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Engineered Compounds to Control Ice Nucleation and Recrystallization

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Abstract

One of the greatest concerns in the subzero storage of cells, tissues, and organs is the ability to control the nucleation or recrystallization of ice. In nature, evidence of these processes, which aid in sustaining internal temperatures below the physiologic freezing point for extended periods of time, is apparent in freeze-avoidant and freeze-tolerant organisms. After decades of studying these proteins, we now have easily accessible compounds and materials capable of recapitulating the mechanisms seen in nature for biopreservation applications. The output from this burgeoning area of research can interact synergistically with other novel developments in the field of cryobiology, making it an opportune time for a review on this topic.

Contents

1. INTRODUCTION

The preservation of cells, tissues, and organs is a critical concern in numerous different research and clinical applications. Commonly leveraged low-temperature preservation strategies can be generally classified into hypothermic (1°C to 4°C), high subzero (−1°C to −20°C), or low subzero (typically < −80°C) preservation, enabling short-term (hours), moderate (days/weeks), or long-term (months/years) storage, respectively (1–3). Large, complex tissues such as organs struggle to endure some of the fundamental stressors associated with subzero storage; therefore, it is conventional that they be stored at hypothermic temperatures. However, the corresponding short preservation times that result from this mode of storage impose logistical strain on the transplant network that contributes to prevailing issues in donor-recipient matching. Subzero preservation of complex tissues would therefore have tremendous clinical benefit, prompting the need to develop technologies and methodologies that can curb the sources of injury that impede its implementation. Controlling the formation or growth of ice is ultimately the crux of this challenge, and strategies that prove efficacious in tissue models may have additional value in existing cell-based preservation methods, which suffer from diminished recovery or loss of function.

The discoveries of glycerol in 1949 by Polge et al. (4) and, subsequently, dimethyl sulfoxide (DMSO) in 1959 by Lovelock & Bishop (5) were paramount in enabling the effective storage of biological material at subzero temperatures and set the stage for a new paradigm of research focused on controlling ice. Initial efforts to control ice formation and growth relied largely on leveraging applied pressure or altering cryoprotectant concentrations to modulate phase diagrams (6). However, there is a delicate balance when implementing such strategies, since the cryoprotection imparted may be offset by the damage caused by the protective strategy itself (7, 8). Commonly used permeating cryoprotective agents (CPAs) (e.g., glycerol, DMSO, propylene glycol, and ethylene glycol), albeit necessary in nearly all subzero preservation approaches, harbor a degree of cytotoxicity. Thus, increasing concentrations of these compounds to reduce the volume of ice, or to completely avoid its formation, has its downsides. Similarly, when applied in excess, pressure could compromise the structural integrity of the biological material. While this delicate balance is relevant irrespective of the complexity or size of the biological material, this invariably becomes more of a consideration during the preservation of organs where heat and mass transfer limitations make subzero preservation significantly more challenging.

The impediments of these historical strategies have led to profound interest in developing methods and technologies that can exert effective ice control without eliciting damaging effects. Much of the inspiration guiding this research stems from naturally occurring organisms that are able to survive internal temperatures in the subzero range through mechanisms that facilitate ice tolerance or inhibit its formation (9). Initial identification of compounds that exert ice control in natural organisms took place in the 1970s (10, 11). This subsequently spurred concurrent avenues of research focused on applying these compounds to biopreservation applications (12–17) and comprehending the mechanisms by which they function (18–21). Such research has identified many of the limitations of these naturally occurring compounds and has enabled development of artificial alternatives that lack some of these limitations (22–26).

This article aims not only to provide an overview of some of the more promising technologies developed to date, but also to contextualize them on the basis of the relevance of specific modes of ice control (i.e., nucleation promotion/inhibition and recrystallization inhibition) in different subzero preservation strategies (for an overview of ice control strategies, see **Figure 1**).

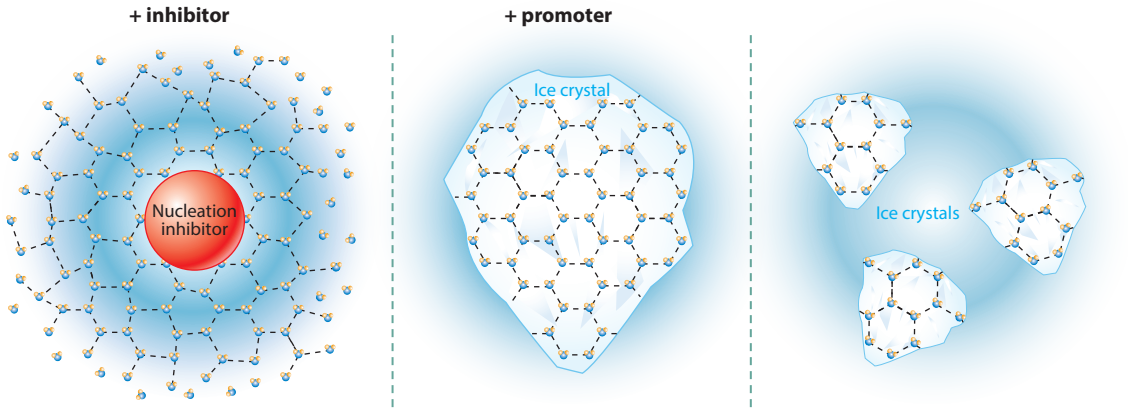
2. PROMOTING ICE NUCLEATION

The temperature of nucleation can alter the location and size of ice crystals formed in a biological system, which can have a fundamental impact on subsequent cellular or structural tissue damage. The water-to-ice transition is known to proceed through a metastable, supercooled state, and it is well established that limiting the extent of supercooling prior to nucleation will reduce damage in any subzero preservation strategy where ice is formed (28). Low nucleation temperatures can reduce the radius of curvature of each individual ice dendrite, increasing the ice crystal's thermodynamic instability and, in turn, the rate at which it grows (29, 30). Low nucleation temperatures can additionally accentuate damage by promoting the formation of ice in smaller regions of a compartmentalized system (when supercooling is limited, ice forms preferentially in the largest regions of a compartmentalized system) (29, 30) (**Figure 2**).

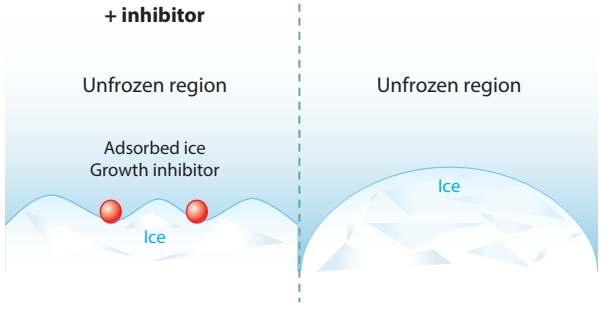
In dilute cell suspensions, high nucleation temperatures limit the onset of intracellular ice formation (IIF), which, apart from a few select cases described by Acker & McGann (31), is associated with cell death (**Figure 2**). In complex tissues, the vasculature can be considered analogous to the extracellular medium in dilute cell suspensions, as any extravascular ice formation, whether it be interstitial or intracellular, is more likely to compromise the structural integrity of the tissue (32–34) (**Figure 2**). Irrespective of the location where ice forms, the speed at which the ice front advances under conditions of high thermodynamic instability can lead to strong compressive stressors at the front of the phase change, which can then compromise vascular integrity (32, 33, 35). In both dilute cell suspensions and complex tissues, increasing the speed at which the ice front advances would decrease the time in which the cells would be able to osmotically equilibrate the difference in chemical potential that is created following extracellular ice formation. This would result in hypertonic stress that can rupture the cell membrane (32, 36).

Initiation of sample nucleation is most often performed by manually generating a cold spot with a precooled probe to seed an ice crystal or by shaking/tapping the container to mechanically perturb the metastable state. An analogous method used in historical attempts to initiate vascular nucleation in organs involved the introduction of small ice crystals through a cannula and directly into a blood vessel (13, 37, 38). More recently, experimental methods have involved the use of ultrasound, as well as electric and/or magnetic fields, to initiate nucleation (39, 40). Passot et al. (41) incorporated ultrasound technology into a modified freeze dryer, whereas Petersen et al. (42) were the first to leverage electric field phenomena first described in 1861 to develop a multisample freezing device with platinum electrodes delivering high-voltage pulses that would initiate

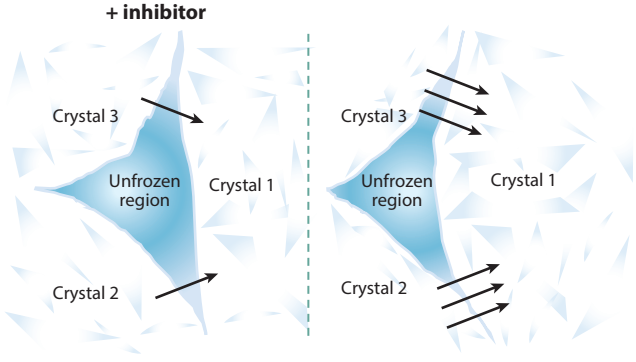
a Modulate ice nucleation



b Control ice growth (i.e., thermal hysteresis)



c Control ice recrystallization



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

An overview of ice control strategies. (*a*) Modulating ice nucleation involves either promoting or inhibiting nucleation, depending on the preservation strategy being sought (i.e., an ice-free or ice-containing strategy, respectively). In the presence of an ice-nucleation inhibitor, the aggregation of water molecules into a hexagonal, ice-like structure is inhibited. In the presence of an ice-nucleation promoter, aggregation of water molecules into a stable crystal conformation occurs at a higher subzero temperature, allowing for larger, more thermodynamically stable ice crystals to form. (*b*) Controlling ice crystal growth (i.e., thermal hysteresis) involves the irreversible adsorption of the ice crystal to specific planes of the ice crystal lattice, causing the surface to become increasingly convex for regions where compounds with thermal hysteresis activity have adsorbed. This diminishes the ability of the ice crystal to expand within the so-called thermal hysteresis gap; however, below the hysteretic freezing point, there is rapid crystal growth (for a detailed description of this process, see Reference 27). (*c*) Controlling ice recrystallization (specifically migratory ice recrystallization) limits the transfer of water molecules from smaller, more convex, and thus more thermodynamically unstable ice crystals to ones that are larger.

