Controlled Ice Nucleation Using Freeze-Dried *Pseudomonas syringae* Encapsulated in Alginate Beads

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Abstract

The control of ice nucleation is of fundamental significance in many process technologies related to food and pharmaceutical science and cryobiology. Mechanical perturbation, electromagnetic fields and ice-nucleating agents (INAs) have been known to induce ice nucleation in a controlled manner. But these ice-nucleating methods may suffer from cumbersome manual operations, safety concerns of external fields, and biocompatibility and recovery issues of INA particles, especially when used in living systems. Given the automatic ice-seeding nature of INAs, a promising solution to overcome some of the above limitations is to engineer a biocomposite that accommodates the INA particles but minimizes their interactions with biologics, as well as enabling the recovery of used particles. In this study, freeze-dried *Pseudomonas syringae*, a model ice-nucleating agent, was encapsulated into microliter-sized alginate beads. We evaluated the performance of the bacterial hydrogel beads to initiate ice nucleation in water and aqueous glycerol solution by investigating factors including the size and number of the beads and the local concentration of INA particles. In the aqueous sample of a fixed volume, the total mass of the INA particles (m) was found to be the governing parameter that is solely responsible for determining the ice nucleation performance of the bacterial hydrogel beads. The freezing temperature has a strong positive linear correlation with $\log_{10}m$. The findings in this study provide an effective, predictable approach to control ice nucleation, which can improve the outcome and standardization of many ice-assisted process technologies.

Keywords: Heterogeneous ice nucleation; Ice-nucleating bacteria; *Pseudomonas syringae*; Encapsulation; Hydrogel; Cryopreservation.

Introduction

Without external nuclei, water and aqueous solutions will maintain a supercooled liquid state well below the melting point until homogeneous ice nucleation occurs. For instance, homogeneous ice nucleation of pure water typically occurs in the range of -35 to -38°C , depending on the cooling rate and the sample volume [21]. However, the initiation of ice nucleation at a relatively higher subzero temperature offers many benefits for a wide range of process technologies. For example, during the freeze-drying of food products and pharmaceuticals, controlled ice nucleation with suppressed supercooling can significantly decrease the primary drying time and improve food texturing and product uniformity [6, 7, 16]. In slow-freezing cryopreservation of isolated rat hepatocytes [9] and human oocytes [22], a higher ice nucleation temperature in the extracellular space also reduces the probability of detrimental intracellular ice formation (IIF). As a result, a number of methods have been developed to minimize the supercooling extent (i.e., the difference between the ice nucleation temperature and the

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melting point), thereby initiating ice nucleation at high subzero temperatures [16]. For example, mechanical perturbation such as shaking and tapping [10] and irradiation of power ultrasound [13, 26] can be very effective at inducing ice nucleation in the supercooled liquid [16]. It has also been a common practice in cryopreservation to induce ice nucleation in the sample by manually generating a cold spot on the outside of the closed cryogenic container, known as “ice seeding”. In addition, magnetic, electric, and electromagnetic fields have been shown to trigger ice nucleation and affect the size of ice crystals and the nucleation rate [2, 18, 20]. A comprehensive review of the approaches to control ice nucleation can be found in the reference [16].

