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Hydration Index—A Better Parameter for Explaining Small Molecule Hydration in Inhibition of Ice Recrystallization

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Abstract: Several simple mono- and disaccharides have been assessed for their ability to inhibit ice recrystallization. Two carbohydrates were found to be effective recrystallization inhibitors. D-Galactose (**1**) was the best monosaccharide and D-melibiose (**5**) was the most active disaccharide. The ability of each carbohydrate to inhibit ice growth was correlated to its respective hydration number reported in the literature. A hydration number reflects the number of tightly bound water molecules to the carbohydrate and is a function of carbohydrate stereochemistry. It was discovered that using the absolute hydration number of a carbohydrate does not allow one to accurately predict its ability to inhibit ice recrystallization. Consequently, we have defined a *hydration index* in which the hydration number is divided by the molar volume of the carbohydrate. This new parameter not only takes into account the number of water molecules tightly bound to a carbohydrate but also the size or volume of a particular solute and ultimately the concentration of hydrated water molecules. The hydration index of both mono- and disaccharides correlates well with experimentally measured RI activity. C-Linked derivatives of the monosaccharides appear to have RI activity comparable to that of their O-linked saccharides but a more thorough investigation is required. The relationship between carbohydrate concentration and RI activity was shown to be noncolligative and a 0.022 M solution of D-galactose (**1**) and C-linked galactose derivative (**10**) inhibited recrystallization as well as a 3% DMSO solution. The carbohydrates examined in this study did not possess any thermal hysteresis activity (selective depression of freezing point relative to melting point) or dynamic ice shaping. As such, we propose that they are inhibiting recrystallization at the interface between bulk water and the quasi liquid layer (a semiordered interface between ice and bulk water) by disrupting the preordering of water.

Introduction

Carbohydrates are prolific in biological systems and are involved in many processes ranging from cellular adhesion, cell signaling, infection, and regulation of the immune response.[1] In addition, carbohydrates are involved in the cryoprotection of organisms inhabiting cold climates.[2] While it is accepted that the hydration of proteins plays an important role in modulating protein function,[3] hydration of carbohydrates and oligosaccharides also tempers their biological activity. Despite the undisputed importance of hydration, assessing the degree of hydration is not a trivial process.[4] While NMR techniques have reportedly been used to assess hydration of complex oligosaccharides, the results of these studies are often subject to a large degree of uncertainty.[5] In contrast, the hydration of simple carbohydrates has been extensively studied during the last few decades.[6] These studies have been limited to small temperature and pressure ranges due to the fact that these molecules possess many different conformations in aqueous solution.[6,7] Nonetheless, these studies demonstrate that carbohydrate hydration is closely correlated to stereochemistry. While the exact reasons for this are the subject of much debate, various hypotheses have been proposed that rationalize hydration characteristics and the subsequent influence of hydration on bulk water.[8−15]

During the last several years, our laboratory has successfully designed and synthesized functional C-linked antifreeze glycoprotein (AFGP) analogues possessing custom-tailored antifreeze activity.[16] These compounds are potent inhibitors of recrystallization and do not possess thermal hysteresis (TH) activity. This is significant as studies demonstrate that cellular damage as a result of recrystallization is the major cause of decreased cellular viability upon cryopreservation.[17] As such, these compounds are novel lead structures representing a new class of cryoprotectants where improved cryoadjuvants and cryopreservation protocols are urgently required to meet the steadily increasing demand for donor organs.[18] We recently described how hydration of a C-linked carbohydrate

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moiety in a C-linked AFGP analogue is a contributing factor to antifreeze activity, specifically recrystallization-inhibition (RI) activity.[19] This work has not only shed new information on the underlying mechanism of action of biological antifreezes, but also highlights the importance of understanding hydration in all biological processes. The current manuscript explores the relationship between hydration of carbohydrates and carbohydrate derivatives with recrystallization-inhibition activity. The ultimate goal of this work is to enable accurate prediction of structures that function as potent inhibitors of ice recrystallization for cryomedical and other commercial applications.

Material and Methods

The monosaccharides used in this study were commercially available and purchased from Sigma-Aldrich. All C-linked pyranose derivatives were synthesized using standard literature procedures (see Supporting Information and ref 19). Isentropic molar compressibility (IMC) values were obtained from ultrasound density measurements reported in the literature.[8] Hydration numbers, n_h, were obtained using the Passynsky equation, eq 1,[8,20]

$$n_h = (n_w/n_s)(1 - \beta_s/\beta_{so}) \quad (1)$$

where n_w and n_s are the mole fractions of water and the solute, respectively; and β_s and β_{so} are the isentropic coefficients of compressibility of the solute and water, respectively.