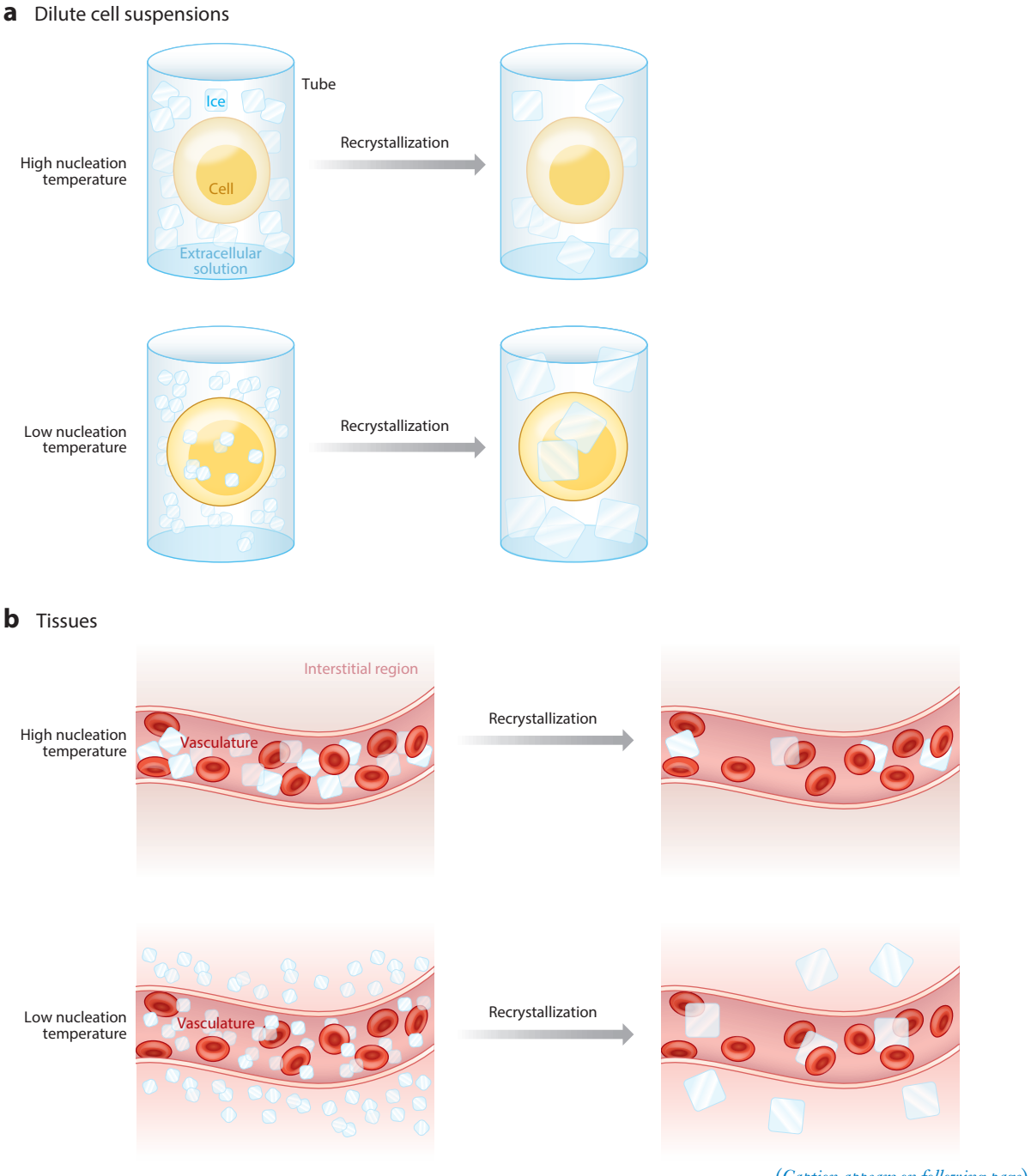
sample freezing. These ultrasound and electrofreezing methods can more effectively standardize the nucleation temperatures attained; however, it is challenging to integrate them into commercial freezers (28). Furthermore, while these strategies are all applicable to the cryopreservation of cell suspensions, they are not tenable for initiating vascular nucleation in a controlled manner when freezing complex tissues. Therefore, using compounds known to reliably initiate nucleation at predefined temperatures would be relevant to the cryopreservation of both cell suspensions and complex tissues.

2.1. Ice-Nucleating Proteins

A large variety of freeze-tolerant insects, marine invertebrates, plants, bacteria, and amphibians possess ice-nucleating proteins (INPs) that function to limit the extent of supercooling prior to nucleation (43). Bacterial INPs are some of the most effective natural INPs, which has led to their regular use in a variety of different industrial (e.g., artificial snow formation, food preservation, etc.) applications (44). In combination with their industrial utility, the ecological impact of INPs, ranging from their roles in the hydrological cycle to crop damage to global climate, has made them a topic of intensive study. Lorv et al. (45) documented and reviewed all 10 types of bacterial species known to synthesize INPs, and we refer the reader to that article for a detailed overview of bacterial ice nucleation. The variants of INPs found in *Pseudomonas syringae* strain 31R (i.e., InaK, InaQ, InaV, and InaZ) are the most effective class of INPs, with mean freezing temperatures as high as −2°C (46). It is these compounds that warrant consideration in biopreservation.

These proteins all contain a distinct hydrophobic N-terminal domain, a hydrophilic C-terminal domain, and a central repeating domain (CRD) consisting of contiguous repeats of an octapeptide (Ala-Gly-Tyr-Gly-Ser-Thr-Leu-Thr) (45). While there is a large and active field of research that is seeking to understand how INPs organize water into preordered patterns to facilitate formation of a stable embryonic crystal, the precise mechanism through which this occurs remains largely unknown. It has long been presumed that the CRD is the predominant structural component that serves as a nucleation template, with some of the most definitive evidence indicating that the hydration and orientation of the CRD β-helix shift as temperatures are lowered in a way that promotes nucleation (47). However, Lukas et al. (48) have suggested that the tertiary and quaternary structures the protein mediates by the other distinct structural domains can play a role in orienting the CRD to facilitate interfacial ordering of the water.

Determining the underlying mechanisms of INP-mediated nucleation is of importance, as it could facilitate the development of synthetic derivatives that are capable of either increasing the nucleation temperature or improving its consistency. Thus far, efforts to improve INP nucleation efficiency have relied on altering solution composition, with one of the most notable studies demonstrating a remarkable improvement with the *P. syringae* INP in pure D_2O (median freezing



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

The relationship between nucleation temperatures and ice recrystallization in dilute cell suspensions and tissues. (*a*) In dilute cell suspensions, high nucleation temperatures facilitate the formation of larger, more stable ice crystals while diminishing the likelihood that ice would form inside the cell. Because these ice crystals are larger, they would have a lower propensity to undergo recrystallization. The opposite proves to be the case when nucleation temperatures are low: Smaller ice crystals form that undergo more recrystallization, and the likelihood that nucleation occurs inside the cell (an event that in most cases is a precursor to cell death) would be higher. (*b*) In tissues, the same general patterns seen in dilute cell suspensions manifest. Low nucleation temperatures would lead to more extensive ice recrystallization due to the formation of more thermodynamically unstable crystals. The vasculature can be considered analogous to the extracellular milieu in dilute cell suspensions, with high nucleation temperatures ensuring that ice is constrained to the vasculature and the probability of extravascular ice formation (a precursor for increased structural damage) increasing when nucleation temperatures are low.

temperature of −4.6°C relative to −8.9°C in pure H_2O) (49). However, the utility of this approach remains unclear as we have yet to fully realize the biological impacts of D_2O (50).

