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Incorporation of Antifreeze Proteins into Polymer Coatings Using Site-Selective Bioconjugation

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Abstract: The diverse functional repertoire of proteins promises to yield new materials with unprecedented capabilities, so long as versatile chemical methods are available to introduce synthetic components at specific sites on biomolecule surfaces. As a demonstration of this potential, we have used site-selective strategies to attach antifreeze proteins found in Arctic fish and insects to polymer chains. This multivalent arrangement increases the thermal hysteresis activity of the proteins and leads to materials that can be cast into thin films. The polymer–protein conjugates retain the ability of the proteins to slow ice growth in subzero water and can inhibit ice formation after attachment to glass surfaces. These inexpensive materials may prove useful as coatings for device components that must function at low temperature without ice buildup. The polymer attachment also allows higher thermal hysteresis values to be achieved while using less protein, thus lowering the cost of these additives for biomedical applications.

Introduction

Protein–polymer hybrid materials afford compelling opportunities for blending the wide functional range of biomolecules with the convenient handling and processability of polymers.[1] The attachment of multiple proteins to each polymer strand could enhance their collective behavior, and polymer attachment could also lead to improvements in solubility and stability. Recently, we reported a general system for activation of proteins at the N- or C-terminus (or both) to allow polymer attachment through oxime formation.[2] The use of site-selective coupling chemistry improves the homogeneity of the resulting material, making it significantly more likely that the individual proteins will function in a uniform manner. These methods are compatible with virtually any desired sequence that can be expressed in *Escherichia coli*, providing ready access to a diverse range of structural, binding, and catalytic functionalities that can be imported into the materials context.

To continue the development of this strategy, we have synthesized surface-attachable polymers that contain “antifreeze” proteins (APFs, also known in the literature as “ice structuring” proteins).[3] Key residues of these sequences bind the surfaces of nascent ice crystals, preventing further growth at temperatures within a characteristic thermal hysteresis range. These proteins also inhibit the subsequent restructuring of small crystals into expanded lattices.[4] These properties are used to advantage in organisms that live in subfreezing environments, slowing the growth of ice crystal embryos that could otherwise cause tissue damage. The ability of these proteins to depress the freezing point of water and delay solid freezing has already been exploited in the context of food additives,[5] and their protective properties are being explored for tissue cryopreservation, vaccine storage, low-temperature surgeries, and the inhibition of gas clathrate formation.[6] We postulated that the attachment of these proteins to polymer chains could provide two advantages over the use of the proteins alone. First, multidomain oligomers of antifreeze proteins have been shown to increase thermal hysteresis beyond that of the single protein subunit,[7] suggesting that the connection of multiple copies of the protein to an extended chain could display further enhancements in these properties. Second, the attachment of polymer chains would facilitate material handling and could serve as a convenient means for preparing surface coatings that discourage ice accumulation on critical machine components. Through the development of a facile site-selective method to attach these

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proteins to polymer backbones, we have found that both of these aspects can be realized.

Results/Discussion

Two different AFPs were chosen for use in this study. The first was a Type III antifreeze protein (T3AFP) from the ocean pout fish, the structure, ice-binding site, and thermal hysteresis of which have been characterized through careful mutagenesis experiments.[8] The second was a snow flea antifreeze protein (SnAFP),[9] which contains two disulfide bonds. Though exact ice-binding residues of SnAFP remain unknown, it possesses one of the highest thermal hysteresis values of any antifreeze protein reported to date.

To allow attachment to the polymer chains, the proteins were modified using expressed protein ligation (EPL)[10] to install a ketone at the C-terminus (yielding **3a**, Figure 1a), as previously reported.[2] Following isolation of the protein, the utility of the ketone group for polymer attachment was verified by exposing the protein to a 2 kDa poly(ethylene glycol) (PEG-2k) chain bearing an alkoxyamine group. As shown for T3AFP in Figure 1c, a gel shift was clearly discernible when the ketone was present, indicating polymer attachment to yield **3c** with over 90% yield. No shift was observed when protein **1c** (which lacked the ketone group) was exposed to the polymer alkoxyamine under identical conditions. The analogous gel-shift assay could not be performed for SnAFP, as in our hands it did not form a well-defined band on the gel.[11] Instead, MALDI-TOF MS analysis was used to confirm modification following exposure to benzyloxyamine alkoxyamine, Figure 1b. Oxime **3b** was observed as the sole product for both SnAFP and T3AFP, confirming that both the EPL and the oximation steps had proceeded with high conversion. In both cases the observed molecular weights indicated that the N-terminal methionine residues had been cleaved after protein expression, as would be expected for proteins with alanine in the second position.[12]

