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Bacterial Ice Nucleation in Monodisperse D₂O and H₂O-in-Oil Emulsions

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Abstract

Ice nucleation is of fundamental significance in many areas, including atmospheric science, food technology, and cryobiology. In this study, we investigated the ice-nucleation characteristics of picoliter-sized drops consisting of different D₂O and H₂O mixtures with and without the ice-nucleating bacteria *Pseudomonas syringae*. We also studied the effects of commonly used cryoprotectants such as ethylene glycol, propylene glycol, and trehalose on the nucleation characteristics of D₂O and H₂O mixtures. The results show that the median freezing temperature of the suspension containing 1 mg/mL of a lyophilized preparation of *P. syringae* is as high as −4.6 °C for 100% D₂O, compared to −8.9 °C for 100% H₂O. As the D₂O concentration increases every 25% (v/v), the profile of the ice-nucleation kinetics of D₂O + H₂O mixtures containing 1 mg/mL Snomax shifts by about 1 °C, suggesting an ideal mixing behavior of D₂O and H₂O. Furthermore, all of the cryoprotectants investigated in this study are found to depress the freezing phenomenon. Both the homogeneous and heterogeneous freezing temperatures of these aqueous solutions depend on the water activity and are independent of the nature of the solute. These findings enrich our fundamental knowledge of D₂O-related ice nucleation and suggest that the combination of D₂O and ice-nucleating agents could be a potential self-ice-nucleating formulation. The implications of self-nucleation include a higher, precisely controlled ice seeding

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Effect of Snomax concentration on the ice-nucleation kinetics of pure H₂O drops and homogeneous ice-nucleation kinetics in drops made of 10% ethylene glycol, 10% propylene glycol, or 0.5 M trehalose solutions in $x\text{D}_2\text{O} + (1 - x)\text{H}_2\text{O}$ (PDF)

Notes: The authors declare no competing financial interest.

temperature for slow freezing that would significantly improve the viability of many ice-assisted cryopreservation protocols.

Graphical abstract

Introduction

Liquid water becomes thermodynamically metastable below its melting point. Supercooled water can maintain its liquid state until spontaneous nucleation occurs in the presence of a stable ice embryo of critical size. Homogeneous ice nucleation of H₂O typically occurs in the range of -35 to -38 °C, depending on the cooling rate and volume.¹ But heterogeneous ice nucleation is typically observed at much higher temperatures and is induced by ice-nucleating agents (INAs). INAs such as mineral dust (e.g., kaolinite) and bacteria (e.g., *Pseudomonas syringae*) can catalyze ice nucleation at a relatively high temperature because of the microscopic structure of the INA particle surface resembling the ice crystalline structure. Silver iodide particles (1 μ m in diameter), for instance, have the same unit cell dimension as ice crystals and therefore can initiate ice formation at temperatures of as high as -4 °C.² In addition to the geometric match mechanism, the electrofreezing of supercooled water has also been put forward to explain many intriguing observations.³ For example, Gavish et al.⁴ studied ice nucleation on the hydrophobic faces of single amino acid crystals that had no structural match with ice. They proposed that the electric fields within cracks on the substrate helped align the water molecules into icelike clusters en route to crystallization. Without a doubt, ice nucleation is of fundamental significance in many areas such as atmospheric sciences, food sciences, pharmaceuticals, and cryobiology.¹ For example, it has been shown that ice formation in upper tropospheric clouds could strongly impact cloud dynamics, cloud radiative properties, precipitation formation, and cloud chemistry.⁵ In slow-freezing cryopreservation, the controlled ice nucleation, or ice seeding, in the extracellular space is an essential step.⁶ Previous studies on the slow freezing of isolated rat hepatocytes⁷ and human oocytes⁸ have shown that the increase in the ice-seeding temperature reduced the probability of detrimental intracellular ice formation (IIF), thereby enhancing the post-thaw survival rate of cryopreserved cells.

