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Vitrification Enhancement by Synthetic Ice Blocking Agents

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Small concentrations of the synthetic polymer polyvinyl alcohol (PVA) were found to inhibit formation of ice in water/cryoprotectant solutions. Ice inhibition improved with decreasing molecular weight. A PVA copolymer of molecular weight 2 kDa consisting of 20% vinyl acetate was found to be particularly effective. PVA copolymer concentrations of 0.001, 0.01, 0.1, and 1% w/w decreased the concentration of glycerol required to vitrify in a 10-ml volume by 1, 3, 4, and 5% w/w, respectively. Dimethyl sulfoxide concentrations required for vitrification were also reduced by 1, 2, 2, and 3% w/w, respectively. Crystallization of ice on borosilicate glass in contact with cryoprotectant solutions was inhibited by only 1 ppm of PVA copolymer. Devitrification of ethylene glycol solutions was also strongly inhibited by PVA copolymer. Visual observation and differential scanning calorimeter data suggest that PVA blocks ice primarily by inhibition of heterogeneous nucleation. PVA thus appears to preferentially bind and inactivate heterogeneous nucleators and/or nascent ice crystals in a manner similar to that of natural antifreeze proteins found in cold-hardy fish and insects. Synthetic PVA-derived ice blocking agents can be produced much less expensively than antifreeze proteins, offering new opportunities for improving cryopreservation by vitrification. © 2000 Academic Press

Key Words: vitrification; polyvinyl alcohol; PVA; antinucleation; ice blocker; antifreeze protein.

Cryopreservation by vitrification (8) requires cooling to below the water/cryoprotectant glass transition temperature and subsequent rewarming without ice formation. Cryoprotectant toxicity is minimized by using the minimum cryoprotectant concentration that is consistent with this objective. Theoretically, this minimum concentration is determined by the concentration at which the homogeneous nucleation temperature, T_h, intersects the glass transition temperature, T_g (Fig. 1) (8). At lower concentrations, the sample must traverse the zone between T_h and T_g in which homogeneous nucleation of ice is unavoidable. At higher concentrations, vitrification is theoretically possible at any cooling or rewarming rate if heterogeneous nucleators are not present.

In practice, heterogeneous nucleators are an omnipresent obstacle to vitrification, even when homogeneous nucleation is minimized or avoided. Evidence for the role of heterogeneous nucleation in ice formation during cooling of vitrification solutions is substantial (5), implying that it is suppression of heterogeneous nucleation rather than homogeneous nucleation that determines the minimum concentration needed to vitrify (C_{vit}) in practical settings. After vitrification, rapid warming rates are necessary (7, 8) to avoid growth (devitrification) of ice that nucleates at very low temperatures near the end of cooling (and beginning of rewarming). Devitrification has been mostly ascribed to homogeneous nucleation near T_g. However, devitrification occurs even if T_h is not crossed, implying that heterogeneous nucleation can also cause devitrification. If heterogeneous nucleation could be prevented, these theoretical issues could be resolved, and cryoprotectant concentrations approaching the theoretical minimum might be usable with less stringent rate requirements for cooling and rewarming. This would be particularly useful for vitrification of large systems, such as organs and engineered tissues.

Polar fish (4) and terrestrial insects (21) have evolved antifreeze proteins (AFPs) and anti-

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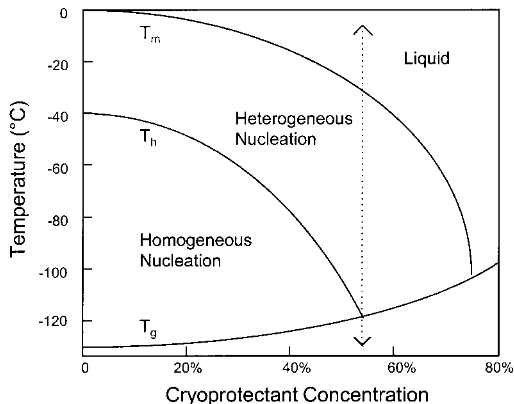

FIG. 1. Supplemented phase diagram of a generic cryoprotectant in water solution. T_m denotes the equilibrium melting temperature, T_h the homogeneous nucleation temperature, and T_g the glass transition temperature. Vitrification typically involves cooling and rewarming at a concentration near the dotted line, making the vitrification process highly susceptible to heterogeneous nucleation of ice. After Fahy *et al.* (8).

