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The Ice-Water Interface and Protein Stability: A Review

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ABSTRACT

The ice-water interface is commonly encountered in our life, and comes into play in a wide number of natural phenomena. Here, attention will be focused on its effects on protein stability, with specific reference to the case of pharmaceutical proteins. This field represents a fascinating, and not yet fully understood, subject of investigation. Some background information on the ice-water phase diagram, as well as to the mechanisms of nucleation and crystal growth, will be provided. We will eventually discuss the effect of ice on protein activity, reviewing the mechanisms of ice-induced denaturation that have been proposed so far and discussing the strategies that may help prevent, or minimize, undesired loss of therapeutic activity.

Keywords: Crystallization, Proteins, Freeze-drying, Stabilization

1. INTRODUCTION

The ice surface is part of our everyday life and represents one of the most routinely encountered solid interfaces. Despite being a very common and abundant material on Earth, ice shows unique and intriguing properties that make it a fascinating subject of investigation. For this reason it plays a crucial role in a variety of fields; it is central in glaciology and snow physics, but is also relevant in astrophysics and atmospheric science, oceanography, cryo-biology and nucleation studies. Thanks to its availability, accessibility and inherent safety (i.e., absence of toxicity, flammability, explosivity), it represents a superb model system for phase transitions and deformation phenomena. The range of different scales involved in ice research, from molecular phenomena to atmospheric events, is impressive, and clearly shows the transversality of this topic.

It is therefore not surprising that the number of studies and publications in this area is already massive, and growing fast. Several books have been written on the physics and chemistry of ice [1, 2, 3, 4, 5, 6]. More than 3 million papers published to date deal with ice chemistry and physics, and an international symposium (the International Conference on the Physics and Chemistry of Ice, PCI [7]) has been focusing on all aspects of ice research for more than 55 years.

Considering the wide range of topics related to ice research discussed above, it would be impossible to satisfactorily cover all of them. This review will therefore focus on the effect of the ice-water interface on protein stability, a specific aspect that is more closely related with the authors' expertise.

Pharmaceutical proteins are becoming increasingly important in the treatment of a variety of human diseases, and are commonly stored in the frozen or freeze dried state to reduce risks of degradation before administration. Lyophilized formulations are generally preferred over frozen ones, because do not require to maintain a controlled cold chain. About 30% of currently marketed biopharmaceuticals are introduced as lyophilized products. Liquid formulations are also commonly used, and comprise about 50-60% of all biologic dosage forms. Liquid formulations are preferred because of the low cost of manufacturing and ease of administration, but cannot be used for very unstable molecules [8, 9].

Both freezing and freeze-drying involve the interaction of the protein with the ice-water surface, and this may affect the final therapeutic potency, as will be discussed in the following. Understanding the mechanisms and effects of protein-ice interaction would be essential for numerous applications, especially in the pharmaceutical industry where this knowledge may help to minimize undesired loss of activity.

However, many questions remain open in this field, and a clear picture of the problem is not available yet. We will show how experimental evidence suggests the central role of the ice surface on protein stability during freezing, even though no definitive explanation is available about the underlying mechanisms.

We aim here to provide an as comprehensive as possible review on this subject, with the hope that it could serve as a basis to further advance knowledge in this area.

In order to do this, some background information will first be provided. The crystal structure of ice will be discussed, focusing in particular on hexagonal ice, which represents the most common form in everyday application. The concept of the quasi-liquid layer (QLL, also called liquid-like layer), i.e., the thin film of liquid water that is formed on the surface of ice crystals and which shows a peculiar behavior, will then be introduced. Some hints to the mechanisms of growth and nucleation will also be provided, introducing the experimental techniques that have been proposed in the literature to monitor these processes.

Finally, the effect of ice on protein stability will be addressed. Experimental evidence of ice-induced destabilization will be presented, covering the extensive literature on this topic. Possible mechanisms at the basis of these observations will be reviewed, also trying to clarify whether direct protein adsorption is necessary or not. In this context, some space will be given to two very peculiar classes of molecules, i.e., antifreeze and ice nucleating proteins, whose unique behavior at the ice surface may help shed some light on the freezing of biopharmaceuticals, as well. Finally, the relevant variables that influence ice-induced denaturation of proteins, and may help to prevent or modulate it, will be described. Among them, the effect of surfactants, cryoprotectants, protein concentration, cooling/thawing rate and nucleation temperature will be covered.

2. CRYSTAL STRUCTURE OF ICE

2.1 Different Ice Polymorphs

Liquid water can freeze into several ice forms, depending on the temperature (T) and pressure (P) at which crystallization is occurring. As a result, at least 17 crystalline ice phases have been experimentally observed to date [10, 11, 12, 13, 14, 15]. The molecular structure of these phases is known, mostly thanks to neutron-diffraction crystallographic studies. The large number of possible structures is mostly due to the open tetrahedral arrangement of water, which can adapt to very different environments.

In these structures, each molecule is hydrogen-bonded to four neighboring water molecules. While in low-pressure forms, such as the common hexagonal ice Ih, the O-O-O angles are close to the ideal value of 109.47°, these angles are distorted in higher pressure structures. For example, they range between 80° and 129° in ice II. When the pressure becomes very high, interpenetrating networks are eventually formed, as is the case, for instance, of ice VII [12].

While the oxygen atoms show topological order (i.e., a tetrahedral coordination) in each phase, all the polymorphs that can be directly crystallized from the liquid are hydrogen-disordered. These include the so-called liquidus phases, namely ices Ih, III, V, VI, VII, and the two metastable phases IV and XII (see Figure 1).

When temperature is reduced, these disordered phases tend to order and transform into fully hydrogen-ordered structures. However, hydrogen ordering needs molecular reorientations processes, which are hindered at low temperature. Therefore, only ices III and VII undergo this transition spontaneously, ordering into ices IX and VIII, respectively. By contrast, the formation of ices XI, XIII, XIV and XV needs to be promoted by doping with bases or acids [16, 14, 17]. In this context, ice II is peculiar, because it is the only hydrogen-ordered phase that shows no hydrogen-disordered counterpart [18]. At the same time, no hydrogen-ordered analogue has been identified so far for ices XVI and XVII.