Homogeneous and heterogeneous ice nucleation related to pure H₂O and H₂O-based solutions have been studied by using a variety of methods such as cryomicroscopy,⁹ calorimetry,^{1,10} electrodynamic levitation,¹¹ and the free-fall freezing tube apparatus.^{12,13} A number of studies have reported that the homogeneous ice-nucleation rate of pure H₂O covering a temperature range of −35 to −38 °C was 10⁴–10⁸ cm^{−3}·s^{−1}.^{1,10,11,14} It was also suggested that the absolute temperature accuracy was the single most important experimental parameter determining the uncertainty of the derived ice-nucleation rates in many previous experiments.¹ Moreover, homogeneous and heterogeneous ice nucleation were investigated for various aqueous solutions of (NH₄)₂SO₄, NaCl, glycerol, propylene glycol, and glucose.^{15–17} It has been revealed that the homogeneous ice-nucleation behavior from supercooled aqueous solutions is independent of the nature of the solute but depends only on the water activity of the solution.¹⁵ Heterogeneous ice nucleation was also studied for most of the above-mentioned aqueous solutions containing different ice nuclei (i.e., nonadecanol, silica, silver iodide, and Arizona test dust).¹⁶ It was found that when the median heterogeneous freezing temperatures of different solutions were plotted as a function of water activity of the solution, the profiles for a given type of INA generally converged onto a single curve, irrespective of the nature of the solute.¹⁶ In addition, drop-specific freezing information was revealed by several on-chip drop freezing experiments that also improved the data throughput. Recently, Edd et al.¹⁸ designed a microfluidic device to characterize the solidification of supercooled aqueous solutions of glycerol by trapping monodisperse drops in pockets along a microfluidic channel. This method enables the specific dynamics of ice nucleation to be observed for more than 100 drops in parallel without any loss of specificity. Stan et al.¹⁹ developed a sophisticated microfluidic device that can record the freezing temperatures of tens of thousands of drops within minutes with an accuracy of ±0.4 °C.

In contrast to the abundant data about ice nucleation of H₂O that are available in the literature, the ice-nucleation phenomenon in D₂O has been studied only in a handful of publications.^{10,11,20,21} D₂O is the isotopic substitution of H₂O. Compared to H₂O, D₂O has higher bond energy, and the mean cluster size in D₂O is larger at a certain temperature. This can be reflected by the fact that D₂O has a melting point of 3.8 °C.¹¹ The calorimetric study by Taborek¹⁰ reported the homogeneous ice-nucleation rate in emulsified H₂O and D₂O drops that were suspended in heptane using sorbitan tristearate as a surfactant. Stöckel et al.¹¹ also investigated the homogeneous ice nucleation of H₂O and D₂O in drops levitated in a cooled electrodynamic balance. The results showed that D₂O had a stronger tendency to nucleate, consistent with the higher intermolecular association in liquid D₂O.¹¹ However, to the best of our knowledge, the ice-nucleation characteristics of mixtures of H₂O and D₂O are still unknown. The heterogeneous ice nucleation catalyzed by ice-nucleating agents contained in these D₂O + H₂O mixtures has not yet been investigated. Filling these gaps can help us better understand the isotopic effect on ice nucleation. More importantly, D₂O and proper ice nucleators may comprise a potential self-ice-seeding formulation. Not only can such a formulation provide a higher, precisely controlled ice-seeding temperature, but it can also lead to uniform ice formation that is highly recommended for successful slow-freezing cryopreservation.

In this study, we investigated the ice-nucleation kinetics of monodisperse drops made of different compositions including 100% H₂O and 100% D₂O with and without ice-nucleating agents, respectively, as well as $x\text{D}_2\text{O} + (1 - x)\text{H}_2\text{O}$ mixtures containing INAs (x is the volume fraction of D₂O). The effect of commonly used cryoprotectants (CPAs) on the ice nucleation of D₂O + H₂O mixtures was also explored. Snomax, the freeze-dried form of *Pseudomonas syringae*, was used as a model ice nucleator in this study because it has been used as the ice nucleator in the external medium when slow freezing mouse oocytes.^{22,23}