freeze glycoproteins (AFGPs) that permit supercooling of body fluids below the equilibrium freezing temperature. An important mechanism of fish AFP and AFGP activity is believed to be inactivation of heterogeneous nucleators by selective binding to nucleators in solution (16, 22). Fahy *et al.* (9) speculated that AFPs might be useful in overcoming the problems of heterogeneous nucleation during the vitrification of large systems. Chang *et al.* (3) noted apparent worsening of devitrification when AFP from *Tenebrio molitor* larvae was added to glycerol solutions. However, Sutton and Pegg (20) reported a spectacular decrease in the critical warming rate necessary to avoid devitrification of 2,3-butanediol and glycerol solutions with the addition of 1% AFP from the Winter Flounder. (They speculated that the negative result of Chang may have been due to impurities in the crude AFP extract used.) O'Neil *et al.* (17) found that adding 0.1% AFGP to an oocyte vitrification solution inhibited devitrification, leading to improved oocyte morphology after vitrification.

Interestingly, Hey and MacFarlane (12) and Fahy (5) observed that fish AFP and AFGP caused no significant change in the low-temperature growth rate of ice crystals in concentrated cryoprotectant solutions. These results suggest that the efficacy of fish AFPs and AFGPs in preventing devitrification may indeed be due to nucleation inhibition rather than ice growth inhibition.

Soon after the original discovery of fish AFPs by DeVries and Wohlschlag in 1969 (4), Klotz (13) speculated that "many polymeric molecules" (not just proteins) ought to be able to inhibit freezing by selective binding to heterogeneous nucleators. In 1983, Caple (1) reported enhanced supercooling of water solutions to which small quantities of polymers with alternating hydrophobic/hydrophilic functionality had been added. However, no results in the presence of cryoprotectants were given. In 1995, Fahy (5, 6) proposed creating synthetic ice interface dopants ("ice blockers") specifically designed to bind to the basal plane and prism faces of ice crystals (and ice nucleators). Molecules were to be designed by spacing polar groups at intervals corresponding to the lattice spacing of water molecules on the crystal faces of ice. Several specific molecules and polymers were proposed, but no data were presented.

In this work we report that the water-soluble synthetic polymer polyvinyl alcohol (PVA), and especially a low-molecular-weight copolymer of PVA, appears to inhibit heterogeneous nucleation of ice in vitrification solutions. PVA has been studied previously as a defined substitute for serum in freezing (2, 15, 18, 19) and vitrification solutions (14, 19). However, no data have been presented on its remarkable ice inhibition properties. We have found that concentrations of PVA copolymers as low as one part in 10^6 visibly inhibit nucleation of ice in vitrification solutions. These results have been previously presented in preliminary form (23).

MATERIALS AND METHODS

Solutions were prepared in either bottled distilled water (Hinckley & Schmitt, Inc., Orange, California, U.S.A.) or ultrapure lab water (Milli-Q Biocel water purification system from Millipore Corp.). All solutions were prepared

gravimetrically. Solution concentrations are therefore always given as weight-by-weight (w/w) percentage, even if not stated explicitly. A.C.S.-certified dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific Co. Reagent-grade ethylene glycol (EG) was obtained from Spectrum Quality Products. Spectrophotometric-grade glycerol was obtained from Aldrich Chemical Co. PVA of various nominal molecular weights ranging from 9 to 150 kDa was also obtained from Aldrich.

PVA is commercially prepared by hydrolyzing polyvinyl acetate, which converts acetate groups to hydroxyl groups. Percentage hydrolysis is therefore a measure of the mole fraction of original acetate groups that have been converted to hydroxyls. To study ice blocking effectiveness as a function of percentage hydrolysis, PVA samples of different percentage hydrolysis were prepared. Partially hydrolyzed (75% nominal) PVA of molecular weight 2 kDa was obtained from Monomer-Polymer and Dajac (Feasterville, PA, U.S.A.). A 10% w/w solution of this PVA in water was prepared and heated to +75°C. The solution was then titrated with NaOH until the pH remained steady above 10, indicating complete saponification of the acetate groups. The quantity of NaOH consumed indicated that the PVA was 73% hydrolyzed at the start of the experiment. Fresh PVA was then treated in hot aqueous solution with NaOH calculated to produce PVA samples of 76, 78, 80, 82, 85, 90, and 98% hydrolysis. Sodium acetate was then added as necessary so that all samples had the same background sodium acetate content at the end of processing.

