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Ice Recrystallization Inhibition Activity of Protein Mimetic Peptoids

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Abstract
Natural antifreeze proteins are known for their excellent control of ice formation and growth, while synthetic molecules seldom have the similar effects. Here we report a series of protein mimetic peptoids with different side chains exhibiting significant ice recrystallization inhibition activity, and their structure–property relationships are also studied. The presence of peptoid can clearly slow down ice growth and decrease ice crystal grain size, but shows no thermal hysteresis, which make peptoids great antifreeze agent candidates in cryopreservation. Peptoids with methyl, ethyl and amino groups on side chains can modulate ice crystal shape, while peptoids bearing hydroxyl and ethyl groups decrease ice growth rate the most. All peptoids can reduce ice crystal grain size and the one with hydroxyl groups give the smallest grain size. This study reveals peptoid structure effects on ice growth and points to the design rules for biomimetic antifreeze agents.

Keywords Peptoid · Antifreeze · Ice recrystallization inhibition · Structure–property relationships

1 Introduction

Ice formation from water is a natural crystallization phenomenon at low temperature. Despite its beautiful looking, ice can cause great damage in most cases [1]. For example, during low temperature storage, ice crystals can cause mechanical damage to cells and reduce their activity. Therefore, how to control the formation and growth of ice crystals is of great importance. Antifreeze proteins (AFPs) found in polar fish enable life surviving in subzero environment [2]. Studies indicated that AFPs can interact with ice through adsorption to specific crystal faces, and depress ice formation and its growth [3–5]. AFPs have two distinct antifreeze activities, thermal hysteresis (TH) and ice recrystallization inhibition (IRI) [6,7]. Ice growth is the major cause of damage in a lot of cases, such as damage during cryopreservation of tissues and food storage [8,9], so IRI plays a very important role in antifreeze activity. AFPs are great antifreeze agents, but the cytotoxicity, low stability and high cost of AFPs greatly limited their application in practice, especially in cryopreservation since TH is not desirable [10,11]. Therefore, synthetic antifreeze materials are urgently needed. Up to now, cheap, easy to synthesize protein mimetic antifreeze molecules are still underexplored.

Peptoids (*N*-substituted glycines) are a class of novel sequence-specific peptidomimetic molecules [12]. They have the same backbone structures compared with peptides except side chains transferred from alpha carbon to amide nitrogen (Scheme 1). Peptoids have a lot of extraordinary characteristics such as biocompatibility and strong proteolytic stability [13]. After 30 years development, peptoid research has made rapid progress in synthesis methods and performance studies, which expand their applications in

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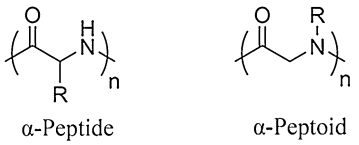


Scheme 1 Chemical structures of α-peptide and α-peptoid

chemical catalysis [14], biomedicine [15–17], nanomaterials [18] and other fields [19]. As a new protein mimetic but robust material, peptoids have great potential in antifreeze research, especially in cryopreservation. However, only a few efforts have explored the effect of peptoids as antifreeze agent [19] and their IRI activity has not been reported.

2 Experimental Section

2.1 Materials

Dichloromethane, anhydrous magnesium sulfate, *N*,*N*′-diisopropylethylamine, methylamine solution, ethylamine solution, methanol and ethanol were purchased from Sinopharm Chinese Reagent Company. Bromoacetic acid, acetone, trifluoroacetic acid, acetonitrile, dimethyldichlorosilane, *N*-Boc-ethylenediamine, β-alanine *t*-butyl ester hydrochloride, 3,4-dihydro-2H-pyran, ether, hexane, pyridinium *p*-toluenesulfonate, *N*,*N*′-dimethylformamide, triisopropylsilane (TIS), rink amide resin (0.3–0.8mmol g^{-1}), 4-methylpiperidine, sodium chloride and *N*,*N*′-diisopropylcarbodiimide were purchased from Aladdin. All solvents and reagents were used without further purification.