Some of the concerns that have been raised regarding the use of bacterial INPs are their biocompatibility, degradability, and toxicity. As the use of these compounds in biopreservation remains a relatively novel area of research, the legitimacy of these concerns warrants investigation. In an effort to preemptively avoid any potential adverse effects associated with INP use, Weng et al. (51) built on an innovative concept initially proposed by Zamecnik et al. (52) to encapsulate INPs in microliter-sized alginate beads. Not only did this strategy impede any contact between the INPs and the biological system but it additionally facilitated their removal and thereby avoided potential issues with downstream degradability. Furthermore, by negating any concerns that arise when the INP is in direct contact with the biological system, there is more flexibility to adjust the nucleation temperature, as no biological impact would be expected when INP concentrations are increased. The authors demonstrated that altering either the size or number of alginate beads in the sample allowed them to modulate nucleation temperatures between −7°C and −3°C for pure water and between −10°C and −6°C for a 10% glycerol solution.

2.2. Nonbiological Ice-Nucleating Agents

Silver iodide, silver bromide, kaolinite, graphite, and potassium-rich feldspars are naturally occurring, inorganic compounds known to facilitate nucleation (53). Of these compounds, silver iodide has been one of the most effective, and thus one of the most intensively studied, inorganic ice-nucleating agents (INAs) (53). In fact, the initial identification of its nucleation properties in 1947, combined with seminal artificial cloud production studies in 1948, heralded an entire field of weather modification research (54, 55). Most of these inorganic compounds have primarily been of interest in the meteorological sciences, with silver iodide being one of the few that has made its way into the biopreservation field (56–59). The most notable studies to leverage silver iodide for nucleation are those of Kojima et al. (56) and Stanzel et al. (57), who demonstrated an ∼5°C higher average nucleation temperature in alginate-encapsulated rabbit embryos and mesenchymal stem cells, respectively. In each case, this led to an increase in post-thaw viability.

Like these inorganic compounds, there are a variety of ice-nucleating organic compounds, which primarily comprise crystallized lipids, steroids, and amino acids (60–62). Crystallized cholesterol has been the most extensively investigated, with ice-nucleating efficiency comparable to silver iodide (61). Massie et al. (63) published what is to our knowledge the only study to leverage this compound for cryopreservation purposes, demonstrating that it raised the nucleation temperature by ∼5°C and facilitated improved post-thaw recovery of liver cell spheroids.

While several of these compounds certainly warrant further consideration and use in cryopreservation, their efficacy spurred research that has aimed to identify surface characteristics that allow for efficient nucleation, as this could enable the design of synthetic ice nucleators. It is well

established that the primary requisites of an effective INA are an insoluble ice-nucleating surface, a strong water–surface interaction (i.e., hydrophilicity), and a crystallographic match between the surface and the ice crystal (64). Interestingly, the dependence of nucleation rates on hydrophilicity has been shown to exhibit a parabolic trend, where water–surface absorption that is either too weak or too strong can limit the ability of water to rearrange into a hexagonal (ice-like) configuration, leading to a loss in nucleation efficiency (65). The complexity of this is further compounded by a distinct interaction that exists between the surface microstructure and hydrophilicity, where changes in the microstructure can in some cases alter the optimal absorption strength (65). It should, however, be noted that Bi et al. (66) found that this interaction only proves relevant under certain degrees of hydrophilicity, as there are some degrees of hydrophilicity (both weak and strong) where the microstructure has a negligible impact on the optimal absorption strength. These concepts only broach the complexity of this field, and enduring deficiencies in our understanding of heterogeneous ice nucleation make it challenging to establish a comprehensive set of molecular principles to design materials that promote nucleation.

Although not a predominant mechanism behind the efficacy of the INAs discussed thus far, local charges on a surface are also known to impact nucleation rates (67). Positively charged surfaces generally act to promote nucleation, whereas the opposite proves to be the case when a surface is negatively charged (67). This would in theory create an opportunity to leverage ionic materials ubiquitous in nature to promote nucleation for biopreservation purposes; however, while ionic surfaces are known to control the dynamics and interfacial structure of water, there has yet to be any evidence to suggest that this corresponds to changes in nucleation kinetics (68). Yang et al. (69) took an innovative approach to promote ice nucleation by modulating surface charge by genetically engineering supercharged unfolded polypeptides rich in positively charged lysine residues and tethering them to a silicon wafer. It was demonstrated that an increase in charge density was correlated with improved nucleation, which introduces an added element of tunability to this approach.

While there are still gaps in our understanding of the underlying mechanism through which compounds that promote ice nucleation function, the general concepts discussed above have recently been used to develop carbon nanotubes, graphene nanoflakes, and surface-modified graphene oxide nanosheets capable of promoting nucleation (70). These represent the first of what are likely to be many synthetic materials capable of functioning in this capacity.

3. INHIBITING ICE NUCLEATION AND RECRYSTALLIZATION

The relevance of inhibiting ice recrystallization (i.e., a reduction in the free energy of the system through an increase in the average crystal size) or nucleation certainly varies on the basis of whether the preservation strategy implemented aims to promote or inhibit the formation of ice. However, several classes of compounds we discuss in this section (with some exceptions) can function in both respects. Antifreeze (glyco)proteins [AF(G)Ps] are the classic example of this, as they are able to prevent the growth of nascent ice crystals and their subsequent recrystallization, respectively offering elements of freeze resistance and freeze tolerance in the natural environment to organisms that express them. In practice, however, recrystallization is often secondary to other forms of ice-induced injury; thus, implementing an ice-free preservation strategy and leveraging agents to limit nucleation tends to be the preferred approach if a biological system proves hard to freeze.

Interest in ice-recrystallization inhibitors (IRIs) has vastly outweighed that of ice-nucleation inhibitors (INIs) due to the predominance of efforts to store biological material in a frozen state (as opposed to ice-free storage strategies), combined with an abundance of recent literature

characterizing the damage recrystallization can induce (71–73). Other than a handful of exceptions, cells for research or clinical use are frozen in DMSO or glycerol-containing solutions at −196°C, and direct relationships have been drawn between temperature fluctuations during the handling or distribution of frozen cells, the degree of recrystallization, and the subsequent reduction in post-thaw viability (74–76). These findings are particularly relevant for novel FDA-approved cell therapies where the necessity of cryopreservation in their supply chains has brought an increasing amount of attention to IRIs (77).

Ice-nucleation inhibition is a more predominant concern for the subzero preservation of specific cell types or complex tissues/organs, where minimizing the damage caused by ice proves to be challenging even in cases where recrystallization is controlled. Vitrification (i.e., storage in an amorphous solid state below the glass-transition temperature of the storage solution) is by far the most popular form of ice-free preservation and has successfully been used to preserve cell types such as oocytes (78), sperm (79), and hepatocytes (80, 81), which are highly susceptible to freezing injury. Although its application to tissues and organs still faces several monumental roadblocks, there have been some promising breakthroughs in recent years that do warrant its continued investigation and consideration (82–84). However, for cases where long-term storage of cells is not necessary or (in the case of complex tissues/organs) not yet feasible, interest has been shifting toward storage in an unfrozen (supercooled) state at high subzero temperatures (85, 86).

Small-molecule carbohydrates, polyol-based polymers, self-assembling molecules, and nanomaterials represent the major classes of synthetic IRIs, with many of them also containing compounds capable of effectively inhibiting nucleation. AF(G)Ps, on the other hand, are nature's ice-recrystallization and -nucleation inhibitors. Thus, they were the first compounds leveraged for these mechanisms of ice control in biopreservation applications.

3.1. Antifreeze (Glyco)Proteins: Thermal Hysteresis and the Development of Small-Molecule Carbohydrate-Based Ice-Recrystallization Inhibitors

AF(G)Ps were first described in the winter flounder (*Pseudopleuronectes americanus*) in 1974 by Duman & Devries (87), following observations by Scholander and colleagues (88) in the early 1960s that marine teleost fish did not freeze despite the water temperature being more than a degree below (−1.9°C) the temperature of their blood serum. These proteins have since been identified in numerous other fish species, as well amphibians, insects, plants, fungi, yeast, and bacteria (89). Furthermore, they represent a remarkable example of convergent evolution where, despite the tremendous variation in molecular weights (of which the higher molecular weight fraction is more active), sequences, and structures of different AF(G)Ps, their function is generally the same. This diversity has made elucidating the critical features that underlie their interaction with ice crystals rather complex, and we touch only very briefly on this topic, as several excellent reviews exist that delve into their structure–function relationships (90–92). It is, however, pertinent that AF(G)Ps be discussed to provide context for the development of compounds that inhibit nucleation or recrystallization, as it is their discovery that allowed scientists to realize that such a feat was possible. Their mechanisms of action have additionally informed the design of several biomimetic antifreeze compounds, particularly in the case of small-molecule carbohydrate-based IRIs (discussed in Section 3.1.2).