Materials and Methods

Materials

H₂O used in this study was cell culture grade water (Gibco WFI for Cell Culture, Thermo Fisher Scientific, Waltham, MA, USA). D₂O, ethylene glycol, and propylene glycol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trehalose was obtained from Ferro Pfanstiehl Laboratories (Waukegan, IL, USA). Snomax (Snomax International, Englewood, CO, USA) was used as the model ice nucleator in this study. For the study of homogeneous ice nucleation, we prepared pure D₂O, pure H₂O, 10% (v/v) ethylene glycol (~1.81 M), 10% (v/v) propylene glycol (~1.39 M), and 0.5 M trehalose solutions made in 100% D₂O, 50% D₂O + 50% H₂O, and 100% H₂O, respectively. Note that the volume fraction (x) of D₂O is based on the solvent only. For the investigation of heterogeneous ice nucleation, a certain amount of Snomax was suspended in mixtures of D₂O and H₂O with the volume fractions of D₂O being 0, 0.25, 0.5, 0.75, and 1 to reach a Snomax concentration of 1 mg/mL. There are on average 23 active ice-nucleating sites in each drop. (See the calculation details in the Supporting Information.) The CPA effect was also studied by preparing 10% ethylene glycol, 10% propylene glycol, and 0.5 M trehalose in 100% D₂O, 50% D₂O + 50% H₂O, and 100% H₂O, respectively, all containing 1 mg/mL Snomax. Snomax was vortex mixed at 3200 rpm for 5 min to achieve a homogeneous suspension.

Experimental Setup

Monodisperse drops of identical composition were generated using the flow-focusing technique on a microfluidic device as shown in Figure 1a. The drops were generated by two continuous streams of immiscible hydrofluoroether oil (Novec 7500; 3M, St. Paul, MN) phases pinching off the aqueous phase with $q_a/q_o = 0.21$. (q_a/q_o is the flow rate ratio of the aqueous phase to the oil phase; see Figure 1a.) The microfluidic device was manufactured with standard soft lithography techniques. Specifically, channels in polydimethylsiloxane (PDMS) were bonded via oxygen plasma to a microscope glass slide. The generated drops have an average diameter of ~35 μm . Despite the fact that different aqueous solutions under investigation may have different viscosities, drops of different compositions have a diameter variation of less than $\pm 6\%$. Six microliters of the drop-in-oil suspension was pipetted onto a no. 2 glass coverslip (0.17–0.25 mm thick) such that a monolayer of drops was formed without contacting the coverslip given that the density of the aqueous drops is smaller than that of the oil (1.614 g/cm³). The oil phase contains 1.5% surfactant Pico-Surf 1 (Dolomite Microfluidics, Royston, U.K.) to stabilize the drop surface and avoid coalescence. Under such conditions, the surface-dependent ice nucleation becomes negligible and the volume-

dependent nucleation is dominant.¹ In this study, we used disposable glass coverslips to avoid potential contamination from previous samples, and no significant difference was found between glass and quartz coverslips in terms of the ice-nucleation kinetics at a cooling rate of 1 °C/min. A second glass coverslip was mounted on top of the sample to prevent the evaporation of the aqueous phase. Meanwhile, a silicone O-ring (1.78 mm high) was placed between the two coverslips with grease sealing to prevent the top coverslip from contacting the drops. The sandwiched sample (as illustrated in Figure 1b) was then placed on the silver block of an FDCS196 cryostage (Linkam Scientific Instruments Ltd., London, U.K.), the temperature of which was controlled by the TMS 94 temperature controller to an accuracy of ± 0.1 °C.²⁴ A PixeLINK PL-A662 camera (PixeLINK, Ottawa, Canada) was used to record the ice-nucleation kinetics. During the freezing experiment, the temperature was first cooled at 20 °C/min to about 5 °C above the temperature at which the first drop in the field was observed to freeze. After thermal stabilization for some time, a cooling rate of 1 °C/min was applied until all drops in the field were frozen. The number of unfrozen drops in the field was counted as a function of temperature in an increment of 0.1 °C. The ice-nucleation event in the drops can be readily identified by the sudden appearance of structure in drops as shown in Figure 1c–e or flashing in drops depending on the composition.¹⁴ Upon ice nucleation, the darkness of the ice-nucleated drops was observed to change continuously from initially dark black to slight darkness. The different darkness of ice-nucleated drops seen in Figure 1e reflects the real-time freezing state within the drops. The ice nucleation is followed by rapid ice growth until it becomes diffusion-limited by the concentrated solute and/or thermally limited by the sudden release of latent heat into the surrounding oil phase. Therefore, the ice-nucleated drops flash black in a bright field because the ice crystals can scatter light. Then the growth of the ice grains can reduce the scattering surfaces over time and result in lighter darkness. Three replicates were performed for each composition, with ~200 drops observed in each. We simulated the heat diffusion in the sandwiched sample system using multiphysics modeling software COMSOL (COMSOL Inc., Burlington, MA, USA). It was found that the temperature on the oil surface was ~0.5 °C higher than the temperature on the silver block surface at –50 °C, indicating a thermal lag of less than 0.5 °C throughout our measurement (data not shown here).

