Chapter 7

Ice Recrystallization Inhibitors: From Biological Antifreezes to Small Molecules

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Additional information is available at the end of the chapter

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1. Introduction

Recrystallization is a phenomenon that is well documented in the geological and metallurgical literature. In metallurgy, the phenomenon can be formally defined as the process by which deformed grains are replaced by a new set of non-deformed grains that nucleate and grow until the original grains have been entirely consumed. A more precise definition is difficult as this process is quite complex. The phenomenon of recrystallization also occurs in ice, where it is similarly defined as the growth of large ice crystals (or grains) at the expense of small ones. Regardless of the definition or context in which recrystallization is discussed, it is a thermodynamically driven process which results in an overall reduction in the free energy of the system in which it is occurring.

While the exact mechanism(s) by which the phenomenon of recrystallization occurs remains controversial, the industrial significance and the benefits of preventing this process have been realized for hundreds of years. Within the context of ice, recrystallization has a direct impact on many areas such as glaciology, food preservation and cryo-medicine. However, it has been considerably less studied than the process of recrystallization in areas like metallurgy, materials and geology. This may not be entirely surprising as ice itself has very unique physical and chemical properties. While ice exists in several forms, ice I_h (pronounced "ice one h") is the most common form of ice found on Earth. The unique properties of ice and the complications these pose for the detailed study of ice will be described in this chapter with particular emphasis placed upon the efforts to identify and/or design inhibitors of the ice recrystallization process. While inhibitors of ice recrystallization have applications in preventing recrystallization processes in other substances, this review will focus on inhibiting ice recrystallization and its impact in cryopreservation.

As the phenomenon of recrystallization has origins in metallurgy, geology and materials a general discussion of this process with reference to these areas is necessary (Section 2.0), followed by a discussion on the structure and properties of ice and ice recrystallization (Section 3.0) and the importance of inhibiting ice recrystallization (Section 4.0). Finally, inhibitors of ice recrystallization and proposed mechanism(s) of action will be addressed, beginning with the first known inhibitors of ice recrystallization, biological antifreezes (Section 5.0), and concluding with novel synthetic peptides, glycopeptides, polymers and small molecules (Section 6.0). This chapter will conclude with a summary of the role of ice recrystallization in cryo-injury and a discussion on the cryoprotective ability of compounds that exhibit the ability to inhibit ice recrystallization, with the benefits and/or drawbacks of their use during cryopreservation (Section 7.0).

2. The phenomenon of recrystallization

As stated in the introduction, the process of recrystallization has been extensively studied and reviewed throughout the metallurgic literature. [1,2] While the mechanism is quite complex, it is generally defined as the thermally induced change in grain structure facilitated by the formation and/or migration of high angle grain boundaries and is driven by the stored energy of deformation. [1] A grain is defined as the microstructure that constitutes metals and alloys. In a metal, each grain consists of an ordered arrangement of atoms (depicted in Figure 1). [3,4] A grain boundary is the interface where two or more grains of different orientations meet and is considered a defect within the crystal structure. A grain boundary contains atoms that are not well aligned with neighboring grains, leading to less efficient packing and a less ordered structure within the grain boundary. [5] Thus, grain boundaries have a higher internal energy than ordered grains. [5,6] At elevated temperatures, atoms within grains are able to transfer between grain boundaries and neighboring grains. [3,4]

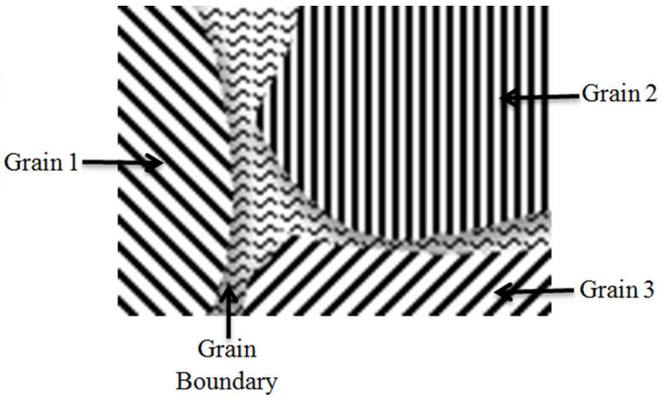

Figure 1. An illustration of grains and grain boundaries in polycrystalline metals and/or alloys.

The process of "plastic deformation" causes a permanent change in the shape of the metal or alloy. During this process, energy is stored mainly in the form of dislocations, ulti-

mately changing the grain shape. [1,2,7] Dislocations are areas where atoms are out of position in the crystalline structure and are linear defects within the grain due to the misalignment of atoms. The amount of dislocations present after deformation is significantly greater than the amount of dislocations prior to deformation. [7] Consequently, the amount of stored energy and the amount of grain strain after deformation is also increased. Heating and annealing of the metal or alloy at or above the recrystallization temperature allows strain-free grains to nucleate and/or migrate within the polycrystalline lattice to minimize the amount of dislocations present within this new set of grains. Thus, the driving force of recrystallization in metals is to eliminate dislocations present in the material to reduce this amount of stored energy in the system. [2]

Recrystallization is an important step in the processing of metals and alloys and can be a desirable or undesirable effect. This is attributed to the fact that recrystallization in metals and alloys ultimately results in a decrease in the strength of the metal. Polycrystalline metals containing smaller grains and more dislocations are significantly stronger than those with larger grains according to the Hall-Petch relationship. [7-9] However, during recrystallization strain-free grains grow to reduce the amount of stored energy from dislocations. As such, the metal is softened and its ductility is increased due to the formation of larger strain-free grains. This process can be a significant problem in metals and/or alloys when these materials are used for structural support where a decrease in metal strength is often detrimental. In contrast, recrystallization can also be beneficial and purposely induced to soften and restore the ductility of metals and alloys that have been hardened by low temperature deformation or cold work, or to control the grain structure of the final metal or alloy product. [1,2,10] For example, metals and alloys that have been deformed by "cold working" (deformation below the recrystallization temperature of the metal or alloy) become stronger and more brittle. [7] Inducing recrystallization will anneal the material to allow it to be deformed further without the risk of cracking or breaking.

3. Recrystallization in ice

Ice has many different polymorphic forms. Individual water molecules in ice can possess different arrangements within three-dimensional space and this is dependent upon temperature and pressure. The most common form of ice below 0 °C and atmospheric pressure is the hexagonal ice I_h lattice unit. [11,12] It possesses a regular crystalline structure in which a single oxygen atom is hydrogen-bonded to two hydrogen atoms. The hexagonal ice I_h lattice unit is characterized by four axes, a_1, a_2, a_3 and c, and the surface of the hexagonal unit has eight faces. [11-14] Two of these faces are normal to the c-axis and are the basal faces, and the remaining six are prism faces. The structure of hexagonal ice is shown in Figure 2. The arrangement of intermolecular hydrogen bonds influences the properties and phases of ice. At 0 °C and atmospheric pressure ice grows most rapidly along the a-axis to give hexagonal shaped crystals which grow as sheets. [11-13,15]

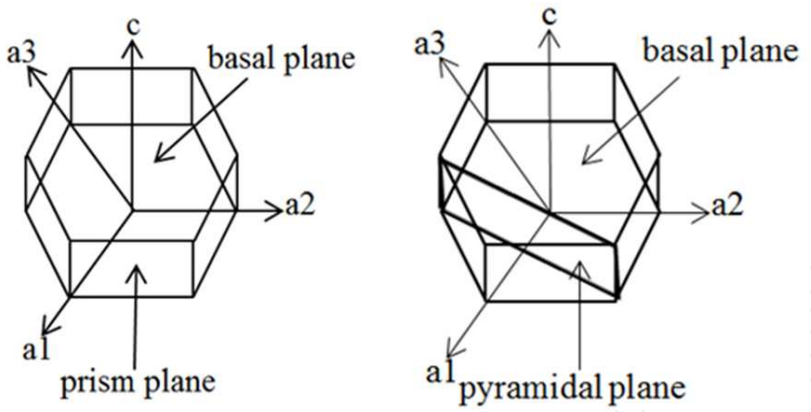

Figure 2. Schematic representation of the hexagonal ice I_h lattice unit illustrating the a_1, a_2, a_3 and c axes and the basal, prism and pyramidal planes.

When ice is in an aqueous solution, the interface between the ice lattice and bulk water is not an abrupt transition. Studies have indicated that a semi-ordered layer exists in between the highly ordered ice lattice and the less ordered bulk water surrounding ice crystals [14,16-22]. This layer has been named the quasi-liquid layer (QLL). While more than 150 years ago Michael Faraday proposed that the surface of ice when near the melting temperature is covered by a thin liquid layer, Fletcher was the first to propose a model for the existence of the QLL in 1962, which was subsequently revised in 1968. [16,17] Important insights on the properties of the QLL was described by Haymet where using molecular dynamic simulations and the TIP4P model of water, the structure and dynamics of the ice/water interface was studied. [18,19] Data from these simulations made it possible to calculate the density profile, molecular orientation and diffusion constants of water molecules in the QLL. The thickness of interface region between ice lattice and bulk water is approximately 10-15 Å thick, but this has been shown to be temperature dependent. [18,19,23] The average density profile, translational and orientational order and diffusion constants of water within the QLL interface also vary depending on the face of ice from which they are calculated. Studies have suggested that the QLL is thicker on the basal and prism faces than on the pyramidal and secondary prism planes. [14]

The exact molecular nature and thickness of the QLL interface has been debated throughout the literature and a wide variety of techniques have been used to study it including atomic force microscopy, [24] X-ray diffraction, [25] infrared spectroscopy, [26] proton-backscattering, [27] Raman spectroscopy, [28] quartz-crystal microbalance measurements, [29] light scattering techniques, [30-32] photoelectron spectroscopy, [33] optical ellipsometry, [22,34,35] optical reflection [36] and mechanical measurements. [37] Ellipsometric studies measuring the refractive index on the basal and prism faces of ice have suggested that the interface is more water-like in nature, rather than ice-like. [20-22,24,33,34] In contrast, other studies have suggested that the orientation and motion of water molecules in the QLL closely resembles that of ice. [25,27,36] The thickness of the QLL has been shown to be temperature dependent, [29,33] such that at temperatures approaching the melting point of ice (at -0.03 °C) the thickness was 15 nm, corresponding to approximately 40 monolayers water. [26] However, below -10 °C the thickness was less than 0.3 nm,

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approximately one monolayer of water. The effect of temperature and thickness also varies depending on the face of ice (prism or basal) from which it is calculated, [20,22] and studies have also reported that there is twice as much anisotropy of the water molecules in the QLL for the prism face than the basal face. [34] Light scattering techniques have shown that ice crystals grow into the QLL and not into the bulk water layer. [38,39]

The recrystallization of ice in polycrystalline aqueous solutions is believed to occur through either grain boundary migration or Ostwald ripening. Grain boundary migration in ice is similar to grain boundary migration in metals and alloys where large ice grains grow larger at the expense of small ice grains. In metallurgy a grain consists of an ordered arrangement of atoms and a grain boundary is the interface where two (or more) grains meet. However, in ice a grain consists of the crystallographic orientation of the water molecules commonly observed in ice I_h (Figure 2). Grain boundaries are therefore the interfaces between different oriented ice grains. [40,41] Grain boundary migration occurs as individual molecules transfer from unfavorably oriented ice grains to favorably oriented ice grains. The boundaries of individual ice grains tend to be curved and the degree of curvature is proportional to the size of the grain. Boundaries of small ice crystals have a higher degree of curvature making them more convex (bulge outwards) and thus have a higher amount of surface energy. Large ice crystals have more concave grain boundaries and have a lower amount of surface energy. Grain boundaries migrate towards their center of curvature to reduce the overall degree of curvature, resulting in ice grains with concave boundaries (larger crystals) growing larger while those with convex boundaries (smaller crystals) decrease in size (depicted in Figure 3). [42,43] Thus, the driving force of grain boundary migration in ice arises from a reduction in grain boundary curvature, which results in an overall reduction in the energy of the system.

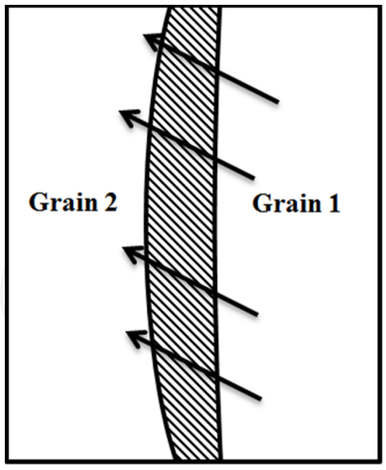

Figure 3. Representation of a liquid-layer (shaded) in a curved boundary between two ice grains. Large ice grains with concave boundaries (grain 2) grow larger while small grains with convex boundaries (grain 1) decrease in size to reduce the overall degree of grain boundary curvature. Arrows indicate the direction of boundary migration.

