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Protein/ice interaction: high-resolution synchrotron X-ray diffraction differentiates pharmaceutical proteins from lysozyme

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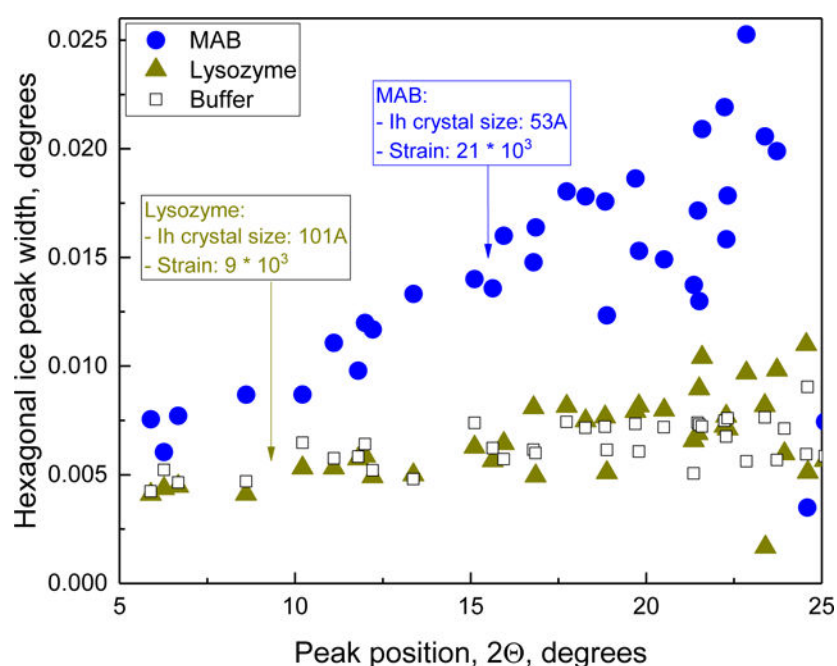
Abstract

While proteins can often be stabilized by maintaining them in the frozen state, water-to-ice transformation can also lead to degradation of protein molecules. A new method to study protein/ice interaction is presented herein, which is based on measuring the characteristic features of X-ray diffraction (XRD) patterns of hexagonal ice (*Ih*). Aqueous solutions of four different proteins and several small molecular weight solutes are studied using high-resolution synchrotron X-ray diffraction at the ID22 beamline at the European Synchrotron Radiation Facility. The beamline is optimized to eliminate the instrumental broadening of diffraction lines and reduce the preferred orientation effects, thereby enabling quantitative analysis of the XRD data. The analysis demonstrates that two pharmaceutical proteins, recombinant human albumin (rHA) and monoclonal antibody (mAb), have a pronounced effect on the properties of ice crystals. In particular, the size of the crystalline domains is significantly smaller, and the microstrain is larger, in the solutions of the pharmaceutical proteins, when compared with a model protein (lysozyme), an antifreeze protein, and sucrose and histidine. Neither of the proteins studied exhibit

Supporting Information. Results of the differential scanning calorimetry tests, instrumental peak broadening for X-ray diffraction peaks, overview of the estimates on freeze-induced pressure, details of the integration procedure for X-ray diffraction peaks, and examples of Williamson-Hall plots are supplied as Supporting Information.

preferred interaction with specific crystalline faces of *Ih*. The results are consistent with indirect interaction of the pharmaceutical proteins with ice, in which protein molecules are accumulated in the quasi-liquid layer next to growing ice crystallization front. Direct interaction would indicate a sorption of protein molecules on ice crystals, whereas “indirect interaction” terminology is used to describe any interference of proteins with ice crystals without sorption involved. Lysozyme molecules, on the other hand, do not exhibit any evidence of interaction (either direct or indirect) with ice crystals. This is the first report, to the best of our knowledge, of major difference in protein/ice interaction between different types of non-antifreeze proteins. In addition, we report an unexpected finding of a second population of ice crystals, with a much smaller (a few nm) size of crystalline domains. The second (minor) population is tentatively identified as a high-pressure form of ice, possibly IceIII or IceIX. This observation highlights a potential role of mechanical stresses and local pressure in freeze-induced destabilization of proteins.

Graphical Abstract



Introduction.

Frozen aqueous systems are ubiquitous in both nature and various industrial processes. Protein drugs and other biopharmaceuticals, for example, are commonly stored in the frozen state or processed by freeze-drying to minimize degradation during shipping and storage. Many food products also require frozen storage or freeze-drying. While rates of physical and chemical processes decrease in the frozen state in the majority of cases, freezing could also destabilize protein molecules.^{1,2} There are multiple pathways for freeze-induced protein destabilization, including freeze concentration, cold denaturation, pH changes, crystallization of a cryoprotector,² to name a few. In addition, ice formation *per se* was shown to cause protein degradation in some cases, with the effects of both freeze-concentration and low temperatures accounted for.³⁻⁵ *Vice versa*, proteins and other solutes

can also influence ice nucleation and growth, and a fundamental understanding of the impact of various solutes on ice crystal properties is essential for numerous industrial applications. In some processes, such as freeze-drying, larger ice crystals are preferred because increased crystal size allows a more efficient freeze-drying process (a higher sublimation rate).^{6,7} On the other hand, many food products, such as ice cream, benefit from smaller ice crystals.⁸ Similarly, cryopreservation of cells and organs requires either complete vitrification or prevention of growth of larger ice crystals from microcrystals formed intracellularly.^{9,10}

The majority of mechanistic studies on protein/ice interactions have been carried out with antifreeze proteins (AFPs). Antifreeze proteins may interfere with one or more specific faces of ice crystals; e.g., inhibition of growth of basal and prism planes by insect AFP has been observed.¹¹ Several hypotheses have been introduced to explain mechanisms of AFP-ice interaction, with AFP-ice hydrogen bonding¹² and hydrophobic interaction being the most prominent. Hydrophobic mechanism, in particular, involves clathrate-like water structure in the ice-binding site, where these water molecules are released during the ice binding resulting in overall gain of entropy.^{13,14} Furthermore, a model was introduced which accounts for both hydrophobic effect and hydrogen bonding contribution to the AFP/ice interaction.¹⁵ The high affinity of AFP to ice has been used for AFP purification.¹⁶ Irrespective of a specific mechanism, AFP/ice interaction was proposed to require partitioning of AFP molecules to and interaction with the quasi-liquid layer on the surface of ice crystals, where the antifreeze molecules are competing with growing ice crystals for water molecules.^{17, 18} The quasi-liquid layer (also known as liquid-like layer) is a thin film of water or solution on the surface of ice crystals, which exists well below the ice melting temperature.¹⁹

Some non-antifreeze proteins can also interfere with ice crystals. Milk proteins (containing predominantly casein, a fibrillar protein) were also shown to reduce ice crystal size,²⁰ and exhibit a tendency to accumulate near the ice crystal surface.²¹ Accumulation of a globular protein, albumin, near the ice surface was also reported.^{22,23} The impact of “common” (i.e., non-antifreeze) proteins on ice crystals is usually attributed to direct protein/ice interaction, i.e., to protein sorption on particular faces of ice crystals. For non-AFP proteins, however, any direct evidence of protein sorption on ice crystals is lacking, and it has been proposed recently that their interference with ice-crystal formation does not necessarily require a direct interaction with ice crystal surface.²⁴ Specifically, it was suggested that pharmaceutical proteins could influence water-to-ice transformations *via* protein partitioning into quasi-liquid layer. Overall, while there are numerous reports of different aspects of protein/ice interaction with AFPs, studies of interaction of pharmaceutically-relevant proteins with ice are lacking.