Nevertheless, the current ice-nucleating methods suffer from a number of disadvantages such as the lack of consistency and standardization in the case of manual operations [16], the cumbersome process for a large number of samples [24], and the necessity of electric and/or magnetic fields. To overcome some of these limitations, ice-nucleating agents (INAs) have been extensively explored, such as mineral dust kaolinite [15, 17], silver iodide [3, 23], and bacterium *Pseudomonas syringae* [19, 24]. It is generally suggested that INAs induce ice nucleation heterogeneously because the microscopic structure of the INA particle surface resembles the ice crystalline structure [24]. For example, silver iodide particles (typically 1 μm in diameter) have the same unit cell dimension as ice crystals, being able to induce the freezing of pure water at a temperature up to -4°C [3, 23]. Due to the highly hydrophilic surface and small size of carbon nanoparticles, they have great potential for enhancing the ice nucleation temperature [8, 25]. For instance, it was reported that the ice nucleation temperature of a 40% ethylene glycol solution in the presence of 0.2% diamond nanoparticles was -49.2°C , about 13 degrees higher than the 40% ethylene glycol alone when subjected to a $1^{\circ}\text{C}/\text{min}$ cooling ramp [8]. Several bacterial strains also serve as biological ice nucleators. The best characterized of these is *Pseudomonas syringae*, a widely distributed bacterial epiphyte of plants [1]. Our previous study has systematically investigated the ice nucleation kinetics of picoliter-sized drops of heavy water (D_2O) and light water (H_2O) mixtures with freeze-dried *P. syringae* [24]. The results showed that the median freezing temperature of the 1 mg/ml *P. syringae* suspension prepared in 100% D_2O was as high as -4.6°C [24]. A recent interface-specific sum frequency generation (SFG) spectroscopy and molecular dynamics simulation study revealed that the hydrogen bonds formed at the water-bacterium interface imposed structural ordering on the adjacent water network facilitating ice nucleation [19].

One of the major advantages of INAs is their self-ice-seeding capability, that is heterogeneously initiating ice formation at their characteristic temperatures without manual or instrumental interruptions [24]. But the use of INAs in biological systems may introduce concerns about their biocompatibility, degradability, toxicity, recovery and ease of use. It is therefore desirable to minimize the direct contact between foreign ice nuclei and biological components in order to avoid the potential toxicity and contamination [1]. In addition, the recovery of used INA particles may also facilitate the downstream processing. Hydrogel encapsulation may provide a promising path to realize these ends. The concept of encapsulating ice-nucleating agents within a hydrogel matrix was adopted by Zamecnik et al. [27] to improve the cryopreservation of plant materials *in vitro*. Their results showed that 10 μl of distilled water containing hydrogel beads embedded with *P. syringae* froze at -2 to -3°C , an ice nucleation activity close to that of a free *P. syringae* suspension with 10^8 - 10^4 cells/ml. However, it was found difficult to count the number of bacterial cells in the hydrogel beads [27], which led to the lack of quantitative characterization. Another drawback of producing bacterial hydrogel beads is the inconvenience of maintaining the bacterial culture [1].

In this study, we encapsulated Snomax particles into microliter-sized alginate beads. Snomax is the freeze-dried form of *P. syringae*, which is readily available and typically used to facilitate snowmaking

for ski resorts. The ice nucleation performance of the Snomax-laden hydrogel beads was characterized by evaluating factors such as the size and number of the bacterial hydrogel beads and the local Snomax concentration c_{local} (i.e., the Snomax concentration within the hydrogel matrix). Pure water and aqueous 10% glycerol solution were chosen as the freezing media. It was found that the total mass of Snomax particles was a governing parameter to determine the ice nucleation ability of the Snomax-laden hydrogel beads in the aqueous sample of a fixed volume.

Materials and Methods

A certain amount of alginic acid sodium salt (Sigma-Aldrich, St. Louis, MO) was dissolved in cell culture grade water (Gibco WFI for Cell Culture, Thermo Fisher Scientific, Waltham, MA) to prepare a stock solution of 4% (w/v) alginate. The Snomax (Snomax International, Englewood, CO) suspension (0.2-30 mg/ml) prepared in WFI water was mixed with an equal volume of the stock alginate solution, yielding a final local concentration of 0.1-15 mg/ml Snomax and 2% (w/v) alginate.