Grain boundary migration of polycrystalline ice assumes that water molecules are transferred directly from the shrinking ice grain to the growing grain. This assumption often neglects the presence of bulk-water or the QLL in between individual ice grains as the system is treated

below -10 °C. [42] However, Ostwald ripening of polycrystalline ice in an aqueous solution considers the whole ice crystal/liquid water system and thus accounts for the presence of bulk-water and the QLL. In ice, Ostwald ripening is the thermodynamically driven process whereby large ice crystals grow larger at the expense of small crystals, resulting in an overall reduction in energy of the ice crystal/bulk-water interface. [44-46] Throughout the Ostwald ripening process a constant ice volume is maintained. Smaller ice crystals have a higher surface area to volume ratio, giving them higher surface free energy since water molecules on the surface are less stable than the water molecules within the ice crystal. [44,45] However, larger ice crystals have a greater volume to surface area ratio and thus are thermodynamically more stable than small ice crystals. As the total overall volume of ice remains constant during the Ostwald ripening process, water molecules transfer from the surface of smaller ice crystals to bulk-water and then are transferred onto the surface of larger ice crystals. The net result is an increase in the average ice crystal size and a decrease in the total number of ice crystals at a constant total ice volume, resulting in an overall reduction in the free energy of the system. [46]

4. Impact of recrystallization

Ice recrystallization is particularly problematic in the areas of frozen foods and cryopreservation of biological samples (cells, tissues etc.). Freezing of foods is a well-established process as it helps decrease the rates of deterioration. In the last 30 years, the frozen food industry has taken significant steps to improve the freezing and storage process of various food products, recognizing that all frozen food products have a finite shelf. [47] Changes in texture, taste and overall quality of a frozen food product are a direct result of the ice recrystallization process. It is well established that ice morphology is an important factor in determining food texture and quality. For example, ice cream containing small ice crystals has better texture and taste. [48]

In medicine, cryostorage is an important process to preserve biological materials or precious cell types such as stem cells (or other progenitors) as well as red blood cells. However, as with any cold storage practice, ice recrystallization remains a major problem and is a significant cause of cellular damage and cell death. [49,50] Section 7.1 of this chapter provides a detailed discussion on the role of ice recrystallization in cryo-injury however, to address these problems effective inhibitors of ice recrystallization are urgently required. Naturally occurring biological antifreezes are very effective inhibitors of ice recrystallization. Biological antifreezes (BAs) are peptides or glycopeptides typically found in organisms inhabiting sub-zero environments. The biological purpose of these compounds is to prevent the seeding of ice crystals *in vivo* and prevent cryoinjury and death.

5. Biological antifreezes as inhibitors of ice recrystallization

The first biological antifreezes were reported in the late 1950s. [51,52] Given their ability to prevent cryoinjury upon exposure to cold temperatures, [53-55] they have attracted a great

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deal of interest in the scientific and industrial communities. [56] Biological antifreezes are a complex class of compounds with dramatically different structures, making it difficult to understand how they inhibit ice recrystallization. Nevertheless, this important class of compounds is the foundation upon which all "rationally designed" novel ice recrystallization inhibitors are based, including the more recently reported small molecule inhibitors of ice recrystallization. [57-62]

5.1. Structures of Biological Antifreezes (BAs)

In the late 1950s and early 1960s it was observed by Scholander and colleagues that marine teleost fish did not freeze during the winter despite the water temperature being -1.9 °C, over a degree below the freezing point of their blood serum. [51,52] DeVries and Wohlschlag later attributed their survival to the presence of circulating proteins and glycoproteins. [53-55] These proteins later became known as biological antifreezes, specifically antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs). A variety of AFPs and AFGPs have since been identified in a number of different fish, insects, plants and bacteria.

There are four classes of structurally diverse fish AFPs that have been identified. These are type I, [13,63-72] type II, [73-81] type III, [73-77,82-87] and type IV AFPs. [88-90] The four types of fish AFPs have a wide variation in their size, which can range from 3-12 kDa, and in their secondary structures, which can be α-helices, β-rolls, random coils and globular structures. AFGPs are also present in fish, and are comprised of a tripeptide repeat of $(Thr\text{-}Ala\text{-}Ala)_n$, in which the secondary hydroxyl group of threonine is glycosylated with the disaccharide β-D-galactosyl-(1-3)-α-*N*-acetyl-D- galactosamine (structure shown in Figure 4). [15,55,60,61,91-95] In general, AFGPs have a homologous structure and have been separated into eight subclasses, AFGP 1-8, based on their molecular masses which range from 2.6 kDa (n = 4) to 33.7 kDa (n = 50). [55] Minor sequence variations have been identified in AFGPs where the first alanine residue is replaced by proline, or where the glycosylated threonine residue is occasionally replace by arginine. [96-101] The solution structure of AFGPs has been debated in the literature. Early circular dichroism (CD) and nuclear magnetic resonance (NMR) studies suggested AFGPs adopt an extended random coil structure. [102-107] However, studies have also suggested that they adopt an ordered helix similar to a PPII type II helix. [106,108-110] It has also suggested that they adopt an amphipathic helical structure, with a hydrophilic face containing the exposed hydroxyl groups of the disaccharide moiety and a hydrophobic face containing the exposed methyl groups of the amino acid residues. [72] However, the most recent studies have indicated that AFGP 1-5 possess no form of long-range order and that AFGP-8 is predominantly random coil with short segments of localized order. [106-108] A brief summary of the key structural differences between AFPs and AFGPs is provided in Figure 4.

A number of other AFPs have been identified in other organisms. Various insect AFPs have been identified such as those from the spruce budworm moth (*Choristoneura fumiferana*, *Cf*AFP), [111,112] the yellow mealworm beetle (*Tenebrio molitor*, *Tm*AFP), [113,114] the fire-coloured beetle (*Dendroides canadensis*, *Dc*AFP), [115] and the snow flea (sfAFP). [116] Plant AFPs have also been identified from carrot (*Daucus carrota*), [117] bittersweet nightshade (*Solanum dulcamara*), [118] perennial ryegrass (*Lolium perenne*), [119-121] Antarctic hair grass

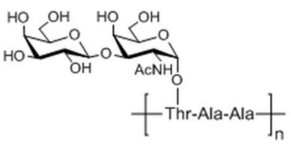
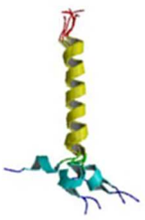
Characteristic	AFGP	Type I AFP	Type II AFP	Type III AFP	Type IV AFP
Mass (kDa)	2.6 - 33	3.3 – 4.5	11 – 24	6.5	12
Key Properties	AAT repeat; disaccharide	Alanine-rich α-helix	Disulfide bonded	β-sandwich	Alanine rich; helical bundle
Representative Structure					
Natural Source	Antarctic Notothenioids; northern cods	Right-eyed flounders; sculpins	Sea raven; smelt; herring	Ocean pout; wolfish; eel pout	Longhorn sculpin

Figure 4. Classification and structural differences between fish antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs).

(*Deschampsia antartica*), [122] and several other species. [123,124] Additionally, AFPs have been identified in fungi and bacteria. [125-130] The secondary structures of the various AFPs from plants and insects are also diverse. [131,132] Regardless of where the AFPs are found or their secondary structure, they are all ice-binding proteins that are crucial for the species survival in the harsh cold environments to which they are exposed.

5.2. "Antifreeze" activities of biological antifreezes: Thermal Hysteresis (TH) and Ice Recrystallization Inhibition (IRI) activity

Biological antifreezes exhibit two types of antifreeze activities. The first and the most studied is thermal hysteresis (TH). This is defined as a selective depression of the freezing point of a solution relative to the melting point. [133-135] TH activity is the direct result of the binding of a BA to the surface of a seeded ice crystal. [136,137] The binding of the BA to the surface of ice facilitates a localized freezing point depression and induces a change in the ice crystal habit. This change in ice crystal habit is referred to as dynamic ice shaping (DIS) and is illustrated in Figure 5A. A more detailed description of this process is described in Section 5.3. The standard assay used to measure TH activity is nanolitre osmometry. [138] In this assay, a single ice crystal in an aqueous solution of the biological antifreeze is obtained, and the growth and behavior of the crystal upon increasing/decreasing the temperature can be observed. TH activity is reported as the difference between the observed freezing and melting points in Kelvin or degrees Celsius.

The second type of antifreeze activity exhibited by biological antifreezes is their ability to inhibit ice recrystallization (referred to as ice recrystallization inhibition (IRI) activity). [41,139]

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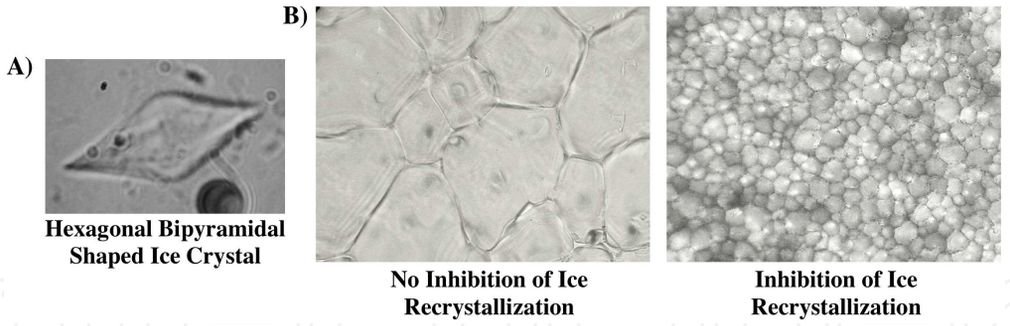

Figure 5. Photographs illustrating dynamic ice shaping (DIS) and ice recrystallization inhibition (IRI) activity. **A)** Ice crystal habit in the presence of 10 mg/mL AFGP-8. The binding of AFGP-8 to the surface of ice crystals induces a change in ice crystal habit, resulting in hexagonal bipyramidal (or spicule) ice crystal shapes. **B)** Photographs of annealed ice grains obtained from a splat-cooling assay. A compound that can inhibit ice recrystallization is able to maintain small ice crystal sizes within a frozen solution.

An illustration of this process is shown in Figure 5B. Inhibiting ice recrystallization results in very small ice crystals within a frozen sample. The ability to maintain small ice crystal size within a frozen solution is a highly desirable property and compounds exhibiting this property have tremendous medical, commercial and industrial applications.

While there are various methods for assessing IRI activity such as the capillary method assay [140,141] or the use of wide-angle X-ray scattering (WAXS) and differential scanning calorimetry (DSC), [142-144] the most commonly used is the splat-cooling assay. [139] In the splat-cooling assay recrystallization can be observed by the change in size of individual ice grains. Briefly, the sample solution is frozen as a thin circular wafer by either dropping a small aliquot onto a precooled (-80 °C) polished aluminum block from a height of approximately 2 meters, [139] or by pressing the solution between two coverslips and freezing. [117] The samples are then annealed at a temperature below 0 °C and the ice crystal size distribution of the sample after a given time is observed. Ice crystal size can be quantified by measuring the mean largest ice grain dimension along any axis [59,145] or by measuring the mean ice grain area. [46,146] Thus, smaller ice crystal sizes represent greater IRI activity. Commonly, analytes are assayed in a salt solution (NaCl, $CaCl_2$ or phosphate buffered saline (PBS)) or a 30-45% sucrose solution, and the solutions without analyte are used as positive controls for ice recrystallization for comparison. The presence of salt or other small solutes is very important as it ensures that liquid is present between ice crystal boundaries and the presence of these solutes negates non-specific IRI effects that can be observed in pure water. [41] While the original version of this assay was subjective in nature, it has recently been improved using Domain Recognition Software (DRS). [146] IRI can now be reliably quantified, providing accurate comparisons between samples and information on small and subtle changes in IRI activity within a series of analogues.