Recrystallization-Inhibition (RI) Assay. Sample analysis for RI activity was performed using the "splat cooling" method as previously described.[21] In this method, the analyte was dissolved in phosphate buffered saline (PBS) solution and a 10 μL droplet of this solution was dropped from a micropipette through a two-meter high plastic tube (10 cm in diametre) onto a block of polished aluminum precooled to approximately −80 °C. The droplet froze instantly on the polished aluminum block and was approximately 1 cm in diameter and 20 μm thick. This wafer was then carefully removed from the surface of the block and transferred to a cryostage held at −6.4 °C for annealing. After a period of 30 min, the wafer was photographed between crossed polarizing filters using a digital camera (Nikon CoolPix 5000) fitted to the microscope. A total of three images were taken from each wafer. During flash freezing, ice crystals spontaneously nucleated from the supercooled solution. These initial crystals were relatively homogeneous in size and quite small. During the annealing cycle, recrystallization occurred, resulting in a dramatic increase in ice crystal size. A quantitative measure of the difference in recrystallization inhibition of two compounds X and Y is the difference in the dynamics of the ice crystal size distribution. Image analysis of the ice wafers was performed using a novel domain recognition software (DRS)[22] program that was developed at the Steacie Institute for Molecular Sciences (SIMS) of the National Research Council of Canada (NRCC). This processing employed the Microsoft Windows Graphical User Interface to allow a user to visually demarcate and store the vertices of ice domains in a digital micrograph. These data were then used to calculate the domain areas. To eliminate the need to fully process each micrograph, an algorithm was developed to randomly display a number of *x*/*y* locations. The algorithm made use of a built-in pseudo random number generator (rand(*x*)) and was written so that no two locations were closer than 1/10th the field of view of the micrograph. The formula for the area of a polygon that is not self-intersecting and contains no holes is then given by eq 2,

$$A = \frac{1}{2}\sum_{i=0}^{N-1}(x_i y_{i+1} - x_{i+1} y_i) \quad (2)$$

where N is the number of vertices, and Σ_{x0},Σ_{y0} to $\Sigma_{xN-1},\Sigma_{yN-1}$ are the vertices circumventing the polygon in a clockwise direction. The point $\Sigma_{x0},\Sigma_{y0}$is assumed to be equivalent to the point Σ_{xN},Σ_{yN}. The software was written in C using Microsoft Visual Studio 6.0 on a Pentium class personal computer running Microsoft Windows 2000 or XP. All data were plotted and analyzed using Microsoft Excel.

Thermal Hysteresis Assay. Nanoliter osmometry was performed using a nanoliter osmometer (Clifton Technical Physics, Hartford, NY) as described by Chakrabartty and Hew.[23] All measurements were made in doubly distilled water. Ice crystal morphology was observed through a Leitz compound microscope equipped with an Olympus 20X (infinity corrected) objective, Leitz Periplan 32X photo eyepiece and a Hitachi KP-M2U CCD camera connected to a Toshiba MV13K1 TV/VCR system. Still images were captured directly using a Nikon CoolPix 5000 digital camera.

Results and Discussion

The "hydration layer" is defined as water that encompasses the carbohydrate and is often bound very tightly. Specific hypotheses to rationalize the observed hydration characteristics of a carbohydrate include: hydration number,[10] anomeric effect,[11] hydrophobic index,[12] hydrophilic volume,[13] and compatibility with bulk water based upon the position of the next-nearest-neighbor hydroxyl groups.[8,9,14] Subsequent to the latter hypothesis, a revised stereospecific hydration model has suggested that hydration of a carbohydrate depends upon the ratio of axial to equatorial hydroxyl groups.[15] In an attempt to generate a unifying hypothesis consistent with the influence of carbohydrate stereochemistry, key thermodynamic parameters thought to dictate hydration were measured by Galema et al.[8,9] By using molecular dynamics simulations, kinetic experiments, and density and ultrasound measurements, the partial molar volumes, isentropic partial molar compressibilities, and hydration numbers of many commercially available hexoses have been determined and correlated to carbohydrate stereochemistry.

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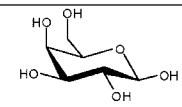
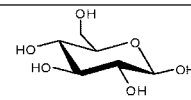
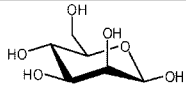
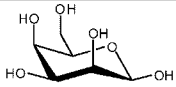
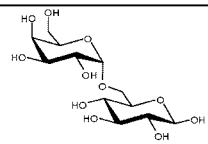
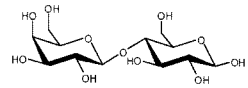
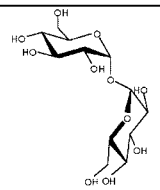
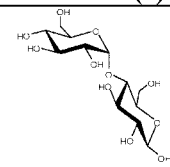
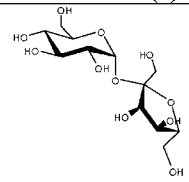
Table 1. Isentropic Molar Compressibility (10^4 $K_2^o(s)$, cm^3 mol^{-1} bar^{-1}) and Hydration Numbers of Various Monosaccharides, **1**−**4**, and Disaccharides, **5**−**9**[a]