3.1.1. Antifreeze (glyco)protein nucleation inhibition and the relevance of thermal hysteresis in biopreservation. AF(G)Ps bind to nascent ice crystals and prevent their growth through what is known as the irreversible adsorption-inhibition mechanism, whereby an increase in the microcurvature between adjacent crystal-bound AF(G)Ps results in depression of the local freezing point through the Kelvin effect (which states that the equilibrium melting point of a

solid is directly related to its surface curvature and, therefore, its interfacial energy) (18, 27). The mechanism through which AF(G)Ps bind ice crystals has not yet been fully resolved, but some of the more predominant ideas have included (*a*) the ordered distribution of hydrophilic (OH) and hydrophobic (CH_3) groups on the ice-binding face (IBF) of AF(G)Ps matching the ice lattice, and (*b*) hydration properties that promote ordered water on the IBF and disordered water on the non-IBF (93–95). Nevertheless, the subsequent noncolligative freezing point (FP) suppression that results following crystal adsorption, termed thermal hysteresis (TH), is not ice-nucleation inhibition per se (as it does not involve inhibiting the formation of ice embryos), but it functions in a similar way within the TH gap [i.e., the gap between the equilibrium freezing/melting point and the nonequilibrium hysteretic freezing point (hFP)] (96). There is also evidence to suggest that some AF(G)Ps can bind foreign particles and perturb their ability to serve as a template for heterogeneous nucleation, offering a direct mechanism of nucleation inhibition (97). However, it is not known if this is consistent among many different types of AF(G)Ps, nor how it would vary on the basis of the type of foreign particle.

This distinction between direct ice-nucleation inhibition and TH would have limited impact on biopreservation outcomes if storage were limited to temperatures above the hFP (e.g., during supercooling preservation). However, below the hFP, the convexity of the regions between adjacent AF(G)Ps reaches a critical point where crystal growth ensues, and during low-temperature, ice-free preservation strategies (such as vitrification), it would be important that nucleation be inhibited well below the hFP. In these cases, TH would have limited relevance, but we do treat it as synonymous to ice-nucleation inhibition for the purposes of the discussion in Section 3, given the sheer predominance of compounds with TH activity and the growing interest in high subzero, ice-free preservation strategies [see Section 3.2 for a description of poly(vinyl alcohol) (PVA), which inhibits nucleation by impeding the formation of ice embryos].

The TH gap of polar teleost fish AF(G)Ps [the most commonly used AF(G)P in biomedical and industrial applications] does not exceed ~2°C at physiologic concentrations; however, it is known that the TH gap is directly proportional to the square root of the AF(G)P concentration (27). While increasing the AF(G)P concentration to achieve stability at any desired degree of supercooling would be an intuitive approach, there is evidence to suggest that AF(G)Ps can promote toxicity in some systems, as evidenced by diminished survival of human embryonic liver and kidney cells following AF(G)P treatment (98). Furthermore, large-scale synthesis of recombinant AF(G)Ps, particularly at volumes necessary for human organ cryopreservation, is an already expensive venture that would only be made more costly if high concentrations were required.

The limited TH activity possessed by fish AF(G)Ps is due to a lack of binding to the basal plane of the crystal lattice (27). Hyperactive AF(G)Ps, most commonly found in insects, bind to all planes of the lattice (prism, pyramidal, and basal), and, as a consequence, possess TH activity 10 to 100 times greater than that seen in commonly used fish AF(G)Ps at equimolar concentrations (99). For example, a hyperactive AF(G)P derived from *Tenebrio molitor*, a species of darkling beetle, produces a TH of 3.5°C at a concentration of 0.1 mM, relative to the type I fish-derived AF(G)P, which produces a TH of 0.6°C at 10 times the concentration (99, 100). Although structure–function relationships have been established for AF(G)Ps with varying degrees of TH activity (91, 101), there have been no published efforts to generate structural analogs that recapitulate the TH activity of these proteins. This contrasts with the extensive efforts to generate synthetic, biocompatible analogs that recapitulate the IRI activity of these proteins (discussed in Section 3.1.2). Nevertheless, in lieu of such efforts, it is instead beneficial to briefly discuss situations that can promote the TH activity of AF(G)Ps.

While the TH dependence on AF(G)P concentration has been studied extensively, there has recently been additional investigation into the role that annealing time has on TH activity

(102–104). Several studies have observed that there is a decrease in the hFP over time, with some authors postulating that this could be a result of progressive AF(G)P adsorption, rearrangement of adsorbed AF(G)Ps, or a combination of the two (102–104). Many moderately active AF(G)Ps tend to reach their adsorption equilibrium within several minutes, whereas hyperactive AF(G)Ps can take significantly more time (up to 4 h); however, the overall hFP depression ends up being much greater (102). A 4-h annealing time with *Tenebrio molitor* AF(G)P can result in a 10-fold reduction in the hFP, in contrast to the 2.5-fold reduction seen with type III fish AF(G)Ps after reaching its adsorption equilibrium (within 10 min) (102). The longer timescale required for hyperactive AF(G)Ps is due to the slow and progressive manner in which they accumulate on the basal plane (102). This occurs despite relatively rapid accumulation on the prism/pyramidal planes [which is also seen in moderately active AF(G)Ps] (102). A mechanism to explain these differences does not yet exist; however, one could posit a strategy whereby the temperature of a biological system with hyperactive AF(G)Ps could be lowered such that it sits comfortably within the initial TH gap and then continuously lowered over time to the point at which the hFP reaches its absolute minimum. Higher degrees of supercooling are thought to decrease the annealing time required to reach maximal hFP due to a temperature-dependent decrease in the basal plane surface area, ultimately offering further support for such a strategy (105).

3.1.2. Antifreeze (glyco)protein analogs, small-molecule carbohydrate-based ice-recrystallization inhibitors, and the importance of separating thermal hysteresis and ice-recrystallization inhibitor activity. AF(G)Ps have proven to both promote and diminish the survival of frozen cells and tissues, raising concerns pertaining to their utility as agents to protect against recrystallization injury (12). Due to the plane-specific adsorption of AF(G)Ps, an increase in their concentration or the degree of departure from the hFP can shift the normal dendritic morphology of ice crystals toward a spicular morphology that can promote structural abrogation of tissues and membranes or result in IIF [due to the increase in the curvature of the ice crystal in accordance with the pore-forming hypothesis of IIF (36, 106)]. This was the primary driver behind a concentration-dependent loss of function seen in several early efforts to cryopreserve rat hearts (107), red blood cells (12), cardiomyocytes (108), and Chinese hamster fibroblasts (109) with AF(G)Ps. It is a phenomenon so well established that it has led to the use of AF(G)Ps for the cryoablation of various tumors (110). However, considering that AF(G)Ps do provide benefit in cases where spicular ice growth is diminished and recrystallization is exacerbated, there has been interest in designing synthetic alternatives to AF(G)Ps that lack these so-called dynamic ice shaping (DIS) characteristics, known to be a product of their TH activity (**Figure 3**).

The motivation for developing synthetic alternatives that maintained potent IRI activity, but lacked TH activity, manifested in response to evidence suggesting a discordance between TH and IRI activity. Moderately active fish AF(G)Ps possess more pronounced IRI activity than many hyperactive AF(G)Ps and, unlike TH activity, IRI activity bears minimal concentration dependence, with many AF(G)Ps capable of inhibiting recrystallization at nanomolar concentrations where TH is absent (111). However, the mechanisms responsible for AF(G)P-mediated inhibition of ice recrystallization were poorly understood at the time, leading to a lack of research progress. In fact, most structure–function studies at this time solely correlated TH activity to structural modifications, with no consideration of IRI activity. In an effort to gain clarity on the mechanisms underlying IRI efficiency, the design of synthetic analogs has largely focused on AFGPs, as their lack of structural variation relative to AFPs enables the study of structure–function relationships.

Initial success at separating IRI and TH activity by Ben and colleagues (112) in 2003 proved largely serendipitous but led to several fundamental discoveries that have altered the landscape of

