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Article

Enhanced Control over Ice Nucleation Stochasticity Using a Carbohydrate Polymer Cryoprotectant

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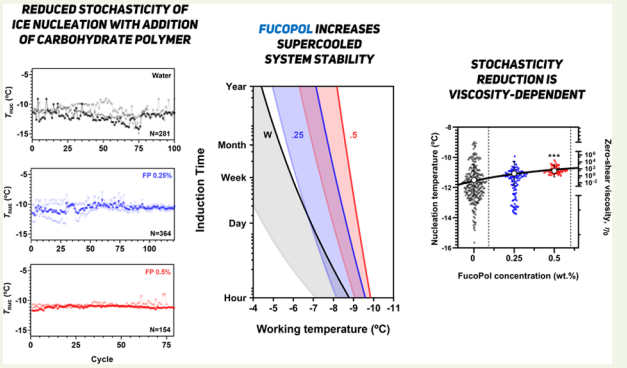
ABSTRACT: Metastable supercooling has emerged as a transformative technique for ice-free biopreservation, but issues of stability inherent to the stochastic nature of ice formation have thus far limited its translation out of the laboratory. In this work, we explore the influence of the bio-based carbohydrate polymer FucoPol on aqueous supercooling using an isochoric nucleation detection technique. We show that FucoPol, a high-molecular-weight, fucose-rich polysaccharide, which has previously been shown to reduce average ice crystal sizes after nucleation, also induces a concentration-dependent stabilization of metastable supercooled water, as evidenced by both a significant reduction in nucleation stochasticity (i.e., the spread in temperatures over which the system will nucleate upon cooling) and a corresponding increase in the predicted induction time of nucleation. FucoPol is found to confine the stochasticity of ice nucleation to a narrow, well-defined band of temperatures roughly one-third as wide as that of pure water under identical conditions. Importantly, this substantial reduction in stochasticity is accompanied by only a minimal (<1 °C) change in the average nucleation temperature, suggesting that this effect is distinct from colligative freezing point depression. Reducing and characterizing the stochasticity of aqueous supercooling is essential to the engineering design of practical biopreservation protocols, and the results reported herein suggest that high-viscosity polymer systems may provide a powerful and largely unexplored lever by which to manipulate metastable-equilibrium phase change kinetics at subzero temperatures.



KEYWORDS: *supercooling, nucleation stochasticity, biopolymer, cryobiology, FucoPol*

1. INTRODUCTION

Aqueous supercooling has shown recent appeal in a range of applications, particularly in the atmospheric,[1] food,[2] and biomedical fields,[3,4] wherein an aqueous system is held in an ice-free state below the freezing temperature of water. Because supercooling is thermodynamically metastable however, stabilizing the supercooled state through chemical, mechanical, or other means is of great importance for the usability of supercooled systems in all manner of applications, and perhaps most namely biopreservation.[5−7]

Current methods for securing the viability of biological matter during subzero temperature preservation rely principally on the use of chemical cryoprotectants (CPAs) capable of reducing ice crystal size, growth potential, or occurrence altogether. However, Rubinsky et al.[8] have shown the efficacy of isochoric (constant-volume) chambers at forcing pressurized, biphasic water-ice systems to undergo continuous and controlled phase transition along the liquidus line, in which the frozen fraction can be manipulated via the final temperature/pressure or chemical modulation and ice-free preservation of biologics in the liquid volume achieved, something unattainable using conventional isobaric freezing.

This methodology eliminates the usage of toxic concentrations of CPAs to achieve an ice-free environment in the biological volume by leveraging the pressure that emerges with the expansion of ice under confinement, but it is limited in temperature by the deleterious effects of pressure.[9] Acknowledging the limitations of high pressure, recent studies have explored the effects of isochoric conditions on aqueous supercooling, in which the aqueous system confined in the isochoric chamber is held in a prenucleation, ice-, and pressure-free liquid state. These studies have identified theoretically several mechanisms by which the stability of supercooling should be enhanced in isochoric systems[10,11] and

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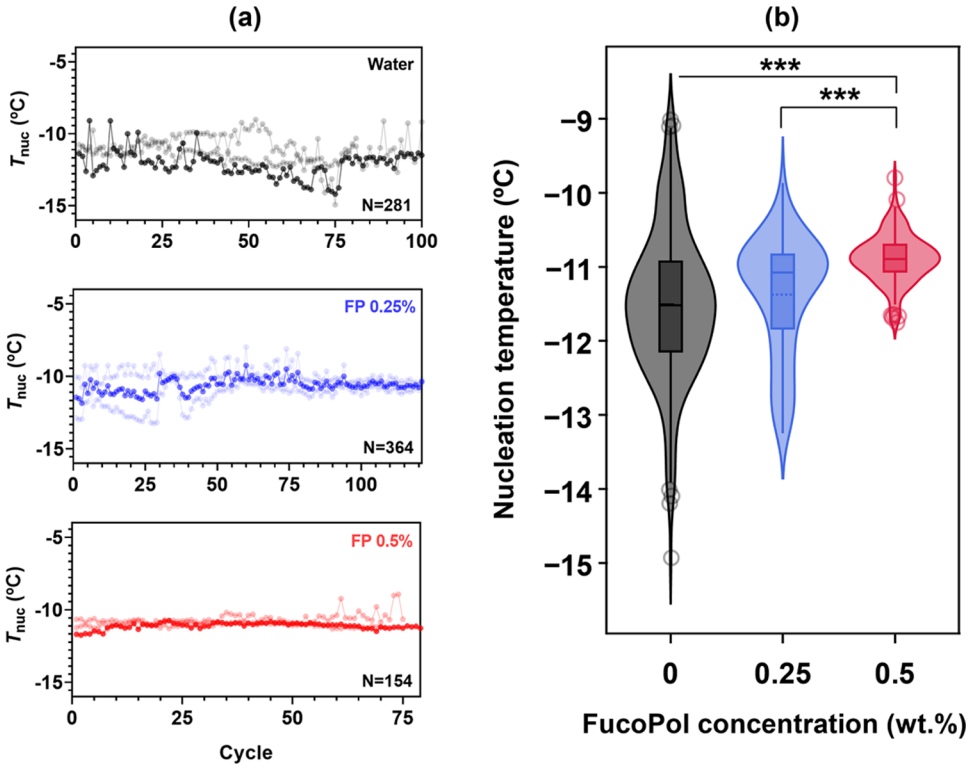

