Zero-sized Effect of Nano-particles and Inverse Homogeneous Nucleation

PRINCIPLES OF FREEZING AND ANTIFREEZE*

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It was found that freezing of water in terms of homogeneous nucleation of ice never occurs even in ultra-clean micro-sized water droplets under normal conditions. More surprisingly, at sufficiently low supercoolings, foreign nano-particles exert no effect on the nucleation barrier of ice; it is as if they physically “vanished.” This effect, called hereafter the “zero-sized” effect of foreign particles (or nucleators), leads to the entry of a so-called inverse homogeneous-like nucleation domain, in which nucleation is effectively suppressed. The freezing temperature of water corresponds to the transition temperature from the inverse homogeneous-like nucleation regime to foreign particle-mediated heterogeneous nucleation. The freezing temperature of water is mainly determined by (i) the surface roughness of nucleators at large supercoolings, (ii) the interaction and structural match between nucleating ice and the substrate, and (iii) the size of the effective surface of nucleators at low supercoolings. Our experiments showed that the temperature of ~40 °C, commonly regarded as the temperature of homogeneous nucleation-mediated freezing, is actually the transition temperature from the inverse homogeneous-like nucleation regime to foreign particle-mediated heterogeneous nucleation in ultra-clean water. Taking advantage of inverse homogeneous-like nucleation, the interfacial tensions between water and ice in very pure water and antifreeze aqueous solutions were measured at a very high precision for the first time. The principles of freezing promotion and antifreeze and the selection for the biological ice nucleation and antifreeze proteins are obtained. The results provide completely new insights into freezing and antifreeze phenomena and bear generic implications for all crystallization systems.

The question of how freezing occurs in micro-sized ultra-clean water droplets is of crucial relevance in agriculture and food and life sciences in phenomena like frost bite (cell damage due to ice freezing) (1–5). In most cases, freezing is mediated by minute foreign particles, including bacterial epiphytes. The understanding of ice nucleation under the influence of minute foreign particles is particularly important for the identification of novel technologies in controlling freezing and antifreeze in general. The eminent antifreeze effect results from antifreeze proteins found in the blood and tissues of organisms that live in freezing environments (2). In these organisms, the effect of freezing proteins is retarded, or the damage incurred upon freezing and thawing is reduced (2–4). Applications of the antifreeze effect of these antifreeze proteins (AFPs), the capacity to inhibit ice crystallization, have been sought for maintaining texture in frozen foods, improving storage of blood, tissues and organs, cryosurgery, and protecting crops from freezing (4). Freezing is a process of ice crystallization from supercooled water. In this process, water should undergo the stage of ice nucleation, followed by the growth of ice (1). Actually, whether or not freezing takes place is determined to a large extent by ice nucleation. In other words, there would be no ice growth if ice nucleation did not occur.

Despite their importance, the microphysical mechanisms responsible for the formation of ice nuclei under the influence of minute foreign particles are not very well understood. Because of the fact that ice nucleation is the initial and one of the most important steps in the freezing process, the question of whether ice crystallization is governed by homogeneous or heterogeneous nucleation bears significant implications for antifreeze, ice nucleation promotion, and freezing-related phenomena and activities.

Spontaneous nucleation of ice from water, in particular below −40 °C, has in most cases been regarded as “homogeneous nucleation” (2–7). This means that water is considered to be clean and “dust free.” It is supposed that, even though there are some minute foreign particles that occur in water, they don’t exert any direct impact on nucleation. The key questions to be addressed are whether the effect of nano-particles can be neglected completely even at T < −40 °C, and if not, how they affect the nucleation and freezing of water. On the other hand, if heterogeneous nucleation inevitably takes place, it is believed (8–10) to be dominant at low supercoolings (or low supersaturations), whereas homogeneous nucleation is believed to occur at very high supercoolings (or very high supersaturations). In this paper, we examine the effect of a trace amount of foreign nano-particles on ice nucleation in ultra-pure micro-sized water droplets and its implications for freezing and antifreeze in general. We also examine how antifreeze proteins change the surface characteristics of foreign bodies. Based on this, we should be able to obtain a new and comprehensive understanding of freezing promotion and antifreeze mechanisms.

EXPERIMENTAL PROCEDURES

The experiments on ice nucleation were carried out by employing the so-called double oil-layer micro-sized microscopic crystallization technique (11). This technology allows the influence of the container on ice

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* This work was supported by National University of Singapore Academic Research Fund R-144-000-080-112. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: AFP, antifreeze protein; DI, deionized; INP, ice nucleation proteins.
nucleation to be minimized, and the examination of ice nucleation kinetics under well controlled conditions. A micro-sized deionized (DI) water (18.2 mΩl) droplet (~0.1 μl) was suspended between two layers of immiscible oil in a circular quartz cell (compare with the schematic illustration in Fig. 2) (11). To minimize the effect of foreign particles, the water and oils were filtered twice to remove foreign particles before they were injected into the cell.

