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Recrystallization inhibition in ice due to ice binding protein activity detected by nuclear magnetic resonance

Jennifer R. Brown [a,e,*], Joseph D. Seymour [a,e], Timothy I. Brox [a], Mark L. Skidmore [b], Chen Wang [c], Brent C. Christner [c], Bing-Hao Luo [c], Sarah L. Codd [d,e]

[a] Chemical and Biological Engineering Department, Montana State University, Bozeman, MT 59717, USA
[b] Earth Sciences Department, Montana State University, Bozeman, MT 59717, USA
[c] Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA
[d] Mechanical and Industrial Engineering Department, Montana State University, Bozeman, MT 59717, USA
[e] Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717, USA

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ABSTRACT

Liquid water present in polycrystalline ice at the interstices between ice crystals results in a network of liquid-filled veins and nodes within a solid ice matrix, making ice a low porosity porous media. Here we used nuclear magnetic resonance (NMR) relaxation and time dependent self-diffusion measurements developed for porous media applications to monitor three dimensional changes to the vein network in ices with and without a bacterial ice binding protein (IBP). Shorter effective diffusion distances were detected as a function of increased irreversible ice binding activity, indicating inhibition of ice recrystallization and persistent small crystal structure. The modification of ice structure by the IBP demonstrates a potential mechanism for the microorganism to enhance survivability in ice. These results highlight the potential of NMR techniques in evaluation of the impact of IBPs on vein network structure and recrystallization processes; information useful for continued development of ice-interacting proteins for biotechnology applications.

1. Introduction

Proteins with ice-interacting activity have been identified in fish, cold hardy plants and insects [1–3], and certain cold-adapted bacteria, diatoms, and algae [4]. The properties of ice-interacting proteins are useful in many areas of biotechnology, including cell line cryopreservation [5] and food manufacturing [6]. Understanding their affect on ice and recrystallization processes is critical for further development in both applied and basic applications. The cold tolerant bacterium 3519-10 (Flavobacteriaceae family), isolated from basal ice recovered from the Vostok 5G ice core [7], secretes an extracellular ice binding protein (IBP) that binds to the ice crystal prism face and inhibits growth along the *a*-axis [8]. The 3519-10 IBP has been shown to increase bacterial viability during freeze and thaw cycling [9]; however, its mechanism of action and impact on the internal pore structure of unfrozen water in ice is not well understood.

Within polycrystalline ice, liquid unfrozen water is located at interfaces between two or three hexagonal ice crystals due to the presence of impurities [10,11]. At triple grain junctions, veins form that may be approximated as cylinders with diameters, d_{vein} which can be related to ice crystal diameters d via liquid water fraction $f = 6\pi\sqrt{2}((1/2d_{vein})/d)^2$ [12]. Where two grains meet, a planar junction of thickness d_{plane} forms on the octahedra faces. As ice ages, it undergoes a thermodynamically driven coarsening, termed recrystallization, whereby larger ice crystals grow at the expense of smaller ones, altering vein dimensions [13,14]. Ice is therefore a complex and dynamic low porosity porous media, where ice crystals compose the solid matrix and liquid veins the pore space. With non-invasive and non-destructive nuclear magnetic resonance (NMR) techniques, the vein network can be directly characterized. With respect to biotechnology applications, Kirsebom *et al.* have shown the utility of NMR to monitor the composition of the unfrozen water phase during the formation of cryogels *in situ* [15,16]. We utilize NMR magnetic relaxation time and molecular diffusion measurements, which are proven robust in probing pore structure in porous media [17] and sensitive to vein

* Corresponding author at: Montana State University, Chemical and Biological Engineering, 306 Cobleigh Hall, PO Box 173920, Bozeman, MT 597173920, USA. Tel.: +1 406 994 7719.
E-mail address: jbrown@coe.montana.edu (J.R. Brown).

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dimensions [18], to provide a novel method for monitoring ice structure and its evolution with time. This provides a new analytical method for quantitative characterization of ice structure during biotechnological freezing processes.

Here we have applied advanced NMR techniques to ice samples, establishing them as methods to physically characterize ice vein network structure. These techniques were then used to examine the impact of IBP on bulk liquid vein network structure in order to improve our understanding of the impact of this ice-interacting protein on recrystallization processes. Our findings have implications for geophysical modelling of frozen systems [4] and in development of IBPs for biotechnology applications [6]. Also, with advances in design of portable NMR systems including Earth's field systems [19], low field permanent magnets [20] and surface NMR [21], our research highlights the potential for using these methods in biotechnology process monitoring.

2. Materials and methods

2.1. Extracellular protein (ECP) and rIBP preparation

Extra cellular proteins (ECP) and the recombinant IBP (rIBP) from isolate V3519 for use in the ice experiments were prepared as follows. For ECP, the V3519-10 bacteria were grown in R2 liquid media at 4 °C until the culture reached an optical density OD_{595} of 0.22 at which time it was centrifuged at 5000 g for 30 min at 4 °C to pellet the cells and recover the supernatant. The supernatant containing the IBP was filtered using Amicon Ultra-15 centrifugal filters with a nominal threshold of 30 kDa to obtain a crude extract of V3519-10's extracellular proteins. Protein concentrations were determined with the Bradford assay using the Coomassie Plus reagent. For the rIBP, the cDNA encoding IBP without the signal peptide but with a 6× His tag added to the C-terminus was cloned into the pET-21a expression vector (Novagen) and transformed into BL21 cells. The BL 21 cells were cultured in LB medium at 37 °C to an optical density of 0.8, when isopropyl β-D-1-thiogalactopyranoside was added to give a final concentration of 1 mM and the temperature was reduced to 18 °C. Proteins were extracted from the cell culture after incubating overnight and the rIBP was purified as described previously [22]. The purified protein size (~52 kDa) was determined via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and its concentration was measured using spectrophotometry (NanoDrop ND-1000).