Figure 1. Nucleation cycle stochasticity and aggregated temperature distribution. (a) Individual nucleation cycle runs for water (black), 0.25% (blue), and 0.5% FucoPol (red). n = 3 independent trials are shown for each condition, each with >50 cycles. An increased experimental deviation of temperature points from the mean is evident in pure water. The spread of temperature data for water is minimized with increasing polymer concentration. (b) Corresponding violin plot distributions show a slight increase in nucleation temperature with increased FucoPol concentration. Violin plots show aggregated data from n = 3 independent trials of at least 50 cycles each for each condition. Total sample size is N = 281, 364, and 154 for water, 0.25% FucoPol, and 0.5% FucoPol, respectively. Labels indicating statistically significant difference (as determined by one-way ANOVA) are shown on top of each violin plot: water *vs* 0.25% FP (no significant difference, p = 0.4495), water *vs* 0.5% FP (***p < 0.0001), and 0.25% FP *vs* 0.5% FP (***p < 0.0001).

shown experimentally that indeed isochoric confinement has the effect of decreasing the likelihood of ice nucleation.[12,13]

Even considering these effects, however, applications of isochoric supercooling are still subject to uncertainties borne of the stochastic nature of ice nucleation. This stochasticity means that the probability of nucleation can be predicted over a range of temperatures, but the random probability distribution means nucleation cannot be pinpointed to a single temperature. The practical consequence is that, under isochoric conditions, the extent and location of ice formation can be fine-tuned, but its physical traits of emergence cannot: even by precisely manipulating thermodynamic phenomenology, this statistical randomness may still result in rogue nucleation, at a temperature at which the system may cascade into unintended freezing and pressurization, thus permanently damaging valuable biological matter.

Therefore, there is a need to find additional methods and molecules capable of reducing nucleation stochasticity. Previous work by Suzuki et al.[14] has shown that viscosity, molecular weight, and rotational correlation time have an influence on the supercooling temperature of water. Thus, supercooling is correlated with intrinsic properties that define molecular structure. Although small molecules have been extensively studied, relatively few polymeric systems have been probed for their potential,[15] and none under isochoric conditions.

FucoPol is a high-molecular-weight (1.7−5.8 × 10^6 Da) fucose-containing polysaccharide secreted by the Gram-negative bacterium *Enterobacter* A47 (DSM 23139),[16,17] whose cryoprotective[18,19] properties were recently demonstrated. FucoPol has a fucose, galactose, glucose, and glucuronic acid hexamer motif (2.0:1.9:0.9:0.5 M ratio), a main chain composed of a → 4)-α-L-Fuc*p*-(1 → 4)-α-L-Fuc*p*-(1 → 3)-β-D-Glc*p*(1 → trimer repeating unit, and a trimer branch α-D-4,6-pyruvyl-Gal*p*-(1 → 4)-β-D-GlcA*p*-(1 → 3)-α-D-Gal*p*(1 → in the C-3 of the first fucose.[18] FucoPol also contains 13−14 wt % pyruvyl, 3−5 wt % acetyl, and 2−3 wt % succinyl in its composition.[20] The presence of glucuronic acid as well as the acyl substituents pyruvyl and succinyl confer a polyanionic character to the biopolymer.[21]

Previous work has shown the wide potential of FucoPol applications. First, it acts as a crystallization initiator, successfully cryopreserving several animal cell lines by promoting earlier ice nucleation at subzero temperatures but reducing ice crystal dimensions to innocuous sizes.[18] Second, it has a rheological shear-thinning behavior[20] reflective of a linearly disposed helical conformation, showing strong analogy to antifreeze proteins,[22] viscoelastic properties comparable to those of guar gum and fucogel,[21] and emulsifying properties[17] that are desirable in the food industry. Lastly, it distinguishes itself from common-use CPAs by its bio-based noncytotoxic nature, very low osmolality, and osmotic regulation effects due to its extracellular location and suspected cell membrane stabilization effects.[23] All of these properties reflect the acute interest in studying the influence of carbohydrate polymer

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Table 1. Summary of Nucleation Temperature Data for the INDe Experiment

sample	mean T_n (°C)	median T_n (°C)	ΔT_{min}^{max} (°C)	σ (°C)	N
water	−11.51	−11.52	5.91	1.01	281
0.25% FucoPol	−11.37	−11.08	3.38	0.79	364
0.5% FucoPol	−10.89	−10.89	1.95	0.33	154

systems in the supercooling behavior of water and under isochoric thermodynamics in general.

In this work, we explore the influence of the bio-based carbohydrate polymer FucoPol on aqueous supercooling under isochoric conditions. We show that a fucose-rich polysaccharide, with proven crystal size reduction[18] and *in vitro* cryoprotective properties,[19] can confine the randomness of ice nucleation to a narrow, well-defined, near-deterministic range of nucleation temperatures. This stochasticity is one of the most defining physical constraints in manipulating success/failure rates in supercooled biopreservation. To the best of our knowledge, this is the first attempt at fundamentally characterizing high-molecular-weight polymer systems under isochoric conditions and at subzero temperatures.