The different structures of ice mentioned so far are also shown in Figure 2. It is evident that the ice crystal phases differ widely. For instance, the crystals structure may be hexagonal (ices Ih and XVII), cubic (ices VII, X, XVI), rhombohedral (ices II and IV), tetragonal (ices III, VI, VIII, IX, XII), monoclinic (ices V, XIII), or orthorhombic (ices XI, XIV, XV). Moreover, while ices Ih, XI, II, and XVII show open structures and relatively low density, the ices VII, VIII and X network is extremely dense.

Amorphous structures of ice were also identified, including amorphous solid water (ASW) [20], hyperquenched glassy water (HGW) [21], very high-density (VHDA) [22], high-density (HDA) [23] and low-density (LDA) amorphous ice [24]. In the past years, neutron-diffraction techniques showed that the three lower density forms, namely ASW, HGW and LDA, share a very similar structure, which resembles a disordered, but tetrahedrally coordinated, fully hydrogen bonded network [25]. In contrast, the two denser forms, HDA and VHDA, are structurally different.

The landscape of possible ice structures is therefore extremely complex. However, the picture seems not complete yet, and many more ice polymorphs may be observed in the future. Several ice phases have been predicted by molecular dynamics (MD) simulations or density functional theory (DFT) computations [26, 27, 28, 29, 30, 31, 32, 33, 34, 13]. For instance, 74963 potential ice structures have been recently identified by DFT [35], even though it is not clear if these structures may be prepared experimentally.

2.2 A Focus on Hexagonal Ice Ih

As previously mentioned, structure Ih is the ordinary form of ice, which is most easily observed in common experimental setup. Ice Ih displays a hexagonal lattice, relatively open and low-density. As a result, the large hexagonal rings characteristics of ice Ih leave almost enough room for an interstitial water molecule. In the ice Ih unit cell, the top and bottom faces are basal planes {0001}, while the six equivalent side faces are called primary prism faces $\{10\bar{1}0\}$. Secondary prism faces $\{\bar{1}2\bar{1}0\}$ are also observed (see Figure 3).

Along with ice Ih, another metastable, hydrogen-disordered phase of ice exists at ambient pressure, namely, ice Ic, which is characterized by a cubic crystal [36, 2, 3, 37, 38], and shows a lower nucleation barrier compared to the Ih form [39]. Ice Ic is generally observed either in the Earth's atmosphere and in extra-terrestrial environments [40], or in small-size droplets at very low temperature [41]. It was further shown that ice Ic can be formed directly in the bulk water phase of a vitrified solution, for instance glucose at low temperature [42]. More recently, it was also hypothesized that ice Ic may be an intermediate phase in the pathway of heterogeneous ice nucleation [43]. However, it is not clear at present if fully cubic ice I (ice Ic) can be prepared. In fact, it was observed from diffraction data that ice Ic samples were not entirely cubic, but contained hexagonal stacking, as well [44]. This structure, where the hexagonal and the cubic stakings are mixed, is called stacking-disordered ice I (ice Isd), and is hydrogen-disordered. Interestingly, it was suggested that Isd may be even more stable than pure Ih ice [45]. No hydrogen-ordered counterpart has been identified so far for ice Isd.

2.3 The Quasi-Liquid Layer (QLL)

Ice interfaces may be disordered even below the equilibrium melting temperature, resulting in the so-called surface premelting [46, 3, 47, 12]. A quasi-liquid layer (QLL), that represents an intermediate state between the solid and bulk liquid, is therefore formed.

The extension of the QLL increases rapidly as temperature approaches the equilibrium melting value [46, 48, 49, 50], because thermal fluctuations of the intermolecular distance become larger in these conditions. The actual thickness of the QLL as a function of temperature is not well known, but, as a rough estimate, it should be comparable to the lattice spacing at T ≈ -10 °C, and should rapidly increase approaching the melting temperature [46].

3. ICE NUCLEATION AND GROWTH

3.1 Fundamentals of Nucleation

During the freezing process, water needs to nucleate first, and the ice nuclei which are formed in this stage subsequently grow to form crystals. In this context, nucleation plays a central role [51].

According to the classic nucleation theory (CNT) [52], the free energy change Δ*G* (see Figure 4) associated with the formation of a nucleus in a supercooled solution is the sum of a negative bulk contribution ($-V\,\Delta g_v$, where *V* is the volume of the nucleus), and a positive surface term ($A\gamma$, where *A* is the surface of the nucleus, and γ the interfacial tension),

$$\Delta G = -V\Delta g_v + A\gamma \tag{1}$$

For pure water, the ice/liquid interfacial tension is generally assumed to be on the order of 0.033 J/m^2 near the triple point, dropping to approximately 0.020 J/m^2 at -40°C [53].

Foreign bodies, e.g. the walls of the container, impurities or other particles present in the solution, can lower the interfacial free energy and reduce the energy barrier for nucleation. In this case, heterogeneous nucleation ensues, and a correlation factor *f* may be used to quantify the decrease in the energy barrier [54, 55, 56],

$$f = \Delta G^*_{heter}/\Delta G^*_{homo} \tag{2}$$

where ΔG^*_{heter} is the heterogeneous nucleation barrier (see Figure 4).

The factor *f* depends on the chemical compatibility between the nucleus and the substrate, which can be described in terms of the contact angle θ. Moreover, it also depends on the radius of curvature r_s of the substrate. Defining two new variables $m = (\gamma_{sf} - \gamma_{sc})/\gamma_{cf} \approx \cos\theta$ and $r' = r_s/r_{cr}$, it is therefore possible to write $f = f(m, r')$. In the previous equation for m, γ_{sf}, γ_{sc} and γ_{cf} are the interfacial free tension values between the substrate and the fluid, the substrate and the crystal or the crystal and the fluid, respectively.

While Ih ice is thermodynamically stable below 0 °C, homogeneous nucleation does not occur spontaneously until very low temperatures. It has been suggested that the onset of homogeneous nucleation may be -40 °C [57, 58, 59].

However, computational simulations predicted that no ice crystals would spontaneously form above -71 °C [60], and also experimental results suggest that homogeneous nucleation scarcely occurs even far below -40 °C [61]. The possibility to reach this high degree of supercooling is due to the high energy barrier for homogeneous nucleation.

Because of this, ice crystallization is generally controlled by heterogeneous nucleation events. In this case, the presence of foreign particles lowers the energy barrier by decreasing the interfacial free energy.

3.2 Understanding the Growth of Ice Crystals

Ice crystals growth is governed by two physical phenomena, namely, surface attachment kinetics and the diffusion processes within the solution [47].