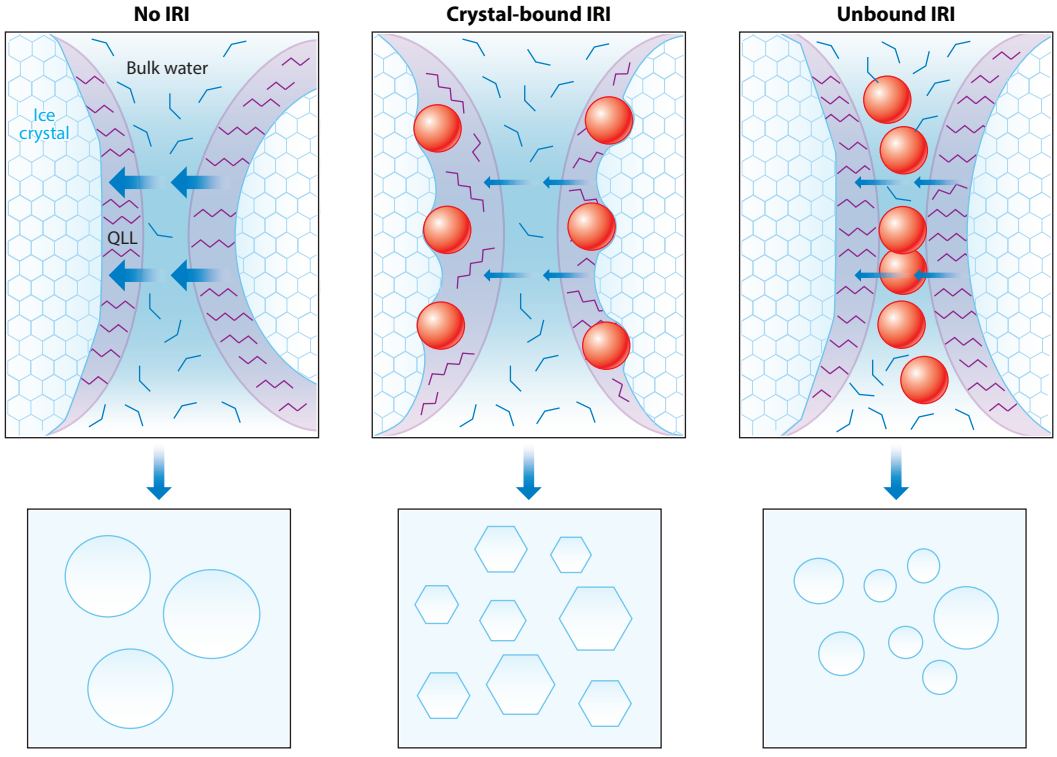


Figure 3

A major distinction between prominent ice-recrystallization inhibitor (IRI) technologies. The unfrozen region between ice crystals consists of the so-called bulk water region (*blue*), containing highly disordered water molecules, and the quasi-liquid layer (QLL) (*purple*) directly adjacent to the crystal surface, in which water molecules are in a semi-ordered conformation. IRI-active compounds (*red spheres*) that do not bind to the ice crystal surface [i.e., C-linked antifreeze (glyco)protein (AF(G)Ps) or small-molecule IRIs] are thought to reside at the bulk water–QLL interface, increase disorder of water molecules at this interface, and thus make it more energetically unfavorable for recrystallization to occur (*right*). Alternatively, IRI-active compounds can adsorb to the surface of the ice crystal and impede water molecule addition (*middle*). The end result with respect to recrystallization inhibition is thought to be similar between bound and unbound IRIs [i.e., reducing rate of water molecule transfer from a smaller to a larger ice crystal relative to a condition lacking an IRI (*left*)], but in the case of the former, dynamic ice shaping would occur. (Note that the shape of the ice crystal would vary on the basis of the planes/axes of the crystal that are bound. Here, an IRI binds to both the *c*-axis and the *a*-axis, resulting in a hexagonal conformation.) Blue arrows indicate water molecule transfer between regions, with their sizes representative of the degree of transfer.

IRI research. Two generations of C-linked AFGPs (which lacked the O-glycosidic linkage found in AFGPs that are highly susceptible to hydrolysis under basic or acidic conditions) were synthesized, and the most potent (i.e., those with the highest IRI activity and lowest TH activity) significantly enhanced the recovery of human embryonic liver cells under conditions that promote recrystallization (113). Although these compounds required intricate and time-consuming multistep synthesis not amenable to large-scale production, their breakthrough design elucidated the independence of TH and IRI activity and revealed several structural features imperative for inhibition of ice recrystallization. Reviews by Ben and colleagues (26, 114) delve into these specific structural features, but the most noteworthy characteristic is the degree of carbohydrate hydration (i.e., the number of nonexchangeable water molecules associated with the solute) (115). Molecular dynamics (MD) simulations revealed that C-linked AFGPs with more hydrated carbohydrate residues caused more disorder in the quasi-liquid layer between the bulk water and the ice crystal, making it

more energetically unfavorable for recrystallization to take place (115). This hydration-dependent control of recrystallization also applied to mono- and disaccharides in solution; however, despite disaccharides having twice the molecular weight, they did not have twice the IRI activity. Thus, it was not solely hydration that was correlated to IRI activity but rather the hydration index, which is defined as the hydration number divided by the partial molar volume of the carbohydrate (116).

These concepts informed the subsequent development of the first nonpeptide or polymer-based small molecules capable of inhibiting recrystallization (117). The molecules described were carbohydrate-based surfactants and hydrogelators, with many of the more potent molecules falling into two main classes: *O*-aryl-β-D-glucosides and *N*-alkyl-D-gluconamides. Aside from hydration indices, amphiphilicity has been deemed one of the key contributing factors to the IRI activity of these compounds, where the hydrophobic portion (aryl or alkyl chain) must be delicately balanced with the hydrophilic portion (carbohydrate). Although these compounds are active at low concentrations, their amphiphilicity can result in low solubility limits. As amphiphilicity is a characteristic of most IRI-active compounds, this issue is not specific to these small molecules. However, given the limited toxicity/biocompatibility issues seen at concentrations approaching the solubility limits, combined with the dose-dependent increase in IRI activity, there is interest in increasing solubility to further improve IRI efficiency. In a promising first step, the incorporation of negatively charged phosphonate moieties on several potent small-molecule IRIs offered a two- to fivefold increase in solubility with limited impact on IRI activity (118).

The use of small-molecule carbohydrate-based IRIs has resulted in improved cryopreservation outcomes for red blood cells (119–121), cord blood-derived hematopoietic stem cells (122), and platelets (123, 124). While these results have been exceptionally promising, one of the major benefits to their small size and amphiphilicity is that they can additionally permeate interstitial regions of tissues and cell membranes. In a first-of-its-kind study, Lautner et al. (125) demonstrated the ability of one highly potent IRI to permeate the entirety of a rat lung within an hour (at 22°C) and linked the corresponding reduction in recrystallization with improved post-thaw viability outcomes. Considering that cells within tissues are highly prone to intracellular ice formation as their capacity to dehydrate is impeded by extracellular connections, limiting intracellular ice recrystallization (one of the major factors that impedes the potentially innocuous nature of intracellular ice) also becomes a pertinent concern. Recent studies have shown potent control of intracellular ice recrystallization in endothelial cells and hepatocytes, a finding that is exceptionally intriguing considering that there are no other IRI-active compounds capable of functioning in this capacity (119, 126).

3.2. Polyol-Based Polymers

A series of studies in the 1980s were the first to demonstrate the ability of polymers to inhibit ice nucleation with similar potency to moderately active AF(G)Ps (127, 128). Knight et al. (129) followed up on this with the first study to investigate the IRI activity of several conventional polymers (poly-L-histidine, poly-L-hydroxyproline, and PVA). Polymers have since remained the most widely studied AF(G)P alternatives (with respect to both their INI and IRI activity), with PVA being the most potent and thus the most widely used. This has become an increasingly attractive area of research in the past two decades, given the advancements in synthetic polymer chemistry that have enabled changes in polymer architecture through precise alterations in chain length/orientation or the addition of functional groups (130).

Interest in the INI activity of PVA has largely occurred in the context of vitrification, as it has proven capable of decreasing the critical warming rate and limiting devitrification (one of the most pressing challenges in vitrification applications) (131). Surprisingly, PVA exhibits limited TH

activity relative to AF(G)Ps, with concentrations of 50 mg/mL (M_w: 13,000–23,000) producing a TH of only 0.18°C (132). Despite this, there is ample evidence of crystal adsorption, as MD simulations have revealed that PVA binds the c-axis of the primary and secondary prism faces (133, 134). Altered crystal morphology is additionally evident within the TH gap of PVA, although the primary mechanism of ice-nucleation inhibition is certainly distinct from the classical TH activity of AF(G)Ps since TH alone does not account for the entirety of its capacity to inhibit nucleation (132, 135).

The backbone of PVA is rich in hydroxyl constituents, which form strong hydrogen bonds with water and thereby increase the viscosity of the solution. Classical nucleation theory posits an increase in solution viscosity to be associated with a reduction in the probability of nucleation and the rate of water molecule addition to a growing ice crystal. Therefore, it is likely that much of the INI activity of PVA is secondary to its effect on viscosity. Leveraging these concepts, Mousazadehkasin & Tsavalas (136) designed potent PVA derivatives by increasing both the backbone flexibility and the number of hydroxyl groups on each monomeric unit. Although the ability of PVA to inhibit nucleation has been previously described to improve with increasing oligomeric chain length, this study demonstrated that the orientation and distance between hydroxyl groups play a greater role in INI activity than the total number of hydroxyl groups (137). The most potent of the PVA derivatives synthesized (glycerol-grafted-PVA) allowed for 2 h of supercooling prior to the onset of nucleation at −17°C, proving far greater than the 30 min seen with its standard counterpart at equimolar concentrations.