2.2 Synthesis of Peptoid Oligomers

Peptoid synthesis was conducted following the previously reported method [20,21]. The P-Nhe amine submonomer was prepared using previously published method [22]. Other amine submonomers were purchased directly. Briefly, Rink amide resin (100 mg) was swelled in 2 mL of dimethylformamide (DMF), stirring by bubbling for 10 min under a stream of N_2 gas. Drain the DMF by vacuum to separate swelled resin. 1 mL of 20% 4-methylpiperidine in DMF (v/v) was added to the swelled resin to deprotect Fmoc group, agitating for 2 min, and draining. Repeat with a 12 min incubation. Rinse the resin by adding 2 mL of DMF (3 × 15 s). The amine-functionalized resin was bromoacylated for 20 min by adding bromoacetic acid (0.5 mL of a 2 M solution in DMF) and then treated with a solution (1 mL of a 1 M solution in DMF) of amine submonomer for 30 min. After above two steps, wash the resin with 2 mL of DMF (5 × 15 s) respectively. The desired oligomers were obtained by repeating steps with bromoacylation and amine displacement. After the final displacement step, the resin was washed with DMF (5 × 15 s) and DCM (3 × 15 s), and stored at − 18°C after dried. Peptoid oligomers were cleaved with 4 mL of trifluoroacetic acid (TFA) cleavage cocktail (TFA/H_2O/TIS = 95/2.5/2.5, v/v/v) for 2 h followed by blowing a stream of N_2 gas to evaporate cocktail. To analyse the crude product, the compound dissolved in 1 mL of acetonitrile/water (1/1, v/v) was characterized by LC–MS (Thermo LXQ) performed on a C18 column (An Pu, 4.6 mm × 250 mm, 5 μm). The analysis was conducted by a solvent gradient (5–95% acetonitrile/water with 0.1% TFA) over 35 min, at a flow rate of 1 mL min^{-1}). Subsequently, the oily products were purified by preparative RP-HPLC and lyophilized. The pure products were identified by ESI-LC–MS analysis. The purity of each final product was confirmed by analytical RP-HPLC using analytical column (SHIMADZU inertsil OSD-SP, 4.6 mm × 250 mm, 5 μm).

2.3 Nanoliter Osmometer Experiments

The ice growth rate, ice morphology and thermal hysteresis (TH) at different temperature were measured through a specially designed and built nanoliter osmometer with the precision of 0.01 °C, achieving to precisely control relative humidity, temperature, and water drop. In order to avoid the impact of the environment such as water condensation from ambient air during the experiments, the chamber was purged with dry purified nitrogen (99.99%, 25 °C). The procedure followed established protocol [23]. In short, sub-microliter volume of peptoid solutions injected into sample filled with silicone oil were quickly frozen at a rate of 40 °C min^{-1} and then slowly heated to the melting temperature. As soon as only a single ice crystal was left, temperature at this moment was defined as melting temperature (T_m). It then slowly cooled again down to a specific temperature recorded as T_f and kept at this temperature, where crystal just begins to grow and ice growth was immediately recorded by digital camera. To determine the growth rate, at least five snapshots must be obtained during the growth course. Ice crystal growth rate at different temperature (ΔT) was obtained as the division of the elongation by the time elapsed during the growth process and the corresponding experiment was repeated at least three times for three different samples, and the final average value was calculated for per ΔT.

2.4 Ice Recrystallization Inhibition (IRI) Experiments

Ice recrystallization inhibition experiments were conducted on a modified splay assay. The apparatus equipped with a Linkam cryostage (C194) and a Nikon polarized optical microscope (LV100ND, Japan), which was sealed to get a certain humidity (~46%) [24]. Droplets of 10 μL of peptoid