Carbohydrate	Molar Compressibility ($K_2^o(s)x10^4$, cm^3 mol^{-1} bar^{-1})	Hydration Number[8]	Carbohydrate	Molar Compressibility ($K_2^o(s)x10^4$, cm^3 mol^{-1} bar^{-1})	Hydration Number[8]
 D-Galactose **(1)**	-20.8 (0.5)[44] -20.4 (0.4)[8]	8.7 (0.2)	 D-Glucose **(2)**	-17.6 (0.3)[8]	8.4 (0.2)
 D-Mannose **(3)**	-16.0 (0.5)[44]	8.1 (0.2)	 D-Talose **(4)**	-11.9 (0.3)[8]	7.7 (0.2)
 D-Melibiose **(5)**	-31.2 (1.0)[8]	15.5(0.3)	 D-Lactose **(6)**	-31.1 (0.2)[8] -30.4 (1.0)[44]	15.3 (0.3)
 D-Trehalose **(7)**	-30.2 (0.3)[8]	15.3 (0.3)	 D-Maltose **(8)**	-23.7(1.0)[44]	14.5 (0.3)
 D-Sucrose **(9)**	-17.8 (0.5)[44]	13.9 (0.3)			

[a] Errors of molar compressibility values and hydration numbers are shown in parentheses.[8,20b,44]

Table 1 reports the isentropic molar compressibility values and hydration numbers for various mono- and disaccharides.[8,9]

As an extension of the stereospecific hydration model,[15] Furuki used differential scanning calorimetry (DSC) to measure the heat of fusion of ice in carbohydrate solutions and subsequently determined the amount of unfrozen water (U_w) surrounding the carbohydrate molecule.[24] These studies suggest that larger amounts of unfrozen water are correlated with poorer compatibility of the sugar with the three-dimensional hydrogen-bonded network of bulk water. Thus, it was proposed that the antifreeze activity of carbohydrates is a function of hydration, which is in turn dependent upon carbohydrate stereochemistry. It should be noted that the technique for determining thermal hysteresis or antifreeze activity in this study was DSC. Although DSC has been utilized to study thermal hysteresis (TH) activity in AFPs and AFGPs, the standardized technique is nanoliter osmometry.[2] However, neither of these techniques assesses recrystallization-inhibition (RI) activity.

TH is defined as a selective depression of the freezing point of a solution relative to a static melting point, whereby the difference between these two temperatures is known as a thermal hysteretic gap. The significance of this gap is that within this temperature range, a seeded ice crystal is stable and does not continue to grow, resulting in a heterogeneous solution containing suspended microcrystals. Thermal hysteresis is always preceded by dynamic ice shaping (DIS), which is a direct result of a solute binding at the interface of the ice lattice and the quasi-liquid layer (QLL). Alternatively, recrystallization inhibition activity prevents (or slows down) the enthalpically driven re-organization of individual ice crystals in an already frozen sample.[2,21,25] While the mechanism of TH activity remains the source of much debate,[26,27] it is ice recrystallization inhibition activity that is the most desirable property of a cryoprotectant, as the majority of cellular damage occurs during the holding and thawing phase of cryopreservation, where recrystallization is a dominant process.[17]

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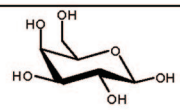
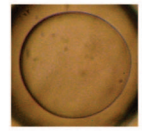
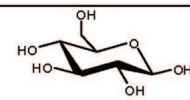
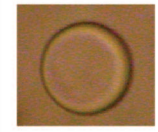
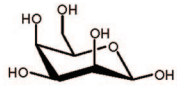
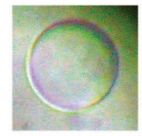
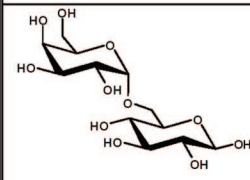
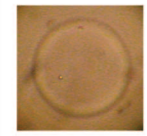
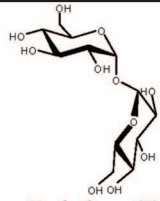
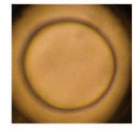
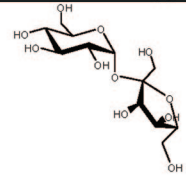
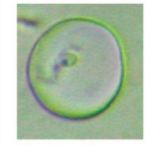
Table 2. Ice Crystal Morphology and Melting Points of 10 mg/mL Solutions of D-Galactose (**1**), D-Glucose (**2**), D-Talose (**4**), D-Melibiose (**5**), D-Trehalose (**7**), and D-Sucrose (**9**) in Double-Distilled Water, Determined Using Nanoliter Osmometry[23]

Carbohydrate	Melting Point (°C)	Crystal Morphology	Carbohydrate	Melting Point (°C)	Crystal Morphology
 D-Galactose (**1**)	-0.069		 D-Glucose (**2**)	-0.055	
 D-Talose (**4**)	-0.22		 D-Melibiose (**5**)	-0.17	
 D-Trehalose (**7**)	-0.015		 D-Sucrose (**9**)	-0.12	