Homogeneous Ice-Nucleation Rate

The supercooled water can maintain its metastable liquid state until a nucleation event occurs in the presence of a stable ice embryo. It is generally accepted that homogeneous ice nucleation, and hence the formation of a critical ice cluster, is a stochastic process.¹ Because our drops have radii larger than the upper limit for surface-dependent ice nucleation (i.e., 4–5 μm),^{25,26} the homogeneous ice nucleation observed in our study should be volume-dependent only. The homogeneous ice-nucleation rate coefficient (J_{hom} , in $\text{cm}^{-3}\cdot\text{s}^{-1}$) under isothermal conditions can be described by the following equation⁹

$$N_2 = N_1 \exp(-J_{\text{hom}} V \Delta t) \quad (1)$$

where the number of unfrozen drops decreases from N_1 to N_2 within a time interval of $\Delta t (= t_2 - t_1)$ and V is the volume of the drops. To obtain J_{hom} as a function of temperature during a cooling ramp experiment, eq 1 has to be applied to a small increment of time (Δt) over which the change in temperature (ΔT) is also small.¹⁴ In this study, ΔT was 0.1 °C and the corresponding Δt was 6 s. In other words, N_1 is the number of unfrozen drops at T , and N_2 is the number of unfrozen drops at $T - 0.1$ °C.

Heterogeneous Ice-Nucleation Rate

There have been several mathematical models to describe heterogeneous ice nucleation, including the stochastic model, singular model, and modified singular model.⁹ The stochastic model is extended from the homogeneous ice-nucleation theory and is therefore time-dependent. The singular model prioritizes the INA-to-INA variability among drops and neglects the time dependence, which is true for atmospheric ice nucleation where the ice-nucleating particles are most likely of different types and concentrations within any given drop. The modified singular model was developed by Vali²⁷ to incorporate the cooling-rate dependence of J_{het} (i.e., the time dependence) with the singular model. Given that Snomax was used as the only ice nucleator and prepared at a single concentration in this study, we assumed that the freezing probability is the same for all drops in a distribution. Such an assumption agrees well with previous findings that heterogeneous ice nucleation activated by a single type of ice nucleator such as kaolinite⁹ and silver iodide²⁸ is consistent with the stochastic model. The single-component stochastic model can be described by the following equation

$$N_2 = N_1 \exp(-J_{\text{het}} \sigma \Delta t) \quad (2)$$

where J_{het} (in $\text{cm}^{-2}\cdot\text{s}^{-1}$) is the heterogeneous ice-nucleation rate coefficient and σ is the nucleating active-surface area that each drop contains. σ was calculated on the basis of the concentration of Snomax in the drops and the surface area per milligram of Snomax (i.e., 44 $\text{cm}^2\cdot\text{mg}^{-1}$).²⁹

Results and Discussion

Figure 2 shows the homogeneous ice-nucleation characteristics in pure H_2O and pure D_2O as well as the heterogeneous nucleation characteristics in 100% H_2O with 1 mg/mL Snomax and 100% D_2O with 1 mg/mL Snomax. As shown in Figure 2a,b, the temperature range over which the fraction of frozen drops increases from 5 to 95% is only about 1 °C for all four compositions when a cooling rate of 1 °C/min is applied. The median homogeneous freezing temperatures of pure H_2O and pure D_2O are, on average, -37.4 and -32.1 °C, respectively. In other words, to freeze 50% of the drops, the average extent of supercooling has to be 37.4 °C for pure H_2O but 35.9 °C for pure D_2O , which is 1.5 °C smaller. The superior ice-nucleation performance of D_2O is mainly due to the stronger hydrogen bond O–D...O compared to O–H...O.³⁰ However, in the presence of Snomax (1 mg/mL), the median heterogeneous freezing temperature is about -8.9 °C or -4.6 °C for potent ice nucleators suspended in H_2O or D_2O , respectively. The corresponding difference in the extent

supercooling is 0.5 °C, which demonstrates the stronger ice-nucleation ability of D₂O even in the presence of foreign ice nucleators. Compared to pure H₂O, the median freezing temperature can be enhanced by 32.8 °C by substituting D₂O for H₂O and adding 1 mg/mL Snomax. Figure 2b shows the ice-nucleation probability derived from profiles of the fraction of frozen drops. It is clear that the median freezing temperature for each composition matches the peak of the corresponding Gaussian fit.

