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Synthesis of star-branched poly(vinyl alcohol) and ice recrystallization inhibition activity

Thomas R. Congdon [a], Rebecca Notman [a], Matthew I. Gibson [a,b,*]

[a] Department of Chemistry, University of Warwick, Coventry CV4 7AL, UK
[b] Warwick Medical School, University of Warwick, Coventry CV4 7AL, UK

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ABSTRACT

Antifreeze proteins are potent inhibitors of ice crystal growth (recrystallization), which is a highly desirable property for cryopreservation and other low temperature applications. It has emerged that relatively simple polymers based on poly(vinyl alcohol) can mimic this activity, but the link between architecture and activity is not known. Here, a trifunctional xanthate was designed and synthesized to prepare star-branched poly(vinyl alcohols) by RAFT/Xanthate mediated polymerization, and their ice growth inhibition activity probed for the first time. The trifunctional agent design affords the formation of well-defined star polymers, with no evidence of star-star linking, even at high conversions, and narrow molecular weight dispersity. It is observed that three-arm stars have identical activity to two-armed (*i.e.* linear) equivalents, suggesting that the total hydrodynamic size of the polymer (diameter three-arm ~ two-arm) rather than total valence of the functional groups is the key descriptor of activity.

1. Introduction

Due to their similar size and diverse functionality, synthetic polymers have been widely explored to mimic the function of biomacromolecules, including proteins and polysaccharides. For example, Tew and coworkers have prepared polymers that can efficiently penetrate cell membranes in a manner similar to cell-penetrating peptides [1]. Synthetic hydrogels can mimic the extracellular matrix to prepare tailored stem cell niches [2], or synthetic glycopolymers can mimic the cell surface glycocalyx [3,4]. Polymers can also be used to mimic protein responses to external stimuli, for example metal concentration [5]. Antifreeze (glyco)proteins (AF(G)Ps) are specialized proteins expressed in extremophile species that enable them to survive in sub-zero climates. These proteins act to reduce the freezing point of the blood serum and show potent ice recrystallization inhibition (IRI) activity; slowing the growth of any ice crystals which form or enter circulation, that leads to a fatal build-up of ice [6,7]. The ability to inhibit ice growth is of huge (bio)technological significance, but especially in the cryopreservation of donor cells and tissue. However, AF(G)Ps are challenging to obtain in large quantities and have had mixed results in cell storage due to unwanted ice-shaping effects.

Inada et al. and Budke et al. have demonstrated that (highly-disperse and partially acetylated) poly(vinyl alcohol) (PVA) has potent ice recrystallization inhibition (IRI) activity despite no real structural similarities to native AF(G)Ps [8,9]. We have used controlled radical polymerization to generate well-defined PVA and elucidated that polymers comprised of as few as

* Corresponding author at: Department of Chemistry, University of Warwick, Coventry CV4 7AL, UK.
E-mail address: m.i.gibson@warwick.ac.uk (M.I. Gibson).

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10–20 units retained potent IRI activity [10]. This potent ice recrystallization inhibition activity was subsequently used to enhance the cryopreservation of red blood cells, by reducing ice crystal growth during the thawing stage [11–13], and also in a solvent free system [14]. Despite these advances, and 40 years of research into AF(G)Ps, there is still much debate on the actual mechanism of antifreeze protein function, which in turn limits the ability to synthesize new biomimetic materials. Recent experiments have suggested that irreversible binding to ice crystal is occurring with antifreeze proteins, but do not prove the link to observable macroscopic effects [15,16]. Ben et al. [17] have prepared synthetic AFGP mimics which do not appear to bind the ice, but rather disrupt the interface between ice crystals; the quasi liquid layer, and posit that this gives rise to potent IRI activity. Star-branched AFPs have been found to retain their IRI activity relative to linear counterparts but show enhanced ice binding, implying a complex relationship between size and activity [18]. However, changing the macromolecular architecture or size of proteins is non-trivial (as it becomes a new protein), requiring site-specific conjugation chemistries. Conversely, due to having only a single chemically distinct repeat unit, synthetic polymers can easily be varied in terms of size and shape. Congdon et al. have shown that addition on a second hydrophilic block to PVA does not affect the IRI activity [19], and Voets and coworkers have developed bottle-brush PVA's for ice growth inhibition [20].