A particularly effective form of partially hydrolyzed PVA (nominal 20 mole percent vinyl acetate content) was synthesized by a proprietary process. This product is available as SuperCool X-1000 from 21st Century Medicine, Inc. (patent pending). The mean molecular weight was determined to be 2 kDa (±500 Da) by osmometry in dilute aqueous solution. The percentage hydrolysis was determined to be 79% (±0.5%) by NaOH titration and ^{1}H-NMR.

Heterogeneous nucleation is a random process that usually occurs at widely spaced intervals in a solution volume. Ice growth resulting from heterogeneous nucleation is therefore best observed in solution volumes of at least several milliliters. A model was therefore developed for observing ice growth in ~10-ml solution volumes; 10-g solution samples were prepared in 20-ml glass scintillation vials (Kimble Glass, Inc., Vineland, NJ, U.S.A.). The vials were then suspended in cold (−160°C) nitrogen vapor 2 to 3 cm above the surface of liquid nitrogen in a MVE TA-60 dewar. This resulted in a mean cooling rate between T_m (equilibrium melting temperature) and T_g of approximately 7°C/min (Fig. 2). Vials containing EG or DMSO solutions were suspended for 16 min, reaching a temperature near −130°C. Glycerol solutions were suspended for only 13 min to avoid fracturing due to the high glass transition temperature of glycerol solutions. Vials were then removed from the dewar, briefly dipped in methanol at room temperature to prevent exterior frost formation, suspended in air at ambient temperature (24°C), and inspected for visible ice. Solutions without any visible ice were considered to be vitrified. (Ice on interior vial walls or the solution/air interface was disregarded in this determination.) Devitrification was studied in EG solutions by continuing visual inspection for several minutes. The mean warming rate to T_m was approximately 8°C/min. The cooling and rewarming curve experienced by a 60% EG solution subjected to this protocol is shown in Fig. 2.

Cooling thermograms were obtained with a Perkin–Elmer DSC 7 differential scanning calorimeter (DSC) running Pyris version 2.04 software. Solution samples of ~10 mg mass were sealed in Perkin–Elmer 0219-0062 aluminum sample pans and placed in the DSC sample oven for analysis. An empty sample pan was kept in the DSC reference oven to balance the instrument. The oven temperature was calibrated by measuring the onset of the crystal transition of cyclohexane at −87.06°C while warming at 1°C/min and the onset of melting of water ice at 0°C. Heat flow was calibrated by measuring the area under the melting curve of a known mass of water ice (334 J/g nominal).