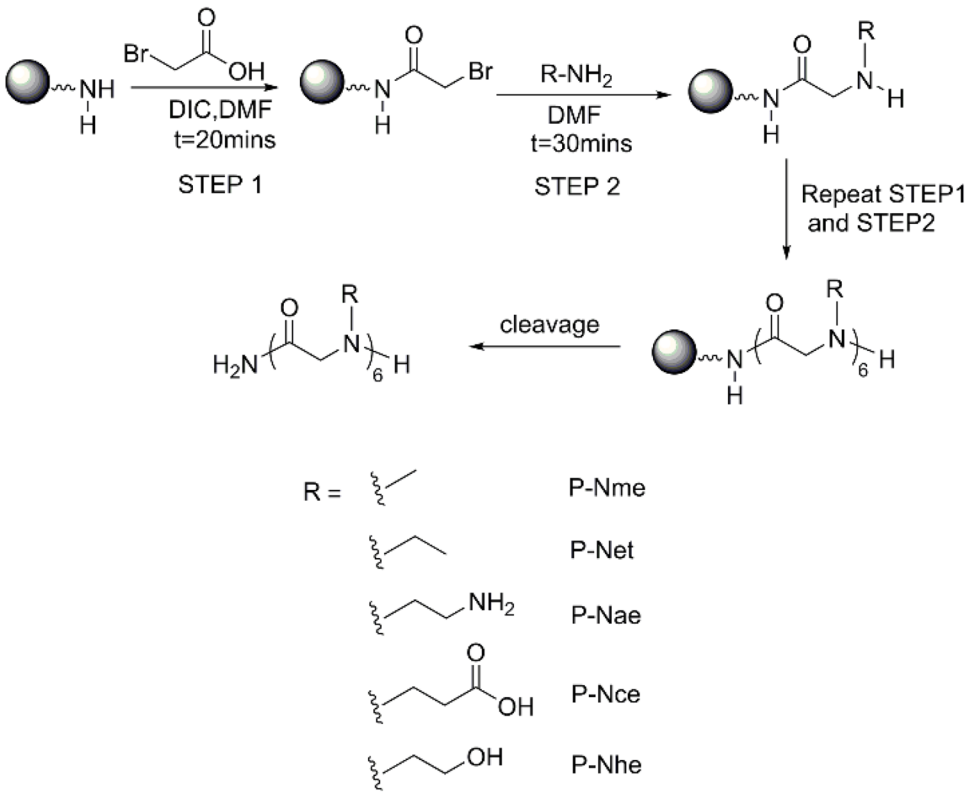
solutions dissolved in PBS buffer at concentrations of 0.01 mg mL^{-1}, 0.05 mg mL^{-1}, 0.1 mg mL^{-1}, 0.5 mg mL^{-1}, 1.0 mg mL^{-1}, 5.0 mg mL^{-1} and 10.0 mg mL^{-1} were dropped onto cryostage pre-cooled to – 60 °C from 1.5 m height to form a thin solid ice film separately. Subsequently, the temperature was increased to – 6 °C at a rate of 5 °C min^{-1}, then the frozen samples were kept at – 6 °C for 30 min to permit recrystallization and evaluate IRI activity. Soon afterwards, microphotographs of the samples were recorded using a digital camera (Nikon Y-TV55, Japan) to get grain size which was defined by the two largest orthogonal dimensions across the ice grain surface. In the observation field, all ice crystal grain sizes were measured by using Image J software, where ten largest grain sizes among them were chosen and subsequently averaged to quantitatively evaluate ice recrystallisation activity. For every sample, corresponding procedure was repeated for three times at a minimum.

3 Results and Discussion

Solid-phase submonomer synthesis method provides an efficient way to prepare peptoids with specific sequence and chain length, and varying side chains can be easily introduced [20]. This method provides great diversity to peptoid structure and makes it highly tunable. To study the effects on ice formation and growth, a series of peptoid hexamers with different side chains (Scheme 2) were synthesized and purified by reverse phase HPLC. Hydrophobic side chains (methyl and ethyl groups) and hydrophilic side chains (aminoethyl, carboxyethyl and hydroxyethyl groups) were designed to compare their structure effects on antifreeze activity.

The obtained peptoid hexamers were dissolved in ultrapure water at a concentration of 10 mg mL^{-1} and studied their control on ice crystal shape and growth. In the presence of P-Nme, P-Net and P-Nae, a representatively hexagonally shaped ice crystal was observed with further growth over time after a disc-shaped single ice crystal was held at − 0.40 °C for a few minutes (Fig. 1c, d, e). For the peptoid solutions with P-Nce and P-Nhe, however, the ice remains a typical flat disc-shaped crystal (Fig. 1a, b), just like pure water [25]. In addition, peptoid oligomers shape ice crystals in the same way at different concentrations and varying supercooling temperatures (the temperature below the equilibrium melting temperature, ΔT). It clearly suggests that not all the peptoid oligomers can regulate ice morphology. The working ones could have hydrophobic side chains (methyl and ethyl) or side chains bearing hydrophilic groups (amino). Hydrogen bonding between peptoid side chains and ice crystals seems not the critical factor for ice morphology modulation.