2. RESULTS

2.1. Raw Data and Reduction of Stochasticity with the Addition of FucoPol. Isochoric nucleation detection was performed according to the methods and protocol of Consiglio et al.[13] under a process of transient supercooling, to assess the influence of varying FucoPol concentrations on the nucleation temperature (T_n) and behavior of pure water (experimental details in Section 5). Figure 1 shows the T_n distributions for increasing FucoPol presence (with Figure 1a showing all raw data recorded and Figure 1b showing data aggregated by FucoPol concentration), and Table 1 summarizes all temperature data recorded.

While pure water nuclei form at an average −11.51 ± 1.01 °C in the 5.3 mL isochoric chamber employed (identical to that described previously[13]), supplementation of FucoPol causes a distinguishable if modest increase in the T_n of water that is correlated with concentration. In the presence of 0.25% FucoPol, water nucleates at −11.37 ± 0.79 °C and subsequently increases to −10.89 ± 0.33 °C at 0.5% FucoPol.

As compared to pure water (Figure 1, black), FucoPol systems are observed to follow an increasingly deterministic pattern of nucleation (Figure 1, blue and red), which is to say that the stochastic range, i.e., the spread of nucleation temperatures, is considerably reduced in the presence of FucoPol. While the pure water distribution has a standard deviation of 1.01 °C, the spread is decreased 1.3- and 3-fold for 0.25 and 0.5% FucoPol, respectively. Considering that the system temperature monitoring has a lower detection limit of ± 0.2 °C, ice nucleation in a 0.5% FucoPol takes on a near-deterministic quality (σ = ± 0.33) at −10.89 °C.

This statistical confinement of T_n can also be interpreted as an enhanced control over ice nucleation stochasticity, and the range of temperature data in Table 1 more evidently shows this change. For pure water, the difference ΔT between the maximum and minimum recorded T_n is 5.91 °C, while for 0.25% it is 3.38 °C and only 1.95 °C for 0.5% FucoPol. In practical terms, a 0.5% FucoPol solution can contain the highly random process of ice nucleation to just under a 2 °C interval, compared to the 6 °C uncertainty of pure water. This stochastic range can be further interpreted as a measure of experimental safety. The smaller the range, the greater the control over ice nucleation will be, enabling predictive planning over which working temperature to employ in an experimental setting to avoid *rogue* nucleation events that could jeopardize the viability of preserved biological material.

2.2. Correlating Viscosity with Observed Nucleation Characteristics. The average nucleation temperatures recorded for each concentration were also correlated with polymer viscosity measurements. According to Suzuki et al.,[15] the effect of molecular structure on nucleation behavior is affected by intrinsic polymer properties, such as viscosity. Figure 2 shows the correlation between the trending

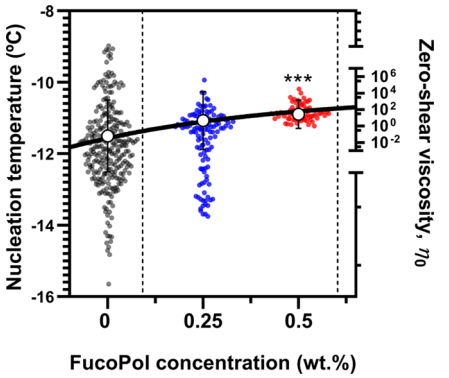

Figure 2. Correlation between polymer zero-shear viscosity, resulting average nucleation temperature, and stochastic range. An increase in concentration results in a statistically significant (p = 0.001) nonlinear increase in water nucleation temperature, which is correlated with the log-viscosity. Zero-shear viscosity data adapted from Torres et al.[20] are fit to a Gompertz exponential growth model (black curve). Swarmplots of collected data points are shown along with error bars showing the standard deviation and the average nucleation temperature (white circle). Data points shown are aggregated from three experiments for each condition. Dashed vertical lines correspond to critical overlap concentrations of 0.09 and 0.6 wt %. The triple asterisks (***) describe a statistically significant difference (p < 0.001) between 0.5% FucoPol and pure water nucleation temperatures.

nucleation temperature as a function of concentration and a Gompertz exponential growth model for viscosity. The high correlation between a concentration-dependent increase in T_n and the log-zero-shear viscosity of FucoPol validates the previous results and suggests an influence of polymeric molecular structure on ice boundary physics.

Swarmplots of nucleation temperatures were also superimposed on the viscosity curve. Note that the relative stochasticity reduction associated with the addition of polymer is always significantly larger than the relative change in average nucleation temperature, and that the slight change in nucleation temperature is positive (see also Table 1). This suggests that the dominating factor affecting stochasticity is distinct from thermodynamic freezing point depression and is likely thus purely kinetic in nature. Future studies should address the precise mechanisms at play, which may reveal ways