A key variable in this context is the degree of supercooling, which is often defined as $\Delta T_{surf} = T_{eq} - T_{surf}$, where T_{eq} is the equilibrium freezing temperature, and T_{surf} the temperature at the ice-water surface during growth. The growth rate v_g can then be written as,

$$v_g \approx K_g \Delta T_{surf} \tag{3}$$

where K_g is a kinetic coefficient. Ice crystal growth is influenced by the different growth rates on each facet, which in turn depends on their different step energies.

The step energy is defined as the energy required to create the edge of a one-molecule-high terrace on a faceted surface. For instance, the ice/water step energy on the basal face near the triple point is about 5.6 x 10^{-13} J/m [62, 63] while is essentially zero for the prism faces. Experimental data suggest that the most stable ice-water interface at 0 °C is the secondary prismatic face (see Figure 3), followed by the primary prismatic plane and, as a distant third, by the basal facet. This results in the typical hexagonal shape of ice Ih [64].

However, the morphology of the ice formed is extremely variable. For instance, it was reported to vary from circular disks when T_{surf} < 1 °C, to dendritic plates when T_{surf} < 2 °C, unbranched needle-like structures if T_{surf} ≈ 4 °C, branched needle-like structures if T_{surf} ≈ 8 °C and platelets if 10 °C < T_{surf} < 30 °C [65]. Either the surface attachment kinetics, or the mass and heat transfer through the system may be the controlling mechanism, thus determining the final expression of the kinetic coefficient.

Figure 5 shows an example of a typical temperature profile during freezing of a pharmaceutical solution. The nucleation and growth phases have been highlighted. The liquid solution is first supercooled to a value below the equilibrium freezing point (segment A-*). Supercooling represents a metastable state, during which water molecules tend to form clusters with long-living hydrogen bonds [66]. However, these clusters are still unstable, and break up quickly, as discussed in section 3.1. When the temperature is low enough to allow crossing of the energy barrier for the nucleation process, ice crystallization occurs rapidly in the whole product (point * in Figure 5). In all pharmaceutical solutions heterogeneous nucleation is observed, and the degree of supercooling often lies in the range of 10-15 °C or more [67]. A sharp increase in product temperature to a value close to the equilibrium freezing point is observed at the onset of nucleation. The ice crystals growth (segment B-C) then proceeds through the addition of molecules to the interface. Here, the latent heat of crystallization is almost compensated for by the heat removed through the already frozen product, and the temperature remains nearly constant. Finally, to ensure complete solidification, the frozen product is typically cooled down to -40 / -50 °C (segment C-D).

4. MONITORING, PREDICTING AND CONTROLLING THE ICE CRYSTAL SIZE

4.1 Monitoring the Freezing Process

Larger ice crystals are generally preferred for protein-based pharmaceuticals [68], as will be discussed in detail in section 5, because this results in a smaller ice/freeze-concentrate surface area, and therefore reduced risk of adsorption.

The extension of the ice/freeze-concentrate surface is, therefore, a fundamental parameter for protein activity preservation during freezing, and should be strictly controlled. However, at present, this task cannot be easily achieved.

Generally, the parameters that mostly influence the ice/freeze-concentrate surface area are the cooling rate used during freezing, and the nucleation temperature. A high nucleation temperature, and/or a low cooling rate, result in larger crystals, and hence smaller surface area [69, 70]. In this context, it should be considered that, in many fields, the range of cooling rates that can be explored is limited by equipment capabilities. For instance, common freeze dryers cannot achieve very fast cooling rates (e.g., > 1 ÷ 2 $K\ min^{-1}$), and are therefore limited to slow cooling protocols.

The temperature profile during freezing of a pharmaceutical solution, which gives information about both nucleation temperature and cooling rate, may be monitored using several tools. For instance, thermocouples or resistance thermal detectors (RTDs) may be used. Thermocouples are typical in lab-scale equipment, while RTDs are common in production-scale ones because are more robust and can be sterilized [71, 72]. Another option for temperature monitoring are the optical fiber sensors [73], where the sensing element shows a temperature-dependent refractive index. Alternatively, if wire connections should be avoided, passive transponders may be used, like the temperature remote interrogation system (TEMPRIS).

However, the presence of a sensor within the sample undergoing freezing could affect its ice nucleation behavior, therefore modifying the ice crystal size and ice-water surface area. For this reason, plasma sputtering was proposed to embed thermocouples within the containers walls, so as to avoid interaction with the product [74, 75]. Optical fiber sensors may be also used noninvasively, for instance fusing the fiber in the container bottom or embedding it in the controlled-temperature shelf where freezing is occurring [73].

Recently, the use of an infrared thermocamera was proposed to noninvasively monitor the freezing process [76], and was successfully combined with mathematical modelling to predict the final ice crystal size [77]. However, this method directly measures the temperature of the vial glass, and a model needs to be used to extract the actual product temperature [76]. Through vial impedance spectroscopy (TVIS) [78, 79] is another promising approach for the noninvasive monitoring of freezing, which exploits the variations in the bulk electrical properties during phase transformations.

These techniques make it possible to monitor the temperature profile, and some of them also the position of the freezing front and therefore the duration of the process. However, coupling with a modelling approach is needed to obtain information on the ice/freeze-concentrate surface area extension, as will be described in section 4.2.

During freezing, liquid water is separated as ice crystals, and the solutes that may be present in the system cryo-concentrate, forming a viscous matrix. If the ice crystals are then removed by sublimation, as it is done in the context of freeze drying, a porous structure is eventually formed in the freeze-concentrated matrix. The few techniques that are currently available to directly measure the extension of the ice/freeze-concentrate interface rely on the fact that the ice crystal size after freezing coincides with the pore size after lyophilization, provided that neither collapse nor shrinkage is observed during drying. Therefore, the specific surface area of the dried cake provides a measure of the extension of the ice-water interface, and could be obtained by BET [80]. Scanning electron microscopy of lyophilized products (see Figure 6) may also be used to obtain information on the average pore size D_p, which could then be linked to the ice-water surface area S_{iw}, for instance assuming that the crystals (and therefore also the pores) have cylindrical shapes,

$$S_{iw} = \frac{4m}{D_p \rho} \tag{4}$$

where m is the mass of crystallizable water, and ρ the ice density. Image analysis could help in the automation of SEM image analysis, as well [81, 82].