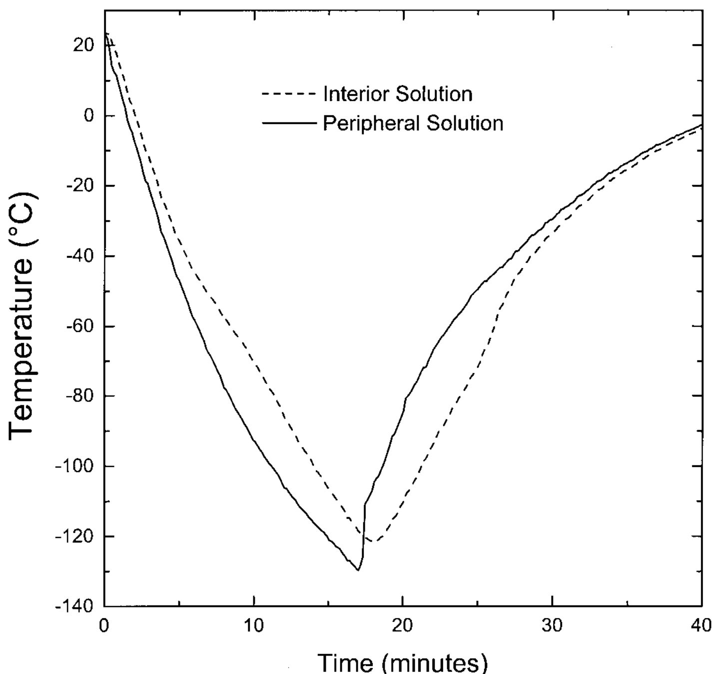

FIG. 2. Cooling and rewarming curves for 10 g of 60% w/w EG solution suspended above liquid nitrogen in a 20-ml glass scintillation vial for 16 min, and then returned to room temperature. The same cooling protocol was followed for all EG and DMSO solutions. Glycerol solutions were cooled for only 13 min to avoid fracture. The separation of the interior and peripheral temperature curves below −50°C is presumably caused by cessation of convective heat transfer at lower temperatures.

RESULTS

All PVA polymers tested were found to visibly reduce the rate of devitrification of 57% EG solutions in distilled water at a concentration of 2%. This effect was observed whether the PVA replaced an equal mass of water or was substituted for an equal mass of EG in the solution. Suppression of devitrification was comparatively poor for PVA of molecular weight >50 kDa, performance being best for PVA <10 kDa. The greater efficacy of low-molecular-weight PVA might be explained by the greater mobility of small molecules and greater number of individual molecules available for binding to heterogeneous ice nucleators.

All fully hydrolyzed (≤2% vinyl acetate content) PVA polymers tested were found to suffer from problems of intermolecular self-association. This resulted in turbid solutions and gel formation if solutions were left standing. The problem was most severe at higher molecular weights. However, even a 1% solution of fully hydrolyzed low-molecular-weight PVA (2 kDa) with 55% EG was found to become turbid within several hours. Solution turbidity was also associated with reduced ice inhibition efficacy, likely because of reduced availability of free PVA molecules. Inclusion of hydrophobic vinyl acetate groups in the PVA polymer (partially hydrolyzed PVA) was found to eliminate the self-association problem in dilute solutions. Vinyl acetate groups also reduced the viscosity of PVA solutions.

The 2-kDa PVA samples of 76, 78, 80, 82, 85, 90, and 98% hydrolysis were evaluated for relative ice blocking efficacy in solutions consisting of 56% w/w EG and 1% w/w PVA in distilled water. (These solutions also contained 45 mM sodium acetate as a by-product of the PVA preparation process.) The solutions were

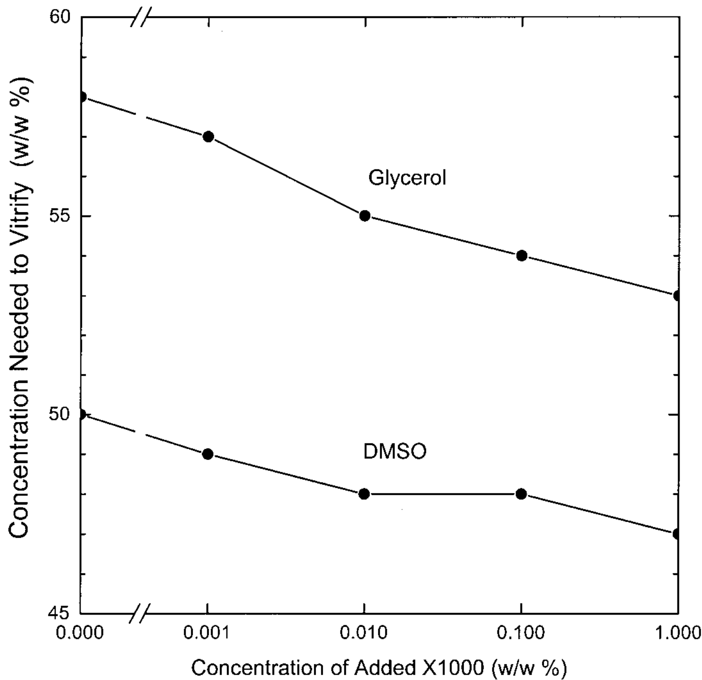

FIG. 3. Minimum concentrations of cryoprotectants in water needed to vitrify by visual inspection (C_{vit}) when 10-g solution samples were cooled according to the protocol of Fig. 2. C_{vit} is plotted as a function of added X1000 PVA copolymer. Very small quantities of X1000 are able to significantly reduce C_{vit} at this slow cooling rate.

cooled and then rewarmed in the standard vitrification model of this work. Devitrification inhibition was similar for solutions containing PVA of 80, 82, 85, and 90% hydrolysis. Approximately twice as much ice formed in solutions with 78 and 98% hydrolyzed PVA and even more ice with 76% hydrolyzed PVA. Evidently, increasing the vinyl acetate content beyond 20% degrades the ice-inhibiting properties of the copolymer.