Thermal hysteresis (TH), another effect of AFPs, is the difference between the equilibrium melting and freezing



Scheme 2 Synthetic route of the peptoid hexamers

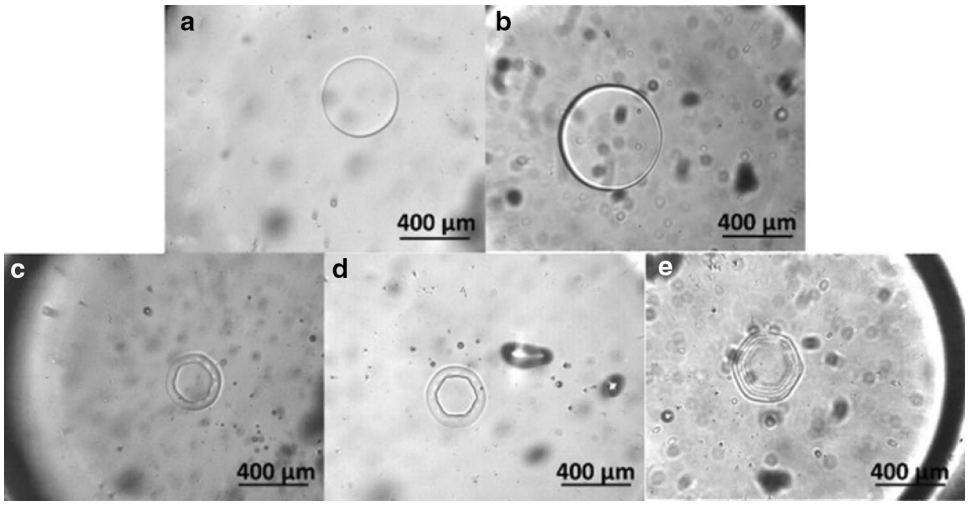


Fig. 1 Optical images present a completely different growth habit and shape of ice crystals with varying side chains of peptoid hexamers dissolved in ultrapure water at the concentration of 10 mg mL^{-1} at − 0.40 °C. The pictures are marked as follows: **a** P-Nhe; **b** P-Nce; **c** P-Nae; **d** P-Nme; **e** P-Net

temperatures of an ice crystal. However, studies suggested that antifreeze agents without TH but IRI is desirable for cryopreservation [26,27]. As a class of biocompatible protein mimetic material, peptoids have great potential application as antifreeze agents in cryopreservation. First, the aqueous solutions of peptoids were characterized and no TH phenomenon were observed for all the samples. In the other hand, the addition of peptoids can slow down the growth of ice crystals obviously (Fig. 2 and Fig. S4). The growth of single ice crystal was monitored for peptoid solutions in ultrapure water (at the concentration of 1.0, 5.0 and 10.0 mg mL^{-1}) at varying ΔT. For all five peptoid oligomers, the ice growth rate decreases with the increase of peptoid concentration at certain ΔT. For example, the ice growth rate drops down from 13.91 to 6.06 μm s^{-1} when P-Nhe concentration enhances from 1.0 to 10.0 mg mL^{-1} at a ΔT of 0.04 °C. When ΔT enhances, i.e. at lower temperature, ice grows faster. Ice growth rate increases from 3.08 to 20.22 μm s^{-1} when ΔT changes from 0.02 to 0.12 °C for P-Nhe solutions at concentration of 10 mg mL^{-1} (Fig. 2a). Among all five peptoid oligomers, P-Net and P-Nhe have the most significant effects (Fig. 2b). It suggests that hydrogen-bonding interactions between peptoid side chains and ice crystal surfaces play an important role in suppressing ice growth, and hydrophobic interactions and length of side chains are also key factors, which agrees with previous reports [28,29]. Kirshenbaum group reported the peptoids as antifreeze agents and found the ones with hydroxyl substituents slowing down ice growth the most [19], which is consistent with our results.