Microscopy observation of frozen products was also suggested [83], and another promising experimental technique is the X-ray micro-computed tomography, which allows the reconstruction of the entire porous internal structure of lyophilized samples [84].

Monitoring the freezing process, and its impact on product morphology, is therefore possible. In the next section, we will briefly describe how these measurements may be integrated, or used to validate, modelling approaches for the prediction of the ice/freeze-concentrate surface area.

4.2 Modelling Approaches to Predict the Ice/Freeze-Concentrate Surface Area

The prediction of ice crystal size generally requires knowledge of the temperature profiles within the solution being frozen. These could be measured, as described in the previous section, or extracted from simulations [85, 86], and may then be combined with empirical [87, 88, 89], or mechanistic [90, 77] models for crystal sizing. A simulation approach, that takes into account the variation in temperature during freezing, as well as cryoconcentration effects and the ice/freeze-concentrate interface formation, has been recently proposed to predict the risk of both cold- and ice-induced denaturation of proteins [91].

Numerical methods have also been developed to reproduce growth morphologies of ice crystals, for instance phase-field models [92, 93, 94], that should apply well to the growth of ice from liquid water [47].

Our understanding of the effect of the ice surface on protein stability may also substantially benefit from a deeper knowledge of the molecular-scale phenomena involved. For this purpose, molecular dynamics simulations represent a promising tool, which has been extensively used to simulate the ice-water interface

[95, 96, 97, 98, 99, 100, 101, 102, 103], and may provide some interesting information about protein/ice interaction, as will be discussed in detail in section 5.

The ability to predict the extension of the ice/freeze-concentrate surface is not enough if we cannot then control it, at least to some extent. This will therefore be the subject of the next section.

4.3 Controlling the Ice/Freeze-Concentrate Surface Area

If we consider that the ice/freeze-concentrate interface is a major source of protein instability during freezing, it is clear that the possibility to control its extension would be extremely beneficial. In principle, adsorption and denaturation phenomena may be minimized by promoting the formation of a small surface area. This could be achieved reducing the cooling rate, and inducing nucleation at a high temperature. However, while the cooling rate can be easily adjusted, the nucleation temperature is generally a stochastic variable, and its value is randomly distributed [104]. This results in huge heterogeneity, and limited possibility to control product morphology. This heterogeneity is hardly compatible with the stringent requirements of the pharmaceutical industry, and several techniques have been developed over the years to address this problem [67, 105, 106, 107].

An approach that may be used to increase the ice crystal size is annealing, which consists in holding the frozen product at a temperature above the glass transition for a given amount of time. During annealing, large ice crystals grow at the expense of smaller ones [108].

A number of techniques have also been developed, that allow control of the nucleation temperature. For instance, electrofreezing [109, 110] uses a high voltage pulse to trigger nucleation in supercooled water. However, individual electrodes need to be inserted in each sample, and in direct contact with the product, which is not compatible with GMP (Good Manufacturing Practice) conditions.

In the case of the ice-fog technique [111, 112, 113, 114, 115] nucleation is triggered by the introduction of small ice particles into the vials. These particles act as foreign bodies and promote heterogeneous nucleation, as discussed in section 3.1.

Another possible solution is ultrasound-induced ice nucleation [116, 117, 118, 119, 120, 121], where ultrasounds are used to start the nucleation process in a noninvasive way. However, it is not yet clear whether this technique may be applied to sensitive proteins, as the localized high temperatures generated by cavitation, and the bubble themselves, may potentially favor aggregation or other denaturation routes.

Two methods have also been developed, that are based on variations in pressure within the chamber. In the high-pressure-shift or depressurization method [122, 123, 124], the pressure is first increased to 1.5-4.5

bar, and then rapidly brought back to the atmospheric value. In the second approach, known as vacuum induced surface freezing, or vacuum induced nucleation [125, 126, 127, 128, 129, 130], pressure is reduced to induce nucleation by promoting evaporation from the surface of the product, and then quickly released to avoid undesired boiling or blow-up of the formulation.

Large ice crystals are obtained when using these controlled nucleation techniques, and a possible impact of that on protein stability will be addressed in section 5.4.

5. ICE AND PROTEIN STABILITY

5.1 Evidence of Ice Effect on Protein Stability

It is well known that the freezing process may have detrimental effects for a protein, leading to denaturation, aggregation and loss of biological activity. Numerous phenomena could contribute to the undesired conformational changes of the protein, including cold denaturation, cryo-concentration, or the formation of ice crystals [131, 132].

The physical environment of the protein is strongly affected by the cryo-concentration process, which induces variations in ionic strength and relative composition of solutes. pH shifts due to crystallization of buffer components [133], or phase separation phenomena [134, 135] may occur. On the one hand, if the protein being processed is stable in a narrow range of pHs, the precipitation of buffer components may lead to undesired denaturation. Actually, it was even shown that freeze-drying may result in remarkable changes in apparent acidity even without buffer crystallization. These changes in the apparent acidity may be related to changes in pK a upon water removal, or preferential inclusion of a basic component by ice crystals, and were observed to correlate with the degradation rates of acid-sensitive compounds [136]. On the other hand, the generation of a new interface during phase separation, as well as the possible partitioning of the protein into a phase with low concentration of stabilizers, may have detrimental consequences. The rate of some degradative reactions, such as hydrolysis and oxidation, may even increase during the freezing process

[137, 138, 139]. Recently it was proposed that the formation of water clusters at subzero temperature catalyzes proton transfer reactions, that are involved in many chemical degradation pathways like hydrolysis and deamidation [140].

At low temperature the hydration of nonpolar residues also becomes less unfavorable, and this leads to the possibility for water molecules to penetrate the protein structure, promoting the exposure of its hydrophobic core and the partial loss of secondary structure. This phenomenon is referred to as cold unfolding, and is driven by the reduced enthalpic barrier against water-hydrophobes interaction that occurs when lowering the temperature. The result is the formation of compact, partially unfolded states of the protein, characterized by a high degree of solvent penetration [141, 142, 143, 144, 145, 146, 147]. A possible way to study the effects of supercooled water on protein stability is the confinement in nanoporous matrices, that inhibits crystallization. Exploiting this technique, it has been shown that the structural transitions of proteins are dictated by the thermodynamic and kinetic modifications of their hydration water [148, 149].