To confirm the activity of the expressed proteins and verify that the C-terminal ketone modification did not interfere with their ice-binding properties, samples of T3AFP and SnAFP were subjected to an ice-recrystallization inhibition assay.[9] In this experiment, capillary tubes containing 25 mg/mL samples of protein (chosen for consistency with previous measurements using these proteins[9b]) were flash frozen and visually recorded as $t = 0$ h. Each sample revealed uniform glassy ice that was nonreflective, Figure 1d. The tubes were held at −6.0 °C for 4 h using a circulating cooling bath and a jacketed beaker. Visual inspection was then used to determine if ice recrystallization had occurred. In the tube containing 25 mg/mL bovine serum albumin (BSA) as a control protein, significant restructuring of the ice was observed, as indicated by the presence of reflective crystalline surfaces. In contrast, the image of the ice in the tubes containing the AFPs remained unchanged, indicating that the

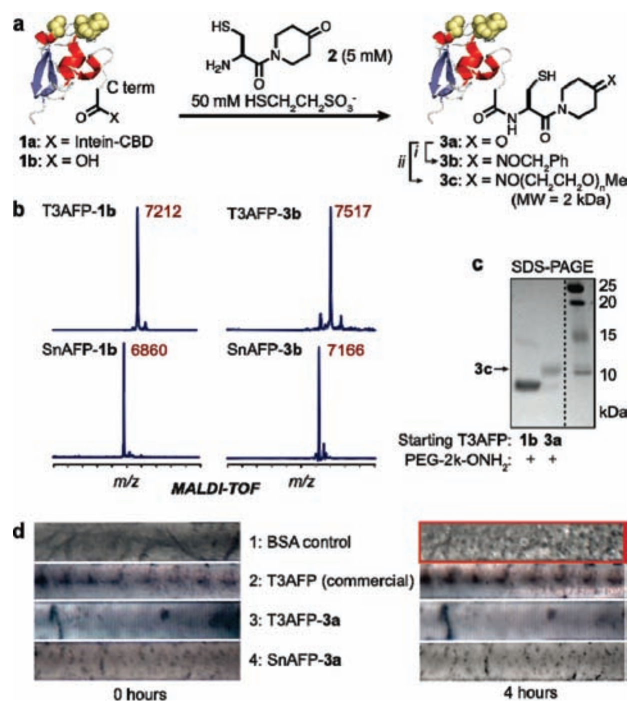

Figure 1. Chemical synthesis of modified ice structuring proteins (or antifreeze proteins, AFPs) for materials incorporation. (a) A native chemical ligation strategy was used to modify the C-termini of AFPs with **2**. The resulting ketones were then coupled to alkoxyamines to yield stable oximes. Conditions: (*i*) 250 mM $BnONH_2$, pH 6.5, 1 h; (*ii*) 50 mM PEG-2k-ONH_2, pH 6.5, 16 h. The type III ice structuring protein (T3AFP) is depicted with ice-binding residues highlighted in yellow. (b) T3AFP samples with (**3a**) or lacking (**1b**) the ketone group were incubated with a 2 kDa PEG alkoxyamine. Analysis by SDS−PAGE indicated a shift corresponding to the formation of **3c** only for the ketone-containing sample. (c) MALDI-TOF analysis indicated complete modification of both T3AFP and SnAFP with **2**, followed by $BnONH_2$. (d) An ice recrystallization inhibition assay was used to confirm activity of the conjugates (each at 25 mg/mL) at −6.0 °C. Images were captured at $t = 0$ and 4 h. Restructured ice crystals were observed only in the BSA case (indicated by the red outline). Expanded versions of these images have been included as Figure S4 in the Supporting Information.

crystalline rearrangement had been inhibited. Similar behavior was observed for modified T3AFP-**3a**, SnAFP-**3a**, and a sample of T3AFP that was obtained from a commercial source, Figure 1d. Additionally, the circular dichroism (CD) spectrum of SnAFP-**3b** was found to match previous reports (see Supporting Information for details).[9b] Taken together, we interpret these data to suggest that the protein expression and intein cleavage protocols yield AFPs in the functional state and that the C-terminal chemical modifications do not interfere appreciably with their ice-binding activity.