Assessing the Carbohydrate−Ice Interaction. Recent reports have suggested that the antifreeze activity of simple mono- and disaccharides is based on complimentarity of hydroxyl groups with the ice lattice. It has been proposed that the optimal distance between hydroxyl groups is 4.2−4.5 Å.[28] To investigate this possibility, we examined three monosaccharides (galactose, glucose, and talose) and three disaccharides (melibiose, trehalose, and sucrose) for antifreeze activity as a function of thermal hysteresis using nanoliter osmometry.[23] From Table 2, it is evident that none of these carbohydrates exhibit dynamic ice shaping, and therefore do not have thermal hysteresis activity. The slight freezing point depressions are due to the colligative properties of each carbohydrate. More interesting is the fact that the ice crystal morphologies of the carbohydrate solutions are consistent with samples of distilled water. This is significant, as it indicates that no direct interaction with the ice lattice is occurring as previously proposed,[28] and that a specific distance between hydroxyls is not the only factor responsible for imparting antifreeze activity. While it has been suggested that the protein−ice interaction has an element of surface complimentarity with antifreeze proteins,[29] recent molecular dynamic simulations have implied that the QLL plays an important role in the binding of biological antifreezes to ice.[30] However, it is still unclear how these biological antifreezes recognize the pseudo-ordered QLL. We propose that a key contributing factor for antifreeze activity is hydration of the carbohydrate residue. To explore this hypothesis, the RI activity of a variety of carbohydrates was examined as a function of their hydration number.

Effect of Concentration on Recrystallization-Inhibition Activity. To determine the optimal working concentration for our RI assay, a concentration scan was performed. Figure 1 shows that a 0.22 M solution of galactose in PBS is an effective inhibitor of recrystallization.

Although this concentration results in reasonable RI activity, the viscosity of this solution was quite high and posed technical difficulties that adversely affected assay performance. However, a 0.022 M solution showed RI activity nearly identical[31] to that of the 0.044 M solution, but did not present viscosity problems. Consequently, concentrations of 0.022 M were employed for all saccharides. The overall relationship between carbohydrate concentration and RI activity appears to be a logarithmic relationship for the five highest concentrations tested, as shown in Figure 2. At concentrations lower than 0.00022 M, there is a limiting effect, whereby the size of the ice crystals in more-dilute galactose solutions approached the size of ice crystals in PBS solution. This suggests that at higher concentrations, the RI activity of galactose is non-colligative, as has been observed for biological antifreezes.[32] This non-colligative relationship has also been reported by Uchida, who utilized field-emission type transmission electron microscopy (FE-TEM) to study ice crystal size as a function of trehalose concentration.[33]

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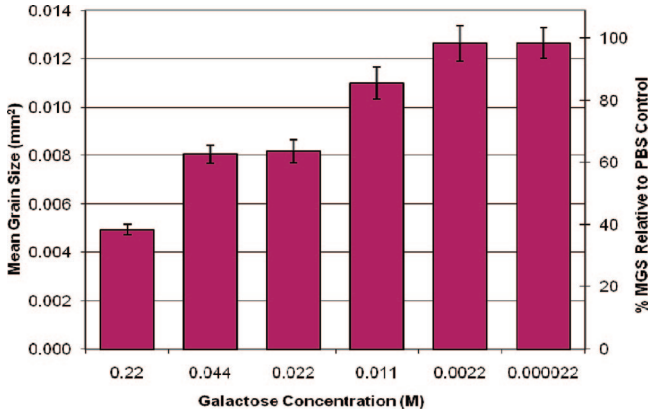

Figure 1. Recrystallization-inhibition (RI) activity of various concentrations of D-galactose (**1**) solutions in PBS.

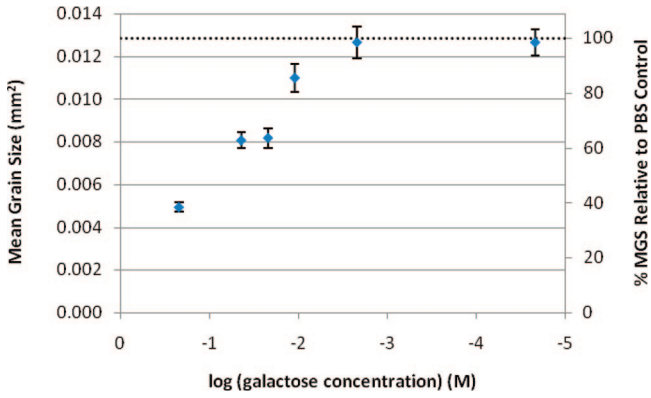

Figure 2. RI activity of various concentrations of D-galactose (**1**) in PBS solution. The dashed line represents the mean grain size of PBS control. X-axis represents the $\log_{10}$(concentration) of D-galactose solution.