The Snomax-containing alginate solution was loaded into a 3 ml syringe (BD Biosciences, San Jose, CA) which was then mounted vertically onto a GenieTouch Syringe Pump (Kent Scientific Corporation, Torrington, CT). As illustrated in Figure 1A, the Snomax-containing alginate solution was then dropped into a 1 % (w/v) CaCl_2 hardening bath as the syringe pump applied a constant flow rate of 100 $\mu\text{l}/\text{min}$. The 30 gauge and 18 gauge flat needles (SAI Infusion Technologies, Libertyville, IL) were used, which have an inner diameter of 0.159 and 0.838 mm, respectively. The alginate beads were gelled in the CaCl_2 bath for 30 min with frequent agitation followed by triple washing with WFI water to remove excessive CaCl_2 and the Snomax particles on the bead surface. The prepared hydrogel beads were added into 0.5 ml WFI water contained in a 2 ml cryogenic vial (Corning Glass Works, Corning, NY). For the samples made of 10% glycerol, the alginate beads were incubated in a stock solution of 10% glycerol at room temperature for 20 hours on a HulaMixer sample mixer (Life Technologies, Carlsbad, CA) before they were added into 0.5 ml fresh 10% glycerol solution contained in the cryovial. It was showed previously that the diffusion of glycerol had reached equilibrium across the 1-5% (w/v) alginate beads after 20 hours [5].

As illustrated in Figure 1B, each aqueous sample contained 1, 5 or 10 beads of a given local Snomax concentration and a given size. The cryovial was placed into a Kryo 560-16 controlled rate freezer (Planer, Middlesex, UK) subjected to a cooling ramp at $1^\circ\text{C}/\text{min}$ until freezing. The temperature of each sample was measured by a Type-K thermocouple (Digi-Sense, Cole Parmer Instrument Co., Chicago, IL) that was inserted into the sample through a drilled hole on the cryovial cap. The temperature was recorded every second by a data logger (RDXL6SD, Omega Engineering, Inc., Stamford, CT). It should be noted that in this study the junction of the thermocouple is enclosed in a smooth stainless steel sheath of 2.5 mm in diameter. Our preliminary study found that when an exposed thermocouple junction was immersed into the liquid, air bubbles might adsorb to the junction and serve as an ice nucleation site, causing uncontrolled and spontaneous freezing [11]. Once ice nucleates in the aqueous sample, the temperature will return to the melting point and then follow the liquidus curve. As seen in Figure 1C, the ice nucleation temperature or the freezing temperature (T_f) can be identified when an abrupt increase appears in the temperature profile. A total of 8-16 replicates were measured for each experimental condition.

Results and Discussion

Morphology of Snomax-laden alginate beads

A range of alginate beads were prepared in this study. Figure 1D shows two examples that were prepared by the 18 gauge needle and had a local Snomax concentration of 0.1 mg/ml and 15 mg/ml,

respectively. As expected, a higher c_{local} of Snomax particles contributed to less transparency of the hydrogel beads. Table 1 summarizes the size of the alginate beads that had different local concentrations of Snomax and were generated by the 30-gauge or 18-gauge needle. With the same c_{local} , the beads generated by the 18-gauge needle are around 3.5 times as large as those generated by the 30 gauge needle in terms of volume. When the same needle size was used, a higher local concentration of Snomax led to a smaller bead volume. This is mainly because a smaller volume is needed for the gravity to overcome the surface tension at the tip of the needle if the local concentration of solid particles is higher. The size of the prepared bacterial hydrogel beads enables easy removal of used INA particles, which may benefit the downstream processing. To illustrate the effect of freezing on the hydrogel structure, Figure 1E and F compared the morphologies of Snomax-free beads before and after they were freeze-thawed. It is clear that the freeze-thawed alginate bead (Figure 1F) is still intact but has a wrinkled surface, which is very similar to those treated by the freeze-drying or air drying process [12, 14]. This wrinkling phenomenon has been suggested to result from the partial collapse of the polymer network due to the loss of water content during ice formation or evaporation [12, 14].