Once the ice-binding properties had been confirmed, the ability of the proteins to react with an alkyoxyamine-labeled polymer was examined. A copolymer of 2-hydroxypropyl methacrylamide (HPMA) and 3-aminooxypropyl methacrylamide was first prepared as previously described [MW = 175 000, PDI = 1.75].[2] A 50 mg/mL solution of this polymer was then incubated with 20 mg/mL T3AFP-**3a** or SnAFP-**3a** at pH 6.5 in 25 mM phosphate buffer for 2 h, Figure 2a. For quantification purposes, some of the AFP samples were labeled using the *N*-hydroxysuccinimide ester of tetramethylrhodamine (TAMRA-NHS) before coupling to the polymers. Remaining free protein (**3a**, MW = 7000) was removed by three rounds of ultrafiltration using a 100 kDa cutoff filter. UV−vis analysis of the retained fraction indicated that 80−85% of the initial

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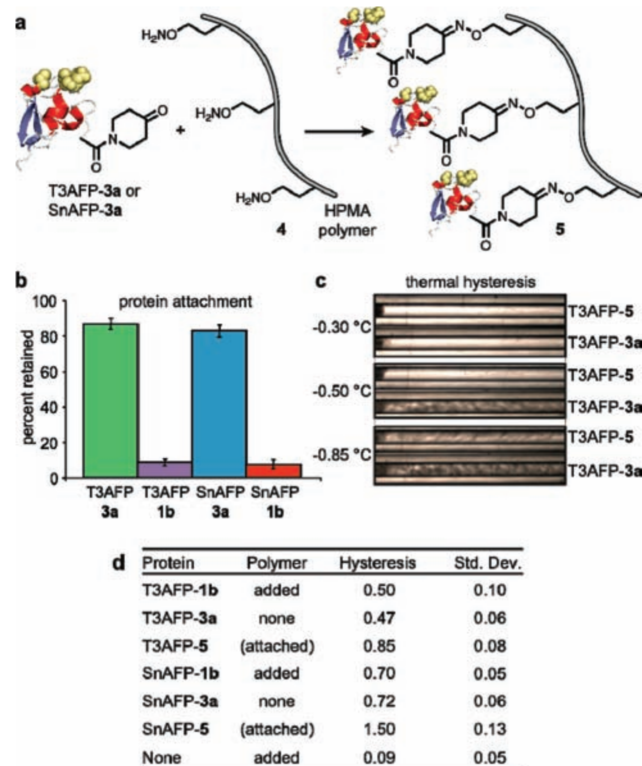

Protein	Polymer	Hysteresis	Std. Dev.
T3AFP-1b	added	0.50	0.10
T3AFP-3a	none	0.47	0.06
T3AFP-5	(attached)	0.85	0.08
SnAFP-1b	added	0.70	0.05
SnAFP-3a	none	0.72	0.06
SnAFP-5	(attached)	1.50	0.13
None	added	0.09	0.05

Figure 2. Polymer attachment for improved thermal hysteresis. (a) Ketone-labeled AFPs (50 mg/mL) were attached to aminooxy-substituted HPMA polymer **4** by oxime formation over a 3 h period. Only the type III ice structuring protein (T3AFP) is depicted, with ice-binding residues highlighted in yellow. (b) To confirm attachment efficiency, TAMRA-labeled protein was attached to **4** under the same conditions. Following concentration of the solution using ultrafiltration (100 kDa cutoff), protein was retained only in samples that displayed a ketone at the C-terminus. (c) Thermal hysteresis was measured for the free proteins (**1b** or **3a**) and the protein−polymer conjugate (**5**). Ice crystals were grown at one end of the tubes by the application of freeze spray. The samples were then subjected to lowering temperatures at a rate of 0.01 °C/min. Thermal hysteresis was determined by the onset of rapid ice crystal growth. (d) Table listing the measured hysteresis values for the T3AFP and SnAFP derivatives.

ketone-bearing, TAMRA-labeled protein had been attached to the polymer, Figure 2b. Protein samples lacking the ketone groups (**1c**) but combined with the polymer under identical conditions were retained only minimally by the concentrators. On the basis of a calculation of relative concentrations and percentage of attachment, we estimate 6−7 protein units per polymer chain.