However, the formation of ice crystals was shown to represent the most critical destabilizing factor. A dramatic decrease in the average phosphorescence lifetime of the Trp-48 residue was detected in solutions of the azurin protein upon ice formation [150]. This suggested that protein destabilization occurred as soon as ice crystals were formed.

The denaturation of several proteins during freeze-thawing, including ciliary neurotropic factor (CNTF), phosphofructokinase (PFK), LDH, glutamate dehydrogenase (GDH), interleukin-1 receptor antagonist (IL-1ra) and tumor necrosis factor binding protein (TNFbp), was found to be mostly related to the increase in the area of the ice-water surface [151].

Lower recovery of catalase, β-galactosidase and LDH activity was observed during fast freezing [152], and the formation of insoluble human growth hormone (hGH) aggregates was also found to increase with increasing cooling rates [153]. This occurs because more rapid cooling leads to smaller ice crystals, which have a greater surface area to volume ratio than larger crystals.

Sarciaux et al. [154] proposed a mechanism of aggregate formation involving denaturation of bovine immunoglobulin (IgG) at the ice/freeze-concentrate interface. This denaturation was reversible upon freeze-thawing, but became irreversible after freeze-drying and reconstitution. Also in this case, quick cooling resulted in increased aggregation. Increasing the protein concentration improved the percentage of recovered protein [152, 154]. This occurs because the extension of the ice/freeze-concentrate interface is finite, and the number of protein molecules adsorbed at the surface cannot exceed a given value. When this value is reached, increasing the bulk concentration reduces the percentage of adsorbed molecules [154].

Exploiting the change in Trp fluorescence between the native and denatured state, it was observed that the stability of the azurin mutant C112S from *Pseudomonas aeruginosa* was remarkably perturbed in ice [155]. Moreover, the extent of destabilization depended mainly on the size of the liquid water pool in equilibrium with ice. At the same time, it was observed that protein-ice interactions increased the solvent accessible surface area of the native fold, and/or decreased that of the denatured conformation [155].

In line with this observation, a remarkable loss of activity was observed in frozen lactate dehydrogenase (LDH) matrices. However, concentrated solutions at the same temperature and composition, but without ice, resulted in no degradation, suggesting that ice formation is the controlling factor for protein denaturation [68].

Similarly, an infrared spectroscopy investigation of LDH and human immune globulin (IgG) revealed an increase in intermolecular β-sheet structures close to the ice crystals, which is indicative of aggregation [156]. In contrast, the infrared spectra for these two protein molecules collected distant from the surface of ice crystals were very similar to spectra collected from the initial solution. This suggests that the vicinity of the ice surface is key to promote conformational changes. In a similar study, the aggregation of IgG2 at -10 °C was related to the formation of the ice/freeze-concentrate interface [157].

Molecular dynamics simulations of hGH also suggested a change in protein conformation at the ice surface, with a significant increase in the non-polar surface area exposed, while no notable conformational change was detected in unfrozen solutions at the same temperature [158]. Partial unfolding of the GB1 hairpin was similarly observed in metadynamics simulations at the ice-water interface [159].

Overall, these results suggest that prevention of ice-induced denaturation represents a key issue whenever a protein is subjected to a freezing process. In the next section, we will review the mechanisms that have been proposed so far in the literature to explain this phenomenon.

5.2 How Does the Ice Interface Affect Protein Stability?

In the previous section, evidence about the negative impact of ice formation on protein stability was presented. However, a question that spontaneously arises from the previous discussion is: what are the reasons at the basis of the ice-induced denaturation of proteins? What makes it difficult to answer is the absence of chemical differences between water and ice. Moreover, the structural differences are also minimal, as both water and ice are composed of a tetrahedral network of hydrogen bonds. Why two substances that are so similar would affect protein behavior in such a different way? This represents an intriguing and complex field of investigation, also because not many experimental techniques are currently available to fully address this problem.

Strambini and Gabellieri [150] suggested that the conformational changes of proteins may arise from the direct interaction between the protein and the ice surface, as schematized in Figure 7a. In this context, accumulation of milk proteins [160] and albumin [161, 162] near the ice surface was detected, using either light and transmission electron microscopy or confocal Raman microspectroscopy. Recombinant human interferon-γ (rhIFN-γ) was found to accumulate at the ice/liquid interface during lyophilization, but with significantly smaller intensity than at the air/liquid surface [163]. Also, accumulation of rhIFN-γ to the ice/liquid surface alone was found to be not responsible for aggregation, and a subsequent drying step was necessary to induce particle formation [164].

However, unless the protein has an antifreeze behavior (see section 5.3), there is no real evidence of direct protein adsorption onto the ice crystals. By contrast, solid-state NMR studies seem to indicate that the hydration shell of non-antifreeze proteins does not freeze below the freezing temperature of the bulk solution [165, 166]. For instance, ubiquitin keeps its entire hydration shell even at -35 °C, and this prevents direct interaction with the ice surface [167]. It is generally believed that most proteins should behave like ubiquitin, and that their hydration shell should remain in the liquid form until a temperature which is much lower than the equilibrium freezing value [168]. No direct interaction with ice would hence be possible above this temperature.

High-resolution synchrotron X-ray diffraction results further suggest that bovine serum albumin tends to partition into the quasi-liquid layer above ice crystals (see section 2.3), but without being directly adsorbed onto the ice surface [169]. A similar behavior was observed for two other proteins, namely recombinant human albumin and a monoclonal antibody, in a more recent investigation [170].

A different behavior is generally observed only in presence of antifreeze proteins (AFPs) [170, 171, 172], whose peculiar behavior will be briefly addressed in section 5.3. Therefore, another explanation for the ice-induced denaturation of proteins, which is not related to direct adsorption at the ice-water surface, should exist.

In this context, it should be remembered that the physical microenvironment of a protein confined within the QLL may be substantially different compared to the bulk. More specifically, the stabilizer may remain in the bulk, and may not be able to exert anymore its cryoprotective effect. In this context, Bhatnagar et al. [170] proposed a picture of the protein/ice interaction where the concentration gradients in the liquid phase adjacent to the ice crystals depend on the mobility of the species. Larger species tend to concentrate closer to the ice surface, because of their lower mobility. At the same time, the pH in the QLL may decrease because of the negative charge on the surface of ice crystals [170, 173]. The electrical double layer which is eventually formed may result in an increased concentration of cations, including protons (see Figure 7b).