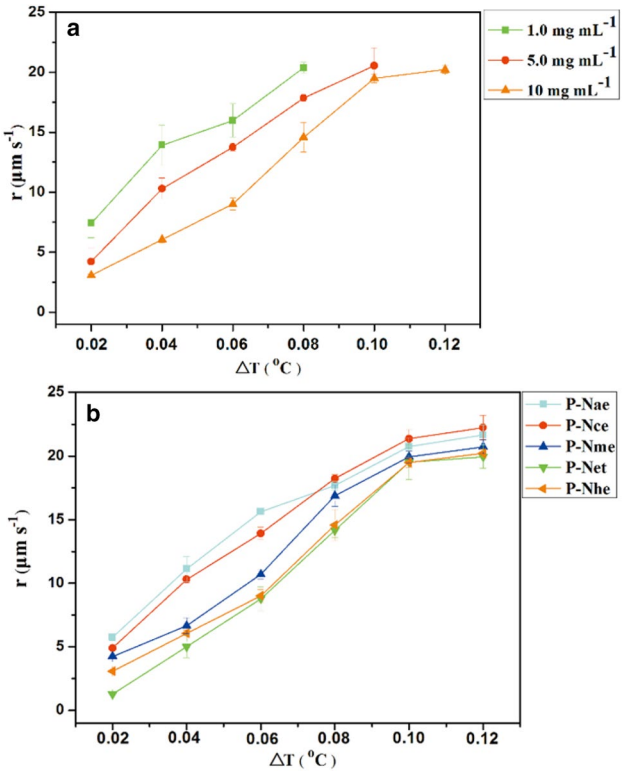


Fig. 2 The effects of peptoids on ice crystal growth rate. **a** The effect of P-Nhe with various concentrations on the growth rate (r) of ice crystals at different ΔT. **b** The effect of peptoid structures on the growth rate (r) of ice crystals with concentrations of 10.0 mg mL^{-1} at different ΔT

Further quantitative evaluation of the peptoid effects on ice recrystallization inhibition were carried out in PBS buffer with the peptoid concentrations of 0.01–10.0 mg mL^{-1}. Solid thin ice films were annealed at − 6 °C for 30 min and then the grain size of ice crystals was characterized. Fig. 3g clearly suggests that all peptoid can reduce ice recrystallization. The grain size decreases 5–73% with the addition of peptoids. The IRI activities are quite stable for P-Nce, P-Nme and P-Nae when concentration increases from 0.01 to 10.0 mg mL^{-1}. The mean largest grain size (MLGS) is around 71% of PBS buffer control. In contrast, the IRI activities of P-Net and P-Nhe are concentration dependent.