Polymer−AFP conjugates were next evaluated for their ability to affect thermal hysteresis using a modified version of an assay reported by De Vries.[13] Thermal hysteresis is a difference in the values of the freezing and thawing points of water. This property results from the ability of AFPs to depress the solution freezing point by binding growing ice crystals while not significantly affecting the thawing temperature. Capillary tubes were filled with 1 mg/mL solutions of the AFPs, and a small (∼1 mm) ice crystal was formed at one end of the tube by application of a freezing spray. The tubes were then placed in a subzero solution, and the temperature was lowered at a rate of 0.01 °C/min. The tubes were imaged using a digital camera on a microscope every 5 min until rapid ice growth was observed. The temperature at which this occurred was taken as the hysteresis value, Figure 2c.

The results of triplicate hysteresis experiments are tabulated in Figure 2d. Compared to the monomeric ketone-labeled proteins (**3a**) at identical concentrations, conjugation to the polymer chains improved the thermal hysteresis value of T3AFP by 60% at 1 mg/mL and doubled it for SnAFP at 0.5 mg/mL. These are the largest hysteresis values reported for these concentrations of protein. The addition of unconjugated polymers had a negligible effect on the thermal hysteresis values (Figure 2d) and did not appreciably change the properties of the free monomeric proteins. We presume that the polymer-conjugated AFPs can reach a higher local concentration on the surface of the ice crystals, thus resulting in a thermal hysteresis value that would be observed only at much higher concentrations of monomeric protein.

The alkoxyamino groups on the polymer that remained after protein attachment provide a convenient means for attaching the material to surfaces bearing aldehyde or ketone groups. As a test case, AFP−polymer conjugates were reacted with commercially obtained glass slides that were coated with aldehyde groups (Schott, Nexterion) at pH 6.5 for 2 h, Figure 3a. The samples were then rinsed with buffer for an additional 2 h to remove any unbound protein. Successful attachment was verified by attaching TAMRA-labeled AFP−polymer conjugates, followed by fluorescence imaging, Figure 3b. Significant fluorescence was observed only in cases in which the polymer was present. Labeling and AFP incorporation were additionally confirmed by observed differences in contact-angle measurements for the glass surfaces, Figure 3c. Additionally, it was possible to quantify the amount of AFPs that were attached to the glass slides using bicinchoninic acid assays (see Supporting Information). The concentration of the applied AFP−polymer conjugates was not found to have a strong effect on the properties of the coated slides, so a standard concentration of 50 mg/mL was used to prepare the surfaces in the experiments that follow.

To examine the antifrost properties of the coated surfaces, a cooled steel surface was constructed (see Supporting Information for a more thorough description). Samples were placed on a surface cooled to −6.0 °C, which was the maximum temperature found to form frost on the glass surface exposed to air at 20 ± 1 °C and 40 ± 3% relative humidity. The glass samples were then observed under a microscope, and the state of frost was recorded every 15 min, Figure 3d. Initially moisture condensed on each surface in small droplets, which slowly coalesced. As would be expected, the morphology of the water drops closely matched what had been seen in the contact angle measurements. After 20 min, the aldehyde- and polymer-coated glass samples had frozen and were continuing to gather frost. In contrast, the glass slides coated with the T3AFP− and SnAFP−polymer conjugates did not freeze after 30 min; instead, the accumulated water droplets continued to expand. We presume that ice nucleation occurs primarily at the cooled metal surface, and thus the protein−polymer conjugates are in an ideal position to bind the nascent crystals and inhibit their growth. After 45 min, ice crystals were observed in the droplets, and the solutions were completely frozen by the next time point (the 75 min result is shown). Presumably, the bulk solution had cooled below its freezing point after this amount of time, and the surface-confined proteins could no longer reach the forming crystals. Thus, the polymeric AFP coating is able to delay frost formation on cooled surfaces, but it cannot prevent it entirely once sufficient water has condensed with no access to the proteins. This material may therefore prove most useful in applications where water ac-