The copolymer with the best ice-inhibiting and viscosity properties was found to consist of approximately 20% vinyl acetate, 80% vinyl alcohol, at a molecular weight of approximately 2 kDa. This copolymer is denoted below as X1000.

Vitrification Enhancement

Figure 3 plots the concentration needed for vitrification (C_{vit}) for glycerol and DMSO solutions under the cooling conditions of Fig. 2 as a function of added X1000 concentration. The data show that glycerol solutions benefit more by addition of X1000 than DMSO solutions. This may be related to the fact that glycerol is an intrinsically poor antinucleation agent compared to DMSO (11, 12).

Figure 4 is a photograph comparing 54% w/w glycerol solutions in distilled water with and without 0.1% X1000 immediately after cooling to near T_g. It is notable that the X1000 stopped ice formation both in the solution and on the interior walls of the borosilicate glass vial. As little as one part per million of X1000 was found to inhibit nucleation of ice on the vial walls.

Effect of Water Purity

Figure 5 is a photograph comparing 54% w/w glycerol solutions in distilled water vs ultrapure water (Milli-Q purified) after cooling to near T_g. The apparent ice nucleation density in ultrapure

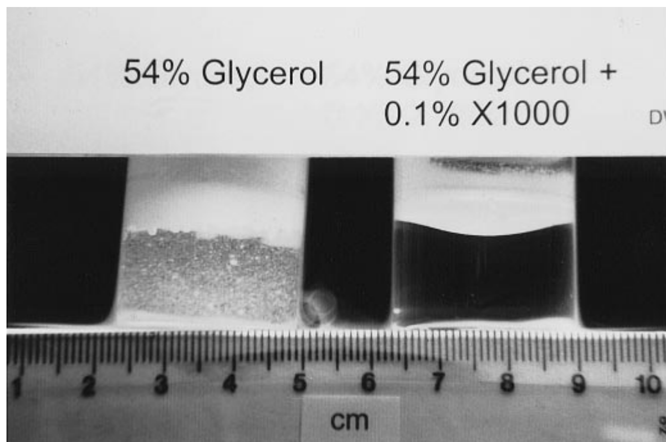

FIG. 4. 54% w/w glycerol solutions in distilled water with and without 0.1% added X1000 after cooling to −110°C as per Fig. 2. The solution with added X1000 vitrified perfectly, while the solution without X1000 has thousands of ice growth sites in the solution and on the container wall. The only ice in the X1000-containing solution is ice that nucleated on the solution surface from ice crystal precipitation in the vapor phase.

water is greatly reduced. This implies that essentially all the ice formation observed in 54% glycerol solutions during cooling is initiated by heterogeneous nucleation. Interestingly, the concentrations of glycerol and X1000 needed to remove all traces of ice from cooled solutions in ultrapure water still follow the curve of Fig. 3. Similar observations were made for DMSO solutions. Figure 3 thus holds for both distilled and ultrapure water. The chief difference is that solutions that fail to vitrify according to the parameters of Fig. 3 fail more dramatically in distilled water than in ultrapure water.