In this study, protein/ice interaction is investigated using quantitative X-ray diffraction (XRD). XRD represents a main experimental tool in the studies of frozen aqueous solutions, e.g.,^{25–27} although it is usually limited to qualitative aspects of freezing behavior, such as confirmation of freezing onset from the appearance of corresponding diffraction lines²⁸ and determination of the ice form, for example, hexagonal (*Ih*) versus cubic (*Ic*).^{29,30} XRD has also been used to study the interaction of antifreeze proteins and their synthetic analogs with specific faces of ice crystals, by detecting the disappearance of particular *Ih*

diffraction peaks, for example, a peak corresponding to basal plane (*002*).^{31–33} It has been suggested recently that additional important information on protein/ice interaction could be obtained from the quantitative analysis of XRD data, by monitoring changes in the extent of crystallinity and microstrain in the crystalline lattice.²⁴ Quantitative XRD analysis for frozen aqueous solutions is usually complicated by the preferred orientation effects, which necessitate additional sample treatment, such as grinding,³¹ and also by instrumental broadening of diffraction lines. With the experimental setup of the present study, in which high-resolution synchrotron X-ray diffraction is used, such limitations are greatly reduced,³⁴ by minimizing the instrumental broadening and preferred orientation effects.

Two pharmaceutical proteins, recombinant human albumin (rHA) and monoclonal antibody (mAb), a common model protein, lysozyme, and an insect AFP (iAFP) are utilized in this study. It is observed that the disorder of *Ih* crystals in frozen solutions of rHA and mAb is significantly higher than in either pure ice or solutions of small molecular weight solutes (such as sucrose and histidine). On the other hand, the impact of lysozyme molecules on the disorder (i.e., on the size of crystalline domains and the microstrain level) in the *Ih* crystals is much less pronounced when compared to samples containing rHA and mAb. The results are consistent with indirect protein/ice interaction, in which larger protein molecules accumulate in a quasi-liquid layer next to the growing ice crystal plane, probably because of their lower mobility. In addition, a second population of ice crystals is detected, with a greater degree of disorder and a much smaller crystal size (a few nm) than the predominant fraction of hexagonal ice crystals. This is the first observation of its kind to the best of our knowledge. The disordered population of ice crystals is tentatively identified as a high-pressure form of ice, possibly IceIII or IceIX. While the experiments are performed at atmospheric pressure, volume expansion during water-to-ice transformation is expected to create mechanical stresses and elevated local pressure.³⁵ To summarize, the paper introduces a novel approach to study protein/ice interaction by quantifying disorder in *Ih* crystals, and also highlights a potential role of mechanical stresses and local pressure in freeze-induced destabilization of proteins.

Materials and Methods.

Compositions of the solutions studied are provided in Table 1. Sucrose, histidine base, and histidine HCl were obtained from EMD Millipore (part number 100892), Ajinomoto (part number 20702), and SAFC (part number H4036), respectively. Lysozyme from chicken egg white (part number L4919) and rHA expressed in rice (part number A9731) were obtained from Sigma-Aldrich as lyophilized powders. A humanized IgG1 monoclonal antibody (mAb) provided by Pfizer, Inc. was also utilized. Solutions of lysozyme and rHA were prepared by dissolving the lyophilized powders in deionized water or the appropriate target buffer (pH 5.8). The monoclonal antibody solution was buffer exchanged into the appropriate target buffer (pH 5.8). The preparation of the iAFP, an isoform from *Dendroides canandensis*, followed the previously published procedure.³⁶ Briefly, the iAFP was expressed as a fusion protein in *Escherichia coli* Origami B cells. The cells were harvested by centrifugation at 4 °C. After the cells were disrupted, the crude protein was purified using immobilized metal ion affinity chromatography (IMAC) (Ni-NTA agarose, Qiagen). The tags of the iAFP were cleaved off with enterokinase (New England Biolabs)

and then the resulting protein was further purified by using IMAC and ion exchange chromatography.

Two series of the high-resolution wide-angle synchrotron XRD experiments were performed, with deionized water, iAFP 1 mg/ml in water, and IgG and lysozyme in histidine buffer studied in both experiments. The experiments were conducted at the European Synchrotron Radiation Facility (ESRF) on the ID-22 beamline at the X-ray wavelength of 0.399960(4) Å (first experiment) and 0.40028(1) Å (second experiment). The instrument angular scale is ensured by a rotary encoder mounted directly on the rotation shaft, with rotational accuracy better than ± 3 microradians, tested by the Belgian Metrology Institute before delivery. The zeropoint and wavelength are calibrated against NIST 640c standard Si at least for each change of wavelength. Intensities and angular offsets between the nine detector channels are calibrated against each other to ensure the signals superimpose using ESRF in-house software.

The solutions were filled in 1-mm borosilicate glass capillaries at ambient temperature and then cooled to 100 K in N₂ flow with ramp rate ~ 360 K/hour. The freezing temperature of 100 K is well below the glass transition temperatures, T_g , of the samples studied, with the lowest T_g for the freeze-concentrated solutions detected to be approx. 218 K (Supporting Information, Table S1); therefore, minimal changes are expected during the XRD data collection. The XRD intensities were sampled every $0.0005^\circ 2\theta$ over a scan range of 0.5 to $40^\circ 2\theta$, normalized and rebinned at appropriate steps (0.001 , 0.01 , 0.05°) depending on the widths of the peaks in the diffraction pattern, using in-house software id31sum.³⁷ The XRD pattern of an empty capillary, which was obtained at 100 K, was utilized for the background subtraction.

Quantification of the X-ray diffraction peak broadening is achieved by calculating the full width at half maximum (FWHM) values for each peak in the range of 5 – $25^\circ 2\theta$, using the peak analysis function of Origin2017 software. In this analysis, the baseline is adjusted manually for each peak, and the peak analysis is performed using the baseline anchor points. Details of the peak analysis are provided in the Supporting Information.

The theoretical XRPD Ih pattern is generated from the reported structure.³⁸ Theoretical X-ray diffraction patterns for high-pressure forms of ice are generated using Mercury software³⁹ with the following parameters: $\lambda = 0.39996$ Å, FWHM (2θ) = 0.01° , step = 0.002° . The initial information on crystal structures for powder diffraction patterns generation is taken from sources described in⁴⁰.