5.3. Biological antifreezes - Mechanisms of action for Thermal Hysteresis (TH) activity

The most widely accepted mechanism for thermal hysteresis (TH) involves an irreversible adsorption-inhibition process. [133-137] In this mechanism, BAs irreversibly bind to specific

planes of a growing ice crystal. Preferential binding occurs on the prism faces of ice, thus inhibiting ice growth along the *a*-axis. [93,147-149] Ice crystal growth continues as the temperature of the solution is decreased below the hysteresis freezing point, however it occurs along the *c*-axis, giving rise to the characteristic hexagonal bipyramidal (or spicule) crystal shapes (illustrated in Figure 6). [133,150] The faces that BAs bind to can be determined experimentally by ice hemisphere etching. [136] In this experiment, a single ice crystal in a dilute solution of the BA is grown into a hemisphere such that all interfacial orientations are present during growth. As adsorption of the BA to ice is irreversible, the BA is incorporated into the crystal during growth. Sublimation of the ice crystal then results in visibly etched regions on the ice surface where the BA adsorbed and the orientation of these regions can be observed. While it has been determined that BAs adsorb preferentially to the prism planes of a seeded ice crystal, various insect and plant AFPs adsorb to the basal planes, and it is postulated this results in the superior TH activity exhibited by these proteins. [131,132,151]

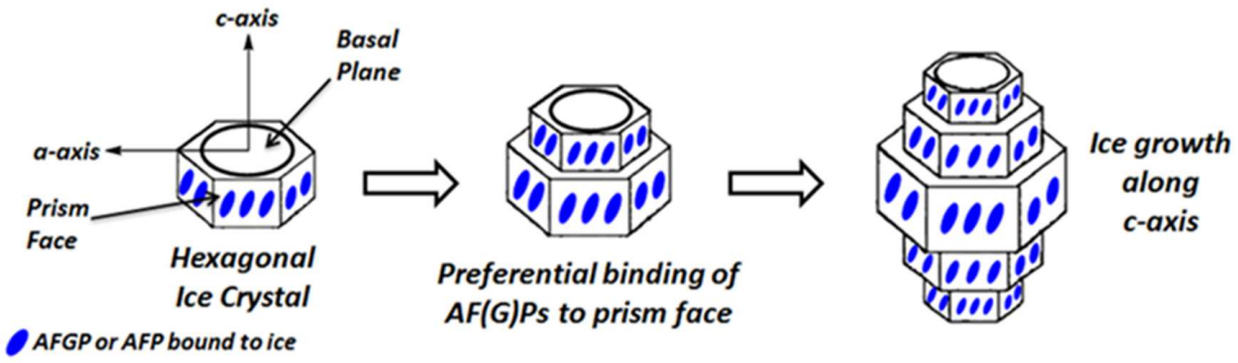

Figure 6. Formation of hexagonal bipyramidal ice crystals by inhibition of growth on the prism faces due to adsorption of BAs.

The irreversible binding of a BA to the surface of ice crystals results in a localized freezing point depression. This occurs *via* the Kelvin (or Gibbs-Thomson) Effect. [135] Given that ice growth cannot occur where the BA has adsorbed, growth occurs on the ice surfaces between adjacent BA molecules, resulting in curved ice surfaces (shown in Figure 7). The energetic cost of adding a water molecule (freezing) to this curved surface is high and it becomes unfavorable for more water molecules to add to this surface, thus a localized freezing point depression is observed. This process does not affect the energetics of the melting process, hence only the freezing point is depressed while the melting point remains constant, resulting in a thermal hysteresis gap (Figure 7A). [135,149,152]

There are two models that described how BAs inhibit ice growth within the thermal hysteretic gap. The first (illustrated in Figure 7B) was proposed by Raymond and DeVries and is known as the step pinning model. In this model, the growth of a step is inhibited by the BA which has pinned ice growth across the ice surface. [133] However, this model assumes that ice crystal growth occurs in steps advancing across the plane that the BA is adsorbed. The second model (illustrated in Figure 7C) is a three-dimensional model known as the mattress model and was

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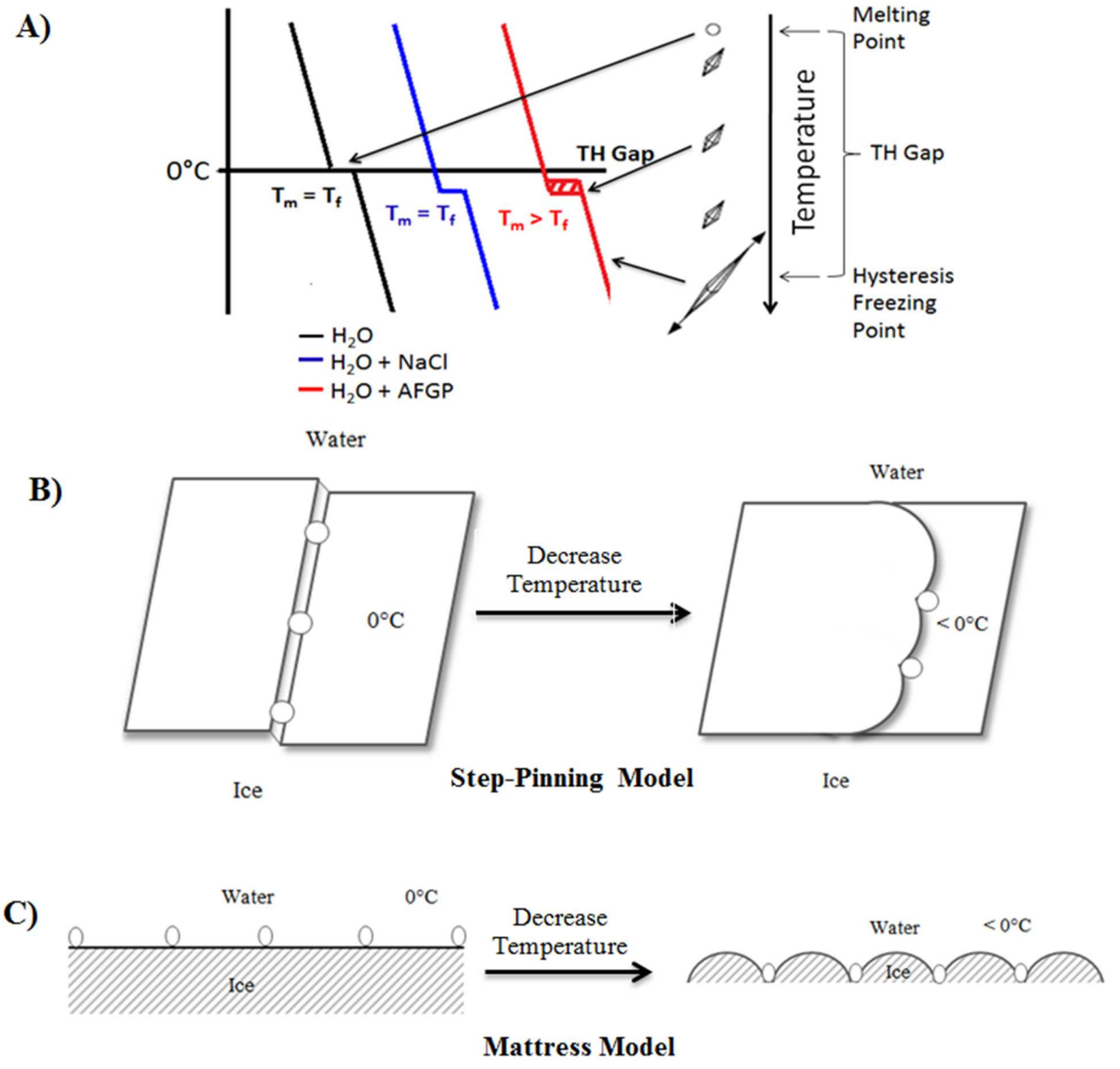

Figure 7. Illustrations of thermal hysteresis (TH) activity and the two models of ice growth inhibition. **A)** BAs have the ability to depress the freezing point of ice crystals relative to the melting point, resulting in a thermal hysteresis gap. **B)** Step-pinning model and **C)** mattress model depicting the irreversible adsorption-inhibition mechanism of BAs.

proposed by Knight and DeVries. In this model, the adsorbed BA molecules exhibit inhibition by pinning ice growth normal (perpendicular) to the ice surface. [136]

Both of these models assume an irreversible adsorption of the BA onto the surface of ice. However, there have been reports suggesting that the adsorption is reversible. The main argument in favour of this is that if adsorption were truly irreversible then significant levels of adsorption would be observed in the presence of very low concentrations of BAs, [67] however this has not been definitively observed. Furthermore, a large free energy of adsorption of BAs would be expected, but it has been observed that the free energy of adsorption is close to zero. [153] Consequently, alternative mechanisms have been proposed describing ice

growth inhibition of BAs. [153-156] Regardless of these alternate mechanisms, sufficient data exists to suggest an irreversible adsorption-inhibition mechanism, and consequently this model is the generally accepted mechanism by which BAs exhibit TH activity.

It should be emphasized that the ability to bind to ice is believed to be a property unique to BAs. However, it has been reported that polyvinyl alcohol (PVA) can bind to ice and exhibit a small degree of thermal hysteresis. [157] It was originally proposed that adsorption of BAs to the surface of ice occurred through the hydrogen bonding of hydrophilic groups to the oxygen atoms in the ice lattice. [12,158] However, this is contradictory to the current mechanism of action for AFPs where the importance of hydrogen bonding between polar residues and ice has been questioned. Alternatively, it has been demonstrated that entropic and enthalpic contributions from hydrophobic residues are crucial for ice binding. [159-161] The importance of hydrophobic residues has been validated with a number of different AFPs through site-specific mutagenesis studies, [82,159,162,163] and in general it is believed that the ice-binding site of these AFPs is hydrophobic and has a discrete complementarity with the planes of ice to which it binds. [82,162-165]

In contrast to AFPs, the current hypothesis of how AFGPs bind to ice involves hydrogen bonding between the hydroxyl groups of the sugars and the ice lattice. [137] A landmark study conducted by Nishimura and co-workers investigated the key structural features of AFGPs that were crucial for ice binding and TH activity. [166] In this study it was reported that three key motifs were required for TH activity (shown in Figure 8): 1) the *N*-acetyl group at the C2 position of the galactosamine; 2) the α-configuration of the *O*-glycosidic linkage between the disaccharide and the peptide chain; 3) the γ-methyl group of the threonyl residue. In addition, the TH activity of homogenous AFGPs is dependent upon the length of the glycoprotein segment. [166,167]

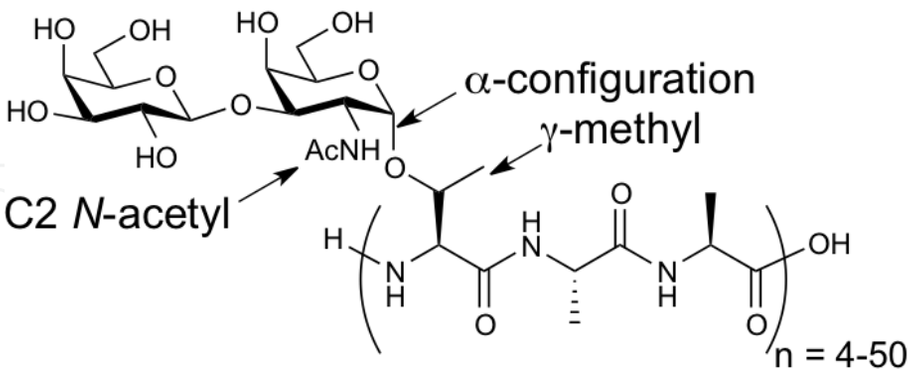

Figure 8. Important structural motifs on AFGPs for TH activity as determined by Nishimura and co-workers. [166]

Despite the tremendous number of structure-function studies conducted on AFPs and AFGPs over the last three decades, in all cases only TH activity has been assessed and correlated to structural modifications. The ability of these analogues to inhibit ice recrystallization has not been assessed, and consequently the structural features necessary for potent ice recrystallization inhibition (IRI) activity are not known. This is unfortunate as IRI activity is a highly

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desirable property for a compound to exhibit due to the many potential medical and industrial applications. Furthermore, while BAs do possess potent IRI activity, they cannot be used effectively as cryoprotectants. The ice binding ability associated with the TH activity of BAs alters the habit of ice crystals, and since the temperatures employed during cryopreservation are outside of the TH gap, this exacerbates cellular damage. [168-170] However, during the last several years considerable amount of progress has been made in discovering novel ice recrystallization inhibitors, some of which are synthetic analogues of AFGPs, and the work that has been conducted in this area will be the focus of the next section.

6. Inhibitors of ice recrystallization

Biological antifreezes are excellent inhibitors of ice recrystallization. However, as stated in the previous section, the dynamic ice shaping (DIS) capabilities prohibits their use in applications where ice recrystallization inhibition (IRI) activity is highly desirable. Thus, the purpose of the following section will be to summarize the progress towards designing molecules that exhibit the ability to inhibit ice recrystallization without the ability to bind to ice, and on understanding the key structural features that are important for the IRI activity exhibited by these molecules.