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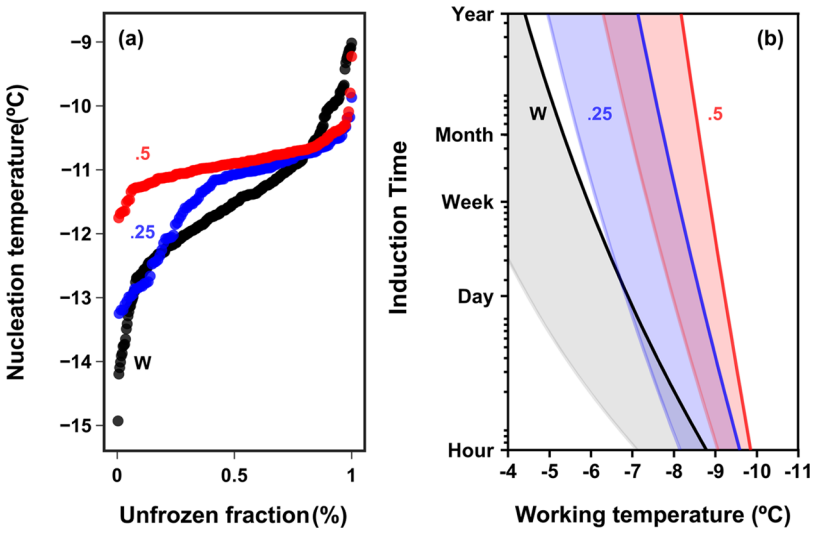

Figure 3. Survivor plot of the unfrozen fraction of water (a) and corresponding Poisson-modeled induction time bands on a practical timescale (b). In panel (a), reduction of stochasticity is evident from a reduced spread of temperature data, characterized by a visible horizontal flattening of the curve. Data points from triplicate independent runs for water (N = 281), 0.25% FucoPol (N = 364), and 0.5% FucoPol (N = 154) are shown aggregated. In panel (b), actionable insight from a realistic timescale perspective can be derived from each nucleation induction time band, calculated from applying eq 4 to separate runs, leaving the working system temperature and solution to choice, depending on the desired applicability.

Table 2. Optimal Poisson-Fit Parameters for a Supercooled System in the Presence of FucoPol

sample	$\gamma\left(\frac{1}{K^n \cdot s}\right)$	n	R^2	τ (−5°C)	τ (−8°C)
water	1.36×10^{-16}	13.0	0.942	∼6 months	∼1 h
	1.04×10^{-13}	8.0			
	7.20×10^{-10}	6.6			
0.25% FucoPol	3.80×10^{-34}	30.4	0.881	>1 year	∼2 weeks
	2.47×10^{-29}	26.8			
	8.60×10^{-21}	18.1			
0.5% FucoPol	4.33×10^{-52}	48.1	0.918	>1 year	>1 year
	8.22×10^{-38}	34.3			
	6.83×10^{-28}	24.7			

in which future polymers could be engineered to specifically elicit this stochasticity reduction effect.

2.3. Effect of Reduced Stochasticity on Induction times and Biopreservation Protocol Design. Ice nucleation is a stochastic process that can be statistically modeled by a Poisson distribution,[13,24] which relates the frequency of nucleation events to the temperature and duration of supercooling (eq 2, Section 5). The experimentally measured survivor curves in Figure 3a (unfrozen fraction *vs* temperature) correspond to the cumulative distribution function of a nonhomogeneous Poisson process (eq 3, Section 5). By assuming a form for an empirical nucleation rate, the survivor function may be fitted to an empirical cumulative distribution function describing the constant cooling rate nucleation process (eq 3, Section 5). The computed nucleation rate parameters for water and FucoPol systems are summarized in Table 2. The method of calculation and fitting are summarized in Section 5 and are detailed rigorously in ref 13.

With knowledge of the nucleation rate, the mean induction time can be computed (eq 4, Section 5). This can physically be understood as the average time necessary for the first ice nucleus to form and kickstart freezing of the whole system or the time period for which the system will remain stable in the supercooled liquid state. Figure 3b shows the induction time for the three tested conditions. The shaded region represents the spread of induction times for the three repeated trials.

This analysis reveals that pure water in an isochoric system is expected to remain in a metastable supercooled state for about 6 months at −5 °C until nucleation initiates but for at most only 5 h at −8 °C. In the presence of FucoPol, water in an isochoric system at −8 °C may remain supercooled 2−3 orders of magnitude longer, lasting 2 weeks with 0.25% FucoPol or more than a year with 0.5% FucoPol without any nucleation.

The reduced stochasticity of ice nucleation when FucoPol is present can be interpreted further. In Figure 3a, the inflections of the survivor curves flatten out to narrower temperature ranges with increasing polymer concentration, which implies that statistical determinism increases and randomness decreases. The overall aspect of the curve is preserved, indicating that the solution still behaves according to the same Poisson distribution, as expected.

Figure 3b shows that the calculated induction time bands for FucoPol concentrations reveal a concentration-dependent increase in the stabilization of the supercooled state of water. With the gradual addition of polymer, the bands steepen, conferring lengthier induction times at a given temperature. Consiglio et al.[13] have shown that DMSO, ethylene glycol, glycerol, and propylene glycol also increase supercooled state

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stability, but do so principally by shifting the curves rather than steepening the curves. At the limit of infinite steepness (i.e., a vertical induction time curve), the system would become fully deterministic. Thus, FucoPol can be interpreted to increase the determinism of the system (and reduce the stochasticity) while only mildly affecting the mean nucleation temperature.

Induction time plots like Figure 3b offer actionable insight into the cryobiologist by acting as a tool to assess the potential stability of a biopreservation scenario under defined conditions of temperature, solute concentration, and duration of preservation. For instance, if a working temperature of −7 °C is chosen, Figure 3b would indicate that an aqueous isochoric solution would last a day without ice. However, by the addition of 0.5% FucoPol, the preservation could be extended to roughly half a year, by conservative estimates.

