



Anomalous high activity of a subfraction of polyvinyl alcohol ice blocker ☆

Brian Wowk *

21st Century Medicine, Inc., 10844 Edison Court, Rancho Cucamonga, CA 91730, USA

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Abstract

Low molecular weight copolymers of polyvinyl alcohol (PVA) are known to be potent inhibitors of ice formation in solutions used for cryopreservation by vitrification, even at concentrations as low as one part per million. Concentrated aqueous solutions of these polymers tend to become turbid after preparation. Condensed particles causing turbidity were isolated from a commercially available PVA-based ice blocker (X-1000) and found to consist of a polymer subfraction that is especially effective at ice blocking. Fifty seven percentage (w/w) of ethylene glycol (EG) in distilled water and 0.025% of the condensate polymer showed similar stability against devitrification as 57% EG + 0.1% ordinary X-1000. At higher concentrations, 56.9% EG + 0.1% condensate polymer was as effective as 56% EG + 1% ordinary X-1000. All solutions containing ice blocker showed much less devitrification during warming than a 57% EG control solution. The condensate polymer was found to be strongly self-associating and less water soluble than ordinary X-1000. The mean molecular weight of the condensate polymer was approximately 1400 compared to 2100 for ordinary X-1000. Proton NMR revealed no large chemical differences. Subtle differences in composition or stereochemistry, perhaps in local regions of molecules, must be responsible for the dramatic differences in physical behavior and ice blocking effectiveness of the condensate polymer.

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Vitrification [2,9,16] is beginning to fulfill its promise as a method of cryopreserving organized tissues more effectively than freezing. Successes

have been demonstrated for vascular grafts [9], cartilage [8], and cornea [1]. Progress continues for the mammalian kidney, with successful recovery reported after cooling to -45°C [4].

There is growing interest in the use of compounds such as antifreeze proteins and synthetic ice blockers (“SIBs”) [3,10,11,14–16] that inhibit ice nucleation and growth by non-colligative

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* Fax: +1 909 466 8618.

E-mail address: wowk@21cm.com.

mechanisms in vitrification solutions. Unlike conventional cryoprotectants that inhibit freezing by interacting with water, ice blockers are believed to act by direct molecular recognition of ice nucleators [15] or perhaps ice itself [10,11,13]. This selective attraction to surfaces of ice growth permits ice blockers to exert significant effects even while present at very low concentrations [14,15]. Small quantities of ice blocker can therefore enhance the vitrification tendency of solutions without adding additional toxicity, or reduce requirements for conventional cryoprotectants while maintaining vitrification tendency, thereby reducing toxicity [3].

The X-1000 ice blocker (21st Century Medicine, Inc., Rancho Cucamonga, CA, USA) is a copolymer of polyvinyl alcohol (PVA) of approximately 2000 molecular weight, with a 20% vinyl acetate content to reduce the viscosity and self-association tendencies of the molecule [14]. X-1000 has been used as an additive in solutions developed for kidney vitrification [3,4], and has also been beneficial in vitrification solutions for tissue engineered bone [7]. It significantly suppresses ice formation in vitrification solutions, even at concentrations as low as one part per 10^6 (Fig. 1). X-1000 has been shown to reduce the activity of several specific ice nucleators [15], although its mechanism of action seems to be more general than just affinity for specific nucleat-

ors. Recent evidence suggests that a direct interaction with ice may be involved [13,17].

A 20%w/w solution of X-1000 in water (Fig. 2A) typically becomes turbid (Fig. 2B) after standing for several hours, especially at refrigerated temperatures. Particles causing the turbidity sometimes condense on the bottom of the container after several months (Fig. 2C). It has been observed that vitrification solutions prepared by adding turbid X-1000 solution are less stable against ice formation than solutions prepared by adding clear X-1000 solution (unpublished). Product instructions therefore recommend that X-1000 solutions be heated to restore clarity before adding to vitrification solutions to ensure that all polymer molecules are adequately dissolved and unassociated.