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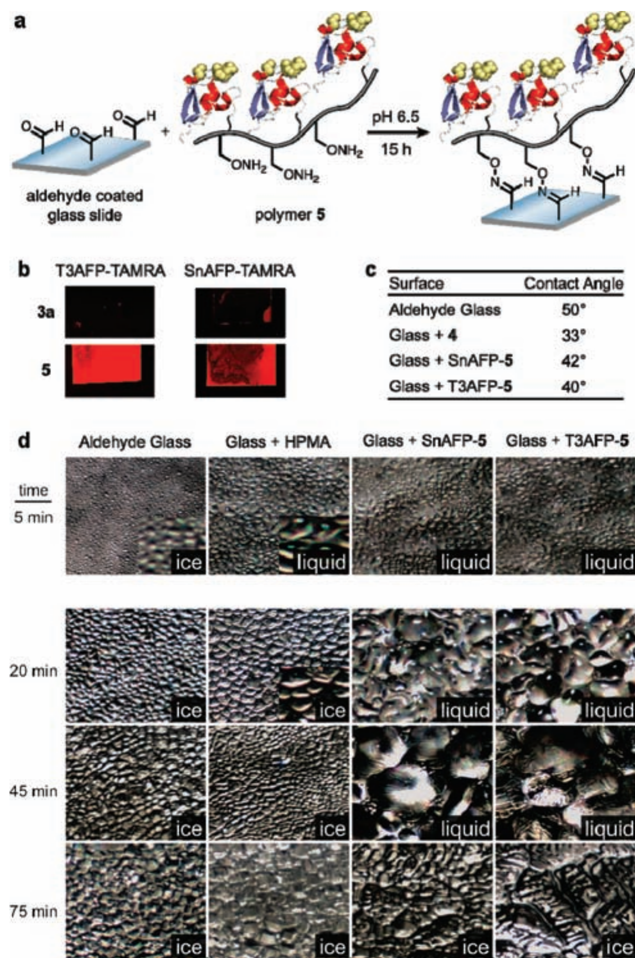

Figure 3. Preparation and evaluation of freeze-resistant surfaces. (a) Free alkoxyamine groups remaining after protein attachment were coupled to aldehyde groups on glass slides (conditions: pH 6.5, 3 h, 50 mg/mL protein). Only the type III ice structuring protein (T3AFP) is depicted, with ice-binding residues highlighted in yellow. (b) To confirm binding, free proteins and protein−HMPA conjugates were labeled with TAMRA. Successful slide attachment was accompanied by red fluorescence. (c) Contact angle measurements were also used to confirm polymer attachment. (d) Coated glass slides were cooled to −6.0 °C at controlled humidity. Condensed water froze significantly faster on samples that lacked the proteins. Images were collected at 40× magnification.

cumulation on a surface is not as pronounced, such as natural-gas pipelines and wind turbines.

Conclusion

These new antifreeze−protein conjugates have demonstrated that protein function can easily be imported for different purposes and onto different surfaces using site-specific oxime formation. Simultaneously, it has been shown that the properties of a protein can be enhanced by the attachment of polymers. Because a specific face of the AFPs binds the growing ice crystals, it is likely advantageous to have a site-selective attachment strategy. It should be possible in the future to test these materials for inhibition of frost and ice formation in a number of different situations. As one possibility, recent work has shown that AFPs can inhibit the formation of gas clathrates,[8] suggesting that these coatings may find use in the natural-gas and energy industries. In parallel to these studies, we will continue to explore the generality of this method for the efficient synthesis of well-defined protein-containing materials.

Experimental Procedures

General Procedure for the Construction of the T3AFP-pTXB1 and SnAFP-pTXB1 Expression Vectors. The T3AFP and SnAFP *E. coli* optimized genes were received from Genscript in pUC57 plasmids. The sequences for the genes were obtained from the literature[8,9] and are listed in the Supporting Information. An alanine−glycine pair (AG) was added to the N-terminus to assist in transamination reactions,[14] and a lysine (K) was added to the C terminus to assist in cleavage from the intein after expression.[15] To allow molecular cloning, an *Nde*I restriction site was placed at the N-terminal coding region of the gene, and a *Sap*I site was introduced at the C-terminal coding region. The gene was removed from pUC57 using a double digestion with *Sap*I and *Nde*I and was ligated into the pTXB1 vector, which contained an ampicillin resistance gene. The resulting plasmid was transformed into super-competent NEB 5α cells (NEB) using electroporation. The cells were then plated and grown overnight, and colonies were selected by virtue of ampicillin resistance. The presence of the AFP coding segment was verified in the resulting plasmids by both restriction digestion and sequencing.