The nature of the excess nucleators in the distilled water is unclear. Preliminary study indicates that they are small (ice nucleation density was found to be unchanged by 0.2-μm filtration of distilled water solutions). It is also

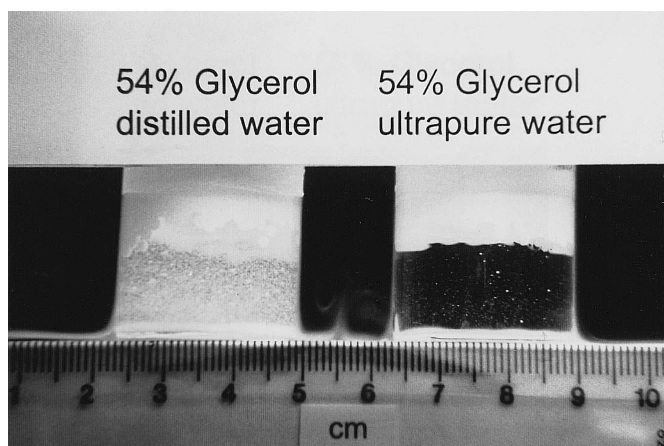

FIG. 5. 54% w/w glycerol solutions in distilled water vs ultrapure water after cooling to −110°C as per Fig. 2. The ice nucleation density is greatly reduced in the ultrapure water, suggesting that all the ice seen during cooling this concentration of glycerol is produced by heterogeneous nucleators.

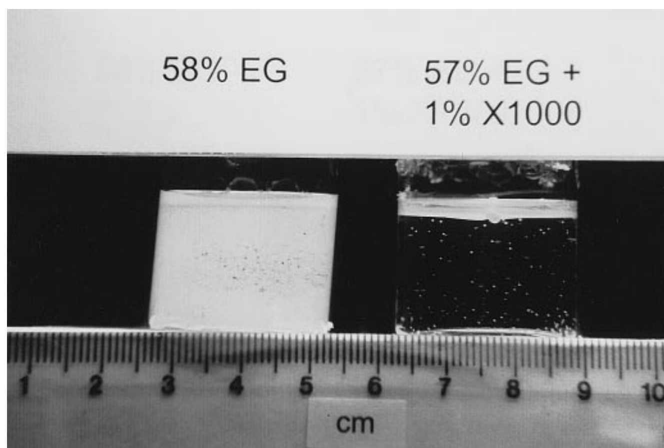

FIG. 6. 58% w/w EG in distilled water with and without 1% substituted X1000 after 5 min of rewarming as per Fig. 2. The solution without X1000 is filled with thousands of ice growth sites that appeared during rewarming. This solution will become completely opaque with devitrified ice in 1 more min. In contrast, the X1000-containing solution has only ~100 tiny ice growth sites.

notable that similar nucleators may be ubiquitous in common laboratory chemicals. Addition of salts, buffers, and polymers to ultrapure water/cryoprotectant solutions resulted in solutions that benefited more visibly from addition of X1000 than ultrapure water/cryoprotectant solutions alone.

Suppression of Devitrification

The effect of X1000 on devitrification was studied in EG solutions in distilled water subjected to the cooling and rewarming protocol of Fig. 2; 57% w/w was the lowest EG concentration at which devitrification could be inhibited essentially completely. This was achieved by adding 1% w/w X1000. Either 57% EG or 58% EG without added X1000 devitrified extensively, becoming optically opaque with ice after several minutes of rewarming. Close examination revealed that the opacity was caused by thousands of individual ice growth sites, each apparently initiated by a discrete nucleation event. In the 57% EG + 1% X1000 solution, approximately 100 small (≤0.3-mm) ice growth sites appeared during rewarming, occupying a negligible solution volume (Fig. 6). The principal difference between 58% EG and 57% EG + 1% X1000 seemed to be the number rather than the size of ice growth sites that appeared during devitrification. Thus, the primary mechanism of ice blocking by PVA copolymers seems to be inhibition of nucleation rather than inhibition of ice growth. One percent of X1000 substituted for EG appeared to inhibit >99.9% of heterogeneous nucleation events that would otherwise appear as ice growth sites during devitrification.

The effect of substituting 2 and 3% w/w X1000 (instead of only 1%) for EG in 58% w/w EG solutions was also studied in the devitrification model. No further benefit was obtained compared to 1% substitution. The ice-inhibiting effects of X1000 thus appear to saturate at about 1% concentration.