In our experiments, antifreeze protein III (AFPIII) was employed to control freezing. AFPIII is a protein with a compact, globular structure in which the overall fold comprises numerous short β-strands and one turn of α-helix (12).

In our experiments, the lower layer of oil (oil 1, Silicon Oil AR 1000 from Fluka), which has a greater density than water, was injected into the quartz cell up to one-half of its volume. Next, a drop of pure water or a drop of an AFPIII solution was carefully injected onto the surface of the oil by using a micro syringe. Then, an oil (oil 2, 200/500 cS fluid from Dow Corning) with a density lower than that of water was injected to fill the cell, covering the water droplet and the oil layer beneath. A glass coverslip was then placed on the top of the cell to prevent evaporation.

Because of the density differences, the water droplet was suspended between the two layers of immiscible oils. The ice crystallization in which the overall fold comprises numerous short β-strands and one turn of α-helix (12). Any ice crystal forming in the drop could immediately be observed with a polarized transmitted microscope (Olympus, BX60-F).

RESULTS AND DISCUSSION

The Role of Minute Foreign Particles in Freezing—For the ultra-pure DI water, the freezing temperature (for a constant droplet volume) decreased progressively from 239.3 K to 235.2 K to 234.1 K as the pore size of the filters was reduced from 200 nm to 100 nm to 20 nm. This implies that, under normal conditions, it is almost impossible to completely remove foreign particles by filtration. The process of “homogeneous ice crystallization,” commonly referred to by authors (13–15), is actually a foreign particle-mediated heterogeneous ice nucleation process promoted by a trace amount of foreign nano-particles.

“Zero-sized” Effect of Foreign Particles—The nucleation process can be regarded as a kinetic process for ice nuclei to overcome the so-called nucleation barrier ΔG° under a given supercooling ΔT (ΔT = Tm – T, where T and Tm are the actual and the melting temperatures, respectively) (16–19). To explore the ice nucleation kinetics, we examined the correlation between the nucleation induction time τ, the time required for the first nucleus to appear in the drop of water with a given volume V, and the supercooling ΔT (ΔT = Tm – T, where Tm is melting temperature and T is freezing temperature) (16–19). According to the generic nucleation model (16–18), the kinetics of nucleation (Fig. 1) can be taken into account by examining the correlation between the induction time of ice nucleation and supercooling as follows (16–19):

\[
\ln(\tau) = \frac{\Delta G^*}{k T \phi} \ln[R/(m, R)]^{1/2} B N^m
\]

where \( \Delta G^* \) denotes the nucleation barrier, \( \phi \) denotes the structural match and interaction between ice and foreign particles, \( B \) denotes the interfacial free energy between water and ice. In Equation 1, the interfacial correlation function (16, 17)

\[
f(m, R^*) = \frac{1}{2} \left( \frac{1}{2} \right)^2 \left( \frac{1-mR^*}{w} \right)^2 \left( \frac{2}{R^*} \right)^3 \left( 2 - 3 \left( \frac{R^* - m}{w} \right) \right)
\]

where \( \Delta G^*_{\text{homo}} (= k T \phi \Delta T^2) \), compare Equations 2 and 8) denotes the homogeneous nucleation barrier. \( f(m, R^*) \) varies from 0 to 1 (see Fig. 3A), depending on \( R^* \) and \( m \) (16–19). \( m \) describes the structural match and interaction between the nucleating phase (ice) and foreign particles and can be approximately estimated as \( \cos(\theta) \) (θ is the contact angle between the nucleating phase and the substrate). \( f^*(m, R^*) \) is a function similar to \( f(m, R^*) \) (16–19). For an optimal interaction and structural match between the nucleating phase and the substrate, \( m \to 1 \) and \( f(m, R^*) \to 0 \) (compare with Fig. 2), meaning that the nucleation barrier is completely eliminated because of the occurrence of foreign particles.