Results

Examples of X-ray diffraction patterns are shown in Figure 1, together with the calculated pattern for Ih . All peaks in the experimental patterns can be matched to the Ih peak, with the exception of a minor peak at approx. $24.5^\circ 2\theta$. The peak is observed in both the frozen water sample and in the solutions, but not in the empty capillaries, and therefore is attributed to ice. Neither proteins nor low-molecular weight solutes (i.e., histidine and sucrose) crystallize at the experimental conditions used in this study. Therefore, the frozen solutions represent

2-phase mixtures of *Ih* and the freeze-concentrate containing all the solutes and unfrozen water. Figure 1b shows also difference in the relative intensities of the diffraction peaks observed between the 1st and 2nd synchrotron experiments for the iAFP samples. The variability in the relative intensity is probably due to the preferred orientation effects, which are difficult to control with ice crystals formed directly in a capillary. Preferred orientation is reduced, but not completely eliminated, under the experimental set-up used in this study.

In order to quantify the disorder in the *Ih* crystals, FWHM values are calculated for each peak in a 2θ range of 5 to 25°. There are two main contributions to X-ray diffraction peak broadening, i.e. instrumental effects and any lattice imperfections. A nominal instrumental contribution to the FWHM is around 0.003° 2θ at low diffraction angles.³⁴ Peak broadening due to lattice imperfections have major contributions from two sources, i.e., reduced crystallite size and microstrain, such as lattice dislocations (linear defects).⁴¹ If only one selected peak is analyzed, it is impossible to distinguish between these two effects. If a 2θ dependence of FWHM is analyzed, then it becomes possible to separate relative contributions of size and microstrain effects. A higher microstrain level influences reflections at higher 2θ values in a greater extent, resulting in a steeper slope of the FWHM vs 2θ dependence.^{41, 42} As shown in Figure 2 and Figure S1 (Supporting Information), non-instrumental peak broadening is observed in all experimental patterns, although the extent of the broadening, and therefore the level of imperfections in the ice crystal lattice, varies between samples studied. The impact of different solutes (that is, proteins or other formulation components such as histidine or sucrose) on the ice crystal disorder is shown in Figure 2a. Peak broadening is lowest in pure water and sucrose solutions, whereas the mAb and rHA solutions exhibit have the highest degree of ice crystal disorder (i.e. broader peaks in the XRD patterns) between the samples studied. A major difference between lysozyme (minor impact on ice disorder) and mAb and rHA (significant line broadening) should also be stressed. This trend is reproduced in a replicate experiment, with mAb showing a much greater extent of the peak broadening than lysozyme (Figure 2b), which is consistent with the 1st experiment.

Broadening of XRD peaks by antifreeze polymers was reported previously,³¹ where width of 3 main *Ih* peaks (100, 002, and 101) in pure ice was visually compared with that in the polymer solution. In a more recent study,²⁴ a quantitative approach to analyze the ice peak broadening was introduced, in which contribution from the finite crystalline size is distinguished from the microstrain contribution by using Williamson and Hall (WH) plot analysis.⁴²

$$\beta^2 = \left(\frac{\lambda}{D \cdot \cos(\theta)} \right)^2 + (4 \cdot \epsilon \cdot \tan(\theta))^2 \quad (\text{Eq. 1})$$

where β is a peak broadening (FWHM), λ is wavelength, D is coherent scattering length related to the size of individual crystallites (i.e., the size of crystalline domains), θ is diffraction angle, and ϵ is a strain component due to defect structure of individual crystallites. Note that D , the size of crystalline domains, is not generally the same as the particle size, due to the presence of grain boundaries and polycrystalline aggregates. Lattice

microstrain, ϵ , is a measure of the distribution of lattice constants arising from crystal imperfections, such as lattice dislocations, interstitials, vacancies, and substitutions.⁴¹

The WH plots, which describe the dependence between $(\beta^2 (\cos(\theta))^2)$ and $(\sin(\theta))^2$, are linearized using Origin2017 software, and $(4 \epsilon)^2$ and $(\lambda^2)/(D^2)$ are obtained from the slope and the intercept values, respectively. Examples of the plots are provided in Supporting Information, Figure S2. D and ϵ values are summarized in Table 1. Both D and ϵ values are reproduced in the two experiments for the protein solutions (i.e., for mAb, lysozyme, and iAFP solutions), while a significant difference in the crystallite size is observed for pure ice sample between the 1st and 2nd experiments.

In addition to strong and narrow peaks of h , several broad and low-intensity peaks are also observed in all the samples (Figure 3). The majority of these peaks coincide with several h peaks, corresponding to 100, 101, 110, 103, and the group of 200, 112, 201 peaks. In addition to the h -associated broad peaks, two or three broad peaks are also detected at 7.5 to $8.5^\circ 2\theta$, and at $12.9^\circ 2\theta$. Since these additional broad peaks were also observed in the ice sample, they most likely correspond to a partially ordered form of solid water, as discussed below.

Discussion.

Protein/ice interaction.

Table 1 provides crystallite size and microstrain for h crystals, which are used to compare the impact of different solutes on ice formation and therefore on the solute/ice interaction. In all non-protein samples investigated in the current study, the microstrain level is consistently lower than in samples containing proteins. The microstrain is the highest in mAb and rHA samples (20×10^3 to 22×10^3), whereas lysozyme and iAFP solutions show lower values of 8×10^3 to 13×10^3 . The microstrain in the non-protein samples ranges from 6×10^3 to 7×10^3 . The higher microstrain level is indicative of a higher concentration of defects in the crystalline lattice, such as dislocations, interstitials, vacancies, substitutions.^{41–43} The higher microstrain in all protein samples, therefore, suggests that all four proteins studied here promote formation of defects during ice crystal growth, although the impact and therefore the extent of protein/ice interaction appears to be dependent on type of the protein. Three proteins, mAb, rHA, and lysozyme, are studied at the same concentration of 100 mg/ml, while protein concentration in the iAFP samples is at least 2 orders of magnitude lower. The higher extent of disorder (i.e., smaller crystallites and higher microstrain) in h observed in rHA and mAb solutions, as compared with lysozyme, is indicative of a stronger interference of rHA and mAb with ice formation, i.e., a stronger ice/protein interaction. Similar microstrain level in ice crystals in lysozyme and iAFP solutions is consistent with a much stronger interaction of iAFP with ice vs. lysozyme, considering that the concentration of iAFP is at least 2 orders of magnitude lower than lysozyme.

The size of the crystalline domains in mAb and rHA solutions is lower than in other protein solutions and in non-protein samples (51 to 66 nm vs 77 to 128 nm). While lysozyme solutions are at the same protein concentration as the rHA and mAb samples (100 mg/ml), the size of ice crystals is larger in lysozyme solutions (94 to 108 nm), and is also not

substantially different from the size of the ice crystals in the non-protein samples. The reduced size of the crystalline domains in the mAb and rHA solutions, combined with the higher level of microstrain, indicates that these proteins demonstrate a greater degree of interference with ice crystals growth than lysozyme. .