3. DISCUSSION

Recent isochoric supercooling studies by Consiglio et al.[13] have shown that aqueous solutions of glycerol, ethylene glycol, DMSO, and propylene glycol have resulted in average nucleation temperatures of −20, −20, −21, and −22 °C, respectively, at 5 mol % (equivalent to 15.4−21.2 wt %). Most small-molecule cryoprotectants and antifreeze proteins display depression of both the equilibrium freezing point and the nucleation temperature.[25] However, FucoPol has consistently shown a distinct effect, in which the nucleation temperature of water increases (albeit mildly) with the addition of polymer. FucoPol was selected as the model molecule for this study due to (i) its previously demonstrated cryoprotective properties,[18] (ii) a very low osmolality-to-function ratio that reduces mass transport and is capable of competing with antifreeze proteins,[26] (iii) a high viscosity associated with high molecular weight,[20] and (iv) intrinsic biocompatibility that eliminates cytotoxicity issues.[19] Previous work under isobaric conditions[18] has shown that FucoPol induces the occurrence of crystallization at a higher temperature, consistent with the results herein, and that there is a concomitant 54% reduction of mean crystal size and a distinguishable effect on crystal morphology. Under isochoric conditions, concentrations of 0.25 and 0.5 wt % FucoPol resulted in a slight increase of the mean nucleation temperature to −11.37 ± 0.79 and −10.89 ± 0.33 °C. FucoPol is thus suggested to affect both the process of initial nucleation and subsequent crystal growth.

The most defining aspect of FucoPol's influence in isochoric water nucleation, however, is the statistical confinement of nucleation temperatures. Here, we have achieved a supercooled state of water that is 1.7−3 times more stable in the presence of FucoPol, and with drastically increased nucleation induction times that range from *ca.* 2 weeks to over a year at −8 °C, compared to only 1 h for pure water (a 2−3-fold order of magnitude increase in temporal stability). This finding has several practical consequences, providing both a *safety net* to biopreservation strategies that rely on the isothermal hold of biological matter in a supercooled state and enabling higher statistical confidence during protocol design.

Both this work and that of Consiglio et al.[13] confirm that polymeric and small-molecule cryoprotectants, respectively, contribute to supercooled state stabilization (SSS), as observed by the shift of their corresponding induction bands toward lower temperatures. However, the dominant manner by which SSS occurs differs. Glycerol, DMSO, ethylene glycol, and propylene glycol have a dominant thermodynamic influence on the system. They significantly influence the nucleation temperature, but its stochastic spread and induction band slope remains comparatively unaltered.

In this work, we focused on the low polymer entanglement regime, where FucoPol appears to have a Gompertzian influence on the ice nucleation temperature of water. However, any slight influence on nucleation temperature is overshadowed by a drastic reduction of nucleation temperature spread and stochasticity. According to Torres et al.,[20] zero-shear viscosity varies nonlinearly with FucoPol concentration due to increased polymer entanglement that changes its flow behavior. These different entanglement regimes are bounded by critical overlap concentrations. Since both studied concentrations are within the low-entanglement regime, situated between 0.09 and 0.6%, we can conclude that stochasticity reduction does not arise from any structural transformation.

These observations suggest that kinetic effects largely outweigh any accompanying thermodynamic effects. From the perspective of water molecules, diffusion is hindered with increasing polymer concentration, reducing the degrees of freedom of the system to induce nucleation in spatially distinct regions. It is also possible that this boundary confinement of water molecules follows a predisposed pattern, defined by the hydrodynamic interactions of FucoPol, that originates similar final states of spatial matter disposition, thus resulting in limited, predictable ways for nucleation to occur. These reductions of molecular interactivity, bond directionality, and local entropy may all contribute to the enhanced viscosity-dependent stochasticity control that is observed.

Proteins derived from mechanisms of selective pressure under subzero temperature habitats, such as antifreeze proteins (AFPs) and ice nucleating proteins (INPs), have independently shown traits of ice growth inhibition and promotion of nucleation, respectively.[25] The accumulated knowledge on FucoPol now reveals that this polymer has the modulatory capability to express both—the former hereby demonstrated, and the latter observed before.[18] Moreover, the ability of FucoPol to provide different flow dynamics due to entanglement creates the possibility to obtain different behavioral regimes in ice physics.

In summary, this substantially different behavior—reduced stochasticity—is hereby highlighted, to the best of our knowledge, for the first time in carbohydrate polymers, with practical application in the design of supercooled biopreservation protocols. FucoPol may be employed to enhance supercooling stability at nontoxic osmolalities, leading to more efficient preservation methodologies.

Future studies should compare the effects on stochasticity elicited by FucoPol to those elicited by other common polysaccharides (for example, ficoll and dextran), high-molecular-weight polymers and biopolymers. Future work should also probe the degree to which the observed effects may correlate with the isochoric conditions employed (and whether or how these effects may change under isobaric conditions); should independently experimentally validate the induction times estimated by the Poisson statistical analysis employed herein; and should probe the potential sensitivity of the observed stochasticity reduction effects to changes in cooling rate.

4. CONCLUSIONS

The potential of implementing polymeric additives in isochoric biopreservation strategies is hereby presented. For the first

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time, a fundamental study on understanding isochoric thermodynamics with polymeric substances was performed. FucoPol, a bio-based, fucose-rich polysaccharide, was shown to be a modulatory molecular additive with very low osmolality that regulates stochasticity by confining ice nucleation to a more deterministic range of temperatures and significantly delaying the onset of nucleation. For the cryobiologist, FucoPol may present a potent tool with which to manipulate the kinetics of ice formation during supercooled biopreservation to increase the stability of supercooling and predictability of ice nucleation behavior.