The particles causing the turbidity can be removed by 0.2 μm filtration, or centrifugation at 40,000g for 30 min at +4 °C. However, the remaining clear solution (which stays clear over time) was less effective as an ice blocker on an equal polymer weight basis than unfiltered X-1000 made clear by heating instead of filtration.

These observations suggest that the polymer molecules constituting the filterable particles are a chemically distinct subfraction of X-1000 that disproportionately contributes to its ice blocking activity. Experiments confirmed that this condensed fraction of X-1000 blocks ice at least four

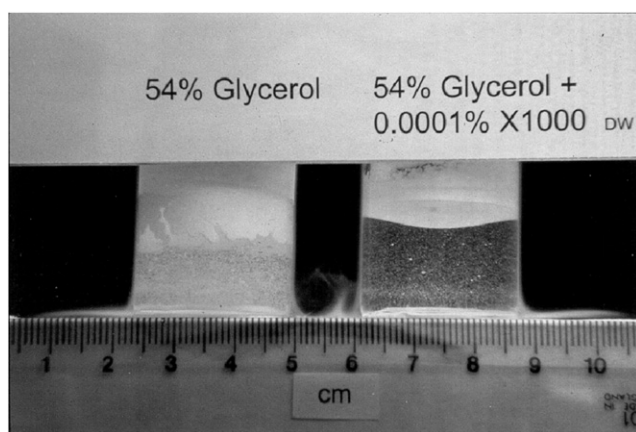


Fig. 1. Ice formed during cooling 10 ml of 54%w/w glycerol solutions in distilled water to a temperature of -110°C with (right) and without (left) the addition of one part per million mass of X-1000 ice blocker. Experimental conditions were as described in a previous study [14]. Inhibition of ice nucleation on glass is now known to be caused by the surfactant properties of PVA copolymers. It can be replicated with trace amounts of other surfactants (unpublished). But reduced ice formation in the solution itself is a phenomenon unique to ice blockers.

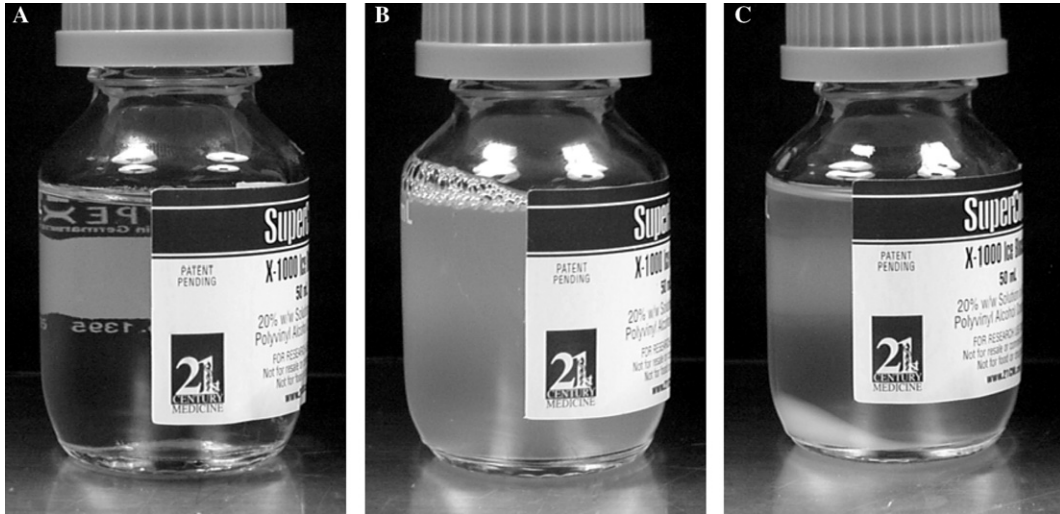


Fig. 2. (A) 20%w/w solution of X-1000 after heating to +80 °C remains a clear solution for several hours at room temperature. (B) After a day of refrigerated storage, the solution becomes turbid. (C) After six months at room temperature, some of the turbid particles condense into an opaque mass at the bottom of the bottle.

times as effectively as ordinary X-1000. The polymer of the condensed fraction also had very different physical properties from ordinary X-1000, although only minor chemical differences. This work was previously presented in brief form [12].