The affinity of a protein or another solute to a specific face of the ice crystal can be monitored by X-ray diffraction, by detecting a reduced intensity of a particular XRD peak. A lower peak intensity (peak height) or, in extreme cases, an apparent absence of a specific peak⁴⁴ would mean a suppressed growth of a corresponding crystallographic plane of ice crystals by a solute.

Several examples of application of XRD to detect affinity of proteins and other solutes to specific faces of *Ih* are summarized in this paragraph as follows. It was observed, for example, that some block copolymers, based on poly-(ethylene oxide)-block-poly(1,4 butadiene) (PEO-b-PB), interfere with *Ih*, resulting in greatly reduced intensity of the 002 peak.⁴⁵ In addition, a shift in the position of 3 main peaks (100, 002, 101) was reported, which was also attributed to the polymer/ice direct interaction.⁴⁵ It was suggested that a specific binding of the OH groups of the polymers to the faces parallel to the c-axis resulted in the suppression of the growth of the basal plane and preferential growth of ice crystals along the c-axis. Similarly, suppression of the 002 peak by synthetic analogs of AFP proteins was also reported.^{31–33} Insect AFPs (iAFP) bind primarily to prism and basal planes,⁴⁶ and such binding should reduce intensities of 100 and 002 reflections (prismatic and basal planes, respectively). It was reported, however, that iAFP interaction with ice could also include other crystal faces. Binding of iAFP to multiple ice crystal planes including basal, prism, and/or pyramidal planes was observed using green fluorescent protein (GFP)-labeled insect AFPs from *Tenebrio molitor* and spruce budworm.⁴⁷ In this case, the relative intensities of a particular peak would not be impacted, because of a strong binding to all crystallographic planes.

In this study, all peaks of *Ih*, including 002 peak, are observed in the frozen protein-containing solutions, indicating that these proteins do not exhibit a strong preferential interaction with any particular face of *Ih*. While a lower intensity of 002 peak is observed in several patterns, including one of the frozen iAFP solutions and mAb and rHA samples (Figure 1), other protein solutions, including several iAFP samples, exhibit a more pronounced 002 peak. Moreover, a lower intensity of 002 peak is also observed in non-protein samples, including sucrose solutions and pure water, in which no specific inhibition of the growth of the basal plane by solute sorption is expected. Therefore, we attribute the lowered intensity of the 002 peak to preferred orientation of *Ih* crystals in these particular samples. Preferential orientation effect is reduced, but not completely eliminated, under the experimental set-up used in this study. Overall, these results do not support a hypothesis of a direct sorption of the proteins on a particular ice crystal face and are consistent with an indirect protein/ice interaction. In this case, protein molecules can impact ice formation by accumulating in the liquid fraction near the growing ice crystals.

An additional consideration, which would favor the hypothesis on indirect protein/ice interaction, could be provided by taking into account the charge on the proteins *vs.* ice.

The pI of ice crystals is approximately 3 to 4,⁴⁸ and therefore ice crystal surface would carry a negative charge at pH 5.8. Similarly, rHA (pI 5.3) has a negative charge at pH 5.8, which would result in electrostatic repulsion from the negatively-charged ice surface and therefore in reduced probability of the protein sorption on the ice crystals. On the other hand, mAb molecules (pI 9), would be positively charged and be electrostatically attracted to ice. Such electrostatic attraction would increase probability of adsorption of the mAb molecules onto ice surface, resulting in a significant impact on growth of the ice crystal. Therefore, if a direct protein/ice interaction is involved in “shaping” ice crystals in these solutions, properties of ice crystals in the mAb solution would be expected to be different from that in the rHA solution. This is not what is observed in this study, with both solutions producing similar crystals of Ih , in terms of both crystallite size and microstrain (Table 1). The similarity in the properties of ice crystals formed in mAb (electrostatic attraction with ice) vs. lysozyme (electrostatic repulsion from ice crystals) indicates that adsorption probably does not play any significant role in the interaction of these proteins with ice. In addition, lysozyme (pI 11.4), which has the same (positive) charge sign as the mAb, demonstrates minimal protein/ice interaction (i.e., a minimal impact on ice crystals). Molecular weight of protein molecules could be more important in defining protein/ice non-specific interaction. In this study, a smaller protein (lysozyme, MW 14.3 kDa) shows much lower level of ice crystal disruption than that observed with the two larger proteins (rHA and mAb, 67 and 150 kDa MW, respectively).

The following description of the protein/ice interaction during freezing is proposed, based on the observation of a strong impact of mAb and rHA on Ih crystals combined with a lack of evidence of any specific sorption of these proteins on ice surface. The growing ice crystals expel all the solutes, creating concentration gradient across the remaining liquid phase, with the elevated local solute concentration in the solution adjacent to the ice crystals. The concentration gradient depends on the mobility of the species, with larger species having a tendency to concentrate in the liquid phase, next to the ice/solution interface, to a greater degree, because of a lower molecular mobility. It was reported, for example, that BSA molecules accumulated in the liquid phase at the ice/solution interface, whereas smaller trehalose molecules were expelled into the bulk freeze-concentrated phase.²² Such accumulation of protein molecules would reduce the local concentration and apparent activity of water in the liquid next to the growing ice crystals, which could hinder their growth, resulting in smaller crystallites with a higher level of microstrain. We suggest, furthermore, that the smaller lysozyme molecules, as well as histidine and sucrose, would diffuse away more readily, and therefore would not interfere with growing ice crystals. In this scenario, larger ice crystals with a lower level of microstrain would form in solutions of small molecular weight solutes and lysozyme, consistent with the results provided in Table 1. Note also that this situation could be more complex, when considering the existence of a quasi-liquid layer (QLL). Quasi-liquid layer (also known as liquid-like layer) is a thin film of liquid water on the surface of ice crystals, which exists below the ice melting temperature.¹⁹ Estimations of the thickness of the layer vary widely, from a few angstroms to up to a micrometer.⁴⁹ An evidence of two populations of ice crystals at sub-zero temperatures was reported in⁵⁰ where 2-step melting was detected in pure ice samples by DSC. It was proposed that a liquid layer exists between ice crystals (the fraction

was estimated to be approx. 1.5%) at temperatures well below 0°C (up to -50 °C).⁵⁰ Accordingly, molecules of rHA and mAb could either be partitioned into the quasi-liquid layer, or accumulated on the interface between the quasi-liquid layer and the bulk freeze-concentrate. Figure 4 provides a schematic representation of the freezing behavior of protein solutions, which reflects partitioning of the proteins onto different compartments during growing of ice crystals.

Second population of ice crystals.