6.1. Peptide and glycopeptide analogues of biological antifreezes as ice recrystallization inhibitors

One of the first studies that examined ice recrystallization inhibition (IRI) activity of peptides and conventional polymers was conducted by Knight *et al.* in 1995. [41] In this study, a type I winter flounder antifreeze protein and six analogues of this protein were investigated for their ability to inhibit ice recrystallization, along with four polypeptides and three polymers including polyvinyl alcohol (PVA). One of the conclusions from this study was that all analogues of the antifreeze protein were completely IRI inactive in 0.1% and 0.5% NaCl solutions, a result that correlated with the reduced TH activity in comparison to the native AFP exhibited by these analogues. [41,171] It was also reported that poly-L-histidine, poly-L-hydroxyproline and PVA exhibited IRI activity at concentrations less than 1 mg/mL in pure water, whereas poly-L-aspartic acid, poly-L-asparagine, polyacrylic acid and polyvinylpyrrolidone were inactive. These polypeptides and polymers were not assessed for IRI activity in NaCl solutions.

This study ultimately suggested there was a correlation between TH and IRI activity in the type I AFP. [41,171] While it is well known that biological antifreezes exhibit both types of antifreeze activity, the relationship between TH and IRI has been debated throughout the literature. It was previously suggested that these two properties were directly correlated and derived from the ability of BAs to bind to ice. [139,153] In contrast, it has been suggested there is little or no correlation between TH and IRI as some plant AFPs typically exhibit a low degree of TH activity but a high degree of IRI activity. [119,120] Furthermore, the elevated TH activity exhibited by hyperactive insect AFPs is often not accompanied by highly potent IRI activity. [141] To date, few studies have emerged examining the relationship between TH and IRI

activity in native BAs, and those that have, report IRI activity using methods other than the traditional splat-cooling assay, [141] making it difficult to ascertain definitive conclusions about the correlation between TH and IRI.

Payne and co-workers recently published a study in 2012 examining the correlation of glycopeptide/glycoprotein mass on both TH and IRI activity for a range of homogeneous synthetic AFGPs (synAFGPs). [167] A native chemical ligation-desulfurization approach was used for the first convergent synthesis of homogenous synAFGPs that ranged in molecular mass from 1.2 – 19.5 kDa (compounds **1**-**6**, Figure 9). Increasing the length of the glycopeptide to eight and twelve tripeptide repeats (synAFGP$_8$ and synAFGP$_{12}$, **3** and **4**) increased TH and IRI activity. However, increasing the number of tripeptide repeats to 16 (synAFGP$_{16}$, **5**) led to reduced TH and IRI activity. Additional elongation of the glycopeptide to 32 tripeptide repeats (synAFGP$_{32}$, **6**) restored the potent TH and IRI activities exhibited by these glycopeptides. Interestingly, while synAFGP$_{16}$ (**5**) exhibited less TH activity than synAFGP$_8$ (**3**), both had similar IRI activities. Furthermore, while synAFGP$_{12}$ (**4**) and synAFGP$_{32}$ (**6**) exhibited similar TH and IRI activities and were three times more IRI active than synAFGP$_8$ (**3**) and synAFGP$_{16}$ (**5**), they twice as TH active than synAFGP$_8$ and four times as TH active as synAFGP$_{16}$. These results support the hypothesis that the two types of antifreeze activities may not be as closely correlated as previously thought as the magnitude of change in TH activity was not reflected in IRI activity with these homogenous synAFGPs. While further work is still required in this area to verify this hypothesis, studies on synthetic structural analogues of AFGPs have shown it is possible to decouple the two types of antifreeze activities from each other, resulting in compounds that exhibit "custom-tailored" antifreeze activity and are only IRI active and not TH active. [57,58]

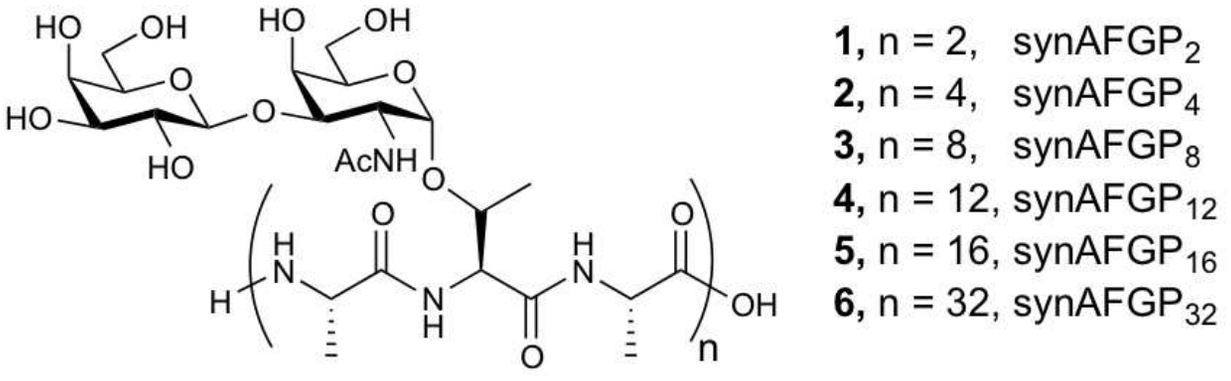

Figure 9. Structures of homogeneous synthetic AFGPs (synAFGPs) reported by Payne and co-workers. [167]

Most of the peptide and glycopeptides that have been assessed for IRI activity have been synthetic structural analogues of AFGPs. The Ben laboratory published the first series of analogues with dramatic structural modifications relative to the AFGP structure, and these analogues maintained the potent IRI activity exhibited by AFGP-8 at equimolar concentrations but did not exhibit TH activity. These analogues were carbon-linked or *C*-linked analogues, and consequently did not possess the *O*-glycosidic linkage found in AFGPs which is suscep-

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tible to hydrolysis under basic or acidic conditions. The first of these analogues was reported in 2003 (shown in Figure 10). [172] In comparison to AFGPs, the terminal galactose unit and the *N*-acetyl group were removed leaving only an α-D- galactosyl unit that was conjugated to lysine residues. Lysine was used due to its structural similarity to an arginine residue, which was occasionally found in native AFGPs (see section 5.1). In addition, the alanine residues present in AFGPs were substituted with glycine residues to avoid racemization encountered during solid-phase synthesis. [172,173] The monomer tripeptide unit (**7**) and the analogue with three repeating tripeptide units (**8**) did not exhibit IRI activity. However, the analogues with six and nine repeating tripeptide units (derivatives **9** and **10**, respectively) were both moderately IRI active. Derivatives **9** and **10** were also assessed for TH activity and both exhibited a small TH gap of 0.06 °C and induced the formation of hexagonal shaped ice crystals.

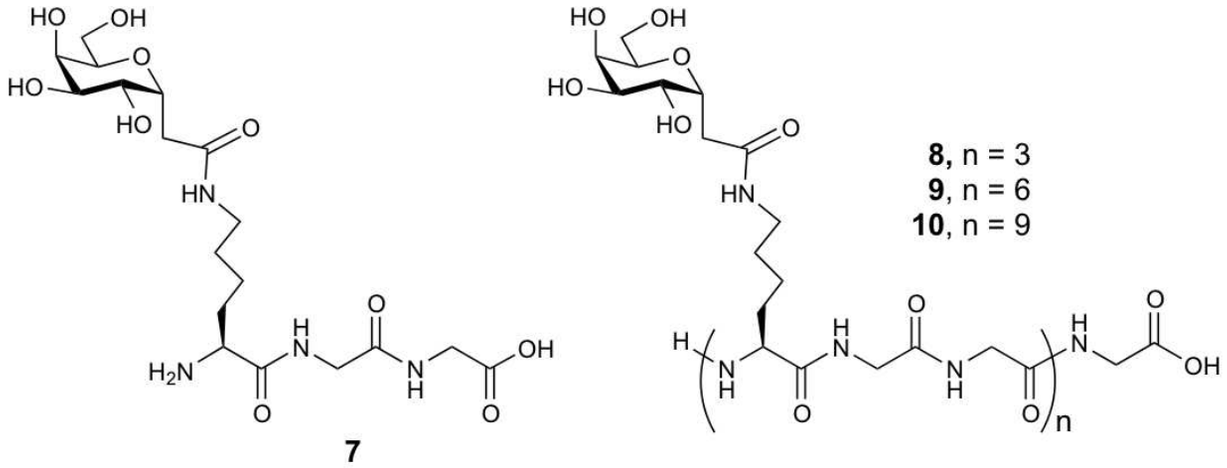

Figure 10. Structure of first-generation lysine-based *C*-linked AFGP analogues reported by Ben. [172]

The Ben laboratory has published two other *C*-linked AFGP analogues that exhibit potent IRI activity. These are derivatives **11** and **12** (Figure 11). Derivative **11** contains four tripeptide repeats, in which a *C*-linked galactosyl unit is incorporated. [57] Derivative **12** also contains four tripeptide repeats, and is structurally similar to lysine derivatives **8-10**, however the *C*-linked α-D- galactosyl unit is conjugated to an ornithine residue. [58] Both of these derivatives exhibited potent IRI activity at 5.5 μM and their activity was similar to that exhibited by AFGP-8 at 5.5 μM. Unlike AFGP-8, neither of these derivatives exhibited TH activity and while **12** exhibited very weak dynamic ice shaping, [58] **11** did not exhibit any ice shaping capabilities. [57] This suggested that the exhibited IRI activity was not likely due to ice binding. These analogues were the first examples where the two properties of biological antifreezes, TH and IRI activity, were decoupled from each other. Additionally, these *C*-linked AFGP analogues were the first compounds that possessed "custom-tailored" antifreeze activity, meaning they exhibited potent IRI activity with little or no measureable TH activity.

Following the discovery of the two novel synthetic ice recrystallization inhibitors **11** and **12**, two studies have been reported that identify the structural features necessary for the potent IRI activity of these *C*-linked analogues. The first structure-function study was conducted on

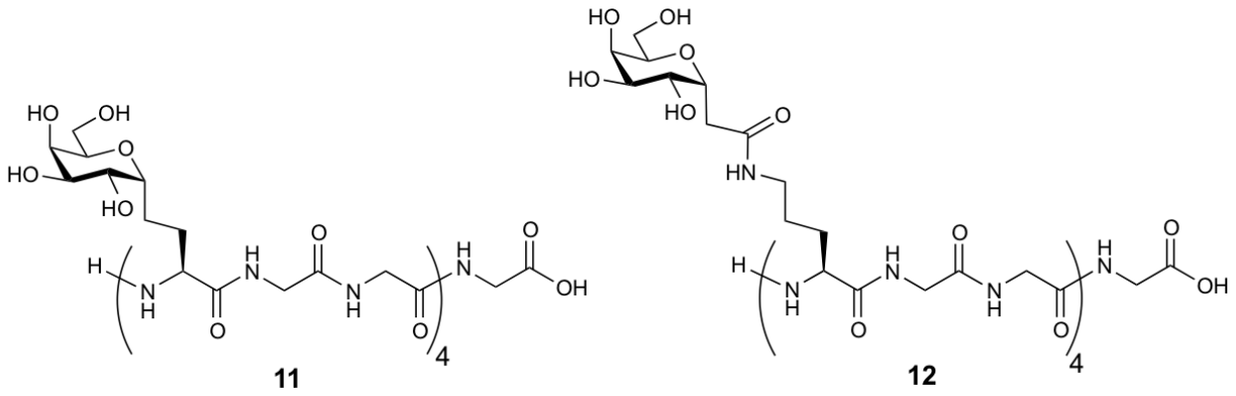

Figure 11. Structures of potently IRI active *C*-linked AFGP analogues **11** and **12** reported by the Ben laboratory. [57,58] Analogues **11** and **12** are the first compounds reported to exhibit "custom-tailored" antifreeze activity, meaning they exhibit potent IRI activity but not TH activity.