5. MATERIALS AND METHODS

5.1. FucoPol Production. FucoPol was produced by cultivation of *Enterobacter* A47 (DSM 23139) in a 2 L bioreactor (BioStat B-plus, Sartorius, Göttingen, Germany) under a fed-batch mode, using 40 g/L glycerol (Sigma-Aldrich, Germany) as the carbon source, according to the procedure previously described.[27] FucoPol was extracted from the cultivation broth by dia/ultrafiltration, as previously described[28] and characterized in terms of sugar monomers and acyl group compositions, and molecular mass distribution, as previously described.[27] The sample had number (M_n) and weight average molecular (M_w) weights of 1.9×10^6 Da and 3.3×10^6 ($\pm 0.3 \times 10^6$ Da), respectively, with a polydispersity of 1.70.

5.2. Sample Preparation. Aqueous solutions of FucoPol were prepared at concentrations of 0.25 and 0.5 wt % using deionized water (type II, SKU S25293) as the solvent. Dissolution was performed at room temperature with a magnetic stir bar under slow agitation, for at least 30 min. Dissolution was considered complete when the solution had a homogeneous beige appearance. The solution was then degassed in a vacuum chamber (Robinair VacuMaster). Degassing was considered complete when no surfacing air bubbles were visible.

5.3. Isochoric Nucleation Detection (INDe). *5.3.1. Setup and Electronics.* The INDe system, as designed by Consiglio et al.,[13] is composed of three main components. The first component is a Python-based control software that runs on a Raspberry Pi 4B single-board computer (Raspberry Pi Foundation, U.K.). The next component consists of two temperature control assemblies, each composed of a two-stage thermoelectric module (CUI Devices CP60H-2 Series) and a fan-cooled CPU heat sink (Cooler Master). The last component is a thermoelectric module controlled by a PID temperature controller (Opt Lasers, TEC-8A-24V-PID-HC-RS232), which is composed of a full-bridge aluminum strain gauge (3147_0), a PhidgetBridge strain gauge DAQ (1046_0B), a thermocouple Phidget DAQ (TMP1101_0), and a USB VINT Hub (HUB0000_0), all purchased from Phidgets Inc. (CA).

5.3.2. Isochoric Chamber Preparation. The internal wall of the isochoric chamber was first coated with a thin layer of petrolatum (Vaseline, Unilever, U.K.) to avoid ice nucleation at the aluminum surface. A small amount of petrolatum (*ca.* 1 mL) was inserted inside the chamber with a stainless-steel lab spatula and heated up for about 5 min using a standard heat gun. After visible melting of the petrolatum, the chamber was inverted and slowly rotated to allow for uniform coating of the walls and remove excess liquid. The chamber was then left to cool at −4 °C until the chamber was at room temperature to allow the petrolatum coating to solidify. Then, the test sample was slowly dispensed to the lateral wall of the chamber using a syringe carefully as to avoid insertion of undesired air bubbles or pockets. The chamber plug was wrapped with Teflon; its bottom surface also thinly coated with petrolatum and threaded into the chamber until the sealing surfaces contacted each other, after which a digital torque wrench (Yellow Jacket 60648) was used to apply a sealing torque of 40 ft-lbs.

5.3.3. Assay and Data Collection. After loading the sealed isochoric chamber between the two Peltier modules and insulating with a 3D-printed Styrofoam cap, the experiment started by holding the system at 5 °C for 5 min. Then, a full cycle is completed which is composed of the following: cooling at 2 °C/min until nucleation is detected by an increase in strain gauge pressure, then fast warming to 5 °C, and hold for 5 min to guarantee full melting of the system. Then, consecutive cycles were performed and the corresponding nucleation temperatures were recorded as a time series.

5.4. Mathematical Modeling. *5.4.1. Polymer Rheology Fitting.* Zero-shear viscosity data on FucoPol (Figure 2) was adapted from Torres et al.,[20] to which a Gompertz growth model was fit, of the form

$$f(t) = a\mathrm{e}^{-\mathrm{e}^{b-ct}} \tag{1a}$$

where a is the asymptote as $t \to \infty$, b is the displacement along the x-axis, and c is the growth rate. The Gompertz growth model is a time series applicable to measuring viscosity, η, as a function of shear stress, $\dot{\gamma}$, that is applied over time.[29,30] However, zero-shear viscosity, η_0, is a measure of intrinsic viscosity decoupled from a time constraint, thus can be directly associated with a concentration value. Substituting mathematical parameters with rheologically meaningful variables, the fitting equation is as follows

$$\eta_0 = \eta_0^{\max} \cdot \left(\frac{\eta_0^{[C]=0}}{\eta_0^{\max}} \right)^{\mathrm{e}^{-k[C]}} = a \cdot b^{\mathrm{e}^{-k[C]}} \tag{1b}$$

where $[C]$ is FucoPol concentration in weight percentage, a = 6201 mL g^{-1}, $b = 5.49 \times 10^{-6}$, and k = 1.921 (R^2 = 0.9985, df = 14, RMSE = 24.82).

5.4.2. Nucleation Rate. Ice nucleation is a stochastic process where the number of critical size nuclei formed per unit time, the nucleation rate, $J(T)$, may be estimated by the following power law

$$J(T) = \gamma \Delta T^n \tag{2}$$

The constant cooling rate experiments presented in this study can be modeled as a nonhomogeneous Poisson process,[24] whereby the fraction of unfrozen samples at a given temperature, $\chi(T)$, can be related to $J(T)$ as follows

$$\chi(T) = \mathrm{e}^{(-1/\beta \int_T^{T_m} J(T)\,\mathrm{d}T)} = \mathrm{e}^{(-\gamma/\beta \cdot (T-Tm)^{1+n}/1+n)} \tag{3}$$

where T is the temperature, T_m is the equilibrium melting point, β is the cooling rate, and γ and n are empirical fitting parameters. This relation (unfrozen fraction *vs* temperature) corresponds to the survivor functions shown in Figure 3a. Here, T_m = 0 °C due to a proven noncolligative effect of FucoPol on water thermodynamics[18] and β = 2 °C/min. Table 2 shows the computed values of γ and n.