Ice nucleation in WFI water facilitated by Snomax-laden alginate beads

Figure 2 shows the freezing temperature of WFI water in the presence of Snomax-laden alginate beads of different c_{local} (i.e., 0.1, 2.5 or 15 mg/ml). Given the stochastic nature of ice nucleation, we presented the data points of all replicates and indicated the median freezing temperature (T_f^{median}) and the interquartile range. In general, the freezing temperature is positively related to three parameters under investigation: the number of beads (N), the volume of beads (V), and the local Snomax concentration (c_{local}). It can be seen in Figure 2A that the presence of a single bead prepared by the 30-gauge needle and containing 0.1 mg/ml Snomax induced the ice nucleation at -5.6°C whereas the median freezing temperature increased to -4.5°C with the addition of four more beads of the same size and c_{local} . As for the effect of V , five beads that were prepared by the 30-gauge needle and contained 2.5 mg/ml Snomax led to a median freezing temperature of -4.0°C . But when the beads were generated by the 18-gauge needle, therefore more than three times as large as their 30-gauge counterparts, the T_f^{median} increased to -3.4°C . As seen in Figure 2B, the presence of ten beads generated by the 18-gauge needle and containing 0.1 mg/ml Snomax led to a median freezing temperature of -3.7°C . When the local Snomax concentration increased to 15 mg/ml, the median freezing temperature became -3.3°C . Table 2 shows that the median freezing temperature of 0.5 ml WFI water alone is -8.0°C while the T_f^{median} of 0.5 ml WFI water containing ten Snomax-free alginate beads prepared by the 18-gauge needle is -7.2°C . Since almost all the data points shown in Figure 2 are higher than -7°C , the Snomax particles played a major role in enhancing the ice nucleation temperature even though they were confined within a hydrogel matrix.

Ice nucleation in 10% glycerol facilitated by Snomax-laden alginate beads

We also investigated the freezing phenomenon in aqueous 10% glycerol solution that was facilitated by the Snomax-laden alginate beads. Glycerol is one of the most commonly used cryoprotectants due to its colligative property, strong hydrogen-bonding ability, and high viscosity at low temperatures [4]. As seen in Figure 3, the freezing temperature of 10% glycerol in the presence of alginate beads of different c_{local} and different sizes presents the similar trends to those found in Figure 2. The addition of the solute glycerol did not change the positive relationship between the freezing temperature and N , V , or c_{local} . Figure 3A shows that the presence of a single bead prepared by the 30-gauge needle and containing 0.1 mg/ml Snomax led to the ice nucleation at -10.0°C while the median freezing temperature increased to -8.7°C with the addition of nine more beads of the same V and c_{local} . It is also shown that the five beads that contained 2.5 mg/ml Snomax and were prepared by the 30-gauge needle contributed to a

median freezing temperature of -7.0°C . When the beads were prepared by the 18-gauge needle, the T_f^{median} increased to -6.4°C . Figure 3B shows that the addition of ten beads each containing 0.1 mg/ml Snomax generated a median freezing temperature of -7.5°C . However, when the beads contained 15 mg/ml Snomax, the median freezing temperature increased to -6.1°C . As summarized in Table 3, the median freezing temperature of 0.5 ml 10% glycerol containing neither Snomax nor hydrogel beads is -11.8°C . The T_f^{median} of 0.5 ml 10% glycerol having ten Snomax-free alginate beads prepared by the 18-gauge needle is -11.1°C . All the data points shown in Figure 3 are above -11°C . Therefore, it is the Snomax particles that significantly enhanced the freezing temperature of the aqueous system.