Small-Scale AFP Expression. T3AFP-pTXB1 or SnAFP-pTXB1 plasmids were transformed into T7 express LysY/I^q competent *E. coli* (NEB) via electroporation utilizing a micro-pulser (Biorad, Hercules, CA) and spread onto Luria broth (LB) agar plates with ampicillin at 100 μg/mL. Cells from selected colonies were grown in 1 L of LB-containing ampicillin at 100 μg/mL at 37 °C until an optical density (OD) of 0.7 was observed at 600 nm. Protein expression was then induced by the addition of 1 mL of 0.3 mM isopropyl β-D-thiogalactopyranoside (IPTG). In the case of T3AFP, the broth cultures were grown for an additional 16 h at 16 °C. For SnAFP, the broth cultures were grown for 4 h at 30 °C after induction. For each sample, the cells were collected via centrifugation for 20 min at 8000 rcf at 4 °C. Protein expression was verified by the presence of the desired AFP−intein−chitin fusion by sodium dodecyl sulfide−polyacrylamide gel electrophoresis (SDS−PAGE). The cells were then resuspended in 10 mL of lysis buffer (0.02 M Tris, 0.15 M NaCl, 5 mM EDTA, pH 8.0) by vortexing. The cells were lysed by sonication using a Branson digital sonifier (VWR Scientific, West Chester, PA) for 20 min with a blunt-ended tip. Debris was removed by centrifugation at 8000*g* for 15 min to give a light brown solution.

Large-Scale AFP Expression. T3AFP-pTXB1 or SnAFP-pTXB1 plasmids were transformed into T7 express competent *E. coli* via electroporation utilizing a micro-pulser and plated on LB agar plates with ampicillin at 100 μg/mL. Cells from selected colonies were grown overnight in 4 L of LB containing ampicillin at 100 μg/mL. This 4 L was then introduced into 120 L of LB containing ampicillin at 100 μg/mL in a 150 L BioFlo Pro industrial fermentor (New Brunswick Scientific Co. Inc., Edison, NJ). Cells were grown at 37 °C until an OD_{600} of 0.6 was reached, at which point IPTG was added to a final concentration of 300 μM. For T3AFP, the temperature was lowered to 16 °C, and the cells were allowed to grow for an additional 18 h. For SnAFP, the temperature was lowered to 30 °C, and the cells were allowed to grow for 4 h following induction. For all samples, the cells were isolated using a Sharples super-centrifuge over the period of 1 h. The cells were scraped from the sides of the centrifuge and heat-sealed in plastic envelopes. The samples were then frozen for storage and transport between two blocks of dry ice. To obtain protein, portions of the cells were thawed and lysed by homogenization using an Emulsiflex-C3 homogenizer (Avestin Inc., Ottawa, Canada) for three

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rounds. Debris was removed by centrifugation at 8000*g* for 15 min to give a murky brown solution. Typical expression yielded 500 g of wet cell mass per 120 L of broth.

