Letter

Enhancement of Macromolecular Ice Recrystallization Inhibition Activity by Exploiting Depletion Forces

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S Supporting Information

ABSTRACT: Antifreeze (glyco) proteins (AF(G)Ps) are potent inhibitors of ice recrystallization and may have biotechnological applications. The most potent AF(G)Ps function at concentrations a thousand times lower than synthetic mimics such as poly(vinyl alcohol), PVA. Here, we demonstrate that PVA's ice recrystallization activity can be rescued at concentrations where it does not normally function, by the addition of noninteracting polymeric depletants, due to PVA forming colloids in the concentrated saline environment present between ice crystals. These depletants shift the equilibrium toward ice binding and, hence, enable PVA to inhibit ice growth at lower concentrations. Using theory and experiments, we show this effect requires polymeric depletants, not small molecules, to enhance activity. These results increase our understanding of how to design new ice growth inhibitors, but also offer opportunities to enhance activity by exploiting depletion forces, without re-engineering ice-binding materials. It also shows that when screening for IRI activity that polymer contaminants in buffers may give rise to false positive results.
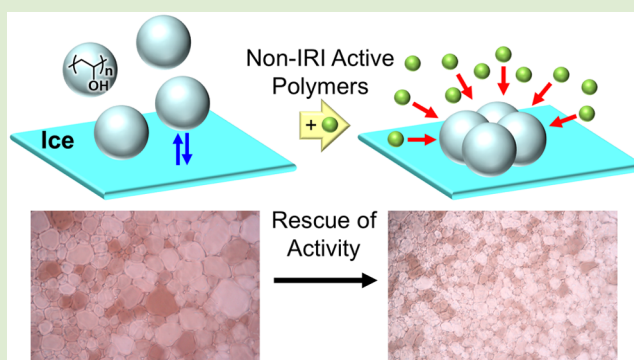
Antifreeze proteins are ice binding proteins that control ice crystal growth and are found in a variety of extremophile organisms.[1] Their ability to modulate ice growth means they have generated interest in application areas where ice growth is a problem, especially in the cryopreservation of donor cells and tissues. Several classes of synthetic mimics[2−5] of antifreeze proteins have been developed, including glycopeptides,[6,7] poly(ampholytes),[8−10] and amphipathic molecules/assemblies.[11−14] Poly(vinyl alcohol) has emerged as a particularly active mimic, shows molecular weight dependent activity,[15−18] and may have application in cryopreservation.[19,20] Molecular modeling suggests that PVA's activity is due to the precise spacing of its hydroxyl groups, which match the spacing in the prismatic plane of ice, and this effect increases with molecular weight due to entropy/enthalpy compensation.[21,22] There have been investigations into whether antifreeze proteins bind to ice reversibly or irreversibly, to account for their concentration dependence. Microfluidic experiments have, however, provided evidence that AFPIII and AFGP bind irreversibly,[23,24] but PVA seems to be a reversible binder and contributes to its lower activity. If PVA is binding reversibly, this means an equilibrium is established between ice-bound and unbound PVA, with only the bound PVA molecules contributing to the observable macroscopic properties. Hence, a large fraction of the added macromolecules does not contribute to activity, but is required to drive the equilibrium toward ice binding. In colloid science,

it is well-known that the addition of noninteracting macromolecules to a colloidal dispersion can cause aggregation and shift them from dispersion to a surface. This is due to the entropically driven depletion force in which polymeric depletants are excluded from the volume between particles, leading to aggregation. This process underpins structural biology, where polymeric depletants (especially poly(ethylene glycol)) are used in protein crystallization buffers to enable structural studies.[25] Castro et al. developed DNA origami devices that refold in response to small depletion forces.[26] Chen and co-workers showed how depletion effects could be used to tune the morphology and self-assembly of block copolymers in the presence of a nonabsorbing homopolymer.[27] Nagao and co-workers utilized depletants to drive the self-assembly of golf ball-like particles to form branched chains.[28] Poly(ethylene glycol) (PEG) has been shown to act as a depletant for colloids.[29] Kuhl et al. were able to directly measure the depletion force induced by PEG on egg phosphatidylcholine vesicles, showing the molecular weight dependence.[30] The depletion effect, also known as macromolecular crowding, is partly responsible for protein

folding,[31,32] the assembly of the cytoskeleton, and whole chromosomes.[33]