Ice nucleation performance parameter

There are multiple factors able to affect the ice nucleation of the aqueous samples containing Snomax-laden hydrogel beads. One may obtain the same freezing temperature by employing different combinations of N , V and c_{local} . Therefore, it will be highly instructive to provide a universal parameter that is solely responsible for determining the freezing temperature. In this study, we identified the total mass of the Snomax particles contained by the aqueous sample of a fixed volume to be such a parameter. In Figure 4, the freezing temperature has been plotted as a function of the total mass of Snomax ($m = N \cdot V \cdot c_{local}$, in a $\log_{10}$ scale) existing in 0.5 ml WFI water. We also measured the freezing temperatures of suspensions of free Snomax particles ranging from 0.005 to 100 mg/ml prepared in WFI water (black data points in Figure 4). It is evident that all the data points shown in Figure 4 share the same trend. The Pearson's correlation between T_f and $\log_{10}m$ yields a coefficient (r) of 0.9497, suggesting a clear positive linear relationship between T_f and $\log_{10}m$. The curve that best fits all the data points is $T_f = 0.6478 \cdot \log_{10}m - 3.052$ ($R^2=0.9019$). This demonstrates that the total mass of Snomax is the parameter to dictate the occurrence of ice nucleation in 0.5 ml WFI water regardless of the form of presence of the INA particles, either in a suspension or in a confined space. In Figure 5, we plotted the freezing temperatures shown in Figure 3, together with the freezing temperatures of the Snomax suspensions (0.005 to 50 mg/ml) prepared in aqueous 10% glycerol solution (black data points in Figure 5), as a function of m in $\log_{10}$ scale. All the data points follow the same trend very well with a Pearson's correlation coefficient of 0.9783, suggesting a strong positive linear relationship between T_f and $\log_{10}m$. The curve described by $T_f = 1.093 \cdot \log_{10}(m) - 5.771$ ($R^2=0.9571$) is the best fit to all the data points shown in Figure 5. It is indicated that the addition of solute did not change the role of the total mass of INA particles as the governing parameter. Therefore, we suggest that one can increase the total mass of INA particles by adjusting the combination of N , V and c_{local} to enhance the ice nucleation temperature.

The experimental conditions investigated so far used identical sample volume (V_{sample}) of 0.5 ml. To investigate the effect of the sample volume on the freezing temperature, Table 4 compares the freezing temperatures of the 0.5 ml and 1.5 ml samples, both of which contain five alginate beads encapsulating 2.5 mg/ml Snomax (i.e., having the same total mass). Although 0.5 ml samples have a slightly higher median freezing temperature than their 1.5 ml counterparts, the unpaired t test revealed that there is no statistical difference in the freezing temperature (T_f) between the 0.5 ml and 1.5 ml groups, with the only exception for the condition of five beads (30 gauge) in WFI ($p=0.0433$). In the latter case, more replicates might be necessary to evaluate the difference more accurately since the combination of WFI and less Snomax contributes to a wider range of data points than either 10% glycerol or more Snomax in WFI. Given such uncertainty, in the current study we conclude that in the case of a constant sample volume the total mass of Snomax is mostly responsible for dictating the ice nucleation temperature.

Conclusions

Although the controlled ice nucleation can benefit multiple process technologies, the ice-nucleating methods available so far suffer from a few disadvantages, especially when employed in biological systems. In this study, we encapsulated freeze-dried *P. syringae* into hydrogel beads to develop a controllable, effective ice-nucleating approach that can minimize the interaction between the INA particles and the biological system and enables the removal of used particles from the biological system. Due to the self-ice-seeding nature of INAs, our approach does not require manual interruption or external fields. For this study, the ice nucleation performance of these bacterial hydrogel beads was characterized. In the aqueous sample of a fixed volume, the total mass of the INA particles was identified as a universal parameter that dictates the ice nucleation temperature. The findings presented here provide the guidance to tune the ice nucleation temperature within a 6-degree window or even larger by adjusting the size and number of bacterial hydrogel beads and the local INA concentration. The ice-nucleating approach proposed in this study can improve the outcome and standardization of many ice-assisted process technologies such as the slow-freezing cryopreservation of stem cells for regenerative medicine, cellular therapies, and drug screening.

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Figure 1 A. The preparation of alginate beads by the ionotropic gelation method using calcium chloride as a crosslinking agent. B. The freezing temperature measurement of a 0.5 ml aqueous sample subjected to the 1 °C/min cooling ramp. C. The temperature profiles of 0.5 ml 10% glycerol solution alone (black) and 0.5 ml 10% glycerol containing ten alginate beads (prepared by 18 gauge needle) and encapsulating 15 mg/ml Snomax (red). D. Two alginate beads prepared by 18 gauge needle and encapsulating 0.1 mg/ml (left) and 15 mg/ml (right) Snomax, respectively. The grid has a dimension of 12.7 mm x 12.7 mm. E. A Snomax-free alginate bead before frozen. F. A Snomax-free alginate bead after freeze-thawed.

