of sugars, then deuterated sugars protein can be used to enhance the scattering from the protein in order to determine the effect of the sugar on the protein interactions.

Acknowledgements

This work utilized facilities supported in part by the National Science Foundation under Agreement No. DMR-0944772.

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