As shown in Fig. 2, \( \ln(\tau) \) versus \( 1/\phi \Delta T^2 \) is plotted for the following different systems: DI water filtered by a 20-nm filter (curve A), 0.5 mg/ml AFPIII DI water solution filtered by a 20-nm filter (curve B), 2.5 mg/ml AFPIII DI water solution filtered by a 20-nm filter (curve C), and DI water filtered by a 200-nm filter (curve D). Let us first focus on DI water filtered by a 20-nm filter (curve A). If freezing were first controlled by heterogeneous nucleation and followed by homogeneous nucleation as \( \Delta T \) increased, we would have obtained for a nucleating system pair-wise intersecting straight-line segments in the \( \ln(\tau) \) versus \( 1/\phi \Delta T^2 \) plot: one segment with a small slope at low \( \Delta T \) (high \( 1/\phi \Delta T^2 \)), the other segment with the highest slope at high \( \Delta T \) (compare Equation 1: the slope is then \( k \phi f(m, R^*) = k \), as \( f(m, R^*)_{\text{max}} = 1 \) (8–10, 16–19). In contrast to this expectation, we obtain at low \( \Delta T \) (high \( 1/\phi \Delta T^2 \)) a straight line segment with the largest slope within the measurable range of \( \Delta T \) whereas at high \( \Delta T \) (low \( 1/\phi \Delta T^2 \)), we obtain a straight line segment with a much smaller slope,
the two straight line segments being joined by a curve (see Fig. 2). This implies that the nucleation barrier rises abruptly to the highest level ($\Delta G^\infty \sim \Delta G_{homo}^\infty$, $f(m, R') = 1$) at low supercoolings, as if the foreign particles had “disappeared” (compare Equations 1, 2, and 8). Notice that the other three curves (B–D) share similar characteristics. Hereafter, we will call such an effect on nucleation revealed by foreign particles at low supercoolings the “zero-sized” effect.

**Inverse Homogeneous-like Nucleation and the Effective Curvature of the Surface Roughness of Foreign Body**—To explain the above surprising results, we will analyze the influence of $R'$ on $f(m, R')$ and, consequently, on ice nucleation kinetics. $R'$ is actually the ratio between the radius of the local curvature of the foreign particles (substrate) and that of the critical nuclei $r_c$ (compare Equation 5 and Fig. 1). For a given crystallization system, $R'$ is proportional to $\Delta T$ (compare Equations 3 and 5). As shown in Fig. 3A, the $f(m, R')$–$R'$ diagram can be divided into three regimes based on $R'$ or $\Delta T$. Regime I (linear regime): $R' \gg 1$ (relatively large supercoolings and/or large $R'$). In comparison to the critical nuclei, the foreign particles can be treated as a flat substrate. $f(m, R')$ then attains its lowest value and becomes a constant with respect to $R'$ (or $\Delta T$), i.e. it assumes the form $f(m)$. According to Equation 1, the plot of $\ln(rV)$ versus $1/\Delta T^2$ is a straight line because $k$ is constant for a given system (compare portion 1 of curve A in Fig. 1). Regime II (nonlinear regime): as $\Delta T$ decreases (or $1/\Delta T^2$ increases), $r_c$ becomes comparable with $R'$ ($R' \sim 1$) (compare Equation 3). The $\ln(rV) \sim 1/\Delta T^2$ plot becomes a curve (compare the curved segment between portions 1 and 2 of curve A) as $f(m, R')$ varies with $\Delta T$ (or $R'$). Regime III (zero-sized regime): further lowering $\Delta T$ (or raising $1/\Delta T^2$) leads to a much larger $r_c$ than the radius $R'$ of the foreign particles or $R' \to 0$. In the case $f(m, R')_{\text{max}} = 1$, one has a straight line with the largest slope (line 2 of curve A in Fig. 1). In this regime, as suggested by the zero-sized effect, the particles behave then as if they had “vanished,” although they are still physically present in water. According to Equation 8, the occurrence of the particles does not lower the nucleation barrier with respect to that of genuine homogeneous nucleation. Notice that the nucleation in Regime III is still not genuine homogeneous nucleation. In the case of genuine homogeneous nucleation, one has $f'(m, R') = f(m, R') = 1$ and $\ln[f'(m, R')/f(m, R')] = 0$. For the four different systems: DI water filtrated by a 20-nm filter (curve A), 0.5 mg/ml AFPIII DI water solution filtrated by a 20-nm filter (curve C), and DI water filtrated by a 200-nm filter (curve D). The curves were obtained based on the data given in Fig. 2.