Materials and methods

Materials

All solutions were prepared gravimetrically (w/w) using bottled distilled water (Hinckley & Schmitt, Orange, CA, USA). Bottled water was previously found to produce a higher ice nucleation density than ultrapure laboratory water [14], permitting more sensitive detection of differences in ice blocking activity. Reagent grade ethylene glycol was obtained from Spectrum Quality Products (Gardena, CA, USA). X-1000 was used as formulated in the product “Supercool X-1000” (21st Century Medicine). Per label directions, turbid X-1000 solution was heated to clarity before addition to cryoprotectant solutions.

The polymer constituting the turbid particles in X-1000 (“condensed fraction”) was isolated from a bottle in which precipitation had previously occurred, as shown in Fig. 1C. The supernatant was poured off, the precipitate evaluated for moisture

content, and then dissolved in hot water to make a 10%w/w stock solution of condensed fraction polymer. The turbid stock solution was always heated to clarity immediately before adding to cryoprotectant solutions.

Ice blocking assay

A devitrification assay was selected because inhibition of ice formation (devitrification) during warming of vitrified samples is an especially important application of ice blockers. Also, unlike cooling where nucleation at different temperatures causes different ice crystal sizes, devitrification is a more uniform and reproducible phenomenon.

The stability of vitrified solutions against devitrification during warming was tested by preparing 10g solution samples in 20ml glass scintillation vials. A concentration of ethylene glycol (57%w/w) was chosen that forms little or no ice during cooling, but that devitrifies extensively during rewarming. The vials were then suspended in cold (−160 °C) nitrogen vapor 2cm above the surface of liquid nitrogen in a TA-60 dewar (MVE), resulting in a mean cooling rate of approximately 7 °C/min, and a peripheral/central solution temperature of (−130 °C, −120 °C) after 16min [14]. The vials were removed from the dewar, briefly dipped in methanol to pre-

vent exterior frost formation, and suspended in room air to warm by passive convection. The vials were photographed after 2 min (-90°C , -115°C), 4 min (-70°C , -100°C), and 6 min (-55°C , -80°C) of air warming. Ice formation by devitrification was maximum after 6 min of warming. The formed ice then melted with further warming.

Molecular weight determination

The number average molecular weight of polymers was approximately determined by measuring the osmolality of a 5%w/v solution with a Osmette A (Precision Systems) freezing point osmometer. The gram molecular weight was calculated by dividing 50 g by 95% of the measured osmolality. With limited material available, it was decided to do a single measurement at 5% concentration to maximize sensitivity to molecular weight differences rather than determine the molecular weight precisely.

NMR analysis

NMR spectra were obtained from a commercial service (Numare Spectralab, Kintnersville, PA). ^1H spectra were acquired at 200 MHz for samples of 30–60 mg polymer dissolved in 0.8 ml $\text{DMSO-}d_6$. The incidence of syndiotactic diads (stereochemical arrangements in which neighboring hydroxyls point oppositely on a Fischer projection) was computed based on the relative areas of the three hydroxyl peaks appearing near 4.4 ppm on the spectra by the formula given by Lyoo et al. [5].

Results

Ice blocking

Various concentrations of the condensed fraction of X-1000 were added to 57%w/w ethylene