5.4.3. Induction Time. The estimated values for γ and n are better descriptors of the supercooling behavior compared to a single value for the average nucleation temperature.[31] Thus, one can accurately grasp how a thermodynamic system will behave at a defined temperature and solute concentration by plotting induction time graphs (Figure 3b). The mean induction time, τ, describes how long a system will remain in a stable supercooled state before the first supercritical ice nucleus emerges. It is inversely proportional to the nucleation rate, $J(T)$, as follows

$$\tau = J(T)^{-1} \tag{4}$$

5.5. Statistical Analysis. All data was collected as three independent samples (different isochoric chambers) of, at least, N = 50 observations (cycles). Results are shown as mean $\pm$ σ for normal data and median $\pm$ σ for n-modal distributed data. Statistical significance was assessed with a one-way ANOVA, with Sidak's multiple comparisons test. Significance is reported in Figures 1 and 2 using the NEJM p-value threshold nomenclature as follows, from ascending order of significance: 0.12 (ns), 0.033 (*), 0.002 (**), and <0.001 (***), where "ns" means not significant for a 95% confidence interval (CI), where α = 0.05.

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Author Contributions

#First co-authorship. Equally contributed to this work. B.M.G. conceptualized study, performed experiments, data analysis, and wrote manuscript. A.C. conceptualized study, performed experiments, reviewed manuscript, and supervised. M.P.P. conceptualized study, reviewed manuscript, and supervised. B.R. conceptualized study, reviewed manuscript, provided resources, and supervised. F.F. provided resources, reviewed manuscript, and supervised. All authors have read and agreed to the published version of the manuscript.

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Notes

The authors declare no competing financial interest.

The data presented in this study is available on request from first authors or corresponding authors.

REFERENCES

(1) Murphy, D. M.; Koop, T. Review of the Vapour Pressures of Ice and Supercooled Water for Atmospheric Applications. *Q. J. R. Meteorol. Soc.* **2005**, *131*, 1539−1565.

(2) Kang, T.; You, Y.; Jun, S. Supercooling Preservation Technology in Food and Biological Samples: A Review Focused on Electric and Magnetic Field Applications. *Food Sci. Biotechnol.* **2020**, *29*, 303−321.

(3) Huang, H.; Yarmush, M. L.; Usta, O. B. Long-Term Deep-Supercooling of Large-Volume Water and Red Cell Suspensions via Surface Sealing with Immiscible Liquids. *Nat. Commun.* **2018**, *9*, No. 3201.

(4) Charpentier, T. V. J.; Neville, A.; Millner, P.; Hewson, R.; Morina, A. An Investigation of Freezing of Supercooled Water on Anti-Freeze Protein Modified Surfaces. *J. Bionic Eng.* **2013**, *10*, 139−147.

(5) Powell-Palm, M. J.; Charwat, V.; Charrez, B.; Siemons, B.; Healy, K. E.; Rubinsky, B. Isochoric Supercooled Preservation and Revival of Human Cardiac Microtissues. *Commun. Biol.* **2021**, *4*, No. 1118.

(6) Berendsen, T. A.; Bruinsma, B. G.; Puts, C. F.; Saeidi, N.; Usta, O. B.; Uygun, B. E.; Izamis, M. L.; Toner, M.; Yarmush, M. L.; Uygun, K. Supercooling Enables Long-Term Transplantation Survival Following 4 Days of Liver Preservation. *Nat. Med.* **2014**, *20*, 790−793.

(7) de Vries, R. J.; Tessier, S. N.; Banik, P. D.; Nagpal, S.; Cronin, S. E. J.; Ozer, S.; Hafiz, E. O. A.; van Gulik, T. M.; Yarmush, M. L.; Markmann, J. F.; Toner, M.; Yeh, H.; Uygun, K. Supercooling Extends Preservation Time of Human Livers. *Nat. Biotechnol.* **2019**, *37*, 1131−1136.

(8) Rubinsky, B.; Perez, P. A.; Carlson, M. E. The Thermodynamic Principles of Isochoric Cryopreservation. *Cryobiology* **2005**, *50*, 121−138.

(9) Wan, L.; Powell-Palm, M. J.; Clemens, M. G.; Rubinsky, B. Time-Dependent Effects of Pressure during Preservation of Rat Hearts in an Isochoric System at Subfreezing Temperatures. *CryoLetters* **2019**, *40*, 64−70.

(10) Powell-Palm, M. J.; Rubinsky, B.; Sun, W. Freezing Water at Constant Volume and under Confinement. *Commun. Phys.* **2020**, *3*, No. 39.

(11) Consiglio, A.; Ukpai, G.; Rubinsky, B.; Powell-Palm, M. J. Suppression of Cavitation-Induced Nucleation in Systems under Isochoric Confinement. *Phys. Rev. Res.* **2020**, *2*, No. 023350.

(12) Powell-Palm, M. J.; Koh-Bell, A.; Rubinsky, B. Isochoric Conditions Enhance Stability of Metastable Supercooled Water. *Appl. Phys. Lett.* **2020**, *116*, No. 123702.

(13) Consiglio, A.; Lilley, D.; Prasher, R.; Rubinsky, B.; Powell-Palm, M. J. Methods to Stabilize Aqueous Supercooling Identified by Use of an Isochoric Nucleation Detection (INDe) Device. **2022**, *Cryobiology*, in press DOI: 10.1016/j.cryobiol.2022.03.003.