Many of the aforementioned factors deemed crucial to the INI activity of PVA have also proved relevant to its IRI activity. In fact, Mousazadehkasin & Tsavalas (136) demonstrated that the PVA derivative with the highest ice-nucleation inhibition activity (glycerol-grafted-PVA) was also the best at inhibiting recrystallization, as it offered up to a 90% reduction in mean grain size at concentrations ranging from 0.1 to 2 mg/mL (after 60 min at −8°C). Using MD simulations, Bachtiger et al. (138) underscored these findings and additionally uncovered new insights into the IRI activity of PVA. It was commonly thought that improving the ability of PVA to bind to the crystal lattice would improve its IRI efficiency [this is the case for rigid IRI-active agents such as AF(G)Ps, and therefore it was also thought to be the case for PVA]; however, several lines of evidence were generated to counter this conventional lattice-matching hypothesis. While the presence of hydroxyl groups that can bind the crystal lattice is necessary, positioning of those functional groups in such a way that they match the ice lattice does not improve their IRI activity, nor is it a prerequisite for PVA to bind the ice crystal. Rather, backbone flexibility plays the predominant role, as demonstrated by the absence of IRI activity in structurally constrained variants. Furthermore, the binding affinity of PVA10 (M_w: ∼10,000) and PVA20 (M_w: ∼20,000) proved similar, despite PVA10 having nearly no IRI activity relative to that of PVA20. This is due to the low surface area–to–volume ratio of PVA10, resulting in it being overgrown and integrated into the ice crystal. Overcoming this was possible by using a PVA10 copolymer (PVA_5-*b*-$PVAm_5$) with a fraction of its hydroxyl groups replaced with NH_3^+ fragments. The nonnegligible IRI activity of this copolymer suggests that the hydroxyl groups on the PVA10 polymer that did not originally bind the ice crystal were likely facilitating engulfment of the polymer into the growing ice front, making the addition of hydrophobic groups favorable. The placement of hydrophobic groups on PVA is, however, an important consideration, as Deller et al. (139) have shown that the random placement of hydrophobic groups on the main chain can, in some cases, decrease IRI activity. Ultimately, considering that there are benefits to limiting the degree of adsorption and that adsorption is not the primary driver for IRI activity, it is likely possible to synthesize highly IRI-active PVA derivatives that display limited DIS characteristics.

The tacticity of hydroxyl groups, speed of adsorption, and degree of acetylation are other pertinent factors to be considered when designing IRI-active PVA derivatives lacking significant DIS characteristics. Atactic forms of PVA have increased activity relative to isotactic forms, as they form more intermolecular hydrogen bonds with water (the identical oriented O atoms in isotactic PVA lead to more intramolecular hydrogen bonds) (140). This causes them to spread out fully at the ice–water interface and more effectively impede the addition of water molecules to the growing ice crystal. In regard to the adsorption speed, Naullage & Molinero (141) showed that there is a direct correlation between the speed of adsorption and IRI efficiency. Therefore, designing individual monomers to have stronger ice-binding free energies (for example, by increasing the density of hydroxyl groups) could be another means of increasing IRI efficiency. Finally, it is valuable that PVA be deacetylated prior to use (142). Commercially available PVA polymers (from which the synthetic variants are made) are polydisperse samples generated from poly(vinyl acetate) and often contain 0–20 mol% acetate groups that break the series of regularly spaced hydroxyl groups, thus diminishing crystal adsorption and, in turn, IRI activity. Removal of acetate groups is possible through controlled radical polymerization methods (which are used to generate PVA of predictable chain length and narrow molecular weight dispersity); however, this requires harsh conditions and does somewhat limit accessibility of PVA (143).

Matsumura et al. (144) reported that synthetically accessible polyampholytes (i.e., polymers with mixed cationic and anionic groups) displayed significant IRI activity, albeit up to 50-fold less than that of PVA. There has also been evidence that poly(butylene succinate) polyampholytes can effectively inhibit heterogeneous nucleation, although structural characteristics that promote nucleation inhibition remain unclear (145). Nevertheless, like PVA, there is a concentration dependence to the IRI activity of polyampholytes (144). However, there are no strong molecular weight trends, which is thought to be due to the lack of obvious ice crystal binding sites (144). The mechanisms underlying the IRI activity of polyampholytes ultimately remain a topic of debate; however, some key design considerations have recently been highlighted. Several groups have indicated that activity is maximal when the stoichiometric ratio of carboxylic acid to amine functional groups is 1:1. Building on this, Stubbs et al. (146) showed that regioregular polymers with alternating positive and negative monomers have more activity than polymers with randomly distributed monomers. In addition, an increase in side-chain hydrophobicity leads to an increase in activity; however, this effect has diminishing returns. For example, despite dimethyl amino side chains being less hydrophobic than ethyl and propyl side chains, they produce the most active polyampholytes (146). This occurs because increased hydrophobicity promotes aggregation and, ultimately, precipitation of the compound. Therefore, there likely exists a potential balance between backbone and side-chain hydrophobicity where optimal IRI activity could be achieved (147). Using these concepts to rationally design IRI-active polyampholytes is, however, not yet feasible considering that the underlying mechanism of activity has not yet been resolved.

3.3. Supramolecules: Engineered and Intrinsic Self-Assembly

Supramolecular chemistry is a flourishing area of research that is now being used to design highly sophisticated materials at a nanoscale level for biomedical, environmental, and information technology applications (148). Its application to ice control technologies has come under investigation only during the past two decades; however, it is an incredibly powerful tool that can be used to creatively design highly functional, yet easily synthesizable, CPAs.

3.3.1. Engineering peptide and polymer self-assembly. As discussed in prior sections, the size of a compound plays a major role in its TH (or INI) and IRI activity. However, there are size limits on what can be obtained using linear polymers, and it is impractical to increase the size of

proteins (e.g., simply generating linear assemblies of repeat units would cause improper folding). Although DIS characteristics are generally enhanced in larger compounds, the 3D conformation of large, self-assembled structures will not necessarily increase the surface area of the crystal that is bound. Rather, such structures are more likely to impede recrystallization by promoting disruption of the quasi-liquid layer above the ice lattice. It is these concepts that make the application of supramolecular chemistry to compounds known to impart antifreeze activities an exceptionally valuable avenue of research.

Engineering self-assembling peptides that can mimic the structure and sequence of AF(G)Ps presents an intuitive starting point for the application of supramolecular chemistry to ice control compounds (**Table 1**). Adam et al. (149) approached this by incorporating perylene bisimide (PBI), a class of polyaromatic dye with programmable helicity and self-assembly properties, into two AF(G)P analogs. While both supramolecular peptides provided less IRI activity than the native compound from which the analogs were derived, IRI activity was significantly higher than when the analogs lacked PBI. As PBI alone does not have IRI activity, aggregation is believed to be the mechanism behind this increase in activity. Rather than using analogs, Xue et al. (150) leveraged knowledge of the structural characteristics underlying the lattice matching of AF(G)Ps to endow IRI activity into 2-naphthylacetylglycine-phenylalanine-phenylalanine (2-NapGFF), a peptide motif that self-assembles into β-sheet structures. The putative ice-binding surface of AF(G)Ps consists of a highly constrained β-helix with threonine residues projecting outward in parallel arrays such that the hydroxyl groups match their binding partners on the crystal lattice (95). Thus, 2-NapGFF was an ideal starting point for this. Installation of either one (2-NapGFFT) or two (2-NapGFFTT) Thr residues at the C-terminal end had minimal impact on the self-assembly of the peptide and in both cases allowed for a distance between Thr residues similar to what is seen in TisAFP6 (~0.68 nm). Both 2-NapGFFT and 2-NapGFFTT promoted efficient, dose-dependent suppression of recrystallization that was not seen when the Thr residue was absent. While some evidence of DIS was evident, it was less than that seen in most AF(G)P solutions at similar concentrations. However, IRI activity on a mass basis was also lower, so it is challenging to conclude whether this supramolecule would confer less DIS than a native AF(G)P at a given level of IRI activity. Nevertheless, this represents the first attempt to utilize supramolecular chemistry to rebuild an AF(G)P active site.

Polymerization-induced self-assembly (PISA) is a versatile method used in the manufacturing of nano-objects used as drug delivery agents or polymersome nanoreactors (151). However, it has recently come under investigation as a strategy to generate nanomaterials capable of inhibiting ice recrystallization (110). Georgiou et al. (152, 153) were the first (and thus far the only) group to publish on this topic, showing that PISA-derived nanoparticles of poly(ethylene glycol), poly(dimethylacrylamide), poly(vinylpyrrolidone) (PVP), and PVA that were not IRI active in their isolated, linear form were able to exert activity when they were assembled into nanoparticles. While activity was less than that of most AF(G)Ps, it could likely be enhanced if polymers that were active in their native state were incorporated into these nanoparticles. Larger particles did prove to be more active than smaller ones; however, the morphology differed in particles of different sizes (larger particles were shaped like vesicles and smaller ones had mixed morphologies of micelles/spheres/worms). Therefore, it is likely that size had a predominant effect on IRI activity of the nanoparticle, although it is challenging to isolate the role that the nanoarchitecture played. Interestingly, other than the PVP-derived nanoparticle, all other nanoparticles provided a slight (1–2°C) rise in the nucleation temperature, suggesting they had dual INA and IRI functionality. There is evidence that some high-molecular-weight AF(G)Ps can promote nucleation; however, the immense structural differences between these nanoparticles and AF(G)Ps limit any conclusions about the mechanism underlying the IRI and INA activity