The homogeneous ice-nucleation rate coefficient was calculated using eq 1 as a function of temperature as shown in Figure 2c. The general trend is evident that the logarithm of J_{hom} (i.e., $\log_{10} J_{\text{hom}}$) increases linearly as the temperature decreases. Both the homogeneous and heterogeneous nucleation rates can increase by about 2 orders of magnitude per degree drop in temperature. Similarly, Pruppacher³¹ observed a 1.2 to 2 orders of magnitude increase per degree when freezing pure H₂O from −34 to −38 °C. According to our results, the relationship between $\log_{10} J_{\text{hom}}$ and T , which is the extent of supercooling ($T - T_m$), can be described as $\log_{10} J_{\text{hom}} = (-1.62 \pm 0.06) T - (54.5 \pm 2.3)$ for pure H₂O and $\log_{10} J_{\text{hom}} = (-1.86 \pm 0.07) T - (60.4 \pm 2.5)$ for pure D₂O. To reach the same nucleation rate, for example, $10^6 \text{ cm}^{-3} \cdot \text{s}^{-1}$, D₂O drops need to be supercooled about 1.4° less than their H₂O counterparts, which is consistent with the stronger intermolecular association in liquid D₂O.¹¹ As seen in Figure 2c, our results of J_{hom} coincide with those reported by Taborek¹⁰ and are 0.3–0.5° offset from the results given by Stöckel et al.¹¹ The results by Stockel et al.¹¹ were suggested to be very precise because each drop was levitated in an electrodynamic trap.¹⁹ In fact, because the absolute temperature is regarded as the most important factor affecting the uncertainty of the derived ice-nucleation rate,¹ our results are actually very close to the results reported by Stöckel et al.¹¹ by considering the thermal lag estimated for our sample setup.

The heterogeneous ice-nucleation rate coefficient was obtained from eq 2 and plotted as a function of temperature in Figure 2d. Similar to J_{hom} , the logarithm of J_{het} ($\log_{10} J_{\text{het}}$) increases linearly as the temperature decreases. To reach the same nucleation rate, for example, $10^4 \text{ cm}^{-2} \cdot \text{s}^{-1}$, the D₂O drops need to be supercooled by 7.8 °C while the H₂O drops have to be supercooled by 8.5 °C if one assumes that the suspended ice-nucleating particles do not affect the melting point of D₂O or H₂O.

We further investigated the concentration effect of D₂O on the heterogeneous ice-nucleation kinetics. Figure 3 illustrates the heterogeneous ice-nucleation characteristics activated by Snomax for suspension in the mixture of $x\text{D}_2\text{O} + (1 - x)\text{H}_2\text{O}$. Overall, the profiles of ice-nucleation kinetics are found to be offset by about 1° as the D₂O concentration increases every 25% (v/v) from 0 to 100%. Such linear relationships can also be reflected in terms of the median freezing temperature, as will be shown later in Figure 5a. This phenomenon actually suggests an ideal mixing character of D₂O and H₂O in terms of ice-nucleation kinetics. Similarly, some thermodynamic properties of $x\text{D}_2\text{O} + (1 - x)\text{H}_2\text{O}$ mixtures, such as the vapor pressure, melting point, and boiling point, were also found to abide by the ideal mixing assumption within the experimental error.³² The ideal mixing rule also holds for the ice-nucleation rate profiles as shown in Figure 3b. We also calculated n_s (i.e., the number of nucleating active sites per unit surface area at a given temperature) for 1 mg/mL Snomax suspended in 100% H₂O based on the singular model as shown in the inset of Figure 3b,

which agrees well with the results reported by Budke and Koop²⁹ in which they investigated slightly different drop sizes and Snomax concentrations for pure H₂O and D₂O.