C-linked AFGP analogue **12** and examined the importance of the carbohydrate moiety. [58] The galactosyl moiety of **12** was substituted with three other monosaccharides: glucose, mannose and talose (analogues **13**, **14** and **15**, respectively, Figure 12). It was found that replacing the galactosyl unit with other monosaccharides was highly detrimental for IRI activity. The glucose analogue **13** exhibited weak activity, whereas the mannose and talose analogues (**14** and **15**) were inactive. The results showed that the stereochemical relationship of the hydroxyl groups on the carbohydrate moiety on the polypeptide has a direct affect upon IRI activity. The stereochemical relationship of the hydroxyl groups on simple carbohydrates (mono- and disaccharides) is known to influence the hydration of carbohydrates. [174-176] This lead to the observation that carbohydrate hydration was important for IRI activity. [58] A more detailed discussion of carbohydrate hydration and its influence on IRI activity is provided in section 6.3 of this chapter. Briefly, carbohydrate hydration influences IRI activity by altering the ordering of bulk-water based on the compatibility of the carbohydrate within the three-dimensional hydrogen-bonded network of water. [58,177] The hydration of a carbohydrate is related to the compatibility of the sugar with the three-dimensional hydrogen-bond network of water. [174-176] Of the monosaccharides assessed, talose is the most compatible and is thought to have the best "fit" into this hydrogen-bond network, whereas galactose is the least compatible and has the worse "fit". It was hypothesized that a poorer "fit" of the carbohydrate into the hydrogen-bond network of bulk water resulted in a more disordered bulk water layer between the semi-ordered quasi-liquid layer and ordered ice crystal layer. Consequently, transferring water molecules from a more disordered bulk water layer to an ordered layer was energetically unfavorable. Thus, carbohydrates that are highly hydrated resulted in greater IRI activity. [177] While the overall hydration of the *C*-linked glycoconjugates **12**-**15** is not known, having a more highly hydrated carbohydrate moiety conjugated on the glycopeptide (ie. galactose) was significantly better for IRI activity than a less hydrated carbohydrate moiety. [58]

The second structure-function study examined how the distance between the galactosyl moiety and the polypeptide backbone influenced IRI activity. In this study, the distance

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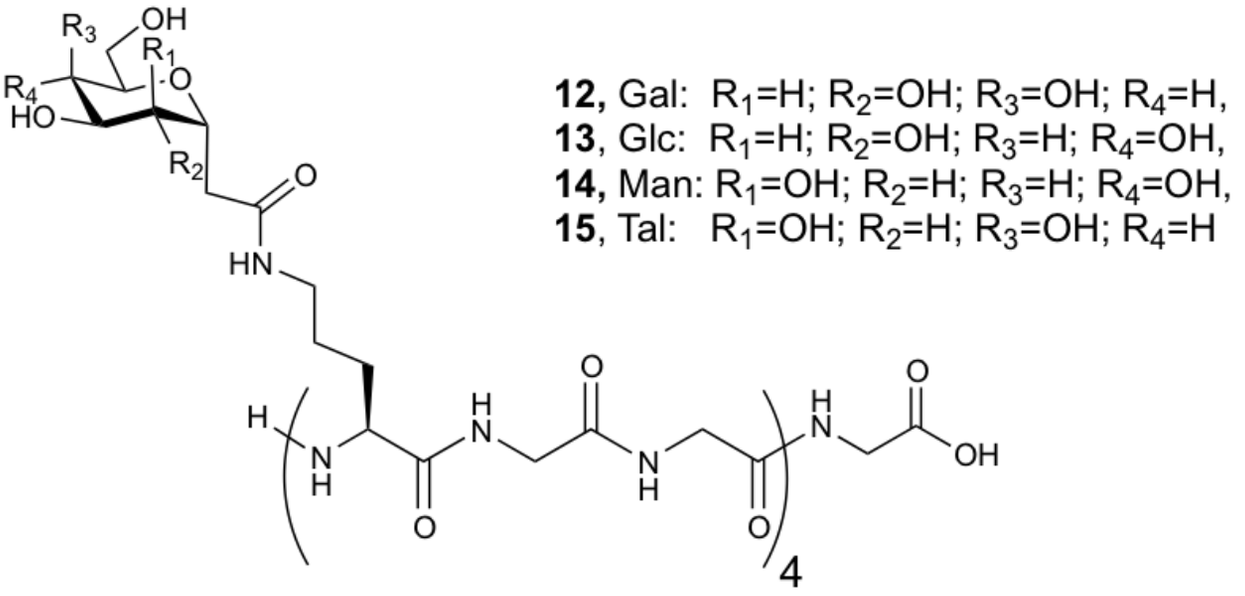

Figure 12. Structures of *C*-linked AFGP analogues containing various monosaccharide moieties. [58]

between the carbohydrate and peptide backbone of derivative **11** was increased such that the side chain linking the carbohydrate to the backbone was two, three or four carbons in length (analogues **11**, **16** and **17**, respectively, Figure 13). [57] The distance between the carbohydrate and peptide backbone of derivative **12** was both increased and decreased such that the side chain linking the carbohydrate to the backbone was a total of four, five, six or seven atoms in length (analogues **18**, **19**, **12** and **20**, respectively, Figure 13). [58,178] All of the analogues in which the side chain lengths were modified failed to exhibit IRI activity. These results indicated that the optimal length of linker between the carbohydrate and peptide backbone is two carbons for analogue **11** and six atoms for analogue **12**. [57,178] Molecular dynamic simulations indicated that **12** adopted a unique conformation in solution that was distinctly different than analogues **18-20**. [178] While **18-20** were found to adopt a conformation in which the carbohydrate moiety was extended away from the polypeptide backbone, the side chain of **12** was folded back on itself. It was speculated this fold formed a hydrophobic "pocket" between the carbohydrate and the peptide, resulting in potent IRI activity.

In addition to *C*-linked AFGP analogues, other synthetic variants of AFGPs have recently been assessed for their ability to inhibit ice recrystallization. In 2010, Sewald and co-workers synthesized analogues of AFGP-8 in which alanine residues were replaced with proline residues and the native disaccharide was replaced with the monosaccharide α-*N*-acetyl-D-galactosamine (Figure 14). [179] It was reported that the glycopeptide analogues containing tripeptide repeats of $(Ala\text{-}Ala\text{-}Thr(GalNHAc))_n$ were found to exhibit IRI activity (compounds **21**-**23**). This activity was dependent upon the length of the glycopeptide, and the compound with five tripeptide repeats (**23**) was found to be the most active at a lower concentration (12.5 μM) in comparison to the compound with three tripeptide repeats (**21**) which was active at a much higher concentration (0.8 mM). AFGP analogues **21**-**23** were found to induce hexagonal ice crystal shaping, suggesting that they are interacting with the ice lattice, however the TH

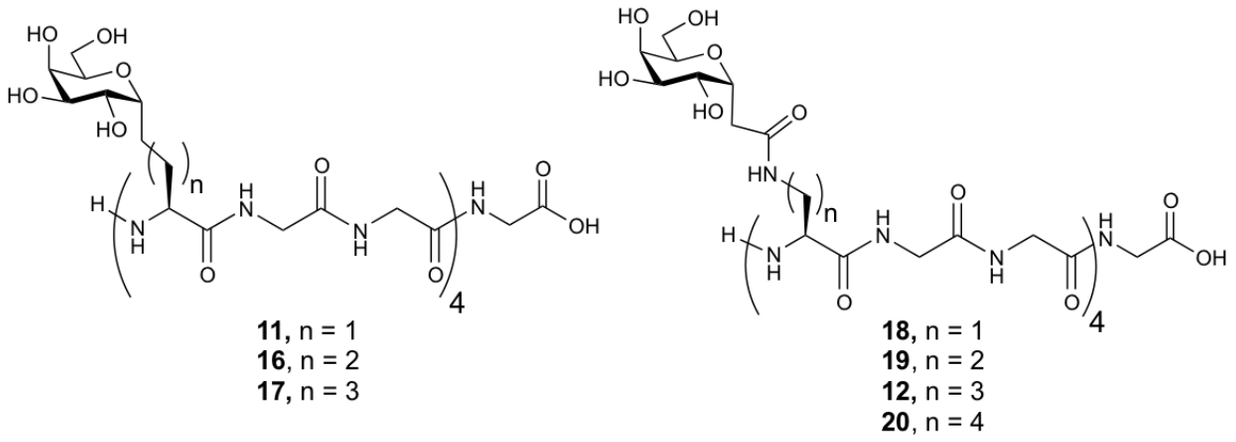

Figure 13. Structures of *C*-linked AFGP analogues containing different side chain lengths between the carbohydrate moiety and the polypeptide backbone, reported by the Ben laboratory. [57,178]

activity of these compounds was not assessed. Irregular incorporation of proline into these derivatives was detrimental to IRI activity as analogues **24**-**26** were only slightly active at a much higher concentration than the alanine-containing derivatives. However, incorporation of proline into a glycopeptide possessing four tripeptide repeats of (Pro-Ala-Thr(GalNHAc))$_n$ (**27**) resulted in similar IRI activity as the analogue containing four tripeptide repeats of (Ala-Ala-Thr(GalNHAc))$_n$ (**22**).

Three studies have been reported where AFGP analogues containing triazole rings have been synthesized and assessed for their ability to inhibit ice recrystallization. The triazole ring was incorporated to provide a convergent synthetic approach to these analogues and to overcome the low yields often associated with glycosylation. The key step in the synthesis of these analogues was the Cu(I)-catalyzed Huisgen azide-alkyne cycloaddition (or "click" chemistry). [180-182] In 2009, the Brimble group described the synthesis of two AFGP derivatives in which a furanose carbohydrate moiety was conjugated to a polypeptide backbone with a triazole-linker (Figure 15, compounds **28** and **29**). [61,180] The IRI activity of these derivatives was not assessed, however neither compound exhibited thermal hysteresis or induced dynamic ice shaping. [61] Sewald and co-workers have also reported the synthesis of a number of triazole-containing AFGP peptoid analogues, three of which were assessed for IRI activity (**30**-**32**, Figure 15), but these analogues failed to inhibit ice recrystallization. [181] Finally, in 2011 the Ben laboratory reported the synthesis of *C*-linked triazole-containing AFGP derivatives **33**-**36** (Figure 15) that were structurally similar to one of their more IRI active glycopeptides reported previously (analogue **12**, Figure 11). [182] While analogues **33**-**36** only exhibited weak IRI activity, this study highlighted the importance of the amide-bond present in the side chain of **12** (Figure 11) and identified this structural feature as crucial for potent IRI activity. Collectively, the result from these three studies suggest that while utilizing "click" chemistry to conjugate the carbohydrate moiety to a polypeptide backbone may offer advantages synthetically, the triazole-linker is detrimental for IRI activity.

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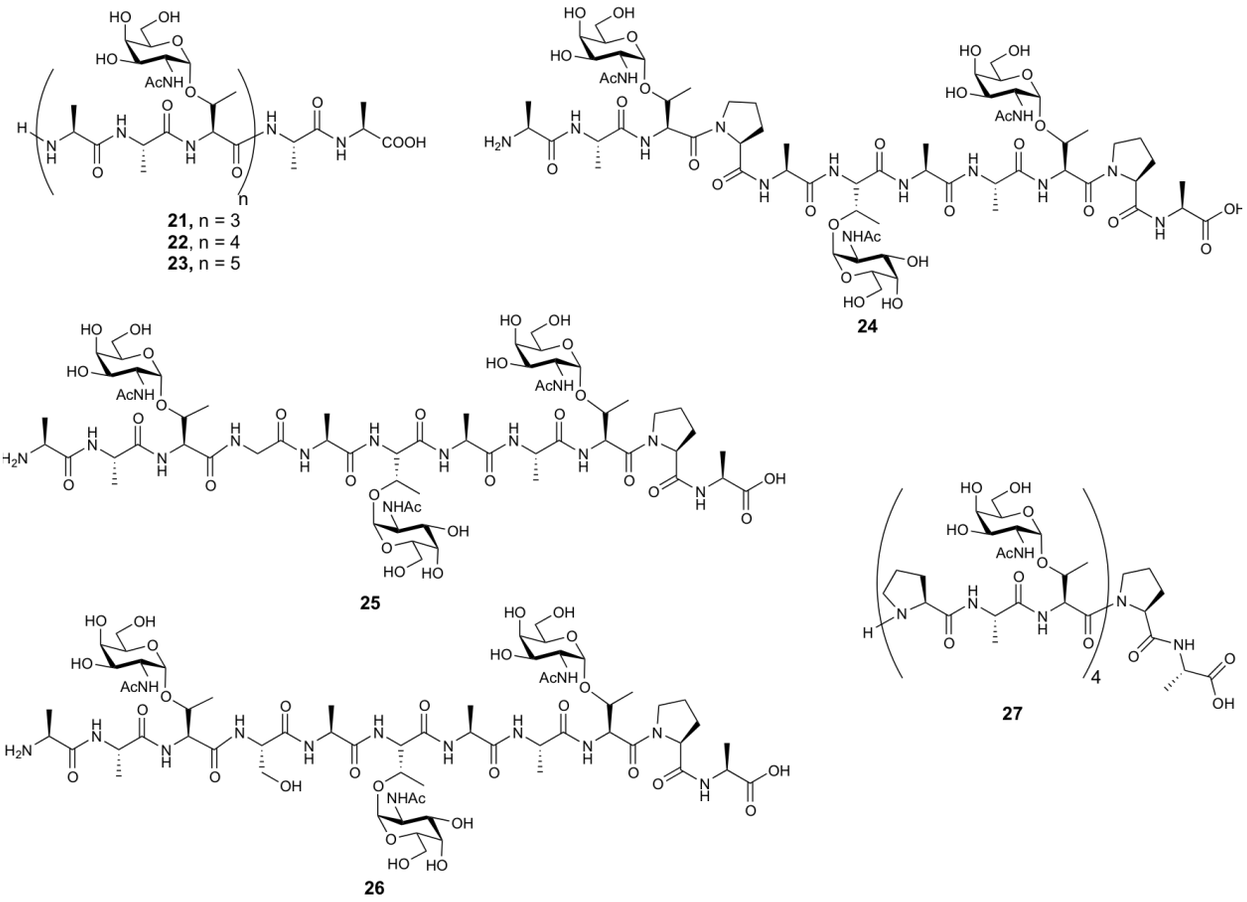