Carbohydrate Stereochemistry. Literature reports have suggested that the compatibility of a carbohydrate with the three-dimensional hydrogen-bonded network of water is governed by carbohydrate stereochemistry.[8,9,34] This observation correlates well with experimentally derived molar compressibility values in that these also vary as a function of hydroxyl stereochemistry at C2 and C4 (Table 1).

Our laboratory previously reported the RI activity of C-linked AFGP analogues.[19] It was observed that analogues containing carbohydrates with low isentropic molar compressibilities (such as galactose) fit poorly into the three-dimensional hydrogen bonded network of bulk water and hence are effective inhibitors of recrystallization. While this hypothesis was based upon isentropic molar compressibilities, this measurement of solute hydration may not be truly representative of the actual hydration state.

Many experimental methods have been reported to study the structure of solutions. These range from near-infrared spectroscopy,[35] density and density ultrasound,[8,36] nuclear magnetic and dielectric-relaxation,[37] quasi elastic neutron scattering,[38] terahertz spectroscopic measurement,[39] and viscosity and acoustic measurements[36,40] to molecular dynamic simulations.[41] The density ultrasound technique is regarded as one of the best experimental methods in that it delineates subtle differences in solution structure, such as water that is tightly bound to a solute versus water that is only loosely associated with a solute.[42] Although this technique is routinely used to obtain accurate adiabatic compressibility coefficients, recent studies have shown that hydration numbers calculated from these coefficients represent a more accurate summary of contributions influencing the hydration of the solute molecule.[43] The hydration number differs from isentropic molar compressibility values in that hydration numbers accurately predict the total number of water molecules hydrogen-bonded to the sugars. However, this is still regarded as a dynamic measurement because it really predicts the number of water molecules that have a relatively long residence time and hence move in solution with the sugars. Given that hydration numbers are a more accurate representation of solute hydration, we plotted the RI activity as a function of hydration numbers[8] (calculated from isentropic molar compressibility coefficients) for simple mono- and disaccharides with varying hydroxyl stereochemistry, Figure 3.

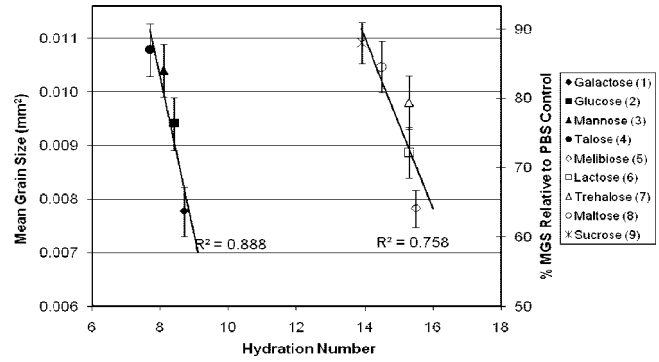

Figure 3. RI activity of various monosaccharides (**1**−**4**) and disaccharides (**5**−**9**) at 0.022 M in PBS solution, plotted against their respective hydration numbers.

From this data, each series of carbohydrates (mono- and disaccharides) show a strong linear trend between hydration numbers and their respective RI activity. However, the general increase in hydration number for disaccharides relative to the monosaccharides did not necessarily correlate to an increase in RI activity. For example, the RI activity of galactose (hydration number = 8.7) and melibiose (15.5) are very similar (mean grain size = 0.0082 mm^2), despite the large difference between their respective hydration numbers. More surprisingly, the difference in hydration numbers between galactose (8.7) and sucrose (13.9) resulted in a decrease in RI activity for the latter. We believe this is due to the difference in steric volume between monosaccharides and disaccharides and have corrected for it in the following manner. Hydration numbers were obtained from the

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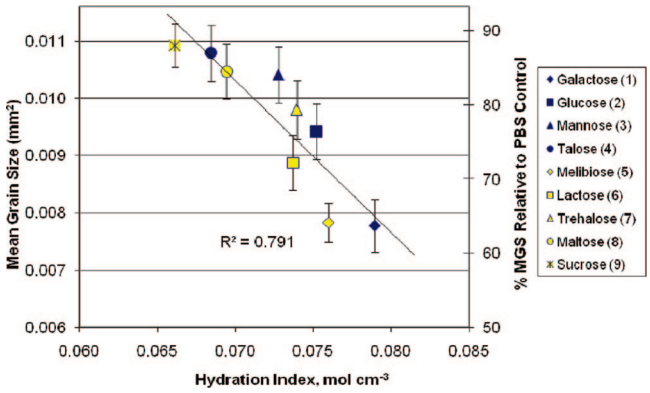


Figure 4. RI activities of carbohydrates (**1**−**9**), plotted against their respective hydration index (hydration number/partial molar volume) (mol^1 cm^{-3}).