Affinity Purification of SnAFP and T3AFP and Modification of the C-Termini through Native Chemical Ligation. The methods used to produce chemically modified SnAFP and T3ASP were based on a literature protocol.[2a] The same procedure was used for both proteins. Before use, all buffers were cooled to 4 °C. The SnAFP- or T3AFP-containing lysate was washed over 200 mL of chitin resin (New England BioLabs, Inc., Ipswich, MA) in a 500 mL centrifuge flask for 1 h. The resin was then isolated by centrifugation at 4 °C. The resulting resin-bound protein was placed in a 500 mL Steritop filter unit (Millipore, Billerica, MA) and washed with 4 L of wash buffer (0.02 M Tris, 0.5 M NaCl, 1 mM EDTA, pH 7.5) that was cooled to 4 °C. Binding to the column was confirmed by SDS−PAGE analysis of the eluent. A 150 mL solution of 50 mM MESNa and 5 mM cysteine-ketone in wash buffer (pH adjusted to 7.5) was flowed over the resin-bound protein using suction. The column bed was then allowed to stand in a minimal amount of this solution at 4 °C for 15 h with protection from light. The protein was eluted from the column with the addition of 200 mL of wash buffer. Purified protein was then buffer-washed three times with wash buffer using Amicon Ultra 15 mL 3000 MWCO (Millipore) and incubated with an additional 25 mL of chitin resin for 1 h. The chitin resin was removed by filtration through a Steriflip filter unit (Millipore). Purified protein was then buffer-exchanged into 25 mM sodium phosphate buffer (pH 6.5) using Amicon Ultra 15 mL 3000 MWCO centrifugal ultrafiltration membranes. The resulting solution was then passed through two sequential PD-10 (GE Healthcare) buffer exchange columns to transfer the protein into 50 mM ammonium carbonate buffer (pH 7.9) at 4 °C. The resulting solution was snap-frozen using liquid N_2 and lyophilized to produce a dried powder that was stored at −20 °C. For all further reactions, the powder was first dissolved in the designated buffer. This purification typically yielded 1 mg/L of purified, modified protein. The entire procedure, except for lyophilization, was performed in a cold room maintained at 4 °C. Protein identity and purity were evaluated by SDS−PAGE with Coomassie staining and/or by using MALDI-TOF MS.

Ice Recrystallization Inhibition Assay. The procedure was based on the methods of Kent et al.[9b] and Knight et al.[16] Lyophilized samples were weighed using a microbalance and dissolved in phosphate-buffered saline (PBS, 10 mM sodium phosphate, 100 mM NaCl, pH 7.5). Samples were then loaded by capillary action into 25 μL microcapillary tubes (Drummond Scientific Co., Broomall, PA), and each end was flame-sealed while leaving at least a 1 cm “air gap” between sample and flamed end. Samples were then snap-frozen for 10 s in 2,2,4-trimethylpentane (isooctane) cooled with dry ice and immediately placed in a jacketed beaker cooled to −6.0 °C using the same solvent. Images were taken at 40× total magnification with lighting from below. Authentic antifreeze protein type III (T3AFP, A/F Protein, Waltham, MA) was used as a positive control. Bovine serum albumin (BSA, Aldrich, St. Louis, MO) was used as a negative control.

Attachment of Polymers to Proteins for Thermal Hysteresis Experiments. Samples of SnAFP and T3AFP were weighed on a microbalance. The proteins were dissolved in 25 mM ammonium carbonate buffer (pH 6.5) at a concentration of 50 mg/mL for T3AFP and 25 mg/mL for SnAFP. The lower concentration was chosen for SnAFP so that measurements of SnAFP and T3AFP could be performed over the same temperature range. Alkoxyamine-substituted HPMA polymer was then added to the solution at a concentration of 25 mg/mL from a stock solution of 50 mg/mL in the same buffer. The two components were allowed to react for 2 h at room temperature. The reaction solution was then diluted to 1/50th the initial concentration and loaded via capillary action into 25 μL microcapillary tubes (Drummond Scientific Co.). Each end was then flame-sealed, leaving at least a 3 cm “air gap” between sample and flamed end. A single ice crystal measuring less than 0.5 cm was then created at the left side of the microcapillary tube by the application of freeze spray (Innovera, Deerfield, IL). Each sample was placed in a jacketed bath that was cooled to −0.2 °C with chilled isooctane. The bath was illuminated from below with a light source that passed through a piece of polarizing film before reaching the samples (polarizing film sheet 30 cm × 30 cm, Arbor Scientific, Ann Arbor, MI). The temperature of the bath containing the samples was lowered at a rate of 0.01 °C/min, and images were captured every 5 min. A second piece of polarizing film was placed between the top of the bath and the lens of the microscope at a 90° angle to the first filter, such that light was unable to pass through the filters unless its polarization had been altered by ice crystals. Images were captured every 10 min with the second polarizing filter in place.