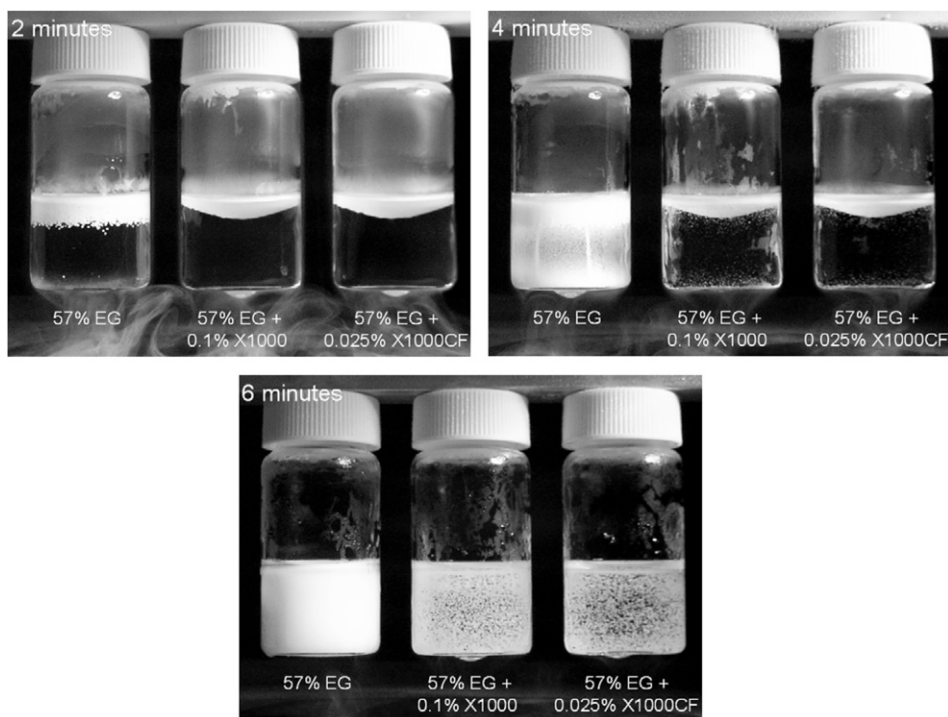


Fig. 3. Ten gram solution samples during warming in room air after vitrification at -130°C . In this experiment, 0.1%w/w ordinary X-1000 (middle) and 0.025%w/w condensed fraction X-1000 (right) have been added to a base solution of 57%w/w ethylene glycol in distilled water (left). Both forms of X-1000 cause similar inhibition of devitrification even though the condensed fraction is present at a lower concentration. Drops under the vials are methanol used to prevent frost from forming during observation.

glycol to determine what concentration would inhibit devitrification with the same effectiveness as 0.1% ordinary X-1000. As shown in Fig. 3, 0.025% of the condensed fraction was found to be equivalent to 0.1% X-1000, indicating a 4-fold greater effectiveness per mass.

An assay in which the ethylene glycol was replaced with ice blocker was also performed. As shown in Fig. 4, 0.1% of the condensed fraction was found to be equivalent to 1% X-1000. The 10-fold greater effectiveness in this mode is related to the fact that X-1000, while a powerful ice blocker, is actually a poor glass former. Replacing a full 1% of ethylene glycol (a moderately good glass former) with X-1000 (a poor glass former) reduces the intrinsic glass forming tendency of the solution (determined by solute–water interaction) even though the net glass forming tendency and stability is raised because of ice blocking (determined by solute–ice interaction). In contrast, 0.1% of the condensed fraction X-1000 gives a strong ice

blocking effect without significantly compromising the intrinsic stability of the glass by reducing ethylene glycol concentration.

Physical and chemical properties

A sample of the opaque precipitate of Fig. 2C was heated to dryness and found to contain 25% dry polymer by weight. This condensed fraction polymer was more difficult to redissolve in water than ordinary solid X-1000 polymer. The measured polymer concentration of the supernatant remained at 20% ($\pm 0.2\%$), indicating that only a small fraction of the total polymer content comprised the condensed fraction.

When the opaque mass containing 25% condensed fraction polymer was heated in a closed container to $+100^\circ\text{C}$ it became a clear liquid. Upon cooling the liquid became viscous, and then gelatinous at room temperature. The gel was difficult to dissolve in a larger volume of water,