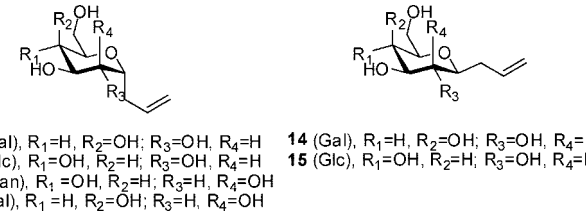
literature and were derived according to the Passynsky equation (eq 1) using molar compressibility coefficients, β, which were obtained from ultrasound and density measurements (eq 3),[8]

$$\beta = 1/u_2 d \tag{3}$$

where u is the speed of sound and d is the density of the solution. Dividing hydration numbers by partial molar volumes[8,44] results in a description of the number of tightly bound water molecules per molar volume of carbohydrate. We refer to this value as a *hydration index*. Similar correlations have been reported by Parke et al. in relating the taste properties of solutes of various masses, volumes, and hydrophobicity to the hydration per unit volume of the solute.[45] Figure 4 shows that plotting the hydration index against RI activity of carbohydrates **1**−**9** results in a single trend for all carbohydrates, compared to Figure 3 which had two distinct correlations.

This is significant in that while the absolute number of water molecules surrounding a solute is important for predicting RI activity, the number of water molecules per unit volume of solute is also important; thus, a higher concentration of water molecules around a solute will increase disorder of the surrounding bulk water[29] which consequently leads to increased RI activity.

RI Activity Comparison between C-Glycosides and O-Glycosides. The AFGP analogues previously synthesized by our laboratory are C-linked in nature.[19] While the conformation of C-linked carbohydrate analogues is generally accepted to be similar to O-linked systems,[46] hydration numbers of C-linked carbohydrates have not been reported and the relationship between hydration and RI activity has not been previously studied. It has been suggested that hydration is largely dictated by substituents at C2 and C4 of the pyranose ring.[8,34] To test this hypothesis, C-allylated derivatives of galactose, glucose, mannose, and talose (**10**−**15**, Figure 5), were synthesized to



10 (Gal), R_1=H, R_2=OH; R_3=OH, R_4=H
11 (Glc), R_1=OH, R_2=H; R_3=OH, R_4=H
12 (Man), R_1 =OH, R_2=H; R_3=H, R_4=OH
13 (Tal), R_1 =H, R_2=OH; R_3=H, R_4=OH
14 (Gal), R_1=H, R_2=OH; R_3=OH, R_4=H
15 (Glc), R_1=OH, R_2=H; R_3=OH, R_4=H

Figure 5. C-allylated derivatives of galactose (**10**, **14**), glucose (**11**, **15**), mannose (**12**), and talose (**13**).

determine the influence of a carbon substituent at C1 and its stereochemistry on carbohydrate hydration and RI activity.

Despite the absence of hydration numbers, the RI activity of C-linked analogues and their respective O-linked monosaccharides is shown in Figure 6. Of all the α-C-allyl pyranoses (**10**−**13**), galactose derivative **10** possesses the most RI activity with mean grain size (MGS) statistically identical[31] to native D-galactose. In fact, the RI activity trend of **10**−**13** is identical to their corresponding native monosaccharides **1**−**4**, respectively. This is consistent with the hypothesis that the nature of the substituent at C1 may have little influence on hydration and hence RI activity. In contrast, β-anomer **14** exhibits a marked decrease in RI activity relative to native O-linked galactose, **1**, and α-C-linked derivative, **10**, suggesting that the anomeric configuration of C-linked carbohydrates may play some role in hydration and therefore RI activity. Comparatively, β-C-allyl glucose, **15**, displayed the same amount of RI activity as its α-anomer, **11**, with no statistically significant difference[31] in MGS. Further studies are required to ascertain the exact effect of anomeric configuration on RI activity and its subsequent relationship to hydration.

Comparison to DMSO. The ability of D-galactose **1** and α-C-allyl galactose derivative **10** to function as cryoprotectants was assessed. This was accomplished using dimethylsulfoxide (DMSO) as a standard. DMSO is regularly used as an additive to cellular suspensions prior to freezing and storage at subzero temperatures.[47] Despite the routine use of DMSO, studies have shown it is cytotoxic and capable of eliciting apoptosis in many different cell types.[47] The generally accepted mechanism by which DMSO imparts cryopreservation is two-fold. First, it is thought to replace water in the cell membrane and thus prevent fractionation of the cell membrane during thermatropic phase transitions.[48,49] Second, molecular dynamics simulations have shown that individual DMSO molecules may cooperatively form extensive ion channels in the cell membrane and thus facilitate the transport of water into and out of the cell to relieve osmotic stress during the freezing event.[48,49] Regardless of the nature of the mechanism, the use of DMSO does result in improved viability of many cell types post freeze−thaw as compared to other protocols, and thus it is routinely employed in cryopreservation.[50] Typically, DMSO concentrations employed in routine cryopreservation range between 1−20 mol%, but recent studies have shown that very little additional cryoprotection is conferred