Figure 14. Structures of AFGP analogues reported by Sewald and co-workers. [179]

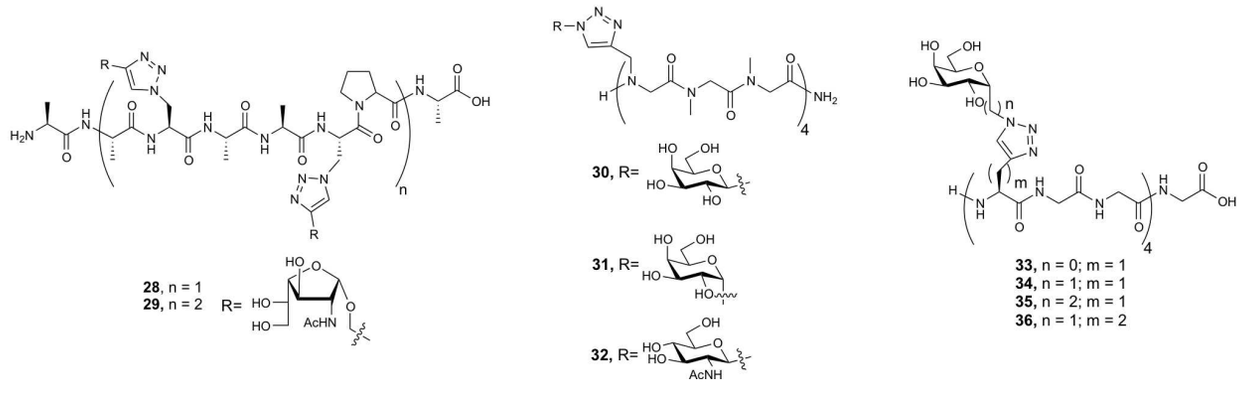

Figure 15. Structures of triazole-containing AFGP analogues reported by the Brimble (**28**-**29**), [180] Sewald (**30**-**32**) [181] and Ben (**33**-**36**) laboratories. [182]

6.2. Synthetic polymers as ice recrystallization inhibitors

All of the compounds discussed thus far that have exhibited the ability to inhibit ice recrystallization have been peptide or glycopeptide-based molecules. While some of these deriva-

tives show great promise for the many applications of ice recrystallization inhibitors, the main limitation is that large-scale preparation of these compounds for *in vitro* or *in vivo* applications is problematic. Thus, interest has arisen in small molecules (section 6.3) and synthetic polymers (described below) that can inhibit ice recrystallization. Such compounds can be more efficiently synthesized. Knight *et al.* in 1995 made the first observation that synthetic polymers could inhibit ice recrystallization. [41] In this study it was found that poly-L-histidine, poly-L-hydroxyproline and polyvinyl alcohol (PVA) exhibited IRI activity at concentrations less than 1 mg/mL in pure water, whereas poly-L-aspartic acid, poly-L-asparagine, polyacrylic acid and polyvinylpyrrolidone were inactive. With the exception of PVA, which retained its IRI activity in a NaCl solution, these polypeptides and polymers were not assessed for IRI activity in a salt solution to negate false positive effects. [41] Following this study, the activity of PVA has been further investigated and various synthetic polymers have been examined for their ability to inhibit ice recrystallization.

In 2003 Inada *et al.* reported an extensive study on the IRI activity of PVA. The activity of PVA was found to be dependent on its molecular mass, with an increase in activity observed with higher molecular weight polymers of PVA. [145] Polymers with an average molecular weight of ~90 000 g/mol were found to exhibit comparable activity to a type I AFP from winter flounder at similar concentrations. However, due to the large difference in molecular weights between PVA and the AFP, the quantity of PVA required to exhibit this activity was significantly higher than that of the AFP. In 2009, Gibson *et al.* re-examined the molecular weight dependence of PVA and showed that PVA with an average molecular weight of ~115 500 has potent IRI activity at a concentration of 5 mg/mL. [59] It was suggested that the ability of PVA to inhibit ice recrystallization is attributed to its ability to interact with the ice crystal lattice. Budke and Koop reported that PVA induces dynamic ice shaping capabilities and suggested this is occurring as the spacing of the PVA hydroxyl groups are closely matched to that of the prism planes of ice, allowing adsorption to these planes. [183] Furthermore, Inada and Lu have shown that PVA exhibits a small TH gap of 0.037 °C at 50 mg/mL, suggesting that an adsorption to ice is occurring. [157]

In addition to PVA, a number of other water-soluble polymers have also been investigated for their ability to inhibit ice recrystallization. [95] In 2009, Gibson *et al.* reported the IRI activity of various structurally diverse polymers (Figure 16). [59] Polyacrylic acid (PAA, **37**), poly(2-aminoethyl methacrylate) (**38**), polyethylene glycol (PEG, **39**), poly-L-Lysine (**40**) and poly-L-glutamic acid (**41**) exhibited only weak IRI activity, and an increase in concentration did not improve activity for any of these polymers. Poly-L-hydroxyproline (**42**) was found to exhibit IRI activity and this activity was dependent on polymer concentration. Poly-L-hydroxyproline has a PPII helical secondary structure [184] similar to the structure AFGPs are suggested to adopt. However, it was suggested this secondary structure is not required for IRI activity as PVA and poly-L-hydroxyproline exhibited similar IRI activities, but PVA is largely unstructured in solution. [59] Two vinyl-derived glycopolymers were also assessed for their ability to inhibit ice recrystallization (**43** and **44**, Figure 16). The highest molecular weight glycopolymer with a glucose residue (**43**, at ~105000 g/mol) did exhibit a moderate ability to inhibit ice

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recrystallization. However, incorporating a different carbohydrate residue (**44**) failed to increase IRI activity. [59,95]

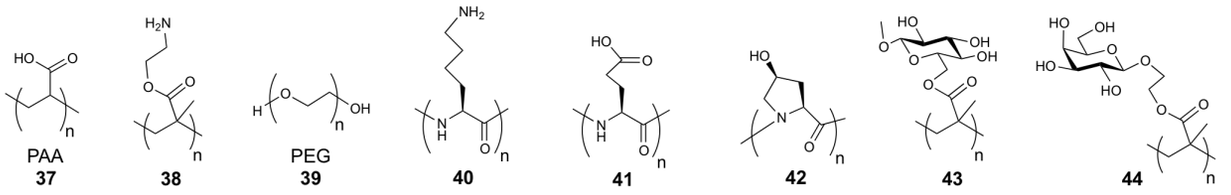


Figure 16. Structures of synthetic polymers assessed for IRI activity. [41,59,95,145]

6.3. Small molecules as ice recrystallization inhibitors

The Ben laboratory was the first group to report that small molecules, which were not peptide or polymer-based, could inhibit ice recrystallization. In 2008, Tam *et al.* reported a study examining the correlation between carbohydrate hydration and ice recrystallization inhibition. [177] This study arose from the observation that having a more hydrated carbohydrate moiety on one of their most active *C*-linked AFGP analogues (**12**) was a contributing factor to its exhibited IRI activity (see section 6.1, Figure 12). [58] Consequently, four monosaccharides and five disaccharides with known hydration parameters [174-176] were assessed for their ability to inhibit ice recrystallization. The structures of the mono- and disaccharides along with corresponding hydration numbers, isentropic molar compressibility and partial molar volume values are shown in Table 1. At a concentration of 22 mM, D- galactose exhibited moderate IRI activity, D- glucose and D- mannose had weak activity while D- talose was inactive. [177] These results showed a strong linear correlation between the hydration number of the monosaccharides and their respective IRI activity. The disaccharides examined also showed this strong linear correlation of their hydration number to IRI activity. Melibiose exhibited moderate IRI activity, while lactose and trehalose showed weak activity and maltose and sucrose were inactive.

The hydration layer or hydration shell of a carbohydrate can be defined as the number of tightly bound water molecules that surround the carbohydrate in aqueous solution. The hydration of carbohydrates has been the focus of many studies, and hypotheses for rationalizing observed hydration characteristics include hydration numbers, [185-188] anomeric effect, [189] hydrophilic volume, [190] hydrophobic index, [191] the ratio of axial versus equatorial hydroxyl groups [192,193] and the compatibility with bulk-water based upon the position of the next-nearest-neighbor hydroxyl group. [194,195] In the early 1990s, Galema *et al.* studied key parameters thought to dictate hydration characteristics and these were correlated to carbohydrate stereochemistry. Using kinetic experiments and density ultrasound measurements, the partial molar volumes, isentropic partial molar compressibilities and hydration numbers were determined for many commercially available mono- and disaccharides. [174-176] The isentropic partial molar compressibility and partial molar volume values of the carbohydrates quantify their "compatibility" with the three-dimensional hydrogen-bond network of bulk-

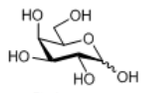
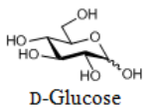
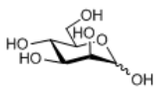
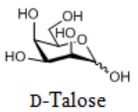
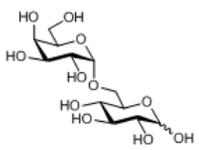
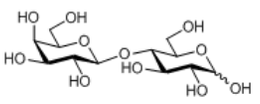
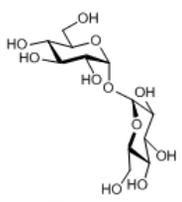
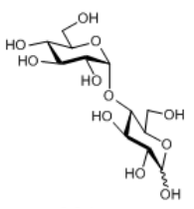
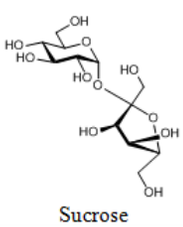
Carbohydrate	Molar Compressibility ($K_2°(s)x10^4$,cm^3 mol^{-1} bar^{-1})	Hydration Number	Carbohydrate	Molar Compressibility ($K_2°(s)x10^4$,cm^3 mol^{-1} bar^{-1})	Hydration Number
 D-Galactose	-20.8 (0.5) -20.4 (0.4)	8.7 (0.2)	 D-Glucose	-17.6 (0.3)	8.4 (0.2)
 D-Mannose	-16.0 (0.5)	8.1 (0.2)	 D-Talose	-11.9 (0.3)	7.7 (0.2)
 Melibiose	-31.2 (1.0)	15.5 (0.3)	 Lactose	-31.1 (0.2) -30.4 (1.0)	15.3 (0.3)
 Trehalose	-30.2 (0.3)	15.3 (0.3)	 Maltose	-23.7 (1.0)	14.5 (0.3)
 Sucrose	-17.8 (0.5)	13.9 (0.3)			

Table 1. Isentropic molar compressibilities ($10^4K_2°(s)$, cm^3 mol^{-1} bar^{-1}) and hydration numbers of various monosaccharides and disaccharides. Errors of molar compressibility values and hydration numbers are shown in parentheses. [175,177]

water as they are related to the size or volume the carbohydrate occupies upon hydration by water. Hydration numbers are calculated using isentropic coefficients of compressibility and they predict the number of water molecules that are hydrogen-bonded to the carbohydrate. In this study, it was observed that the compatibility of the carbohydrate with the three-dimensional hydrogen-bond network of bulk-water was directly related to the stereochemical relationship of the hydroxyl groups on the carbohydrate. D- Talose, with axial hydroxyl groups on C2 and C4, had a higher isentropic molar compressibility value and a lower hydration number, and fit well into the three-dimensional hydrogen-bonded network of bulk-water. In contrast, D- galactose, with an axial hydroxyl group on C4 and equatorial hydroxyl group on C2, had a lower isentropic molar compressibility value and a higher hydration number, and had a poor fit into the three-dimensional hydrogen-bonded network of bulk-water. Thus, D-

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talose was the most compatible and caused the least disturbance on the hydrogen-bonded network of bulk-water, whereas D- galactose was the least compatible and caused a greater disturbance on the hydrogen-bonded network of bulk-water. The carbohydrates with an equatorial C4 hydroxyl and either an equatorial or axial C2 hydroxyl group (ie. D- glucose and D- mannose) had a moderate fit and caused a moderate disturbance of the three-dimensional hydrogen-bonded network of bulk-water.