In addition to the well-defined and narrow peaks of *Ih*, very broad peaks are also detected, as shown in Figure 3. These peaks are unlikely to be an experimental artifact, as they are not observed when empty capillaries are measured under identical conditions in the same experiment. The fact that the broad peaks are observed in the pure ice sample (without any solutes) suggests that these peaks are associated with water, and are not due to a disordered crystalline phase of a solute. In the literature, broad peaks of ice were reported in various aqueous systems, e.g., in concentrated solutions of sugars,⁵² water nanodrops⁵³ and in mesoporous systems with pore size of several nm (e.g.,^{54,55}). In these cases, the peak broadening was attributed to formation of very small (a few nm) crystals of *Ic*. In one report, XRD patterns with relatively narrow peaks and an elevated baseline around peaks corresponding to 100 of *Ih*, and 002 of *Ih* or 111 of *Ic* were observed in frozen droplets of approx. 900 nm size.⁵⁶ The XRD patterns were shown to be neither purely cubic, nor hexagonal, nor *Ih+Ic* mixture, but were instead identified as stacking-disordered ice *Isd*.⁵⁶ It was proposed that homogeneous nucleation of ice in the sub-micron water droplets resulted in growth of ice crystals in which cubic and hexagonal stacking sequences are randomly arranged, i.e., *Isd* ice structure. Furthermore, similar XRD patterns, with sharp peaks and broad bases, can be observed in nanocrystalline systems such as with very long needles or very thin disks.^{57,58}

In the present study, the majority of the broad peaks are located exactly at the base of several *Ih* peaks, corresponding to hkl 100, 101, 110, 103, and the group of 200, 112, 201. Note also that the 112 *Ih* peak would coincide with the 311 plane of *Ic*. Several broad peaks, at 7.5 to 8.5° 2 θ and 12.9° 2 θ , could not be matched to either *Ih* or *Ic* patterns. This observation indicates that these broad peaks could not be attributed to the diffractions from *Ih* or *Ic* crystals with major difference in the dimensions of certain crystallographic planes, such as very thin needles. Moreover, while a portion of the XRD patterns (that is, around *Ih* hkl 100, 002, and 101) resembles the patterns reported previously⁵⁶ for stacking-disordered ice *Isd*, the *Isd* patterns described in a study by Malkin et al⁵⁶ do not reveal peaks which could be matched with the peaks observed at 7.5 – 8.5° 2 θ and 12.9° 2 θ . Therefore, the broad peaks observed in this study are unlikely to represent *Ih*, *Ic*, or *Isd*.

The detection of two types of peaks, i.e., narrow peaks as well as very broad peaks (Figure 3), could indicate co-existence of two populations of ice crystals, with *Ih* crystals producing the narrow X-ray diffraction peaks while the broad peaks are related to a disordered crystalline form of water. As an approximate estimate of the lengthscale for the disordered crystalline structure, Scherer equation⁵⁹ could be applied:

$$D = K\lambda/\beta\cos\theta \quad (\text{Eq. 2})$$

where D is the mean dimension of crystalline domains, λ is the wavelength, θ is the diffraction angle, β is width of the diffraction peak on the 2θ scale in radians, and K is a constant approximately equal to unity.

Based on the peak width of approx. 0.5° in the experimental patterns, the corresponding size of crystallites would be in a few nanometers scale range. This is obviously an oversimplified approach, which does not account for peak broadening from microstrain. Nevertheless, the Scherer equation provides useful order-of-magnitude estimate for the lengthscale of this highly disordered, but still crystalline, ice.

It is widely accepted that, even in pure water samples, two populations of water molecules coexist at sub-zero temperatures, that is, *Ih* crystals, and quasi-liquid layer, as briefly discussed above. Therefore, it is possible that the broad peaks, which represent a minor portion of the total water in the samples studied, belong to solidified (crystallized) quasi-liquid layer confined between *Ih* crystals. Note that these confined spaces would probably experience significant transient local pressure and mechanical stress due to volume expansion during freezing. Several reports provide estimates of the freeze-induced pressure of 2 to 3.5 kBar (see Supporting Information for details).^{35, 60–62} Such conditions could promote formation of nanocrystals of high-pressure ice forms. For example, IceIX could form at pressure of a few kBar in the temperature range of 100 to 200 K.⁶³ The experimental XRD patterns are therefore compared with the theoretical patterns of various high-pressure forms of ice. Among the forms considered, IceIII and IceIX exhibit peaks in the position corresponding to the first broad peak observed in the experimental patterns ($\sim 8^\circ 2\theta$) (Figure 5). IceIII also has a peak corresponding to the 2nd broad peak in the experimental pattern ($\sim 13^\circ 2\theta$), whereas there is no IceIII peak at the position of the third broad peak ($\sim 13.8^\circ 2\theta$). IceIX, on the other hand, exhibits peaks at all three locations. It is possible, therefore, that these 3 broad peaks, observed in the XRD patterns in the current study, indicate the formation of disordered crystals of ice with a structure resembling IceIX. It should be emphasized, however, that a number of other IceIX peaks are not observed in the experimental patterns, whereas the majority of the strongest IceIII peaks could be related to the experimental patterns (Figure 5).

Hypothesis: Protein/ice interaction and freeze-induced destabilization.

The rates of many physical and chemical processes decrease in the frozen state. However, freezing has been shown to destabilize protein molecules,² and there are several reports of increased rate of some chemical reactions, including hydrolysis and oxidation, in partially frozen aqueous solutions.^{64–67} While there are multiple stresses associated with freezing, such as freeze-concentration, pH changes, mechanical stresses, and protein cold denaturation due to low temperature *per se*, freeze-induced protein destabilization and increased chemical reactivity was shown to be directly related to ice formation rather than to low temperatures and freeze concentration.^{4,68–70} Conformational changes and unfolding of several globular proteins as the result of water-to-ice transformation were observed by monitoring the phosphorescence emission of tryptophan residues.⁵ In addition, thermodynamic stability of

a protein, azurin mutant C112S from *Pseudomonas aeruginosa*, was observed to correlate with the size of the liquid solution pool coexisting with ice crystals, with greater stability associated with the larger pool size and lower ice fraction.⁷¹ Furthermore, the stability of an enzyme, lactate dehydrogenase (LDH), in a partially frozen solution was compared to that in a solution of the same composition as the freeze-concentrate, but without ice, where both systems were exposed to the same sub-zero temperatures and for the same duration. LDH was inactivated in the partially frozen samples, whereas its activity was totally preserved in the freeze-concentrated solution (without ice).⁴ While these studies provided convincing evidence that formation of ice resulted in the destabilization of a protein, it is not immediately obvious that such destabilization was caused by the sorption of protein molecules on ice crystals, as other mechanisms can be invoked, as described below.

One possible alternative freeze-induced destabilization pathway is related to protein partitioning into the QLL. The microenvironment of protein molecules in the QLL can be very different from that in the bulk freeze-concentrate. In particular, the local acidity in QLL could increase (i.e., pH decrease) due to the negative charge on the surface of ice crystals.³⁹ The ice surface exhibits a negative charge when in equilibrium with solution at pH values higher than pH 4; the negative charge is balanced by an elevated local concentration of cations, including protons, in the quasi-liquid layer next to ice surface, resulting in a corresponding increase in the local acidity.