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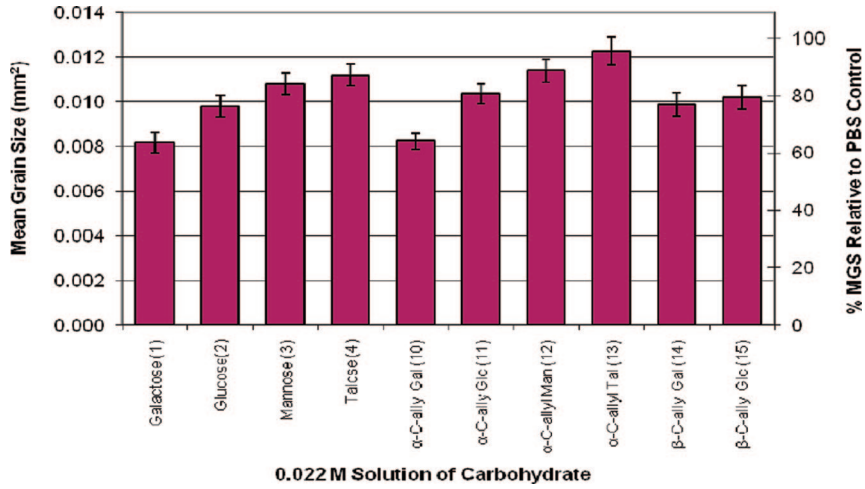

Figure 6. RI activity of native O-linked monosaccharides (**1**−**4**) and their C-glycoside derivatives (**10**−**15**) at 0.022 M in PBS solution.

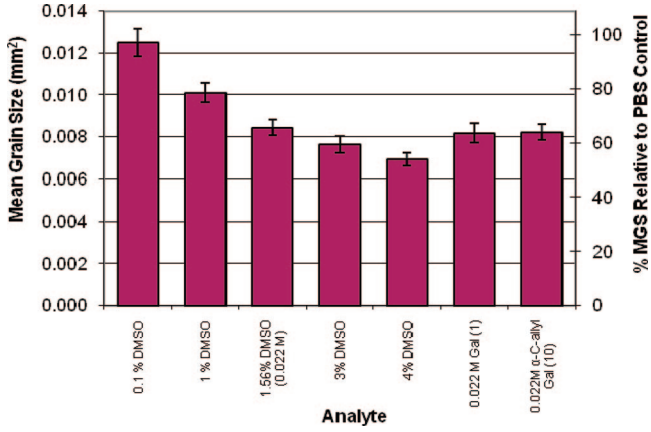

Figure 7. RI activity of various concentrations of DMSO and 0.022 M solutions of compounds **1** and **10** in PBS solution.

at concentrations above 5 mol%.[51] To the best of our knowledge, the ability of DMSO to function as an inhibitor of recrystallization has not yet been explored. Consequently, an initial concentration scan was performed using our RI assay. The results of this study are shown in Figure 7.

As illustrated in Figure 7, concentrations of 0.1 to 4% (v/v) DMSO were assessed. In practice, concentrations above 6% (v/v) were very difficult to use in this assay and produced inconsistent results due to large portions of unfrozen solution in the ice wafer. Consequently, only concentrations less than that of 6% were reliably assessed. A 0.1% DMSO solution failed to inhibit recrystallization and yielded ice crystals identical in size to the PBS control. In contrast, a 4% solution of DMSO exhibited RI activity higher than a 0.022 M galactose solution. As a benchmark for comparison between DMSO concentration and galactose concentration, a 0.022 M solution of galactose is as efficient at inhibiting recrystallization as a 3% (v/v) DMSO solution, Figure 7. These results suggest that galactose may be a suitable cryoprotectant.

Proposed Recrystallization-Inhibition Mechanism of Action with Ice. The mechanism of recrystallization has been studied extensively in the metallurgical literature within the context of inorganic composites.[52] In ice, there are two key issues that need to be considered. First, a small amount of bulk water is present between adjacent ice crystals. Second, the interface of the ice lattice and bulk water is not an abrupt transition. Independent studies have proven that a layer of semi-ordered ice (quasi-liquid layer, QLL) exists between the highly ordered ice lattice and bulk water.[53] During the last several years, the nature and properties of this interfacial domain have been studied extensively and implicated in the ability of antifreeze proteins to recognize ice.[30] Consequently, a mechanism of action for inhibitors of ice recrystallization must account for the QLL. On the basis of the results of the current study, a direct interaction between the carbohydrate and the ice lattice is unlikely since no thermal hysteresis or dynamic ice shaping is observed (Table 2).

In the splat-cooling RI assay, the wafer is frozen and contains very little unfrozen water. Furthermore, as solutes are excluded from the ice lattice, the carbohydrate will be concentrated at the interface between two adjacent ice crystals and their QLLs. The QLL is expected to be approximately 3−10 Å in thickness, although this thickness is known to be temperature-dependent.[53] Taking all of this into account, a representation of a carbohydrate solvated in bulk water between two adjacent ice crystals and their QLLs is shown in Figure 8.