Table 1 An overview of pertinent ice control compounds

Mechanisms of ice control	Compound or class of compounds	Important structural considerations	Applications in biopreservation
Promoting ice nucleation	Organic crystals (e.g., crystallized cholesterol)	Presence of flexible hydrophilic surfaces forming hydrogen bond cages and a diverse topography of surfaces that offer efficient nucleation over a broad range of supercooling	Liver spheroids (63), embryos (56), mesenchymal stem cells (57)
	Silver iodide	Lattice match between the surface and the ice crystal, high atomic surface roughness, and charge distribution (positive charges localized above or in plane with negative charges promotes nucleation)	Liver spheroids (63)
Promoting ice nucleation, inhibiting recrystallization, and thermal hysteresis	Graphene oxide	Decrease in size and oxidation promotes nucleation; lattice match, the differential in the water versus ice hydrogen bond strength (stronger hydrogen bonds with water), and an increase in the carbon-to-oxygen ratio promote IRI activity	Sperm (166)
	PISA-derived nanoparticles	Size, morphology, and type of constituent polymer impact nucleation and IRI activity	Red blood cells (172)
Inhibiting ice recrystallization with no dynamic ice shaping	C-linked AFGP analogs	High hydration index of the carbohydrate residue and high amphiphilicity	Embryonic liver cells (113)
	Small-molecule carbohydrate-based IRIs	High hydration index of the carbohydrate residue and high amphiphilicity	Platelets (123, 124), red blood cells (119–121), endothelial cells, hepatocytes (126), hematopoietic stem cells (122), liver tissue (173), lungs (125)
	Nanocelluloses	Low surface charge density, high fibril length, and high amphiphilicity	A549 cells (162)
Inhibiting recrystallization and thermal hysteresis	Safranine O chloride	Large size of supramolecular complex (increases with increased concentration) and the presence of alternating hydrophilic amine and hydrophobic methyl groups in the complex	NA
	Quantum dots (quasi-carbon nitride quantum dots and glucose-derived carbon dots)	Lattice match and the density of hydrogen bonding sites	Red blood cells (166, 169, 170)
Inhibiting recrystallization, thermal hysteresis, and inhibiting nucleation	Poly(vinyl alcohol)	High backbone flexibility, high chain length, and a greater number of hydroxyl groups on each monomeric unit improve IRI and INI activity	Embryos (174, 175), oocytes (175), sperm (176), platelets (177), red blood cells (178), hepatocytes (179), A549 cells (179)
	Polyampholytes	Equal ratio/distribution of cationic to anionic functional groups, high amphiphilicity, and high chain length promote IRI activity; structural considerations relevant to INI activity are not well studied	Red blood cells (180), mesenchymal stem cells [isolated (144) and as monolayers (181)], L929 cells (144)

Abbreviations: AFGP, antifreeze glycoprotein; INI, ice-nucleation inhibitor; IRI, ice-recrystallization inhibitor; NA, not applicable; PISA, polymerization-induced self-assembly.

(154). Nevertheless, possessing both forms of activity would be highly beneficial, particularly in efforts to cryopreserve organs where manually inducing vascular nucleation is challenging.

In contrast to the aforementioned efforts that introduced an intrinsic ability to self-assemble, there has also been interest in introducing self-assembly into IRI-active compounds in such a way that it can modulated by external stimuli. In research performed by Phillips et al. (155), a modified form of PVA10 bearing a catechol end group allowed the compound to form complexes following the addition of Fe^{3+}. These Fe^{3+}-catechol complexes are known to have high mechanical stability and reformation kinetics, making them an ideal choice for modulating the assembly and dissociation of compounds (156). Addition of Fe^{3+} to the catechol-terminated polymers promoted the formation of two- and three-armed star polymers (based on the octahedral assembly around the metal ion), which led to a significant enhancement in IRI activity relative to the nonassembled polymers (155). Although this represents what is to our knowledge the only published effort using external control to induce self-assembly into an IRI-active compound, other known supramolecular triggers, such as changes in pH, temperature, or redox status, or introducing different chemical signals, biological inputs, or magnetic fields, could also be used (157). External control of IRI activity can ultimately be used in creative ways to shed light on the mechanisms underlying the IRI activity of different compounds.

3.3.2. Self-assembling compounds. For the past 150 years, organic dyes have been a mainstay in crystallographic literature, with examples of dyes binding to different regions of the ice crystal and producing striking patterns of color that are representative of the ice crystals' morphology (158). However, it was not until the past decade that these concepts began to receive interest in the biopreservation sciences (158, 159). Safranine O chloride (a dye used commonly in histology and cytology staining) is a chromonic mesogen (i.e., small molecules that aggregate when placed in solution to form lyotropic liquid crystals) that has been reported to display TH activity at low concentrations (<3 mM). This activity is similar to that of several moderately active AF(G)Ps, although significantly lower than hyperactive AF(G)Ps even when used near their solubility limit (66 mM) (159). The mechanism also appears to parallel that of moderately active AF(G)Ps, where complete inhibition of crystal growth is seen along the a-axis, but not along the c-axis. Safranine O additionally exhibits profound dose-dependent IRI activity, with almost complete cessation of recrystallization seen over the course of 120 min at −6°C when used at a concentration of 4.2 mM. Aggregation theory and metadynamics simulations suggest that safranine O was preorganized in stacks of up to 30 molecules at concentrations where ice growth inhibition was evident. This, combined with the fact that aggregates of safranine O were found to include regularly spaced amino and methyl substituents [similar to the ice-binding interface of many AF(G)Ps], could explain how a small molecule can recapitulate the ice control of AF(G)Ps that are 10–100 times its size. Dyes with similar self-assembly properties (methylene violet and phenosafranine chloride) that lack these hydrophilic amine and hydrophobic methyl groups do not have detectable IRI activity, therefore suggesting that the presence of these functional groups is necessary (160).

An improved understanding of the mechanisms that guide the ability of safranine O chloride to control ice will invariably allow for the identification of other similar compounds that may even exhibit greater antifreeze properties. Thus far, it is the only small molecule with an intrinsic ability to self-assemble whose antifreeze properties have been studied.

3.4. Nanomaterials

As with supramolecules, the ice control afforded by nanomaterials is an incredibly novel avenue of research. Despite the limited work completed in this field and the lack of relation to other technologies we have discussed, there are many promising findings that make nanomaterials deserving of their own section in this review.

3.4.1. Nanocelluloses. Nanocelluloses are by far the most heavily researched IRI-active nanomaterial. This is due to their biocompatibility, biodegradability, low cost, low toxicity, and ease of access. Cellulose is the most abundant renewable organic material produced in the biosphere, with annual production rates that are estimated to be more than 7.5×10^{10} tons. Initial efforts to investigate the IRI activity of nanocellulose were prompted by recent advancements in our understanding of its facial amphiphilicity (i.e., hydrophilic and hydrophobic groups localized on two opposite faces). The relevance of hydrophobic interactions to the structure and physiochemical properties of cellulose is a relatively novel idea, as cellulose is not historically thought of as being amphiphilic (161).

Natural cellulose is present as highly crystalline nanofibrils that can be isolated and altered into various forms using mechanical treatments, acid hydrolysis, or chemical oxidation. Initial investigations found that two forms of nanocellulose, cellulose nanocrystals (CNCs) and 2,2,6,6-tetramethylpiperidine-1-oxyl oxidized cellulose nanofibrils (TEMPO-CNFs), expressed IRI activity that was potent, albeit 10-fold less than that of PVA on a mass basis. This was the case in a 0.1-M NaCl solution, but when the ionic strength was increased to isotonic, CNCs and TEMPO-CNFs experienced extensive aggregation and, in turn, lost IRI activity. Using well-described methods to offer electrostatic stabilization to CNCs under high ionic strengths, Li et al. (162) demonstrated that this modified CNC both retained IRI activity in isotonic solutions and did not precipitate out of solution during the increase in ionic strength experienced during cryopreservation (163). Furthermore, they demonstrated that these CNCs were capable of improving the post-thaw viability of A549 cells under freezing and thawing conditions expected to exacerbate recrystallization.

Neither TH nor DIS has been observed with nanocelluloses, and thus it is possible that they exert IRI activity without binding to the crystal surface (164). While further insight into the mechanism of action is warranted, there is evidence that a reduction in the surface charge density (up to a threshold level immediately before fibril aggregation would occur), and an increase in fibril length, can improve activity (164). Together, this suggests that an increase in total hydrophobicity (and therefore amphiphilicity) is associated with IRI activity.