Considering the above, we reasoned that, if the PVA was forming colloids, then addition of a secondary polymer, which has no IRI activity, could act as a depletant to drive the PVA to the ice and, hence, shift the equilibrium to favor ice binding, enabling activity at lower concentrations.

Here we report that the addition of secondary polymers which do not target any ice crystal faces can enhance the IRI activity of poly(vinyl alcohol) by forcing the equilibrium toward binding due to depletion forces. The enhancement is shown to require a polymer, not small molecules, and is universally applicable.

Figure 1A illustrates the calculated depletion force for PVA aggregation in the presence of PEG. When the saline ([NaCl]
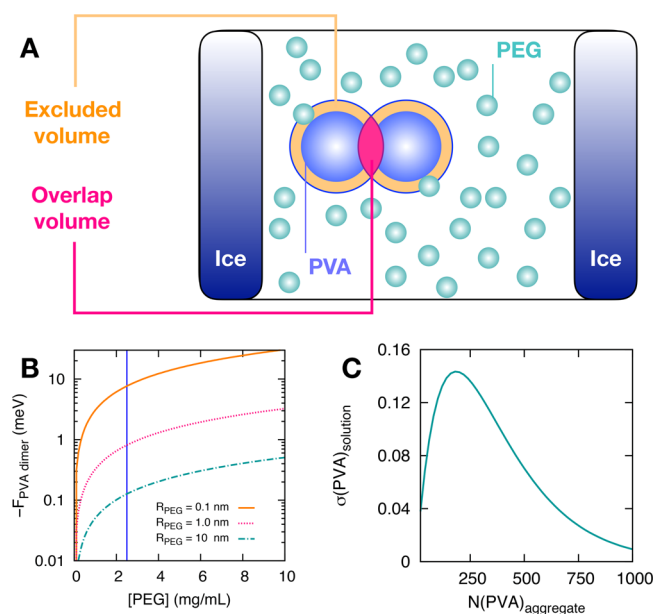
= 0.14 M) solutions used to assess IRI activity freeze, ice crystals exclude other solutes and hence concentrate them between ice grains to give unfrozen water channels.[34] As seen using DLS (dynamic light scattering), PVA aggregates under these conditions (see ESI), but PEG (which has no IRI activity) does not. As depicted in Figure 1A, the aggregation of PVA particles reduces the excluded volume of the system which is not accessible to PEG (modeled as a small particle). This leads in turn to a net entropic gain for the system. The Asakura–Oosawa model for depletion forces was used to estimate the free energy gain resulting from the aggregation of (radius = 10 nm) PVA particles in the presence of (nonaggregated, radius = 1 nm) PEG particles. Full details of the model can be found in the Supporting Information. As shown in Figure 1B, the free energy gain due to depletion forces is strongly dependent on the size of the depletant, which has been observed experimentally by others using a PEG system.[29,30] Smaller PEG (but still a polymer) leads to much stronger PVA–PVA interactions, where a larger (radius 10 nm) depletant has less effect, showing a macromolecular, but not-particulate, depletant is required (confirmed experimentally). In Figure 1C we report the volume fraction of PVA particles participating in PVA aggregates containing N-(PVA)$_{aggregate}$ particles; Here, we have assumed a PVA concentration of 2.5 mg·mL^{-1} and a volume fraction of PEG of 0.38 (assuming a 100-fold increase of both PVA and PEG concentration upon confinement between ice crystals during the experiments). In this case, most of the PVA can only be found as aggregates of substantial size ($\sim$250 polymers). While estimates, these results provide a simple motivation for the aggregation of PVA particles due to the depleting action of PEG.