In the study conducted by Tam *et. al* which investigated the IRI activity of several mono- and disaccharides, a correlation was observed between IRI activity and carbohydrate hydration. [177] As none of the carbohydrates exhibited thermal hysteresis or dynamic ice shaping, it was unlikely that the IRI activity exhibited by the carbohydrates was due to an interaction with the ice lattice. This lead to an alternative proposed mechanism for the inhibition of ice recrystallization based upon the compatibility of a solute with bulk-water. As described in detail in section 2.0 of this chapter, a semi-ordered quasi-liquid layer (QLL) exists between the highly ordered ice lattice and bulk-water. For ice recrystallization to occur, bulk-water molecules transfer to the QLL, then subsequently from the QLL to the growing ice lattice. [38,39] Tam *et al.* have suggested that the carbohydrates are concentrated at the bulk-water-QLL interface. [177] A carbohydrate that had a poor fit into bulk-water will cause a greater disturbance to its three-dimensional hydrogen-bonded network, increasing the energy associated with the transfer of bulk-water to the QLL. It was therefore hypothesized that the inhibition of ice recrystallization observed with carbohydrates occurred at the bulk-water-QLL interface as more highly hydrated carbohydrates, such as D- galactose, disrupted the pre-ordering of bulk-water making it energetically unfavorable for water molecules to transfer to the QLL. Less hydrated carbohydrates, such as D- talose, fit well into bulk-water and caused less of a disturbance to the pre-ordering of bulk-water, thus inhibition of ice recrystallization was not observed.

The disaccharides assessed in this study also showed a strong linear correlation of their IRI activity to their hydration numbers (values give in table 1). [177] However, the increase in hydration numbers for disaccharides relative to monosaccharides was not reflected with an increase in IRI activity. For instance, melibiose has a hydration number of 15.5, yet it exhibited similar IRI activity to D- galactose, which has a hydration number of 8.7. Furthermore, D-galactose was significantly more IRI active than maltose, despite maltose having a much larger hydration number (8.7 for D- galactose and 14.5 for maltose). This was attributed to a difference in total steric volume between the monosaccharides (containing one carbohydrate unit) and disaccharides (containing two carbohydrate units). By dividing the carbohydrate hydration number by their partial molar volumes an indication of the degree of hydration per molar volume of carbohydrate was obtained. This value was referred to as the hydration index (HI) and it provided the degree of hydration of the substrate as a function of its size or volume. This metric was useful in justifying why highly hydrated monosaccharides exhibited similar IRI activity as highly hydrated disaccharides at 22 mM, despite hydration numbers for monosaccharides being almost half the value of disaccharides. [177] However, at higher carbohydrate concentrations, such as 220 mM, the disaccharides were twice as IRI active as the monosaccharides. [196] Thus, hydration numbers, not hydration indices, were better

predictors of IRI activity at this concentration, but ultimately IRI activity still correlated with carbohydrate hydration.

Following the report that simple commercially available carbohydrates exhibit moderate IRI activity, the Ben laboratory has reported the ability of various other carbohydrate derivatives to inhibit ice recrystallization. Most of these compounds have been derivatives of D- galactose. [177] *C*-allylated derivatives of galactose (**45** and **49**), glucose (**46** and **50**), mannose (**47**) and talose (**48**) were assessed for ice recrystallization inhibition activity (Figure 17) to investigate the influence of a carbon substituent at the C1 position as their most IRI active AFGP analogues were *C*-linked glycoconjugates (see section 6.1). [177] The α-*C*-allyl-glycosides (**45**-**48**) had similar activities as the native monosaccharide units (ie. D- galactose and α-*C*-allyl-galactopyranoside exhibited similar IRI activities), and the trend of activity for these *C*-linked derivatives was identical to the trend observed with the corresponding native monosaccharides (ie. galactose was most active and talose was least active). However, the β-*C*-allyl-glycosides (**49**-**50**) showed a significant decrease in activity in comparison to the native monosaccharides (D- galactose and D- glucose) and the α-linked derivatives. Other D- galactose derivatives have been assessed for their ability to inhibit ice recrystallization, including compounds **51**-**57** (Figure 17). [197,198] All of these derivatives had weak to poor IRI activity, and were less active than native D- galactose.

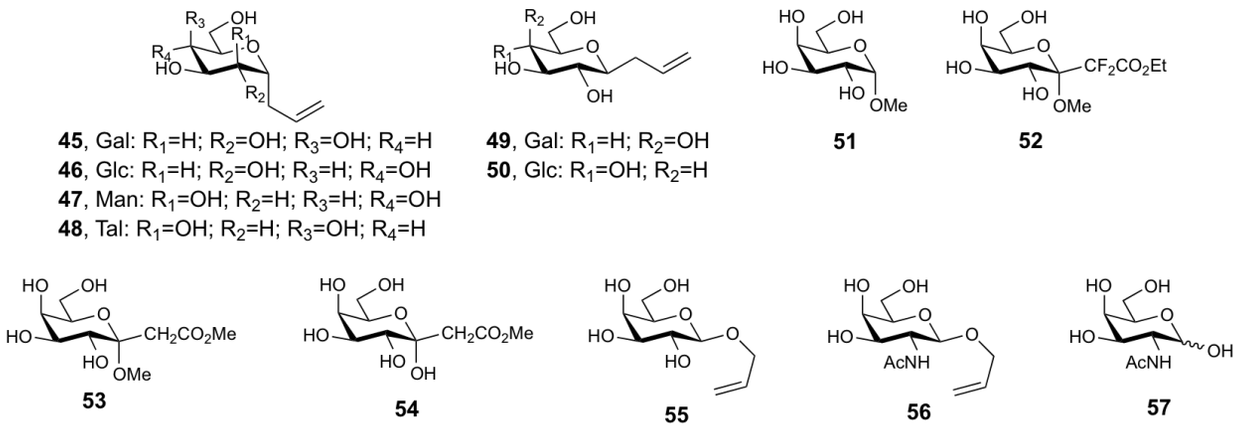

Figure 17. Structures of D- galactose-based analogues assessed for IRI activity by the Ben laboratory. [197,198]

In addition to monosaccharide derivatives, structural analogues of the disaccharide β-D-galactosyl-(1-3)-α-*N*-acetyl-D- galactosamine found in native AFGPs were investigated for IRI activity. These include disaccharide **58** (Figure 18), a close analogue of the disaccharide found in native AFGPs, regioisomers of **58** where the terminal β–D- galactosyl unit was linked to the C4 or C6 hydroxyl group of the *N*-acetyl-D- galactosamine moiety (**60** and **61**, respectively), and disaccharide **59**, in which the C2 *N*-acetyl group was replaced with a hydroxyl group. [197] These four disaccharides were assessed for IRI activity at 22 mM, and interestingly the most active disaccharide was not the analogue of the disaccharide found in native AFGPs. The β-(1,4)-linked disaccharide **60** was the most active disaccharide analogue assessed. The β-(1,6)-linked disaccharide **61** and both β-(1,3)-linked disaccharides, **58**-**59**, exhibited similar IRI

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activity and were less active than the β-(1,4)-linked analogue. These disaccharides were not conjugated to the native polypeptide backbone (Ala-Ala-Thr) found in AFGPs to investigate if the same trend was observed with the glycoconjugates. However, this study highlighted how the structural features necessary for TH and IRI activity may be different as the functional groups which were required for the TH activity of AFGPs (see section 5.3, Figure 8) [166] were not required for the IRI activity of the disaccharide analogues. [197]

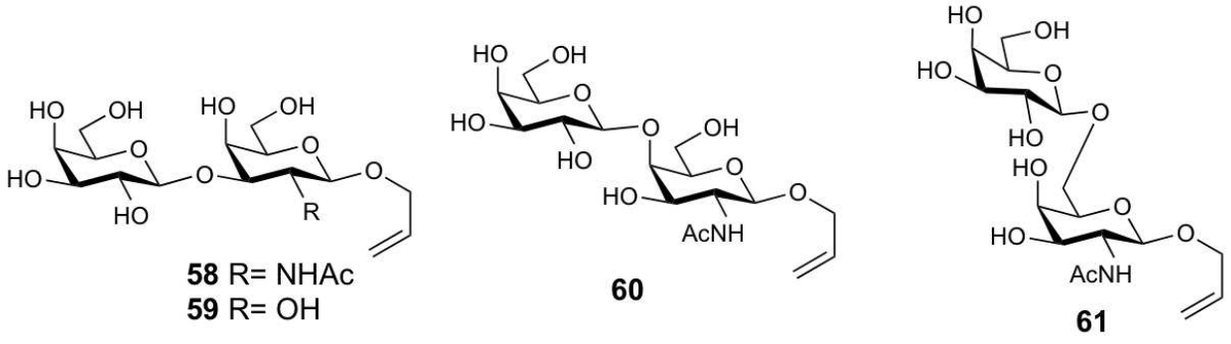

Figure 18. Structural disaccharide analogues of the native β-D- galactosyl-(1-3)-*N*-acetyl-D- galactosamine disaccharide found in AFGPs. [197]

While the small molecules described above had the ability to inhibit ice recrystallization, all exhibited only weak to moderate activity at much higher concentrations than those of the potently IRI active glycoconjugates. However, in 2012 the Ben laboratory reported the first examples of small carbohydrate-based molecules that were extremely potent inhibitors ice recrystallization, some that were highly IRI active at concentrations much lower than 22 mM. To date, these are the most potent IRI active small molecules. The molecules investigated were carbohydrate-based surfactants and hydrogelators (structures shown in Figures 19), two of which were found to exhibit potent IRI activity. [62] The carbohydrate-based non-ionic surfactant β-octyl-D- galactopyranoside (**62**) was highly IRI active, with potent activity reported at 11 mM. In contrast, carbohydrate-based non-ionic surfactant β-octyl-D- glucopyranoside (**63**) was only weakly active even at 44 mM. These results were in agreement with previous studies were that derivatives of the more highly hydrated D- galactose were significantly better inhibitors of ice recrystallization than derivatives of the less hydrated D- glucose. [58,177] While these carbohydrate-based surfactants were known to form micelles in solution, it was concluded that micelle formation was unrelated to IRI activity. β-octyl-D- galactopyranoside (**62**) was highly active at a concentration well below its critical micelle concentration (CMC) of 30 mM, where as β-octyl-D- glucopyranoside (**63**) did not exhibit an ability to inhibit ice recrystallization even well above its CMC value of 22 mM. [62] Furthermore, other structurally different non-ionic and anionic surfactants exhibited weak to moderate activity at concentrations well above their respective CMC values. None of the non-ionic carbohydrate-based surfactants assessed in this study possessed TH activity or dynamic ice shaping abilities, suggesting that the activity exhibited by these compounds was not due to an interaction with the ice lattice.

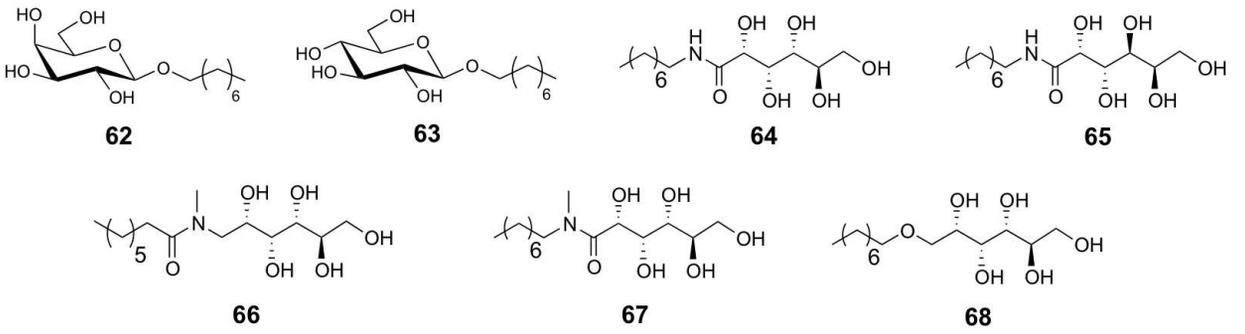

Figure 19. Structures of carbohydrate-based non-ionic surfactants and hydrogelators assessed for IRI activity by the Ben laboratory. [62] β-octyl-D- galactopyranoside (**62**) and *N*-octyl-D- gluconamide (**64**) are the first report of potent small molecule ice recrystallization inhibitors.