(14) Kimizuka, N.; Suzuki, T. Supercooling Behavior in Aqueous Solutions. *J. Phys. Chem. B* **2007**, *111*, 2268−2273.

(15) Kimizuka, N.; Viriyarattanasak, C.; Suzuki, T. Ice Nucleation and Supercooling Behavior of Polymer Aqueous Solutions. *Cryobiology* **2008**, *56*, 80−87.

(16) Alves, V. D.; Freitas, F.; Torres, C. A. V.; Cruz, M.; Marques, R.; Grandfils, C.; Gonçalves, M. P.; Oliveira, R.; Reis, M. A. M. Rheological and Morphological Characterization of the Culture Broth during Exopolysaccharide Production by *Enterobacter* sp. *Carbohydr. Polym.* **2010**, *81*, 758−764.

(17) Freitas, F.; Alves, V. D.; Gouveia, A. R.; Pinheiro, C.; Torres, C. A. V.; Grandfils, C.; Reis, M. A. M. Controlled Production of Exopolysaccharides from *Enterobacter* A47 as a Function of Carbon Source with Demonstration of Their Film and Emulsifying Abilities. *Appl. Biochem. Biotechnol.* **2014**, *172*, 641−657.

(18) Guerreiro, B. M.; Freitas, F.; Lima, J. C.; Silva, J. C.; Dionísio, M.; Reis, M. A. M. Demonstration of the Cryoprotective Properties of the Fucose-Containing Polysaccharide FucoPol. *Carbohydr. Polym.* **2020**, *245*, No. 116500.

(19) Guerreiro, B. M.; Silva, J. C.; Torres, C. A. V.; Alves, V. D.; Lima, J. C.; Reis, M. A. M.; Freitas, F. Development of a Cryoprotective Formula Based on the Fucose-Containing Polysaccharide FucoPol. *ACS Appl. Bio Mater.* **2021**, *4*, 4800−4808.
(20) Torres, C. A. V.; Ferreira, A. R. V.; Freitas, F.; Reis, M. A. M.; Coelhoso, I.; Sousa, I.; Alves, V. D. Rheological Studies of the Fucose-Rich Exopolysaccharide FucoPol. *Int. J. Biol. Macromol.* **2015**, *79*, 611−617.
(21) Freitas, F.; Alves, V. D.; Torres, C. A. V.; Cruz, M.; Sousa, I.; Melo, M. J.; Ramos, A. M.; Reis, M. A. M. Fucose-Containing Exopolysaccharide Produced by the Newly Isolated *Enterobacter* Strain A47 DSM 23139. *Carbohydr. Polym.* **2011**, *83*, 159−165.
(22) Marshall, C. B.; Chakrabartty, A.; Davies, P. L. Hyperactive Antifreeze Protein from Winter Flounder Is a Very Long Rod-like Dimer of α-Helices. *J. Biol. Chem.* **2005**, *280*, 17920−17929.
(23) Shurer, C. R.; Kuo, J. C.-H.; Roberts, L. M.; Gandhi, J. G.; Colville, M. J.; Enoki, T. A.; Pan, H.; Su, J.; Noble, J. M.; Hollander, M. J.; O'Donnell, J. P.; Yin, R.; Pedram, K.; Möckl, L.; Kourkoutis, L. F.; Moerner, W. E.; Bertozzi, C. R.; Feigenson, G. W.; Reesink, H. L.; Paszek, M. J. Physical Principles of Membrane Shape Regulation by the Glycocalyx. *Cell* **2019**, *177*, 1757−1770.
(24) Lilley, D.; Lau, J.; Dames, C.; Kaur, S.; Prasher, R. Impact of Size and Thermal Gradient on Supercooling of Phase Change Materials for Thermal Energy Storage. *Appl. Energy* **2021**, *290*, No. 116635.
(25) Chang, T.; Zhao, G. Ice Inhibition for Cryopreservation: Materials, Strategies, and Challenges. *Adv. Sci.* **2021**, *8*, No. 2002425.
(26) Fletcher, G. L.; Hew, C. L.; Davies, P. L. Antifreeze Proteins of Teleost Fishes. *Annu. Rev. Physiol.* **2001**, *63*, 359−390.
(27) Torres, C. A. V.; Marques, R.; Antunes, S.; Alves, V. D.; Sousa, I.; Ramos, A. M.; Oliveira, R.; Freitas, F.; Reis, M. A. M. Kinetics of Production and Characterization of the Fucose-Containing Exopolysaccharide from *Enterobacter* A47. *J. Biotechnol.* **2011**, *156*, 261−267.
(28) Ferreira, A. R. V.; Torres, C. A. V.; Freitas, F.; Reis, M. A. M.; Alves, V. D.; Coelhoso, I. M. Biodegradable Films Produced from the Bacterial Polysaccharide FucoPol. *Int. J. Biol. Macromol.* **2014**, *71*, 111−116.
(29) Tjørve, K. M. C.; Tjørve, E. The Use of Gompertz Models in Growth Analyses, and New Gompertz-Model Approach: An Addition to the Unified-Richards Family. *PLoS One* **2017**, *12*, No. e0178691.
(30) Gaber, S. M.; Johansen, A.-G.; Skeie, S. B.; Rukke, E.-O.; Schüller, R. B. Analysis of Rheological Time Series Data. *Annu. Trans. Nord. Rheol. Soc.* **2019**, *27*, 127−131.
(31) Wilson, P. W.; Haymet, A. D. J. The Spread of Nucleation Temperatures of a Sample of Supercooled Liquid Is Independent of the Average Nucleation Temperature. *J. Phys. Chem. B* **2012**, *116*, 13472−13475.

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