Another potential mechanism for freeze-induced protein destabilization involves mechanical stress associated with the water-to-ice transformation. The density of liquid water is approx. 9% higher than that of hexagonal ice,¹⁹ and water-to-ice transformation results in a corresponding volume expansion. The volume expansion could generate significant pressure build-up and mechanical stresses in the sample (see Supporting Information on the pressure estimates), which could lead to destabilization of higher-order structure of proteins. While pressure-induced unfolding is usually reversible,⁷² unfolded protein molecules are more prone to unwanted chemical and physical changes than the native forms.

Based on the results of the present study, it is proposed that non-AFP proteins, including typical pharmaceutical proteins, interact with ice crystals indirectly, by accumulating in the liquid portion of the sample near the growing ice crystals. These protein molecules would experience different microenvironment and destabilizing stresses, which include exclusion of the cryoprotector molecules from the protein and the acidity gradients between the surface of ice crystals, quasi-liquid layer, and the bulk. Furthermore, the detection of a second population of ice crystals, which is tentatively assigned to a high-pressure form of ice, highlights potential contribution of mechanical stresses in freeze-induced destabilization of proteins.

Conclusion

A novel approach to study protein/ice interaction is introduced in this study. The approach is based on a quantitative analysis of the disorder of *Ih* crystals formed in the presence of different proteins during freezing. The disorder is expressed as elevated microstrain and reduction in the size of crystallites. Experimental data, which are used for the *Ih* disorder

quantification, are obtained by high-resolution X-ray diffraction. The results show that, while lysozyme exhibits very minor (if any) interaction with *Ih*, rHA and mAb demonstrate a significant impact on ice crystal formation, and therefore, a strong protein/ice interaction. At the same time, elimination of specific XRD peak(s) of *Ih*, which would be typically associated with protein sorption (most commonly, on the basal 002 plane) and therefore a direct protein/ice interaction, is not observed. These two features of XRD patterns (i.e., no major reduction in the intensity of a specific *Ih* peak and a significant increase in the ice crystal disorder), when coupled, indicate that these pharmaceutical proteins interact with ice in a non-specific and indirect manner, i.e., without direct sorption onto the surface of ice crystals. We hypothesize that such indirect protein/ice interaction involves partitioning of the protein molecules into the quasi-liquid layer. In this case, proteins can interfere with ice crystal growth by competing for water molecules, which need to be transported from the liquid phase to the ice phase to support growth of the ice crystals.

Finally, the study reports, for the first time, on an existence of two population of ice crystals in pure ice and frozen aqueous solutions, comprising of predominant *Ih* phase and a minor phase of solid water. The second (minor) phase probably represents a highly disordered ice with crystallite size of the nanometer scale. The disordered ice phase is tentatively identified as a high-pressure form of ice, exhibiting close resemblance with IceIII or/and IceX.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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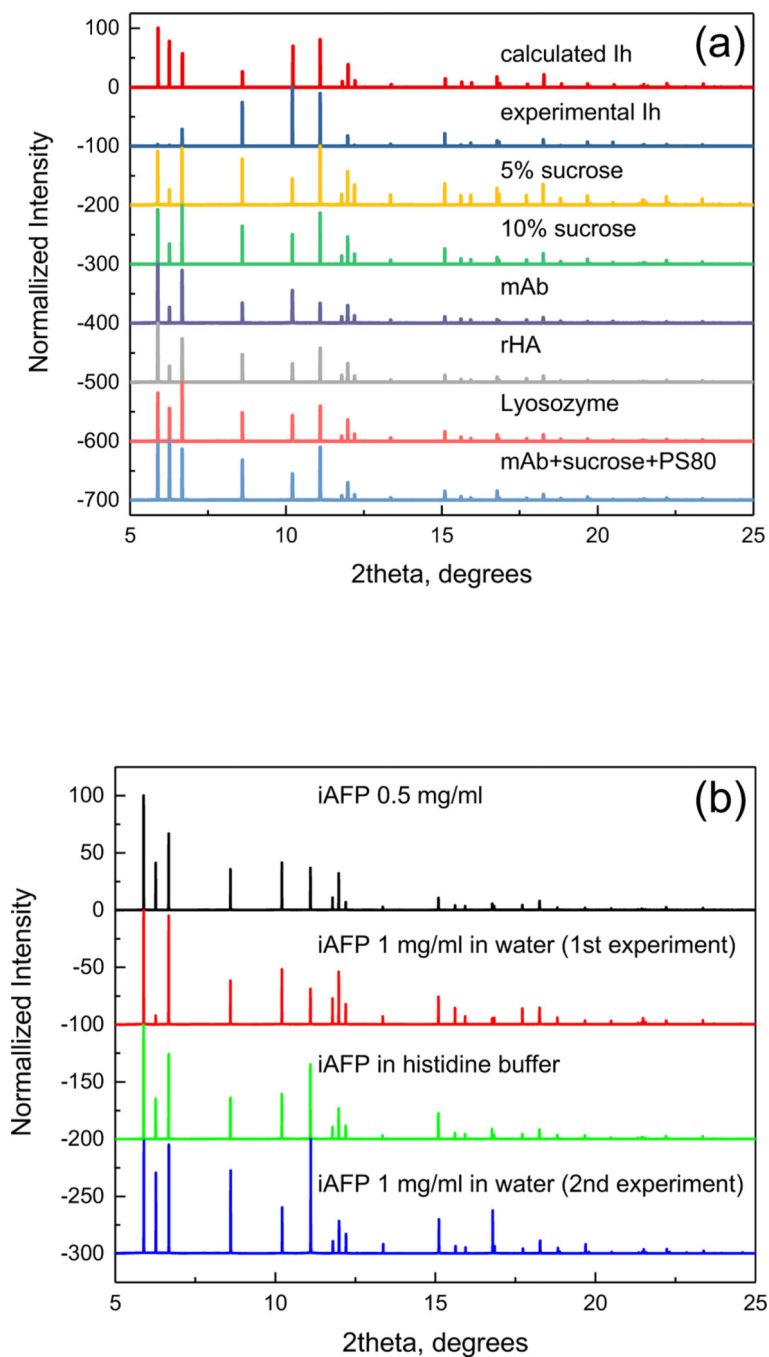
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**Figure 1.**

Examples of XRD patterns at 100 K for solutions containing iAFP (b) and other proteins and low molecular weight solutes (a). The intensities are normalized to the most intense peak. See Table 1 for the composition of the samples, and Materials and Methods for the description of 1st and 2nd experiments.