To enable experimental investigation of this depletion effect, a range of polymers were synthesized by RAFT (reversible additional fragmentation chain transfer) polymerization to be tested as depletants along with commercial PEG. Full details of the synthesis are in the Supporting Information, and the polymers are shown in Table 1.

PVA with DP = 85 (PVA$_{85}$) was selected to ensure the polymer was well above the critical minimum length required for ice growth inhibition.[16,17,22] The "splat" assay was used to evaluate its concentration-dependent IRI activity; This involves seeding a polynucleated ice wafer, which is annealed at -8 °C for 30 min, and the average crystal size compared against a negative control. The mean grain size (MGS) is reported and smaller numbers indicate greater IRI activity. A concentration of PVA$_{85}$ of 0.025 mg·mL^{-1} gave 50% MGS and was used from this point on to ensure that any changes in activity can be easily observed. Figure 2A shows the MGS of PVA$_{85}$ supplemented with/out PEG4k. PEG4k alone shows no IRI

Figure 1. Depletion effects as the driving force for PVA aggregation in the presence of PEG particles. (A) Schematics of the origin of depletion forces: as the PEG particles cannot overlap with the PVA particles, the former cannot access a certain excluded volume around the latter. However, the excluded volume is reduced by the overlap volume created when two PVA particles get close enough. Thus, the aggregation of PVA increases the volume available to the PEG, which in turn leads to an entropic gain for the system. (B) Calculated free energy gain due to the aggregation of two PVA particles into a "dimer", as a function of PEG concentration. (C) Calculated volume fraction $\sigma(PVA)_{solution}$ of PVA particles participating in PVA aggregates of size N(PVA)$_{aggregate}$. The details of all these calculations are reported in the ESI.

Table 1. Polymers Used in This Study

code[a]	[M]:[CTA][b] (−)	conv[c] (%)	Mn$_{Theo}$[d] (g·mol^{-1})	Mn$_{NMR}$[e] (g·mol^{-1})	Mn$_{SEC}$ (g·mol^{-1})	Đ[f]
PVAc$_{85}$	100	90.0	7700	8200	7300	1.50
PAA	100	94.2	6800	12000	1040[g]	N/A[g]
PMA	100	86.6	7500	7700[h]	14176	2.72
PVP$_{12}$	20	74.3	1700	1300	1300	1.40
PVP$_{206}$	300	65.4	22000	15000	23000	1.51