3.4.2. Carbon-based nanomaterials. Graphene oxide (GO) is a derivative of graphene that is arranged as a honeycomb hexagonal scaffold of carbon rings with coexisting oxidized and unoxidized regions. The hexagonal arrangement of hydroxyl groups localized in the oxidized region of GO is reminiscent of the arrangement of hydroxyl groups on the putative IBFs of many AF(G)Ps, and several studies have shown that some of the antifreeze effects of GO do in fact recapitulate that of AF(G)Ps (165–168). Geng et al. (166) demonstrated that GO possesses IRI activity and attributed the mechanism to be in part a result of the hydrogen bonds it forms with ice exceeding the strength of the bonds it forms with water [a carboxyl-functionalized graphene (GCOOH), which showed the opposite trend in hydrogen bond strength, had no observable IRI activity]. IRI activity additionally decreased as the ratio of carbon to oxygen decreased, suggesting that an increase in the density of hydroxyl groups and a decrease in hydrophobicity is preferential (166). While crystal adsorption and subsequent evidence of TH was seen, the size of the TH gap and the planes of crystal adsorption were not identified in this study. Surprisingly, the IRI activity of GO exceeded that of many other AF(G)P-derived biomimetics, proving to even exceed that of PVA at low concentrations of 0.01 mg/mL (although this tapers off above 5 mg/mL) (166). GO is distinct from many other IRI-active compounds in that it is able to also promote nucleation in a manner that is dependent on its size (must be greater than that of the critical ice nucleus) and oxidation (lower oxidation reduces the free-energy barrier for nucleation) (165, 167). As mentioned in Section 3.3.1, dual IRI and nucleation promotion activity is beneficial, and it is therefore valuable

to test whether structures of GO that promote nucleation are also able to effectively inhibit recrystallization. Nevertheless, in a promising indication of the cryoprotective efficacy of GO, Geng et al. (166) demonstrated that GO can increase the post-thaw motility of horse sperm from 24.3% to 71.3% when used at a concentration of 0.01% w/v.

Inspired by the crystal adsorption and ice control properties of GO, there has been interest in generating easily synthesizable carbon-based quantum dots (i.e., highly tunable nanoscale crystals with semiconductor properties) with similar properties. Bai et al. (169) published the first study on this topic, investigating the influence of two graphitic-carbon nitride (g-CN) derivatives, oxidized g-CN quantum dots (OCNs) and quasi-carbon nitride quantum dots (OQCNs), on ice crystal growth. The distance between neighboring tertiary N atoms (i.e., hydrogen bond acceptors) of the OQCNs (7.42 Å; lattice mismatch of 0.99%) had a better match with the hydrogen bond donors of the ice crystal lattice (7.35 Å) than that of OCNs (7.13 Å; lattice mismatch of 2.99%). This let the OQCNs adsorb to the lattice while the OCNs did not, which was in turn thought to be the main reason why a dose-dependent increase in the TH gap and recrystallization inhibition was seen. In a follow-up study, the importance of the density of hydrogen bonding sites was emphasized by using sulfur doping to partially replace N atoms with S atoms (169). The S atoms were unable to serve as hydrogen bond acceptors during binding to the crystal lattice because the smaller electronegativity relative to N atoms made them less likely to serve as hydrogen bond acceptors and the distance between S atoms and N atoms was 7.81 Å, corresponding to a lattice mismatch of 6.25%. As would be expected, the sulfur-doped OQCNs had diminished binding efficiency, and this amounted to a lower IRI activity (169). While there have not yet been efforts to improve the IRI or TH activity of OQCNs, glucose-derived carbon dots (G-CDs) have recently been proven to have IRI activity and adsorption characteristics similar to those of OQCNs, which could open the door to using carbohydrates with higher hydration indices to achieve more effective inhibition of recrystallization (116, 170). Both OQCNs and G-CDs allowed for 55–60% recovery of red blood cells rapidly frozen in liquid nitrogen in the absence of any other CPA, which is a compelling indication of their efficacy considering the high levels of stress incurred under this freezing condition (169, 170).

Given these promising initial results with GO, OQCNs, and G-CDs and the rate at which nanomaterials are advancing, other carbon-based nanomaterials will invariably begin to be studied and developed for biopreservation purposes.

5. GENERAL CONSIDERATIONS FOR FUTURE RESEARCH

Although several of the technologies we have discussed are highly effective, even the more established ones have yet to expand to industrial biomedical applications. The reason for this is highly multifaceted but can be distilled down to the following key points:

1. A lack of education and acceptance of the damage incurred when ice nucleation or recrystallization is not adequately controlled
2. Limited direct connections being made between the ice control afforded by a specific technology and improved subzero storage outcomes
3. Inadequate characterization of the biological impact of ice control technologies, irrespective of the biophysical protection they afford during cryopreservation

Points one and two will inevitably diminish as this field continues to advance. However, for all the technologies we discussed (even the more established ones for which point two is less of a concern), interactions with cell membranes or cell metabolism have not been adequately studied. Polyampholytes are one of the few exceptions, having been proved to reduce the phase transition temperature of the plasma membrane, thereby limiting the transient increase in permeability

that occurs during the phase transition from liquid crystalline to gel and the subsequent buildup of nonphysiological, damaging ion gradients during hypothermic or supercooled storage. This still pales in comparison with the thorough understanding that exists of how conventional CPAs such as DMSO and glycerol impact cell biology. Similar investigation and publication of cell-specific interactions of the ice control technologies discussed throughout this article are warranted, regardless of whether these interactions are protective or deleterious.

Despite many recent developments in ice-free preservation strategies and their relevance to tissue/organ preservation, the development of compounds that inhibit recrystallization continues to outweigh the development of those that inhibit nucleation. For several of the IRI-active technologies discussed, we did not mention their INI activity for the sole reason that these mechanisms were not investigated. While dual IRI and INI activity is not preferential if ice is present, it is highly beneficial for ice-free preservation strategies, considering that if ice were to form, then inhibiting its recrystallization could provide protection. However, given the alternate mechanisms through which ice can promote damage when it is nucleated in a metastable system (see Section 2) and the limited number of compounds that effectively inhibit nucleation, there is value in tailoring IRI-active compounds to inhibit nucleation, even if it be at the expense of their IRI activity.

Finally, the combined ability to promote nucleation and inhibit recrystallization is invaluable, as both mechanisms of action are pertinent in any biopreservation strategy where ice is present (**Figure 2**). Integrating them into a single compound would therefore be highly economical. Of all the technologies we discussed, only the PISA-derived nanoparticles (see Section 3.3.1) were able to exert both mechanisms of ice control; however, their ability to promote nucleation was limited, and this was ultimately more of a serendipitous observation than an intentional design consideration (152, 153). Although we did not go into the topic in depth, there is evidence that certain concentrations of specific types of AF(G)Ps can enhance nucleation while still retaining the ability to inhibit recrystallization (154, 171). This is specifically seen under conditions where the IBF of AF(G)Ps is exposed to liquid water (154, 171). The contrasting ability of AF(G)Ps to promote or inhibit nucleation is one that has only recently begun to be appreciated, and thus the underlying mechanism for simultaneous nucleation promotion and recrystallization inhibition is not well described. Additional research into this topic will invariably yield an opportunity to design novel biomimetics concurrently exhibiting these mechanisms of ice control.

6. CONCLUSION

The abundance of biomimetic technologies that can effectively recapitulate the activity of nature's mechanisms of ice control is incredibly impressive, especially considering that only five decades have passed since the initial discovery of AF(G)Ps. In a review of the literature on this topic, there is no apparent plateau in advances, and a constant influx of new ideas is apparent. One could certainly envision that five decades from now, these technologies will outperform ice-binding proteins [both INPs and AF(G)Ps] with respect to the degree and selectivity of the specific mode of ice control and will perhaps even possess functionality that is currently unforeseen. In such a scenario, they would be poised to fundamentally shift the landscape of cryobiology.

SUMMARY POINTS

1. Biomimetic ice control technologies are preferred over their natural counterparts [i.e., ice-nucleating proteins and antifreeze (glycol)proteins], whose use in biopreservation applications is hampered by biocompatibility, immunogenicity, and accessibility concerns.

2. The development of biomimetic compounds that control ice recrystallization has vastly outweighed the development of those that either promote or inhibit nucleation. A growing interest in storing complex systems such as tissues and organs at subzero temperatures requires careful control of nucleation (either promoting or inhibiting it depending on the preservation strategy being sought), making the compounds capable of doing this increasingly valuable.
3. The following points are suggestive that the surface of a compound or material could lower the free-energy barrier for nucleation: highly water insoluble, hydrophilic, and having an arrangement of molecules or atoms that closely matches that of an ice lattice. The careful introduction of positive charges or specific topological defects can additionally promote nucleation, although these are not prerequisites for nucleation.
4. Thermal hysteresis is always predicated on adsorption to the crystal, whereas the dependence of ice-recrystallization inhibition on crystal adsorption differs on the basis of the class of ice-recrystallization inhibitor (IRI) (e.g., many synthetic polymers must bind to the ice crystal to exert their IRI activity, while small-molecule carbohydrate-based IRIs, on the other hand, do not). The dynamic ice shaping that results from crystal adsorption can induce a damaging crystal morphology at temperatures below the hysteretic freezing point, making it preferable that adsorption is either absent or minimized in IRI-active compounds.
5. Although the molecular-level mechanisms responsible for ice-recrystallization inhibition remain ill defined, the overall number of hydrophilic versus hydrophobic groups as well as their 3D positioning have dictated the IRI activity of nearly all biomimetic IRI technologies developed thus far.

DISCLOSURE STATEMENT

J.P.A. is the CEO of PanTHERA CryoSolutions, a company that manufacturers ice-recrystallization inhibitors; R.N.B. and J.P.A. are cofounders and shareholders of PanTHERA CryoSolutions.

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