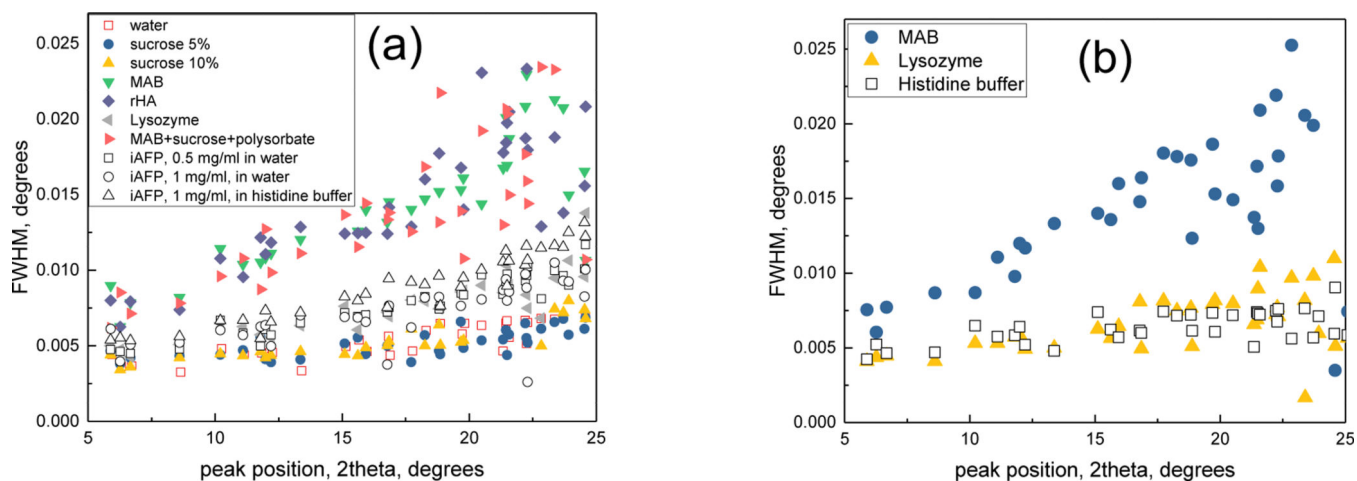
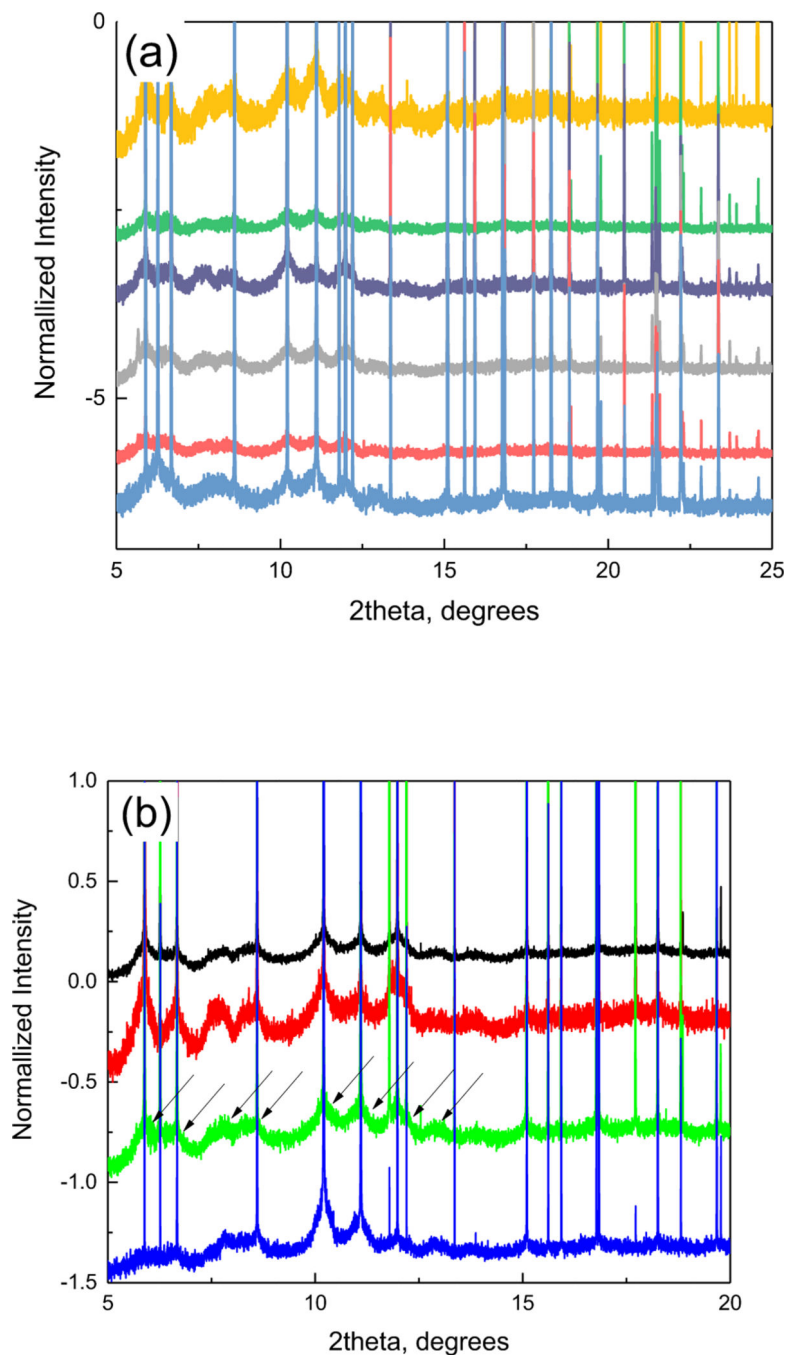


Figure 2.

Full width at half maximum (FWHM) of l_h peaks as a function of the diffraction angle.

Results from the 1st and 2nd experiments are presented in panels (a) and (b), respectively.

**Figure 3.**

Enlarged portions of representative X-ray diffraction patterns. (a) Top to bottom: 5% sucrose, 10% sucrose, mAb, rHA, lysozyme, mAb+sucrose+ PS80; (b) Top to bottom: iAFP 0.5 mg/ml; iAFP 1 mg/ml in water; iAFP in histidine; ice experimental. The arrows point to broad peaks which are observed in all patterns.