Water-soluble compounds are shown to have different effects on the ice nucleation of aqueous solutions. The homogeneous freezing temperatures of aqueous salt solutions (e.g., NaCl, MgCl₂, LiCl, Ca(NO₃)₂, etc.) were shown to decrease with increasing salt concentration.¹⁵ It is the same case for the heterogeneous freezing temperature of these solutions containing different ice nucleators.¹⁶ Also, a positive linear relationship between freezing-point depression and the reduction of nucleation temperatures was found for aqueous sucrose solutions both with and without freeze-dried *P. syringae*.³³ However, some macromolecules that are dissolved in water but have a critical ice embryo of comparable size can efficiently induce ice nucleation.³⁴ These macromolecules can be diverse in chemical structure and origin and initiate ice formation either as singular molecules or in an aggregated form.³⁴ In this study, we investigated the effects of several commonly used cryoprotectants such as polyols and disaccharide on the ice nucleation of D₂O + H₂O mixtures. As shown in Figure 4, compared to the heterogeneous freezing of Snomax samples without CPA, 10% ethylene glycol depresses the ice-nucleation phenomenon the most whereas 0.5 M trehalose has the least effect. This is consistent with the melting-point depression of these CPA concentrations. The median freezing temperatures are shown in Figure 5a. For example, for CPAs dissolved in 100% D₂O, the median freezing temperature for the ethylene glycol-containing composition is 4.4° lower than $T_{f,het}$ of 1 mg/mL Snomax suspended in D₂O without CPA. In comparison, $T_{f,het}$ for 0.5 M trehalose dissolved in 100% D₂O containing Snomax is -6.0 °C, representing only a 1.4° depression of the freezing point. In general, all of the CPA molecules investigated in this study depress the freezing point perhaps because they are small molecules of sizes much smaller than the critical ice embryo. We also investigated the homogeneous ice nucleation of these aqueous CPA solutions without Snomax. The resulting trends are similar to their corresponding heterogeneous counterparts (Figure S2 in the Supporting Information). Figure 5a shows the median freezing temperatures of these homogeneous phenomena.

Previous studies by Koop and co-workers proposed that the ice-nucleation behavior from supercooled aqueous solutions was independent of the solute nature and depended only on the water activity of the solution.^{15,16} Given the rarity of information available in the literature about the water activity related to D₂O, we evaluated only the role of water activity in the ice nucleation of aqueous CPA solutions made in H₂O. Several equations have been developed to describe the relationship between a_w and T_m , but the difference between them is insignificant within the range of $a_w = 0.85-1$. A thermodynamic model was used in this study as described by eq 3³⁵

$$\ln a_w = \frac{T_m - T_m^0}{(R/\Delta \bar{s}_f) T_m} \quad (3)$$

where T_m^0 is the melting point of pure H₂O (273.15 K), R is the universal gas constant, and $\bar{s}_f$ is the standard molar entropy change for the fusion of ice (22 J/(mol•K)). On the basis of

eq 3, the $T_m(a_w)$ curve for aqueous solutions made in 100% H₂O is shown in Figure 5b. The water activity of aqueous 10% ethylene glycol, 10% propylene glycol, and 0.5 M trehalose solutions can be calculated on the basis of the equation $a_w = \exp(-\pi M_w)$, where M_w is the molar mass of H₂O and π is the osmolality of these solutions, which can be estimated on the basis of the osmotic virial equation $\pi = m + Bm^2$.³⁵ The values of B for ethylene glycol, propylene glycol, and trehalose are 0.02, 0.039, and 0, respectively.³⁴ We assume that the molality (m) is interchangeable with the molarity because of the relatively low concentration. In Figure 5b, we plotted both homogeneous and heterogeneous median freezing temperatures ($T_{f, \text{hom}}$ and $T_{f, \text{het}}$, respectively) as scattered points corresponding to the water activity of each solution. The $T_m(a_w)$ curve was shifted by a_w (a_w is 0.31 for homogeneous and 0.09 for heterogeneous, respectively). The shifted curves are found to match well with the trends followed by the scattered points of $T_{f, \text{hom}}$ and $T_{f, \text{het}}$, respectively. We obtained a_w by $(a_w(T_m) - a_w(T_f))$ for pure H₂O. Overall, the information in Figure 5b demonstrates that for CPAs investigated in this study both homogeneous and heterogeneous freezing behaviors from supercooled aqueous solutions depend on the water activity and are independent of the solute nature.