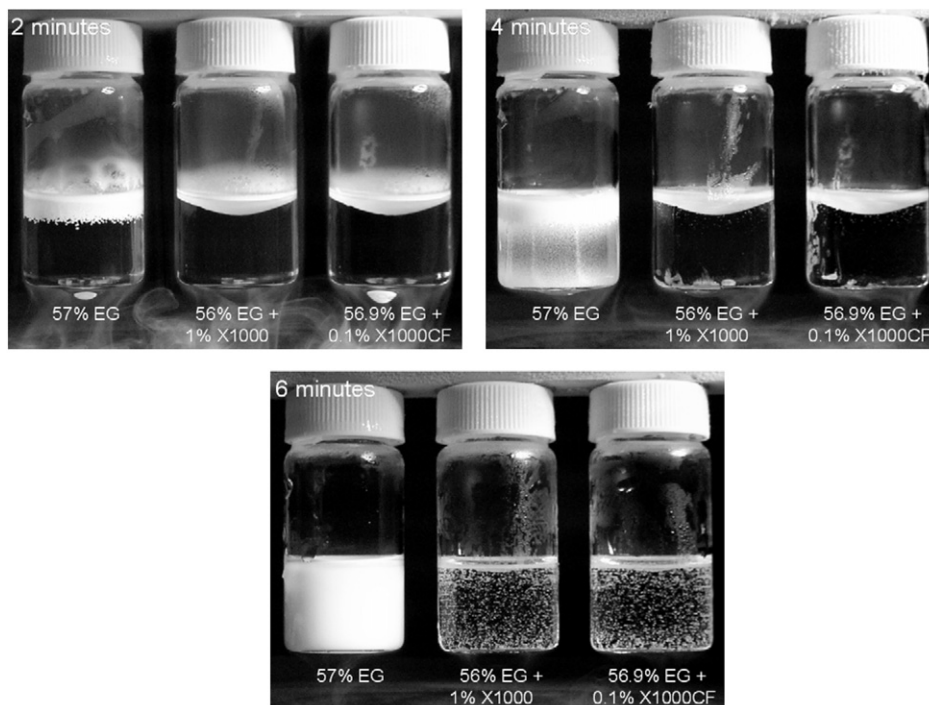


Fig. 4. Ten gram solution samples during warming in room air after vitrification at -130°C . In this experiment, a base solution of 57% ethylene glycol in distilled water (left) has either 1% of the ethylene glycol substituted with ordinary X-1000 (middle) or 0.1% of the ethylene glycol substituted with condensed fraction X-1000 (right). Both substitutions result in similar inhibition of devitrification.

required stirring for 20 min at greater than +80 °C to dissolve. This is very different behavior from 25% ordinary X-1000, which is fluid at room temperature, and easily dissolves in a larger volume of water. Evidently, the X-1000 condensed fraction polymer molecules have a strong intermolecular self-association.

The approximate molecular weight of the condensed fraction was found to be 1400. This compares to a molecular weight of 2100 found for ordinary X-1000.

Surprisingly, proton NMR of the condensed fraction polymer and the polymer in the non-precipi-

tated supernatant of X-1000 (Fig. 2C) revealed no dramatic differences. 18.9% mole percent of hydroxyl groups in the condensed fraction were acetylated vs. 18.4% in the supernatant polymer. Stereochemically, the condensed fraction possessed 53.6% syndiotactic diads vs. 53.0% in the supernatant polymer. Visually, the spectra were essentially identical (Fig. 5).

Discussion

Polymer molecules that tend to aggregate and ultimately precipitate from 20% solutions of X-