2.2. Ice sample preparation

Five ice samples were prepared. All samples contained a 7 g/l NaCl solution, which is a salt concentration comparable with measurements in Antarctic basal ice [23]. IBPs were added to three samples to monitor concentration effects and the difference between naturally secreted extracellular protein (ECP) and purified recombinant IBP (rIBP). The ice sample containing a crude preparation of the IBP consisted of 7 g/l NaCl solution with 10 μg/ml of 3519-10 ECP (>30 kDa with an unknown IBP fraction) and will hereafter be referred to as ice with ECP. The two samples containing 7 g/l NaCl and 2 and 4 μg/ml recombinant IBP will be referred to as ice with rIBP(2) and ice with rIBP(4) respectively. Two control samples were also prepared: (i) the ice control, a 7 g/l NaCl solution without protein and (ii) ice with bovine serum albumin (BSA), a 7 g/l NaCl solution with 10 μg/ml BSA. The second control was used to examine ice binding activity from colligative effects due to the presence of a similar macromolecule, since BSA is of similar size (~64 kDa) to the 3519-10 IBP (~52 kDa), but does not exhibit ice binding activity. All samples were prepared by filling 13 mm OD (11.7 mm ID) standard NMR tubes with solution, placing them in a polystyrene sample holder, insulated on the sides and bottom, and freezing them in a Revco ULT-750 chest freezer at −13.5 °C. To ensure hexagonal ice crystal structure consistent between sample types, multiple samples of each concentration were frozen and inspected by eye and those with cloudiness and/or air bubbles which would indicate supercooling and subsequent rapid freezing were discarded. Samples were transferred from the chest freezer in a cooler filled with gel freezer packs stored in the same freezer. Transfer time of the ice from the cooler to being in the RF coil with cold nitrogen gas flow was minimized to ~3 min. The MR magnet electronics were always pre-cooled at the set temperature before sample insertion and the set temperature equilibrized within ~5 min. The samples were allowed to equilibrate at the set temperature for 45 min before measurements were performed. Samples were analysed via NMR at multiple time points over 1800 h, and stored in the freezer at −13.5 °C in between NMR measurements.

2.3. NMR methods

NMR measurements were performed on a Bruker DRX250 spectrometer with a 5.8 T superconducting vertical wide bore magnet and Micro2.5 gradient imaging probe capable of producing maximum gradients of $1\,T\,m^{-1}$. Temperature was controlled via flow of cooled nitrogen gas along the vertical axis of the NMR sample tube using a Bruker variable temperature control unit. The 13 mm OD (11.7 mm ID) frozen ice samples were centred in a 20 mm diameter radiofrequency coil using polyether ether ketone (PEEK) spacers to allow for constant and consistent air flow around the sample. The set point temperature was −15 °C at the base of the coil and the temperature increase of the cooled nitrogen gas was ~7° C across the full coil length. Unfrozen water content was calculated from the NMR signal magnitude after calibration with a known volume of water at the same receiver gain as a function of temperature. Signal from the solid ice crystals was not detectable. The FID decay was single exponential, i.e. from liquid water only, no solid state Gaussian signal from the ice phase was detected due to the rf excitation and signal acquisition digitization time scales. Cross-relaxation between the solid ice crystal phase and liquid water in veins can be neglected based on this and the large difference between the water diffusivity $\sim 10^{-10}\,m^2\,s^{-1}$ and the spin diffusion $\sim 10^{-15}\,m^2\,s^{-1}$ [24]. T_2 relaxation time distributions were obtained using a standard Carr–Purcell–Meiboom–Gill (CPMG) echo train with echo time $t_E = 403$ μs. A standard pulsed gradient stimulated echo (PGSTE) sequence was used to measure diffusion for displacement observation times Δ ranging from 10–1000 ms at a constant echo time t_E of 8 ms and gradient duration $\delta = 2$ ms. Gradients were applied in the horizontal y-direction, perpendicular to the tube walls, in order to eliminate the impact of any anisotropy on the measurements from crystal elongation in the z-direction due to the top-down freezing process. Diffusion coefficients were calculated from a standard Stejskal–Tanner plot and the fit was linear with no indication of multiexponential decay. The mono-exponential decay was also confirmed by performing an inverse Laplace transform which resulted in a single diffusion coefficient. Images were obtained with a standard 2D multi-slice spin echo sequence and had a spatial resolution of 55 × 55 μm (256 × 256 matrix size and 14 × 14 mm field of view) over a 0.5 mm slice centred in the middle of the rf coil.

3. Results

Fig. 1, top row, shows cross-sectional magnetic resonance images acquired for ice with BSA at various time intervals after freezing. Definitive ice crystal growth during recrystallization was observed over 1800 h, with crystal diameters growing from