The second class of compounds investigated were carbohydrate-based hydrogelators, as in aqueous solution they were known to aggregate and sequester bulk-water forming fibres and hydrogels. D- glucose hydrogelator derivative *N*-octyl-D- gluconamide (**64**) was found to be a potent inhibitor of ice recrystallization at 0.5 mM, a concentration much lower than that of other reported carbohydrate derivatives. [62] However, the D- galactose hydrogelator derivative *N*-octyl-D- galactonamide (**65**) was only weakly IRI active at this same concentration. *N*-octyl-D- gluconamide (**64**) is the first example of a small molecule exhibiting potent activity at a concentration much lower than 22 mM, and it was also the first example of a glucose-based derivative exhibiting better activity than a galactose-based derivative. Structure-function work conducted in this study suggested that the amide bond in **64** is an essential structural feature for its activity as **66**-**68** (Figure 19) were significantly less active at much higher concentrations. While *N*-octyl-D- gluconamide (**64**) was able to form hydrogels in solution, it was concluded using solid-state NMR studies and characterization of the hydrogels that the ability to form a hydrogel was not a prerequisite for potent IRI activity. This conclusion was further supported by the fact that *N*-octyl-D- galactonamide (**65**) also formed hydrogels in solution, yet it did not possess IRI activity. Finally, these studies also suggested that ice binding was not a prerequisite for potent activity as solid-state NMR studies and TH measurements failed to indicate an interaction with the ice lattice. To date, the report that small molecules can exhibit potent IRI activity remains a significant discovery that will facilitate the rational design of small molecule ice recrystallization inhibitors suitable for medical, commercial and industrial applications.

7. Cryopreservation

Cryopreservation is a very attractive process for the preservation of biological materials. While vitrification and hypothermic storage each offer their own unique advantages and their own limitations, cryopreservation has a major advantage. At the temperatures associated with cryopreservation (typically -190 °C) all biochemical processes are effectively stopped. However, cryopreservation is a complex process during which careful attention to sample volume, cooling rates and cryoprotectants (dimethyl sulfoxide and glycerol) are extremely important

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to ensure cells survive the process. Unfortunately, all cryoprotectants exhibit cytoxicity and this complicates the cryopreservation process as the cryoprotectant must often be removed during the thawing cycle. Indeed there is a common myth that cooling rates of 1 ^{o}C/min with 10% dimethyl sulfoxide (DMSO) is sufficient for all cryopreservation applications. Unfortunately, this is incorrect and there is an urgent need for novel cryoprotectants, especially in light of the recent developments in the field of regenerative medicine where the supply of various progenitor cells is problematic for the many clinical applications. To highlight the complexity of this process and the need for new and improved cryoprotectants a brief description of cellular injury during cryopreservation will be presented in the following section.

7.1. The complex mechanisms of cryoinjury

Traditionally, there exist three characterized mechanisms of cell death that occur during cryopreservation. These are cell rupture due to damage to the external cell membrane, necrosis and cold induced apoptosis. Cell rupture is usually the result of osmotic imbalance causing a loss in membrane integrity. [50] Cell necrosis is characterized by cellular swelling (due to an increase in immune response), compromised cell membrane integrity, random DNA fragmentation by cellular endonucleases, cell lysis and the release of cytokines. Apoptosis (programmed cell death) is a highly complex and closely regulated biochemical pathway (the details of which will not be covered in this chapter). It may appear at first that cell death due to apoptosis is not related to cryopreservation however, it has been demonstrated that cold-induced apoptosis is common in cryopreserved cells. [50,199]

The formation of ice under typical cryopreservation conditions is inevitable, but cooling rates become extremely important in mitigating the damage associated with ice formation. For every cell type there is an optimal cooling and warming rate that is determined by the permeability of the cell membrane to water and the cryoprotectant. Hence, cryopreservation is performed with either slow or fast cooling rates depending on cell type. In most instances, ice will prefer to form outside of the cell. [200] Formation of extracellular ice creates an increased osmotic pressure across the cell membrane. This "osmotic flux" intensifies as ice growth continues after the nucleation event. As the ice crystal grows all solutes are excluded from the ice lattice [201] and are concentrated in the extracellular medium. Cells with less permeable membranes will rupture with increasing osmotic pressure if they cannot dehydrate fast enough.

The process of dehydration during freezing is somewhat of a "double-edged sword". In one instance, the amount of intracellular water decreases, reducing the chance for intracellular ice formation – a lethal process. However, it has been shown that dehydration and exposure to excessively high concentrations of electrolytes is also lethal to the cell. [202] This is referred to as solute damage or the "solute effect" and it facilitates damage to the cell membrane that is irreparable. [202] Conversely, when cells are frozen very slowly, dehydration and excessive cell shrinkage facilitates cell death. Excessive dehydration can be prevented using cryoprotectants. Two classes of cryoprotectant are commonly employed. Non-penetrating cryoprotectants do not cross the cell membrane and hence remain outside the cell, thereby increasing the osmolality of the extracellular solution, facilitating dehydration of the cell prior to freezing and preventing formation of intracellular ice. Penetrating cryoprotectants, such as DMSO and

glycerol, readily cross the cell membrane and decrease the concentration of intracellular electrolytes while maintaining greater cell volumes. The major problem with penetrating cryoprotectants is cytotoxicity due to the disruption of intracellular signaling. [203] In summary, cryopreservation of cells using slow-freezing results in dehydration of the cell in response to increasing osmotic pressures as electrolytes are concentrated outside the cell during extracellular ice growth. While dehydration of the cells helps to prevent intracellular ice growth, it is also detrimental to cell survival.

Cryopreservation using high cooling rates traps water inside the cell promoting the formation intracellular ice. [204] The exact mechanism by which this occurs is not clear [205] however, most cryobiologists believe that intracellular ice formation results in cell death. Hence, practical fast-freezing protocols must dehydrate cells prior to freezing in order to mitigate intracellular ice formation. [206] Of course cryoprotectants are necessary to accomplish this, but the role of the cryoprotectant during fast cooling is different than during slow cooling. Non-penetrating cryoprotectants are employed in an effort to dehydrate the cell and minimize the chance of intracellular ice formation. Interestingly, while the correlation between intracellular ice formation and cell death has been recognized, there is evidence to suggest that formation of intracellular ice does not directly kill cells. [200] Studies have shown that survival of cells post-cryopreservation is dependent upon the rate at which the cells are warmed during thawing and that cell death associated with intracellular ice formation is not caused by the initial nucleation of ice but by an alternate process during warming. [207,208] Possible mechanisms by which intracellular ice damages cells have been reviewed extensively in the literature and it has been concluded that cell death is occurring as a result of ice recrystallization. [202,209] This hypothesis is supported by the fact that may freeze-tolerant organisms inhabiting sub-zero environments produce large quantities of recrystallization-inhibitors *in vivo* to ensure survival. [139,210] In addition, mechanical damage to cell membranes from ice recrystallization has been identified as a primary cause of cell injury during cryopreservation. [50]

7.2. Preservation of biological materials using biological antifreezes and their analogues

Cellular damage due to ice recrystallization occurs during the storage and thawing cycles of cryopreservation and, given the cryoprotective nature of BAs, it is not surprising that they have been investigated as cryoprotectants to increase cell viability post-thaw. In principle, BAs have the advantage of being relatively non-toxic compared to common cryoprotectants such as DMSO and glycerol. While BAs seem like ideal cryoprotectants, they have not been very effective and often fail to protect mammalian cells from cryoinjury at temperatures outside of the TH gap. This section will discuss specific examples where BAs were used to cryopreserve biological materials, including the benefits and problems associated with their use.

BAs have been examined as protective agents for the hypothermic storage and cryopreservation of various biological materials. AFPs have been reported to protect cell membranes during hypothermic storage. For instance, Rubinsky and co-workers demonstrated that AFPs [211] and AFGPs [212] of various molecular weights and in concentrations ranging from 1-40 mg/mL can successfully preserve the structural integrity of pig oolem-

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ma and bovine immature oocytes. Furthermore, these oolemma and oocytes underwent successful *in vitro* maturation and fertilization. [211,212] In addition, it has been shown that AFPs can stabilize plasma membranes. [213] Crowe and co-workers demonstrated that while a 1 mg/mL solution of AFGP prevented cold-induced activation of human blood platelets following hypothermic storage, a type I AFP had no effect. [214,215] Despite these promising examples, toxic effects during hypothermic storage from the BAs during hypothermic storage have also been reported. Both AFPs and AFGPs have exhibited significant toxic effects and have compromised cell viabilities in spinach thylakoids, [216] ram spermatozoa [217] and chimpanzee spermatozoa. [218]

In addition to hypotherminc storage, BAs have also been utilized for cryostorage of biological materials. Several studies have reported benefits of using AFPs and AFGPs as cryoprotectants. Rubinsky and co-workers observed dramatically improved morphological integrity of immature oocytes and two-cell-stage embroys of mice and pigs that were subjected to vitrification in the presence of 40 mg/mL AFGPs. [219,220] Similar results were observed with mature mouse oocytes [221], bovine and ovine embryos at the morula/blastocyst stage, [222] ram spermatozoa, [217] chimpanzee spermatozoa [218] and porcine oocytes. [223] While post-thaw viabilities were increased in the presence of BAs with ram and chimpanzee spermatozoa and porcine oocytes, cytotoxic effects during cooling were also observed. [217,218,223]

In contrast, other investigations have reported that BAs fail to protect cells during cryopreservation and actually facilitate cellular damage during cryopreservation. For instance, no specific benefits were observed in survival rates of vitrified bovine blastocysts, [224] two-step-cryopreserved oyster oocytes [225] and equine embroys using various AFPs. [226] Freezing of red blood cells in the presence of glycerol with AFPs (at concentrations between 25 and 1000 μg/mL) [227] and AFGPs (at 40 μg/mL) has been reported to damage cells during cryopreservation. [228] A similar result was also observed during the cryopreservation of hematopoietic cells with AFPs in DMSO. [229] Additionally, this cellular damage during cryopreservation with BAs has also been reported with spinach thylakoids, [216] intact rat heart (from cardiac explant) [230] and cardiomyocytes. [231] This damage has been attributed to the change in ice crystal morphology that is induced in the presence of BAs (dynamic ice shaping). [228,231] Furthermore, it has been suggested that BAs may also increase the incidence of intracellular ice formation, thereby decreasing cell viabilities post-thaw. [232] Finally, reports have demonstrated both beneficial and detrimental effects with BAs during cryopreservations, depending on AFP concentration and type. [233] At low concentrations AFPs were reported to increase the survival rate of red blood cells however, at higher concentrations where the ice recrystallization inhibition ability of the AFP was significantly enhanced, they decreased survival rates. [234,235]

In contrast to native biological antifreezes, the benefit of analogues possessing "custom-tailored" antifreeze activity for cryopreservation has been demonstrated. In 2011, the Ben laboratory demonstrated that *C*-linked AFGP analogues that exhibit potent IRI activity but not TH activity function as effective cryoprotectants. Using a human embryonic liver cell line, 1.0-1.5 mg/mL of *C*-linked AFGP analogues **11** or **12** doubled cell viability relative to the negative control (cell medium only). [236] The post-thaw viability was comparable to that

obtained with a 2.5% DMSO solution. This effect was attributed to the IRI activity of these *C*-linked AFGP analogues. This conclusion was validated when it was demonstrated that IRI active carbohydrates exhibiting minimal cytotoxicity significantly increased cell viabilities post-thaw. [196] To date, these are the only examples where potent inhibitors of ice recrystallization not displaying thermal hysteresis activity or dynamic ice shaping capabilities have been successfully utilized as cryoprotectants.

8. Conclusions and outlook

While Nature has provided various organisms with peptides and glycopeptides to mitigate cellular damage during exposure to cold temperatures, these compounds have failed to be effective cryoprotectants in various medical and commercial applications. This is somewhat ironic as these compounds are potent inhibitors of ice recrystallization, a process that contributes significantly to cellular injury. The recent discovery that IRI activity can be selectively enhanced while suppressing TH activity in various analogues of biological antifreezes is a significant advancement towards the rational design of novel cryoprotectants. Some of these molecules have even demonstrated the ability to enhance cell viabilities post-thaw. While these compounds do not yet exhibit viabilities comparable to 10 % DMSO solutions, it is feasible that with a better understanding of the structural features necessary for potent IRI activity future analogues will be efficient cryoprotectants replacing conventional ones such as DMSO and glycerol. The recent discovery that small molecules are extremely potent inhibitors of ice recrystallization represents a "quantum leap" forward in this area. Further studies with these compounds *in vitro* and *in vivo* will elucidate their effectiveness as cryoprotectants while overcoming the problems of high cost and large-scale synthesis associated with the higher molecular weight analogues of biological antifreeze that exhibit the potent IRI activity, a property necessary for an effective cryoprotectant.

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