Another possible explanation involves the entrapment of air at the ice surface. Using fluorescence microscopy [156], it was observed that several air bubbles may remain trapped between ice crystals. Air bubbles have also been observed by optical microscopy [174] and small angle neutron scattering [175].

Therefore, proteins may denature at this hydrophobic air-water interface, as outlined in Figure 7c. A similar mechanism was also proposed in a recent work [132], where it was pointed out that the growth of ice crystals leads to an increase in the concentration of dissolved air gasses. The cryo-concentration of oxygen may accelerate oxidation reactions during freezing [176, 177]. It was also estimated that the extension of the air bubbles interface may be significantly larger than the already existing interface on the top of the container [132].

Recently, a disordered population of ice crystals was detected by high-resolution synchrotron X-ray diffraction in frozen solutions of recombinant human albumin, lysozyme, an insect AFP and a monoclonal antibody [170]. This disordered population of ice crystals was identified as a high pressure form of ice, tentatively Ice III or Ice IX (see section 2.1). This observation was explained by the volume expansion that may occur during ice formation, and which may result in mechanical stresses and high local pressure. Values of freeze-induced pressure exceeding 2 kBar have been theoretically estimated [132]. Pressure-induced unfolding of the protein may hence occur [178], and the combination of elevated pressure and low temperature may have a synergetic effect on protein unfolding [179] (see Figure 7d).

Finally, in a recent molecular dynamics investigation of protein L [180], the effect of the ice surface was explained as an enhancement of cold denaturation phenomena. The protein was found to be destabilized in presence of an ice slab, compared to the bulk solution at the same temperature but without ice. No direct interaction between the protein and the surface was evidenced, but the observed denaturing effect seemed to be mediated by the nearby layer of liquid (or, better, liquid-like) water molecules. These molecules were significantly slowed down by the presence of ice, and could form a significantly larger number of hydrogen bonds with the protein, especially with the nonpolar patches that are generally poorly hydrated. These hydrogen bonds were also remarkably strong, and promoted the solvent-penetration of protein L and consequent exposure of its hydrophobic core, which is a common feature of cold denaturation [147] (see Figure 7e).

In the next sections, we will first present two very peculiar classes of molecules, namely, antifreeze and ice-nucleating proteins, whose unique behavior may help us understand the phenomenology of ice-peptide interaction. Afterwards, possible approaches to counteract ice-induced denaturation of proteins will be discussed.

5.3 The Peculiarity of Antifreeze (AFPs) and Ice-Nucleating (INPs) Proteins

In this section, attention will be devoted to two specific classes of proteins, namely, antifreeze (AFPs) and ice-nucleating (INPs) proteins. These molecules have peculiar characteristics, and our general understanding of protein-ice interaction may substantially benefit from a deeper analysis of their behavior.

AFPs have the ability to bind to nascent ice nuclei, therefore inhibiting their growth [181, 182]. For instance, it was reported that AFPs can inhibit the growth of both basal and prism planes of ice Ih [183]. They represent the natural way many organisms protect themselves against freezing damage, by depressing, in a noncolligative way, the freezing point of water. In a broader perspective, AFPs represent a central element in the problem of ice recognition by biomolecules. What makes their role and action even more puzzling is the remarkable difference of AFP structure between different classes of organism. This indicates that the depression of the freezing point can be exerted by extremely different structural motifs.

A spontaneous question about AFPs is what mechanisms drive their interaction with ice. Since the ice surface is characterized by the presence of OH groups, it was first proposed that the hydrophilic groups on the ice-binding sites (IBS) of AFPs may hydrogen-bond with ice [182, 184]. However, many AFPs do not expose H-bonding groups at their IBS. On the contrary, it was observed that hydrophobic residues are largely present on the IBS [185]. It was therefore suggested that hydrophobic groups were crucial [186, 187], together with a flat-binding surface [188]. However, neither H-bonding nor hydrophobic groups can be the only explanation. H-bonding groups would provide affinity, but no specificity, as they tend to interact more with liquid than ice water [189]. At the same time, hydrophobic groups would make it possible to achieve specificity, but are not compatible enough with the hydrophilic ice surface. This implicates that both groups should be involved [190].

Exploiting a simulation approach, it was suggested that the arrangement of water molecules in proximity of the IBS may play a role in the action of AFPs [191, 192, 193]. In fact, both a slight increase in tetrahedral order [194] and slower relaxation dynamics were observed in the hydration water of AFPs [195, 196]. These water molecules seem to adopt a highly ordered structure, forming a clathrate-like structure, similar to ice, around the ice-binding site of AFPs [190, 189]. This structure surrounds the hydrophobic groups of the IBS, while being anchored at the edges by H-bonds to the polar groups. Therefore, the AFPs may bind to ice thank to this ice-like structure that is formed at their IBS. These ordered layer of water molecules may be released upon binding, leading to a net entropic gain [197, 198]. NMR suggests that only the portion of AFPs hydration shell at the IBS is in direct contact with the ice surface. The remaining part of the hydration shell does not undergo freezing, behaving similarly to the hydration shell of non-AFPs proteins [167].

A further question that may arise is whether this ice-like structure is always present, or, on the contrary, forms only close to the ice surface. Molecular simulations of TmAFP (a hyperactive insect AFP, which shows the best lattice matching with ice Ih, see Figure 8a) indicate that the second option is correct [199], suggesting that a preordering of hydration water is not necessary for ice recognition. The formation of the clathrate-like structure at the IBS is therefore probably induced by the close presence of an ice layer. This suggests that ice has an effect on the surrounding liquid water layers [172].

Another class of interesting molecules is represented by ice-nucleating proteins (INPs), that are used by several bacteria, such as *Pseudomonas syringae*, to trigger ice formation at temperatures close to the equilibrium value [200].

INPs are structurally and chemically similar to AFPs, but are considerably bigger and can result in the formation of molecular clusters. The larger surfaces that are eventually formed are capable of nucleating ice. A common example of INP is InaZ, produced by *Pseudomonas syringae*, which is approximately 1200 residues-long [201]. Its structure includes degenerate octapeptide repeats, a subpopulation of which includes the sequence GSTXT(A/S), where X is an unconserved amino acid. Recently, *ab initio* structures of the GSTSTA segment were resolved by electron microdiffraction [202]. It was discovered that both homochiral and racemic GSTSTA (see Figure 8b-c) form amyloid-like protofibrils, that display antiparallel β-sheets.