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Figure 2. The freezing temperature (T_f) of WFI water in the presence of alginate beads of different sizes and containing different local concentrations of Snomax (0.1, 2.5, or 15 mg/ml). A: The beads were generated by a 30 gauge needle; B: The beads were generated by an 18 gauge needle.

Figure 3. The freezing temperature (T_f) of aqueous 10% glycerol in the presence of alginate beads of different sizes and containing different local concentrations of Snomax (0.1, 2.5, or 15 mg/ml). A: The beads were generated by a 30 gauge needle; B: The beads were generated by an 18 gauge needle.

Figure 4. The freezing temperature (T_f) profile that is plotted as a function of the total mass of Snomax (m) existing in 0.5 ml WFI water. The black data points correspond to the freezing temperatures of the Snomax suspensions of 0.005-100 mg/ml. The curve that best fits all the data points is $T_f = 0.6478 \cdot \log_{10}m - 3.052$ ($R^2=0.9019$) as represented by the solid line.

Figure 5. The freezing temperature (T_f) profile that is plotted as a function of the total mass of Snomax (m) existing in 0.5 ml aqueous 10% glycerol. The black data points represent the freezing temperatures of the Snomax suspensions of 0.005-50 mg/ml prepared in 10% glycerol. The solid line described by $T_f = 1.093 \cdot \log_{10}(m) - 5.771$ is the best fit to all the data points ($R^2=0.9571$).

Table 1. The diameter (D) and volume (V) of the alginate beads containing different local concentrations (c_{local}) of Snomax generated by the 30 gauge or 18 gauge needle

c_{local} (mg/ml)	D (mm)		V (μ l)	
	30-gauge	18-gauge	30-gauge	18-gauge
0	1.91 $\pm$ 0.03	2.92 $\pm$ 0.05	3.7 $\pm$ 0.2	13.1 $\pm$ 0.6
0.1	1.94 $\pm$ 0.05	3.03 $\pm$ 0.03	3.8 $\pm$ 0.3	14.6 $\pm$ 0.5
2.5	1.89 $\pm$ 0.04	2.86 $\pm$ 0.02	3.6 $\pm$ 0.2	12.2 $\pm$ 0.3
15	1.78 $\pm$ 0.02	2.71 $\pm$ 0.05	3.0 $\pm$ 0.1	10.5 $\pm$ 0.5

Table 2. The freezing temperature (T_f) of 0.5 ml WFI water in the absence of any beads or containing ten Snomax-free beads

	T_f		
	WFI	WFI w/ 10 beads (30 gauge)	WFI w/ 10 beads (18 gauge)
Median	-8.0 °C	-8.1 °C	-7.2 °C
(Min, Max)	(-9.8 °C, -7.2 °C)	(-8.8 °C, -8.1 °C)	(-7.5 °C, -7.0 °C)

Table 3. The freezing temperature (T_f) of 0.5 ml aqueous 10% glycerol in the absence of any beads or containing ten Snomax-free beads

	T_f		
	10% glycerol	10% glycerol w/ 10 beads (30 gauge)	10% glycerol w/ 10 beads (18 gauge)
Median	-11.8 °C	-11.5 °C	-11.1 °C
(Min, Max)	(-13.0 °C, -10.4 °C)	(-12.0 °C, -10.9 °C)	(-11.8 °C, -10.6 °C)

Table 4. The effect of the sample volume (V_{sample}) on the freezing temperature (T_f). The local concentration of Snomax was 2.5 mg/ml and five beads were added into each sample.

Medium	Needle size	Median (Min, Max) / °C		p -value
		$V_{sample}=0.5$ ml	$V_{sample}=1.5$ ml	
WFI	30 gauge	-4.0 (-4.3, -3.8)	-4.2 (-4.4, -3.9)	0.0433
	18 gauge	-3.4 (-3.8, -2.7)	-3.6 (-3.9, -3.3)	0.0582
10% glycerol	30 gauge	-7.0 (-7.3, -6.7)	-7.3 (-7.4, -6.9)	0.0527
	18 gauge	-6.4 (-7.0, -5.9)	-6.7 (-7.6, -6.5)	0.0817

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