The ice seeding temperature (T_{seed}) has been an important parameter in the field of slow-freezing cryopreservation. The importance of a precisely controlled seeding temperature for reliable cryopreservation protocols has been repetitively emphasized. Harris et al.⁷ studied the kinetics of intracellular ice formation in isolated rat hepatocytes when cooled at different rates. In general, for the cooling rates studied (50–150 °C/min), lower seeding temperatures resulted in a higher maximum cumulative incidence for IIF. For a cooling rate of 50 °C/min, the maximum cumulative incidence of IIF increased from 0% at $T_{\text{seed}} = -2.0$ °C to 100% with only a 1.7° drop ($T_{\text{seed}} = -3.7$ °C). Similarly, Trad et al.⁸ investigated the effect of T_{seed} on intracellular ice formation and the survival of human oocytes when cooled at 0.2 °C/min. It was found that when using 1.5 M propylene glycol and seeding temperatures of -8.0, -6.0, and -4.5 °C, the incidences of IIF were 78, 33, and 0% and the 24 h post-thaw survival rates were 32, 56, and 93%, respectively. This implies that by increasing the ice seeding temperature from -8.0 to -4.5 °C (i.e., a 3.5° decrease in the supercooling extent) the survival rate can be increased nearly 3-fold.⁸ It has been demonstrated here that the extent of supercooling can decrease by 1.5 and 0.5° for homogeneous and heterogeneous ice nucleation, respectively, by completely substituting D₂O for H₂O in the absence of CPA. The supercooling depression of aqueous CPA solutions could be numerically similar given the ideal mixing behavior of D₂O and H₂O. Therefore, the composition containing D₂O and proper ice-nucleating agents is proposed to be a self-ice-seeding formulation that can lead to a lower extent of intracellular supercooling. The increase in the ice-nucleation temperature also indicates that the self-ice-seeding formulation can initiate the osmotic efflux of intracellular water earlier, compared to other H₂O-based formulations when being subjected to the same cooling rate. Thus, the preserved cells will dehydrate more at a given subfreezing temperature. Both less supercooling and more dehydration can reduce the probability of intra-cellular ice formation, which can positively affect the post-thaw survival rate after slow freezing. In addition, by distributing ice nucleators throughout the preserved sample, extracellular ice nucleation can occur within a range of 1° and without the need for the cumbersome mechanical ice-seeding technique.⁶ Therefore, one can improve the

homogeneity of the thermodynamic status of biological cells suspended throughout the preserved system. Because much larger sample volumes are generally used in the laboratory for slow-freezing applications, the temperature at which ice nucleation can actually happen could be even higher than the results determined for the picoliter volumes of drops investigated in this study.

With regard to the effect of drop volumes on T_f , it was shown that the change in the drop diameter from 172 to 409 μm did not result in a statistically significant increase in the median freezing temperature of H_2O drops containing kerosene-burner exhaust particles.³⁶ Similarly, the homogeneous freezing temperature of pure H_2O drops increased only about 2° by increasing the drop diameter from tens of micrometers to hundreds of micrometers.³¹ However, when the increase in the drop diameter is dramatic, for example, from 10^{-6} to 10 cm, the $T_{f,\text{hom}}$ of pure H_2O drops increased from -44 to -32°C .³¹ For heterogeneous ice nucleation, it has been proposed that with the same concentration of ice-nucleating agents a much larger drop may simply contain more ice-catalyzing surfaces and therefore freeze at higher temperatures.⁹ But further studies of substantially larger samples (e.g., on the order of milliliters) formulated with Snomax in D_2O are necessary to demonstrate the potentially significant increase in the ice-nucleation temperature compared to that observed for picoliter-sized drops in this study. Furthermore, the benefits and toxicity of D_2O to biologics are still under debate.³⁷ The solvent isotope effects and deuterium isotope effects have been shown to be D_2O -concentration-dependent.³⁸ Thus, the toxicity of different concentrations of D_2O to cryopreserved biologics has to be explored in more detail in the future.