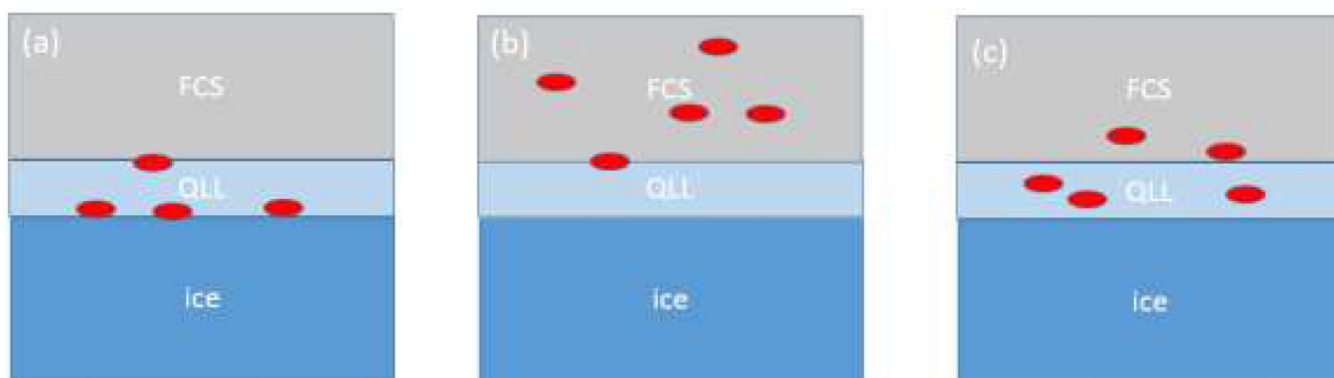


Figure 4.

Schematic presentation of freezing behavior of solutions of proteins. The red circles represent protein molecules. iAFP (a), which can directly interact with ice by sorption on growing ice crystals, is distinguished from non-AFP proteins, which are either partitioning predominantly in the QLL (mAb and rHA, (c)) or in the freeze-concentrated solution, FCS (lysozyme, (b)). The FCS can be either liquid, if the sample temperature is above the corresponding glass transition temperature, T_g , or solid. See Table S1 (Supporting Information) for the T_g values. The QLL has been reported to be present in a wide temperature range from the equilibrium ice melting point down to 200 K.⁵¹

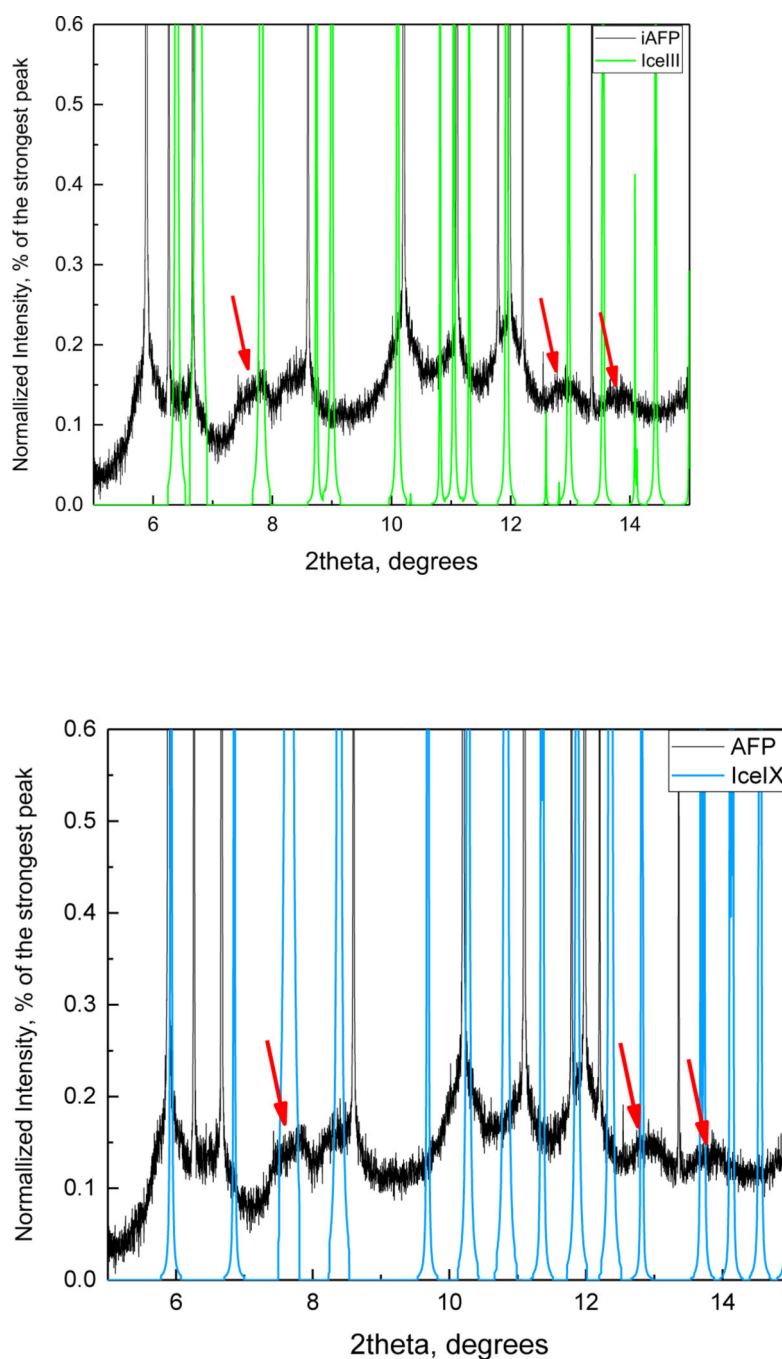


Figure 5.

An example of experimental X-ray diffraction patterns of a frozen iAFP solution (0.5 mg/ml) overlaid with theoretical patterns of IceIII (a) and IceIX (b). Red arrows show broad peaks without matching *Ih* peaks.

Table 1.

Composition of the samples and the results of the Williamson-Hall plot analysis, including ice crystallite size, D , and microstrain, ϵ .

Sample composition		<i>Ih</i> crystal properties	
Protein type & concentration	Buffer and other solutes	D, Å	Strain $\times 10^3$
mAb 100 mg/ml	20 mM Histidine, pH 5.8	55 $\pm$ 6 51 $\pm$ 7 *	20 $\pm$ 8 21 $\pm$ 9 *
mAb 100 mg/ml	20 mM Histidine, pH 5.8, 5 % w/v sucrose, 0.02 %w/v PS80	66 $\pm$ 14	21 $\pm$ 10
rHA 100 mg/ml	20 mM Histidine, pH 5.8	60 $\pm$ 8	22 $\pm$ 8
lysozyme 100 mg/ml	20 mM Histidine, pH 5.8	108 $\pm$ 11 94 $\pm$ 8 *	11 $\pm$ 4 8 $\pm$ 4 *
iAFP 1 mg/ml	Water	89 $\pm$ 6 78 $\pm$ 4 *	9 $\pm$ 4 9 $\pm$ 4 *
iAFP 1 mg/ml	20 mM Histidine, pH 5.8	105 $\pm$ 6	13 $\pm$ 3
iAFP 0.5 mg/ml	Water	104 $\pm$ 5	11 $\pm$ 3
-	5 %w/v sucrose	104 $\pm$ 3	6 $\pm$ 2
-	10 %w/v sucrose	128 $\pm$ 7	7 $\pm$ 2
-	Water	119 $\pm$ 5 77 $\pm$ 4 *	7 $\pm$ 2 7 $\pm$ 4 *
-	20 mM histidine buffer, pH 5.8	78 $\pm$ 2	6 $\pm$ 3

* Values determined from the replicate experiment