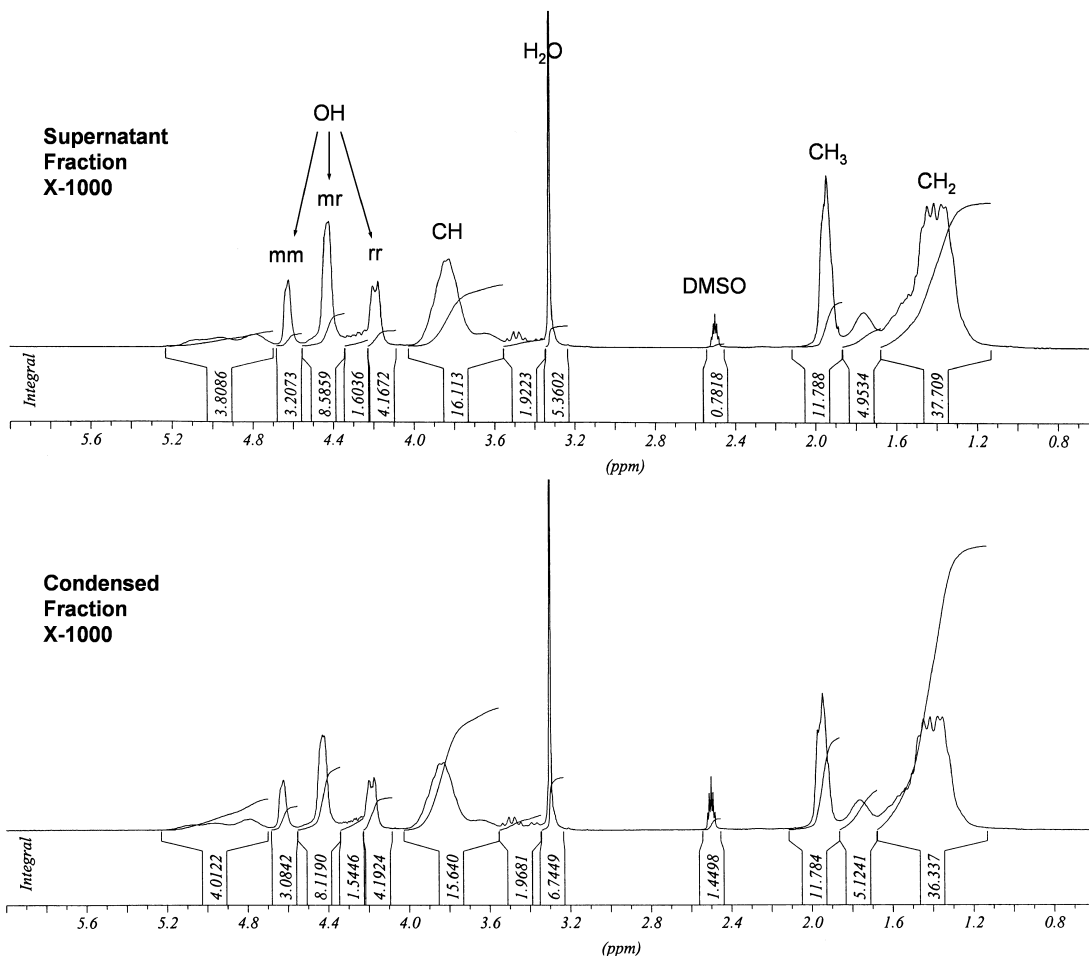


Fig. 5. Proton NMR spectra of non-condensing X-1000 and condensed fraction X-1000 polymer dissolved in deuterated DMSO. Trace amounts of water and non-deuterated DMSO are present. There are three hydroxyl peaks corresponding to three different possible stereochemical positions of neighboring hydroxyls. The syndiotactic fraction was computed by the formula $(rr + mr/2)/(mm + mr + rr)$.

1000 ice blocker inhibit ice more effectively than the majority of molecules in the solution that do not self-associate as strongly. It is remarkable that strong self-association and enhanced ice blocking properties do not correlate with large differences in molecular weight or NMR spectra.

A possible explanation is that the chemical differences responsible for the different physical properties are confined to a small region of the polymer, such as chain branch points, or end groups. (The nominal termination of the polymer is hydroxyl based on the synthesis method.) In support of the end-group hypothesis, turbidity of 20% aqueous solutions of PVA copolymers (10–25% vinyl acetate content) was only observed for polymers of mean molecular weight less than 6000, becoming progressively greater with decreasing molecular weight down to 1000. Ice blocking has also been observed to increase with decreasing molecular weight [14], also correlating with the number of polymer molecule ends in the solution.

It is possible that both self-association and ice blocking interactions occur primarily near the ends of polymer molecules, where rotational reorientation around backbone carbons into favorable intermolecular or ice-binding conformations is most rapid. In PVA, both polymer self-association (aqueous insolubility) [6] and ice blocking [13] are known to be related to syndiotactic stereochemistry. The stereochemistry near polymer ends may be particularly important.

The mechanism for enhanced ice blocking activity by the self-associating fraction of X-1000 is not currently well understood. Better understanding of this mechanism will no doubt improve understanding of ice blockers generally.

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