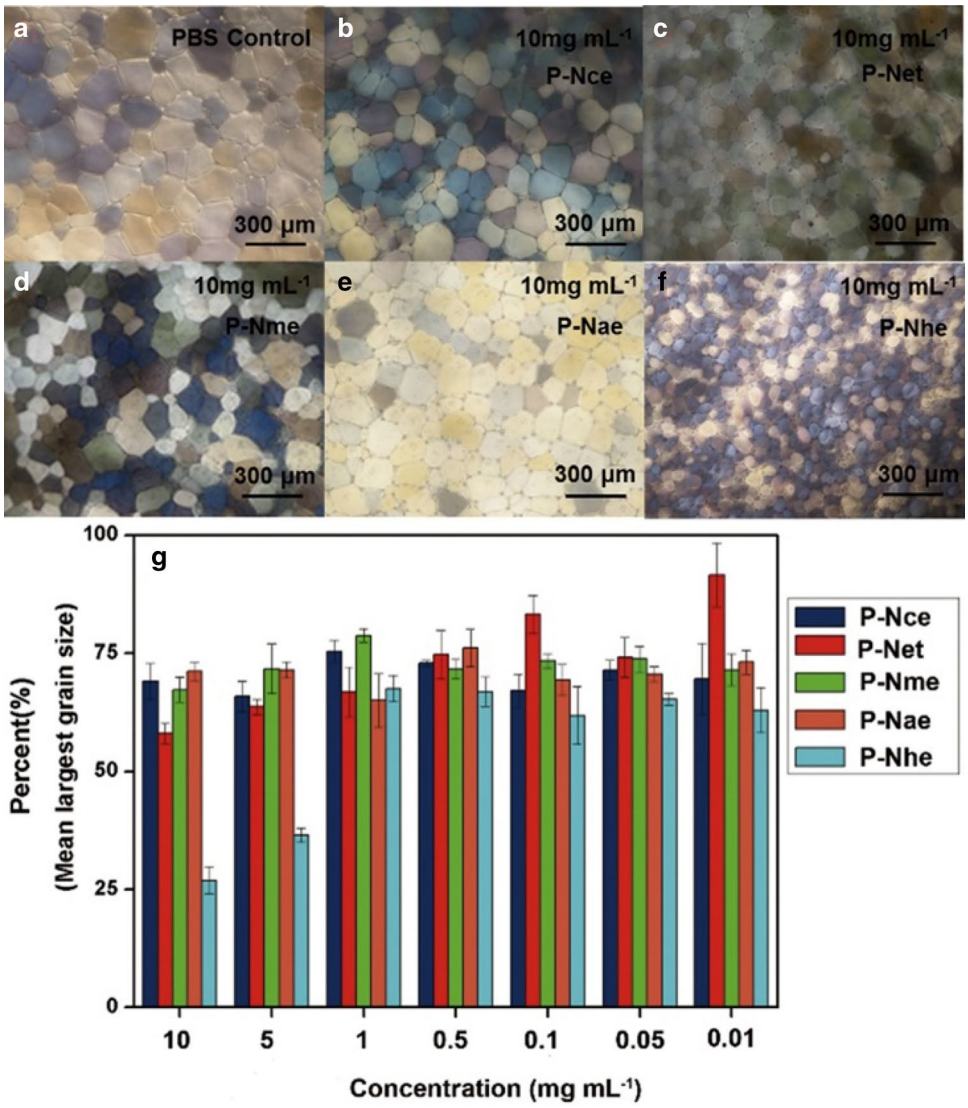


Fig. 3 Ice recrystallization inhibition of peptoids. **a** Microscopic images of ice crystals grown in the PBS buffer as a negative control; Microphotographs of ice crystals grown in the **b** P-Nce, **c** P-Net, **d** P-Nme, **e** P-Nae, and **f** P-Nhe solutions dissolved in the PBS buffer at 10.0 mg mL^{-1} after annealing at − 6 °C for 30 min; **g** Mean largest grain size (MLGS) is presented as a percentage of PBS buffer

Ice crystals become smaller when P-Net/P-Nhe concentration enhances. In the presence of P-Nhe, MLGS decreases from 68 to 27% of PBS buffer when concentration increases from 0.01 to 10.0 mg mL^{-1}. The addition of P-Nhe to a PBS buffer at concentration of 10.0 mg mL^{-1} can greatly reduce the average MLGS to 50 μm (Fig. 3f and Fig. S5), indicating the best IRI activity of P-Nhe among all the peptoid hexamers, consisting well with the results of ice growth rate study.

4 Conclusions

In conclusion, we investigated the ice recrystallization inhibition activity of peptoids and studied the structure–property relationships. Peptoid hexamers with side chains bearing typical hydrophobic groups (–CH_3 and –CH_2CH_3) and hydrophilic groups (–OH,–COOH, –NH_2) were designed and prepared by solid-phase submonomer synthesis method. All peptoids exhibit no TH but IRI effects, i.e. slowing down ice crystal growth and decreasing ice crystal grain size, indicating the ability of peptoids as antifreeze agents in cryopreservation. However, the morphology study of single ice crystal indicates that only peptoids with methyl, ethyl and amino groups on side chains have the ice modulation effect, suggesting these three side chains have special interactions with ice crystal growth interface. Peptoid hexamer equipped with hydroxyl substituents on side chains shows the best IRI activity, giving the lowest ice growth rate and smallest ice crystal grain size. Theoretical modelling, which can provide deeper understanding of the structure dependence of peptoid on IRI acitivities, is currently underway. We believe this study will facilitate the development of synthetic antifreeze agents that can mimic the structure and function of AFPs in cryopreservation application.

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Compliance with ethical standards

Conflicts of interest There are no conflicts to declare.

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