Conclusions

We investigated the ice-nucleation kinetics of drops of multiple compositions including D_2O and H_2O mixtures containing Snomax, the freeze-dried form of ice-nucleating bacteria *P. syringae*. The effects of several cryoprotectants on the freezing characteristics of D_2O and H_2O mixtures were also studied. The results show that the median freezing temperature of 1 mg/mL Snomax suspended in D_2O is as high as -4.6°C , compared to -37.4°C of pure H_2O . By completely substituting D_2O for H_2O in the absence of CPA, the extent of supercooling can be decreased by 1.5 and 0.5° for homogeneous and heterogeneous ice nucleation, respectively. Also, the profiles of ice-nucleation kinetics of D_2O and H_2O mixtures containing Snomax are found to be offset by 1° toward a higher temperature as the D_2O concentration increases every 25% (v/v), suggesting an ideal mixing behavior of D_2O and H_2O . In addition, the cryoprotectants investigated in this study depress the freezing temperature. Both homogeneous and heterogeneous freezing behaviors from these aqueous solutions depend only on the water activity. The findings in this study suggest that compositions containing D_2O and potent ice-nucleating agents can potentially be an excellent self-ice-nucleating formulation for slow-freezing cryopreservation, providing a higher, precisely controlled ice-seeding temperature with a lower likelihood of intracellular ice formation and improved post-thaw viability of preserved biologics including cells, tissues, and even organs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Flow focusing: drops were generated by two continuous oil phases pinching off the aqueous phase. (Average drop size: $35\ \mu\text{m}$ in diameter). (b) Experimental setup of the cryostage and a zoomed-in view of the sandwiched sample. (c) Drops containing 0.5 M trehalose and 1 mg/mL Snomax in 100% H_2O at $5\ ^\circ\text{C}$. (d) Drops containing 0.5 M trehalose and 1 mg/mL Snomax in 100% H_2O at $-10\ ^\circ\text{C}$ when $\sim 10\%$ of drops were frozen. (e) Drops containing 0.5 M trehalose and 1 mg/mL Snomax in 100% H_2O at $-15\ ^\circ\text{C}$ when all of the drops were frozen (scale bars in c–e: $100\ \mu\text{m}$).

Figure 2.

(a) Fraction of frozen drops as a function of temperature in 100% H₂O, 100% D₂O, 100% H₂O with 1 mg/mL Snomax, and 100% D₂O with 1 mg/mL Snomax. (b) Corresponding ice-nucleation probability. Gray lines represent the Gaussian fits. (c) Homogeneous ice-nucleation rate coefficient (J_{hom}) of 100% H₂O and 100% D₂O. (The solid lines represent the corresponding literature values.) (d) Heterogeneous ice-nucleation rate coefficient (J_{het}) of 100% H₂O with 1 mg/mL Snomax and 100% D₂O with 1 mg/mL Snomax.

Figure 3.

(a) Heterogeneous ice-nucleation kinetics of drops containing 1 mg/mL Snomax suspended in $x D_2O + (1 - x) H_2O$. (x is the volume fraction of D_2O based on the solvent.) (b)

Corresponding heterogeneous ice-nucleation rate coefficients. (Inset) The heterogeneous freezing was evaluated on the basis of n_s , the number of active surface sites per unit area at a given T .

Figure 4.

Heterogeneous ice-nucleation kinetics (a, c, and e) and nucleation rate coefficients (b, d, and f) of drops containing 1 mg/mL Snomax and 10% (v/v) ethylene glycol (a and b), 10% (v/v) propylene glycol (c and d), or 0.5 M trehalose (e and f) in $x\text{D}_2\text{O} + (1-x)\text{H}_2\text{O}$.

Figure 5.

(a) Median freezing temperature (T_f) of drops containing 1 mg/mL Snomax and no CPA (gray), 10% (v/v) ethylene glycol (blue), 10% (v/v) propylene glycol (red), or 0.5 M trehalose (black) in $x\text{D}_2\text{O} + (1-x)\text{H}_2\text{O}$. (b) Melting point and median freezing temperature ($T_{f,hom}$ and $T_{f,het}$) of drops made of H_2O plotted as a function of water activity. Squares, no CPA; circles, 0.5 M trehalose; triangles, 10% propylene glycol; and crosses, 10% ethylene glycol.