[a]Polymers are named according to the DP from SEC. [b]Monomer to CTA ratio. [c]Determined by NMR compared to an internal standard. [d]Determined by target MW multiplied by conversion. [e]Determined by end group analysis. [f]Determined by SEC. [g]Significant tailing was observed in the aqueous GPC, leading to high dispersity values (>4). [h]Determined from NMR conversion as end groups not visible.
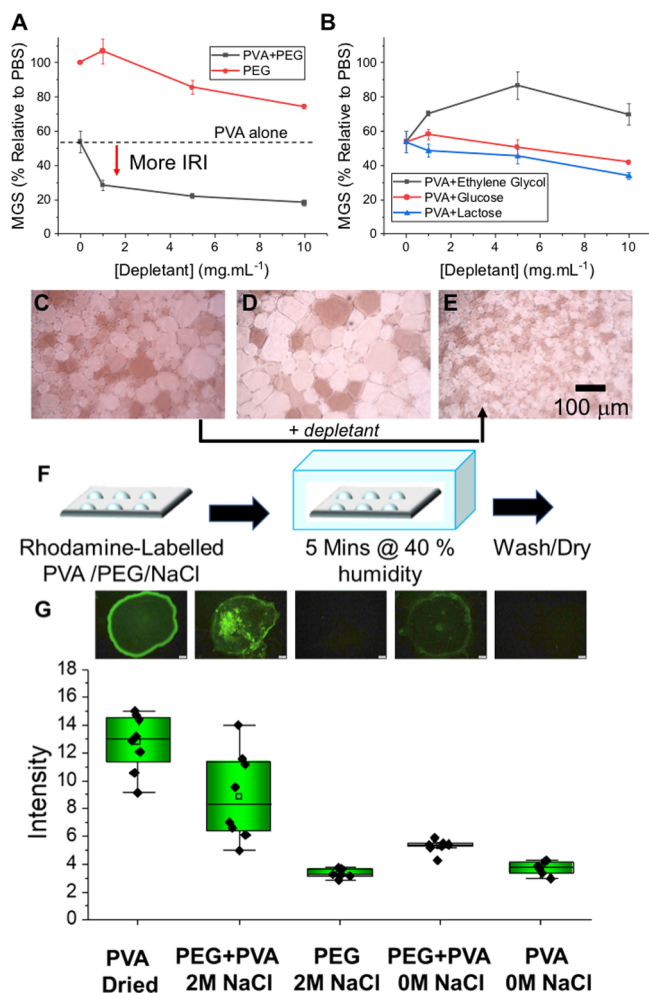
Figure 2. Effect of PEG as a depletant to modulate IRI activity. (A) IRI activity of PVA$_{85}$ (0.025 mg·mL^{-1}) with PEG 4k; (B) PVA$_{85}$ IRI activity with small molecule additives; Example cryomicroscopy images for (C) PVA$_{85}$ (0.025 mg·mL^{-1}), (D) PEG 4k (10 mg·mL^{-1}), (E) PVA$_{85}$ (0.025 mg·mL^{-1}) + PEG 4k (10 mg·mL^{-1}), (F) Schematic of depletion testing onto glass slides, and (G) Fluorescence microscopy and image analysis of Rhodamine-labeled PVA being depleted by PEG and NaCl. Average intensity values from minimum of 6 droplets. Scale bar = 100 μm. MGS = mean grain size.

activity, confirming it has no direct ice interaction. However, addition of PEG4k to PVA$_{85}$ enabled the rescue of activity, with 10 mg·mL^{-1} rescuing all activity with a MGS of 20%. The observed activity profile is in close agreement with the theoretical calculations (Figure 1). Theory predicts that only polymers can act as depletants, which was confirmed by the lack of enhancement when mono/disaccharides and ethylene glycol are added. To visualize the depletion effect, Rhodamine-labeled PVA was incubated with PEG, with and without NaCl (to mimic the conditions between ice wafers) on a glass slide, Figure 2F. In the presence of a depletant, the polymer will be forced onto the interface between the glass and the droplet, adhering to the surface. Image analysis (Figure 2G) confirmed that PVA was depleted and forced to the glass surface only when PEG and NaCl were present, in agreement with the ice growth data. It can be seen in Figure 2G that when fluorescent PVA is dried on the glass surface, the majority of the polymer accumulates at the air/water/glass interface.

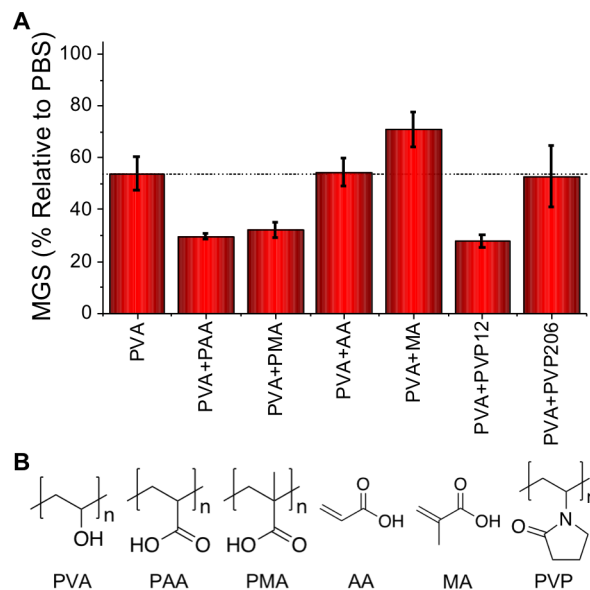If the depletion hypothesis is true, it should also be valid for other polymers. Figure 3 shows the observed MGS for PVA$_{85}$