Molecular dynamics simulations suggested that INPs may be anchored to the cell surface in such a way to expose ice-active sites to the surrounding water [204, 205]. It was proposed that threonine and serine amino acids, that are largely represented on these sites, should mimic the basal plane of ice. This is possible because of their OH groups and the presence of clathrate water molecules that effectively induce nucleation [190, 206].

Later on, sum frequency generation (SFG) spectroscopy was used to study the INP of *Pseudomonas syringae*, namely, the protein inaZ [207]. This study demonstrated that hydrogen bonding at the water-bacteria contact promotes structural ordering on the adjacent water network. The unique hydrophilic-hydrophobic motifs at the ice-nucleating site, together with the effective removal of latent heat due to nucleation, are highly effective in triggering the formation of stable ice crystals. Later on, it was also suggested that only large surfaces, like INPs clusters, may effectively order hydration water into ice-like structures [199].

Pseudomonas syringae may also be added to a solution being cooled down to trigger nucleation. This was done to promote the formation of nuclei at higher temperature [69, 208, 209, 210]. However, this approach cannot provide a real control of the nucleation temperature, and cannot therefore substitute the techniques discussed in section 4.3.

5.4 How to Counteract Ice-Induced Denaturation of Proteins

From the previous discussion, it is evident that the ice-induced denaturation of proteins should be adequately controlled to maximize the recovery of therapeutic activity after freezing. In this context, two main classes of strategies could be adopted; the first relies on the selection of suitable operating conditions, while the second involves optimization of the formulation.

From the point of view of the operating conditions, the two variables that mostly affect protein stability during freezing are cooling rate and nucleation temperature. As previously mentioned, a low cooling rate and a high nucleation temperature are associated with the formation of larger ice crystals, and therefore smaller ice/freeze-concentrate surface area. This, in turn, should minimize ice-induced denaturation of proteins. In line with these considerations, a correlation has often been observed between cooling rate and protein stability, with higher recovery at lower cooling rates [151, 152, 153]. However, this is not true for all proteins. In the case of particularly unstable molecules, such as myoglobin at low pH, a fast freezing protocol was found to maximize protein stability [91]. In this case, the formation of a cryoconcentrated matrix at high viscosity, where cold unfolding of the protein is kinetically inhibited, should be promoted as quickly as possible.

While the cooling rate can easily be controlled and adjusted, the nucleation temperature is a stochastic variable. However, as discussed in section 4.3, several strategies have been developed to address this problem. The impact of controlled nucleation techniques on protein stability has also been investigated in recent works. For instance, the controlled nucleation strategy based on the depressurization method was found to improve LDH stability after freeze-thawing [211]. The depressurization method applied to a highly concentrated monoclonal antibody also suppressed glass fogging, which is the undesired migration of protein solutions up on the inner walls of glass vials [212]. Another controlled nucleation technique, namely, the ice-fog method, was applied to monoclonal antibody formulations [213], and resulted in reduced particle formation in highly concentrated systems. However, the addition of polysorbates was more effective in decreasing particle level. Also, the authors observed no difference in particle formation between the controlled and spontaneously nucleated samples at low concentration [213]. The benefits of vacuum induced surface freezing (VISF) on the stability of the human growth hormone (hGH) were also investigated [214].

In this case, both HPLC-SEC and a cell-based potency assay indicated that there was no dramatic difference in the behaviour of hGH at low concentration when either VISF or spontaneous nucleation were used. In the case of very unstable molecules, such as myoglobin at low pH, the application of VISF proved to be detrimental. In this case, the benefits of a smaller ice/freeze-concentrate interface are offset by the necessity to introduce two holding steps at low temperature and low product viscosity during application of the VISF. During these equilibration stages, cold denaturation of myoglobin may quickly occur [91].

Finally, annealing is another technique that may affect protein stability. While it does not make it possible to control the nucleation temperature, it nevertheless promotes the formation of larger ice crystals. The resulting decrease in specific surface area of the ice/freeze-concentrate interface may improve protein stability. This was the case, for instance, of bovine IgG as model protein [154]. It should also be considered that the thawing process may also be dangerous for protein activity.

In this context, the thawing rate is a crucial parameter, as slow thawing promotes the recrystallization process, with small ice crystals growing into larger ones. While the growth of larger ice crystals during freezing, for instance by annealing, was found to be beneficial, recrystallization has been observed to be detrimental during thawing, as it promoted undesired interfacial or shear stress for proteins at the ice-water interface [215]. Moreover, during the process of ice melting, the amorphous cryo-concentrated phase will still not have the same composition as the initial fully-liquid solution. Problems due to unequal distribution of solutes may therefore occur during thawing, as well. For this reason, it is generally advisable to perform a fast thawing [132].

For what concerns the choice of the formulation, different possibilities exist to improve protein recovery after freezing. Typical biopharmaceutical formulations include cryo/lyo-protectants (e.g., sugars, polyols, amino acids, polymers), buffering species, to control the pH, and in many cases also surfactants, to counteract surface-induced denaturation, and bulking agents (e.g., mannitol, glycine), to improve cake resistance. A comprehensive description of the type/amount of excipients found in commercial products is out of the scope of this review. We will therefore focus on those case studies where formulation components have been shown to affect protein stability at the ice surface, starting from surfactants. For instance, it was observed that the addition of small amounts of Tween 80 or Tween 20 protected proteins from ice-induced denaturation [151, 163, 216]. Addition of the same surfactant to LDH and a human IgG formulation resulted in a decrease in intermolecular β-sheet structure formation at the ice surface, which is indicative of diminished aggregation [156]. Surfactants are amphiphilic molecules that tend to locate at interfaces, thereby sterically preventing protein adsorption [217, 218]. This may explain why they have often been reported to prevent ice-induced denaturation of proteins. However, as discussed in section 5.2, there is increasing evidence that non-AFP proteins do not bind to the ice interface, but rather remain confined in the QLL above the surface. The steric repulsion exerted by surfactants that coat the ice interface may, therefore, be not enough to account for the observed stabilizing effect. Molecular dynamics simulations of the GB1 peptide suggest that surfactants tend to surround the protein at the ice surface, with their hydrophilic heads oriented towards the peptide [159]. On the one hand, this orientation prevents denaturation, because the exposure of the protein hydrophobic core is unfavorable in these conditions. On the other hand, the formation of a protein-surfactant complex hinders aggregation, as well. The stabilizing role of surfactants may hence be explained according to an orientation-dependent mechanism.