The question of where the carbohydrate localizes with respect to the QLL is a difficult question to answer given current inabilities to study the QLL. However, there are two possibilities. In the first, the carbohydrate is concentrated at the bulk water−QLL interface while in the second, the carbohydrate is actually incorporated into the QLL. The latter does not seem probable, as we are not aware of any precedent for incorporation of a carbohydrate into the QLL. Furthermore, Uchida and co-workers have studied the RI activity of trehalose using TEM and proposed that trehalose functions at the bulk water−QLL interface.[33]

Our studies are consistent with others[24,28,34] in that they suggest that carbohydrate stereochemistry does play some role

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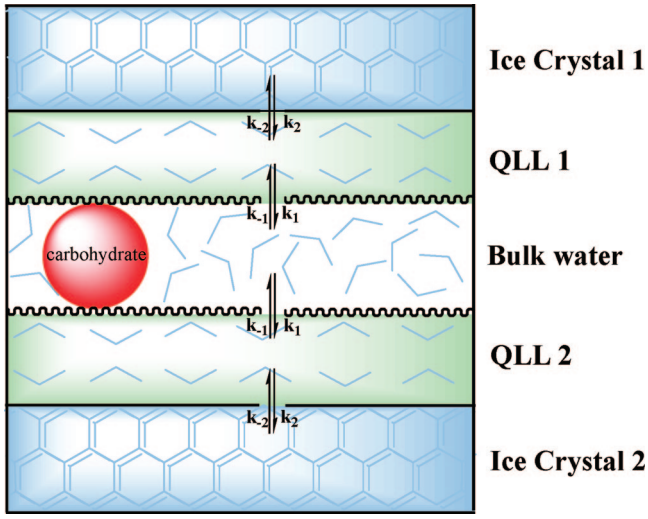

Figure 8. Schematic representation of the proposed mechanism for inhibition of ice recrystallization. Carbohydrates reside at the QLL–bulk water interface between two adjacent ice crystals. Water molecules surrounding a carbohydrate with a high hydration index will be more disordered than in cases with a low hydration index carbohydrate or in the absence of solute. The area shaded red represents hydrated solute, QLL = quasi-liquid layer.

in inhibiting the process of ice recrystallization, but we propose that due to the interaction with the QLL, hydration of the carbohydrate is a significant factor for inhibiting recrystallization of ice.

Our previous work tentatively linked isentropic molar compressibilities and carbohydrate stereochemistry to RI activity.[19] The work in the present paper suggests that *hydration index* (hydration number per molar volume of carbohydrate) constitutes a better predictor of RI activity, Figure 4. Carbohydrates with larger hydration indices (such as galactose and melibiose) positioned at the interfacial domain cause a greater disruption on the ordering of bulk water surrounding the hydration shell, which leads to slightly increased energies associated with the transfer of bulk water to the QLL. Given that the thickness of the QLL is small and the entropy of the first hydration shell can extend out to the third hydration shell,[39] this energy difference would be significant. Heyden et al. have recently confirmed this long-range effect on bulk water using terahertz absorption measurements.[39] This study demonstrates that solvated carbohydrates alter the long-range motion of water molecules by increasing the number of water−carbohydrate hydrogen bonds that can interact with water. Consequently, carbohydrates with larger hydration indices function as better ice recrystallization inhibitors. Larger absolute hydration numbers for disaccharides relative to smaller-sized monosaccharides do not necessarily translate to an increased inhibition of ice growth, Figure 3; a disaccharide with a small hydration index, such as sucrose, has little effect upon ice recrystallization despite a larger absolute hydration number and volume than that of a monosaccharide such as galactose. The ultimate significance of this relationship is that there needs to be a large number of water molecules in the hydration shell, and these must be highly concentrated around the solute, helping to increase the entropy of surrounding bulk water.[29]

Conclusions

In summary, we have demonstrated that the hydration of a carbohydrate is correlated to RI activity. The relationship between carbohydrate concentration and RI activity is noncolligative, and the *hydration index* modulates the ability of a sugar to function as a potent inhibitor of recrystallization. Among the monosaccharides examined in this study, galactose possesses the most RI activity, whereas in the disaccharides, melibiose is the most potent. While C-linked derivatives of monosaccharides appear to parallel the RI activity of their O-linked native structures, the importance of anomeric stereochemistry in C-linked derivatives requires further investigation. A 0.022 M solution of D-galactose, **1**, and C-linked galactose derivative, **10**, inhibit recrystallization as well as a 3% DMSO solution. On the basis of the fact that the carbohydrates examined in this study did not possess any TH activity or dynamic ice shaping, we propose that they are inhibiting recrystallization at the bulk water−QLL interface by disrupting the pre-ordering of water. The concept of a hydration index will greatly facilitate the rational design of novel inhibitors of recrystallization for medical, commercial, and industrial applications.

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Supporting Information Available: Procedures and characterization of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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