The effect of substituting X1000 for EG in EG solutions of lower concentration (≤55% w/w) was also studied during devitrification. Substituting 1% EG with X1000 was found to markedly worsen devitrification in this EG concentration regime. This is likely because devitrification at lower cryoprotectant concentrations is driven by homogeneous nucleation, and the T_h suppressing power of EG is more valuable than the heterogeneous antinucleation effect of X1000 under these conditions. Replacing water with X1000 (instead of replacing EG with

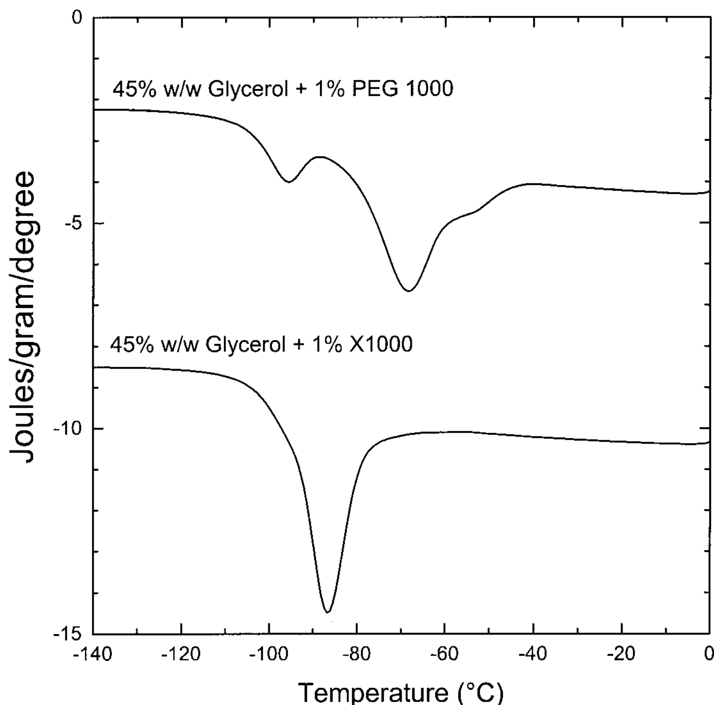

FIG. 7. Thermograms obtained while cooling DSC samples at 40°C/min. The control solution with PEG 1000 freezes heterogeneously between −40 and −90°C, with only a small peak near −95°C caused by homogeneous nucleation. Heterogeneous nucleation is greatly postponed in the solution with X1000, which shows only a single freezing peak that begins at −80°C.

X1000) was found to inhibit devitrification at all EG concentrations tested.

DSC Measurements

DSC cooling runs were performed with samples of 45% w/w glycerol in distilled water with either 1% w/w X1000 or 1% polyethylene glycol of mean molecular weight 1000 (PEG 1000) added to the solution. Figure 7 shows thermograms obtained while cooling at 40°C/min. This concentration of glycerol is intrinsically unstable ($T_h \gg T_g$) and is prone to freezing both heterogeneously and homogeneously during cooling. The control solution with PEG 1000 shows two exothermic peaks at temperatures identical to those reported for the maximum heterogeneous and homogenous ice formation rates in 47% w/w glycerol (11). However, the solution with X1000 shows only one low temperature peak caused by greatly postponed heterogeneous nucleation that merges with the homogeneous nucleation peak.

DISCUSSION AND CONCLUSION

Small concentrations of PVA inhibited the formation of ice in vitrification solutions during cooling and rewarming. Low-molecular-weight PVA copolymers containing vinyl acetate were particularly effective, as well as possessing the desirable physical properties of decreased viscosity and optical clarity in solution. Ice blocking effectiveness was found to rapidly decrease with more than 20 mole percentage vinyl acetate content. This suggests that a minimum number of contiguous vinyl alcohol units (perhaps four) may be necessary for the ice blocking efficacy of PVA copolymers.

Very-low-molecular-weight PVA copolymer visibly decreased nucleation of ice in cryoprotectant solutions at only 0.001% concentration

and eliminated visible nucleation of ice on glass at only 0.0001% (one part per million) concentration. For such small concentrations to be effective, the ice inhibition mechanism must be noncolligative in nature. Preferential binding to heterogeneous nucleators and/or nascent ice crystals in a manner similar to that of natural antifreeze proteins seems to be the most likely mechanism.

As a simple synthetic molecule, PVA copolymers can be produced in large quantities much less expensively than antifreeze proteins. These compounds (and likely other synthetic ice blocking agents yet to be discovered) open new possibilities for controlling ice during cryopreservation of biological systems.

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