Figure 3. (A) IRI activity of PVA$_{85}$ with indicated polymers added at 5 mg·mL^{-1} compared to combinations of PVA and other polymers or monomers. The horizontal line at 53% activity corresponds to the IRI of PVA alone. (B) Structures of depletants screened.

with addition of poly(acrylic acid), poly(methacrylic acid), and poly(vinyl pyrrolidone). In all cases the IRI activity was rescued, showing that these synergistic enhancements are not limited to PEG. These observations are particularly interesting as it has been shown that PVA/PEG mixtures are potent protein and bacterial cryopreservation agents, and this depletion-induced enhancement might be a factor.[35,36] Control experiments were conducted using acrylic acid and methacrylic acid (monomers) and there was no IRI enhancement. Interestingly, high molecular weight PVP did not show activity. In the depletion model (Figure 1B), if the MW of the depletant is similar to the colloid, activity is lost, which these results would support. DLS analysis also supported that PVP$_{206}$ aggregates (Supporting Information).

It is important to highlight that the ammonium salt of poly(acrylic acid) can enhance the thermal hysteresis gap (another measure of antifreeze protein activity) of Type I antifreeze proteins and PVA. This was hypothesized to be due to specific basal face binding by poly(acrylic acid) and, in these reports, was only undertaken at saturation concentrations of the PVA.[37,38]

Our hypothesis for this activity enhancement relies on PVA being in a colloidal state (as proved by DLS) to enable the depletion effect to be valid. To provide evidence of our mechanism, two ice binding proteins[23,24] were explored. AFP Type III was produced by recombinant expression in *E. coli* and AFGP8 (antifreeze glycoprotein from Antarctic Tooth-fish). As with PVA, the concentration of protein required to give ~50% MGS was determined, and the impact of supplementing with PEG4k was evaluated, Figure 4. There was little or no enhancement in either case, in contrast to PVA. DLS of the AFPIII confirmed that it was not aggregating in up to 2 M NaCl, and hence, no depletion effect is possible, as colloids of the IRI active material are required.
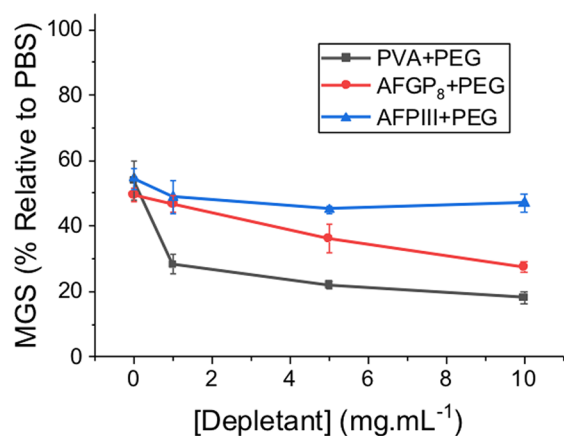
Figure 4. IRI activity with addition of PEG 4k: [PVA] = 0.025 mg·mL^{-1}, [AFGP$_8$] = 0.00008 mg·mL^{-1}, [AFPIII] = 0.00015 mg·mL^{-1}.

In conclusion, we have introduced a simple approach to rescue the ice recrystallization inhibition activity of PVA by exploiting colloidal depletion forces. Due to the high saline concentration between frozen ice grains, PVA aggregates to form colloids. Theoretical calculations showed that polymeric depletants can provide a driving force to deplete PVA colloids from solution and onto the ice. Experiments support this, showing that addition of polymers with no ice recrystallization inhibition activity enhanced the apparent activity of IRI, apparently due to the depletion effect. In line with theory, small molecule additives had no impact on the IRI activity and antifreeze proteins (which did not aggregate in saline) could not be enhanced due to the lack of colloids. These results are significant, as they present an accessible route to enhance the activity of synthetic IRI agents through simple addition of low-cost, water-soluble polymers.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmacro-lett.9b00386.

Full experimental details (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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