Confirmation of Polymer Attachment Using Ultracentrifugation. Samples that were 100 μM in T3AFP or SnAFP were reacted with 500 μM TAMRA-NHS (Invitrogen, Carlsbad, CA) in 0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA. The reaction proceeded for 3 h and was quenched by elution through a NAP-5 column (GE Healthcare) to remove unreacted TAMRA-NHS. The eluted protein (in 50 mM ammonium carbonate (AC) buffer, pH 6.5) was then concentrated to 50 mg/mL for T3AFP and 25 mg/mL for SnAFP. A 50 μL aliquot was reacted with alkoxyamino-substituted HPMA polymer in a manner identical to that described above. A fluorescence emission spectrum was obtained from 550 to 700 nm (the integration over this wavelength range was recorded as I) from 1 mL of sample. Samples were then subjected to three rounds of ultrafiltration (100K MWCO, Millipore). The retentate was collected and brought to a volume of 500 μL with AC buffer, and then the same fluorescence measurement was performed (yielding an integrated spectral value of R). The percent retention was calculated by the equation $(I - R)/I \times 100$ and is graphed in Figure 2b. The error bars represent ±1 standard deviation from the mean for three independent measurements.

Attachment of Polymer−Protein Conjugates to Aldehyde-Coated Glass Slides. Lyophilized samples of SnAFP or T3AFP were weighed on a microbalance, with a typical weight of ∼2 mg, and dissolved in PBS (10 mM sodium phosphate, 100 mM NaCl, pH 6.5) to a concentration of 50, 25, 10, or 0 mg/mL. The solution was then reacted by the addition of 5 mg aliquots of alkoxyamino-substituted HPMA polymer from a stock solution of 100 mg/mL. The samples were allowed to react for 1 h at room temperature. The solution was diluted to 600 μL and then pipetted onto a cut piece of aldehyde-coated glass (Nexterion Slide AL, Schott-Nexterion, Jena, Germany) typically measuring 0.983 × 0.350 in. The solution covered the entire surface. The glass had been previously cleaned by sonication in H_2O for 30 min. The glass sample was placed in a micro Petri dish (Millipore) that was then sealed with parafilm. The reaction on the glass surface was allowed to proceed for 2 h at room temperature. The liquid containing the remaining polymer and protein was then removed via pipet, and the glass was washed first in PBS for 30 min and then with H_2O for 30 min by rotary shaking. The glass was air-dried and subsequently used for the frosting inhibition experiments.

Confirmation of Polymer Attachment Using Fluorescence. Samples of T3AFP or SnAFP were prepared at 100 μM in 0.1 M sodium phosphate buffer, pH 8.0, containing 1 mM EDTA. TAMRA-NHS (Invitrogen) was added to a concentration of 500 μM. After 3 h, the reaction was quenched by elution through a NAP-5 column (GE Healthcare) to remove unreacted TAMRA-NHS. The eluted protein (in PBS) was then concentrated to 50 mg/mL for both T3AFP and SnAFP in a volume of 40 μL, reacted with alkoxyamino-substituted HPMA polymer, and subsequently pipetted onto aldehyde-coated glass as noted above. All samples designated “**3a**” in Figure 3b were incubated with T3AFP, SnAFP, or rhodamine-labeled ketone **S1** (see Supporting Information) while

(16) Knight, C. A.; Wen, D.; Laursen, R. A. *Cryobiology* **1995**, *32*, 23–34.

excluding polymer **4**. They were then subjected to identical washing and sonication conditions. Fluorescent measurements were obtained using a Typhoon 9410 (GE Healthcare) flat bed fluorescent scanner. The emission filter was set at 588-Cy3,TAMRA using a green laser (532 nm). Images were false colored (red) and identically contrast/brightness adjusted using ImageQuant software.

Procedure for Frosting Inhibition Experiments. All experiments were conducted on a steel surface that was cooled from below using a circulating bath (Neslab RTE-111). The surface was first cooled to −6.0 °C. The atmosphere surrounding the surface was at ambient laboratory room temperature (20 ± 1 °C), pressure, and relative humidity (40 ± 3%). Protein-modified glass slides were removed from the washing solution, dried, and then placed on the steel plate. Images were captured every 5 min for the first 30 min and every 15 min thereafter. All images are reported at 40× total zoom. The general trend for each plate was to accumulate water condensation and then freeze after a certain amount of time. The T3AFP- and SnAFP-coated plates were observed to resist ice formation until a substantial amount of water had accumulated over the surface (see Figure 3d).

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Supporting Information Available: Supporting Figures S1−S4 and general experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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