Apart from surfactants, cryoprotectants may be added, including sugars (for instance sucrose, trehalose, glucose, lactose), polyols (e.g., sorbitol and glycerol), polymers (albumin, dextran, polyvinylpyrrolidone and hydroxyethyl cellulose are common examples), and amino acids (such as glycine, proline, arginine etc.). For instance, Strambini and Gabellieri [150] observed that the addition of glycerol and sucrose reduced the protein conformational changes induced by ice-formation during freezing. They suggested that the stabilizing action of these excipients may be considered as a combined effect of decreasing the freezing temperature and reducing the adsorption affinity of the protein by coating the surface of ice. However, sterical exclusion from the ice interface can hardly be the only mechanism to explain the role of cryoprotectants. Actually, the opposite was often observed, with excipients being expelled in the bulk freeze-concentrated solution and promoting a closer approach between the protein and the ice surface [170, 162].

The crystallite size and microstrain for Ih crystals was also evaluated for different protein formulations [170]. A higher microstrain, compared to pure water, was observed in all protein-containing samples, indicating that proteins may promote the formation of defects during ice crystal growth. In contrast, the microstrain level was consistently lower in all non-protein samples (containing, for instance, sucrose or histidine), suggesting that the effect of excipients on the ice structure should be minimal. At the same time, SEM analysis of freeze-dried cakes shows that the choice of the formulation strongly affects the pore size, and, therefore, the extension of the ice/freeze-concentrate interface. It has been hypothesized that this may be related to a different energetic cost for forming the ice/freeze-concentrate interface (i.e., different interfacial tension) in presence of different cosolutes [90].

It was also suggested [150] that the cosolutes may exert their protective action by being preferentially excluded from the protein surface. This should make the unfolding process thermodynamically unfavorable [219]. In particular, molecular dynamics simulations suggested that exclusion from specific patches on the protein surface should be crucial for protein stability at the ice surface [158, 180]. However, the mechanism of preferential exclusion should be prevailing only at the beginning of the freezing process, when cryoconcentration is not yet complete. During the last stages of freezing, the matrix that separates from ice is so concentrated that other mechanisms come into play [220, 221]. At high excipient concentration, the vitrification theory [222, 223], the water replacement scenario [224, 225] and the water entrapment hypothesis [226, 227, 228] apply. Vitrification invokes the formation of a viscous, glassy matrix [229] where protein movements are hindered, while water entrapment envisions the formation of a cage of excipient molecules around the protein, where water is entrapped and slowed down. While these two are kinetic mechanisms, the water replacement theory suggests that a thermodynamic stabilization applies, where the excipient substitutes water in satisfying the hydrogen-bonding requirements of the protein. All these mechanisms of stabilization are effective only if the stabilizer remains in the same amorphous phase where the protein is. Therefore, crystallization of the cryoprotectant may reduce protein stabilization, also because it results in another surface onto which the protein may adsorb and denature [216].

Another typical component of a protein formulation is the buffer, which should maintain the pH in a range of values where the active ingredient is stable. In this context, it should be remembered that some buffer species may undergo selective crystallization, and this could result in undesired pH shifts. This is the case, for instance, of sodium and potassium phosphate [230, 231].

Finally, as previously mentioned, a high protein concentration is beneficial to minimize the effect of the ice-water interface [152, 154]. On the one hand, the percentage of protein molecules that are in the region perturbed by the presence of ice decreases when the concentration is increased. On the other hand, volume exclusion effects may arise at higher concentration. For instance, the cold denaturation temperature of β-lactoglobulin was found to decrease significantly when increasing the protein concentration [232].

6. CONCLUSIONS

In this work, an overview of the chemical and physical properties of the ice-water interface, and of its effects on protein stability, was provided. Rather than giving answers, the objective of this work was to raise questions that, according to the authors opinion, are worth further investigation.

More specifically, the following points should be addressed:

i. Is the Ih form of ice the only crystal structure that is relevant for pharmaceutical applications? As evidenced using high-resolution synchrotron X-ray diffraction [170], other structures of the ice phase diagram described in section 2.1 may be important, and this aspect should be further investigated.

ii. Do the advantages of controlled nucleation approaches make them worth application in the manufacturing of pharmaceuticals and biopharmaceuticals? At present, the controlled nucleation techniques discussed in section 4.3 are not applied by pharmaceutical industries in the production of commercially available drugs. Further investigations should be added to evaluate their effect on protein stability, and the first steps in this direction have been discussed in section 5.4 .

iii. What are the mechanisms at the basis of ice-induced denaturation of proteins? Some hypotheses in this context have been described in section 5.2, and should further by addressed. The answer to this question may benefit from a deeper understanding of the peculiarity of antifreeze and ice-nucleating proteins, as discussed in section 8.

iv. What are the conditions that minimize the denaturing effect of ice on protein stability? Some possible strategies to prevent, or minimize, ice-induced denaturation of proteins have been described in section 5.4. However, a more robust approach to the selection of appropriate conditions for the freezing of protein-based therapeutics would be desirable. It is evident that many variables come into play, and it is therefore probably impossible to give a unique answer, valid for all classes of proteins. In this regard, the application of mathematical models, such as those mentioned in section 4.2, may help to reduce the number of experiments to be performed, providing at the same time a better understanding of the underlying phenomena. The modelling approaches should be multiscale, covering both the molecular mechanisms of protein conformational changes and the effect of process variables on the critical attributes of the product.

The hope of the authors is that this work may help to focus the attention of both industries and academies in this direction, indicating possible objectives and providing a basis for future investigations.

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LIST OF FIGURES

Figure 8: (a) Cartoon representation of TmAFP (PDB structure 1EZG [203]), where β-sheets are in yellow, turns in cyan and coils in white. The N-terminus and C-terminus are highlighted in red and blue respectively. (b-c) L-GSTSTA (PDB code 6M9I [202]) (b) and racemic-GSTSTA (PDB code 6M9J [202]) (c) from inaZ (INP protein from *Pseudomonas syringae*). Glycine amino acids are shown in white, serine in yellow, threonine in red and alanine in blue.