Due to the advances in controlled radical polymerization methods, it is now not only possible to readily access well-defined materials, but also polymers of variable architecture which enables their properties to be tuned [21,22]. Star branched polymers typically have smaller solution dimensions and lower intrinsic viscosity compared to the corresponding linear polymer, and also display more end-groups, which may affect their ice interactions [23,24]. For these reasons, probing the effect of branching and viscosity will give a greater understanding as to the factors affecting ice recrystallization inhibition. The effect of viscosity is especially intriguing, as with linear polymers viscosity is dependent on polymer size, whereas with star polymers it is possible to access comparable viscosities, but at higher molecular weights. Stenzel and coworkers have developed multifunctional xanthates to enable (star) polymerization of lesser activated monomers such as vinyl acetate, which are typically harder to polymerize then methacrylate monomers. These multifunctional xanthates displayed a tendancy to form star-star couples, leading to an increase in dispersity and poorly defined polymer products. This was due to the configuration of the xanthates on the multifunctional agent [25–27].

Considering the above, this manuscript describes the design, synthesis and use of a novel multifunctional MADIX agent designed with a configuration that allows the polymers to grow from the core. This approach affords well-defined three-armed polymers with no star-star coupling. When using these stars as an IRI agent, activity is maintained, opening the door to increasingly complex IRI active materials and tools to understanding the ice/water interface.

2. Experimental section

2.1. Materials

4,4′-azobis(4-cyanovaleric) acid (80%), benzyl bromide (98%), deuterated chloroform (99.8 atom% D), deuterium oxide (99.9 atom% D), potassium ethyl xanthate (96%), 1,3,5-tris(bromomethyl)benzene (99%), vinyl acetate (99%), and all solvents were purchased from Sigma Aldrich. Hydrazine hydrate solution (80%) was purchased from Fisher Scientific. Phosphate-buffered saline (PBS) solutions were prepared using preformulated tablets (Sigma-Aldrich) in 200 mL of MilliQ water (>18.2 Ω mean resistivity) to give [NaCl] = 0.138 M, [KCl] = 0.0027 M and pH 7.4. Methyl(ethoxycarbonothioyl)sulfanyl benzene was prepared according to literature methods [28].

2.2. Analytical and physical methods

^{1}H and ^{13}C NMR spectra were recorded on Bruker DPX-300 and DPX-400 spectrometers using deuterated solvents purchased from Sigma-Aldrich. Chemical shifts are reported relative to residual non-deuterated solvent. Size exclusion chromatography (SEC) was used to examine and differentiate between the molecular weights and dispersities of the synthesized polymers. SEC analysis was performed on a Varian 390-LC MDS system equipped with a PL-AS RT/MT autosampler, a PL-gel 3 μm (50 × 7.5 mm) guard column, two PL-gel 5 μm (300 × 7.5 mm) mixed-D columns held at 30 °C and the instrument equipped with a differential refractive index and a Shimadzu SPD-M20A diode array detector. The mobile phase was THF with 5% triethylamine (TEA) eluent at a flow of 1.0 mL/min, and samples were calibrated against Varian Polymer Laboratories Easi-Vials linear poly(styrene) and poly(methyl methacrylate) standards (162–2.4 × 10^5 g/mol) using Cirrus v3.3. Ice wafers were annealed on a Linkam Biological Cryostage BCS196 with T95-Linkpad system controller equipped with a LNP95-Liquid nitrogen cooling pump, using liquid nitrogen as the coolant (Linkam Scientific Instruments UK, Surrey, UK). An Olympus CX41 microscope equipped with a UIS-2 20x/0.45/∞/0-2/FN22 lens (Olympus Ltd, Southend on sea, UK) and a Canon EOS 500D SLR digital camera was used to obtain all images. Image processing was conducted using Image J, which is freely available from http://imagej.nih.gov/ij/.

2.3. 'Splat' (ice recrystallization inhibition) assay

Ice recrystallization inhibition was measured using a modified splay assay [29]. A 10 μL sample of polymer dissolved in PBS buffer (pH 7.4) was dropped 1.40 m onto a chilled glass coverslip sat on a piece of polished aluminum placed on dry ice.