**Fig. 3.** A, dependence of the interfacial correlation function $f(m, R')$ on $m$ and $R'$: experimental $f(m, R') \sim R'$ for the four different systems: DI water filtrated by a 20-nm filter (curve A), 0.5 mg/ml AFPIII DI water solution filtrated by a 20-nm filter (curve C), and DI water filtrated by a 200-nm filter (curve D). The curves were obtained based on the data given in Fig. 2.
The measured transition temperature ($\Delta T_{\text{mid}}$) and the radius of the local curvature of foreign particles for different systems

| R' | $\kappa$ | $\langle \Delta T \rangle_{\text{mid}}$ | (r_c)_{\text{mid}} (= R') |
|----|--------|----------------|----------------|
| DI water (20-nm filter) | 1.2 x 10^6 | 39.1 | 2.16 |
| DI water (200-nm filter) | 1.1 x 10^6 | 33.9 | 2.43 |
| AFPIII 2.5 mg/ml (20-nm filter) | 0.32 x 10^6 | 40.3 | 1.35 |

ln($\nu$) = -1/T($\Delta T$)^2 plot is $s f(m, R')$. If the inverse homogeneous-like nucleation takes place (Regime III, $f(m, R') = 1$), one then has $s f(m, R') = \kappa$. It follows that the experimental $f(m, R') - \Delta T$ curve can be obtained from the slope of the ln($\nu$) = -1/T($\Delta T$)^2 plot and the corresponding $\kappa$ at different $\Delta T$. Because $r_c$ can be obtained from Equation 3 and $\kappa$, one of the most challenging and important steps in obtaining $f(m, R') - R'$ is to estimate the average local radius $R'$ of the rough surface of the foreign particles (compare Equation 5 and Fig. 2). As illustrated by Fig. 3A (e.g. $m = 0.8$), one has approximately $R' \approx 1$ or $r_c(\text{mid}) \approx R'$ at the midpoint of the ln($f(m, R') - \ln(R')$) curve (ln($f(m, R') - \ln(R')$) plot), enabling one to estimate, according to Equation 5, $R'$ from $r_c(\text{mid})$, which can be obtained from $\langle \Delta T \rangle_{\text{mid}}$ (compare Equation 3; $\langle \Delta T \rangle_{\text{mid}}$ is the supercooling of the midpoint (−ln($f(m, R') - \ln(\Delta T)$) plot). The values of $R'$ for various systems are listed in Table I. The ln($f(m, R') - \ln(R')$) plots for these systems are given in Fig. 3B.

To check the above approach, we will compare the estimated $R'$ ($= r_c(\text{mid})$) with known $R'$ values for some specific foreign particles. Note that any intentionally added foreign particles could not serve for this purpose for the following reasons: (i) because the shape of the foreign particles is irregular, $R'$ cannot be equated with the size of these particles (compare Fig. 1); and (ii) the pre-existing foreign particles should be inactivated to eliminate their interference in the course of the investigation and subsequent analysis. The AFPIII (from A/F Protein Canada, Inc.) was selected as a specific foreign particle. Each AFPIII molecule has both a dominant hydrophilic and a dominant hydrophobic portion (compare Fig. 4A; Ref. 20). Our latest experiments show that AFPIII molecules adsorb onto and cover the surface of foreign particles by orienting their dominant hydrophobic portions outwards (compare Fig. 4B), thus interrupting the interaction between the nucleating phase (ice) and the foreign particles. This can be seen from the rise in $f(m)$ (from curve 1 → curve 2 in Fig. 3B) after adding 0.5 mg/ml AFPIII to the DI water filtered by a 20-nm filter. As the concentration of AFPIII begins to exceed C_{AFPIII} = 2.5 mg/ml, the molecules start to aggregate into some hydrophilic-like clusters (Fig. 4C) with an average diameter (2$	ext{R}'$) $\approx 2.8$ nm (see our light scattering results in Fig. 5, A and B). In their role as new foreign particles, these hydrophilic clusters lower the nucleation barrier (Fig. 3B, compare 2 with 3). In Table I, for C_{AFPIII} = 2.5 mg/ml, the radius $R'$ of the foreign particles (AFPIII clusters) is estimated to be 1.42 nm, based on the ln($f(m, R') - \ln(R')$) plot, which is in excellent agreement with the results given in Fig. 4B.

The "Infinite" Nucleation Energy Barrier of the Inverse Homogeneous-like Nucleation and the Freezing Point of Water—The roughness ($r_c$) of the pre-existing foreign nano-particles and their surface structural match with ice nuclei exert a profound impact on the freezing and antifreeze process, which has never been taken into account before. If the inverse homogeneous-like nucleation becomes a dominant process (Regime III), the nucleation barrier will rise drastically (compare Figs. 2 and 3B). For DI water (filtrated by a 20-nm filter), the nucleation barrier ($\Delta G^* / kT$) rises from 222 to 11,123 at $\Delta T = 40$ K (compare with Fig. 4). Such a high nucleation barrier is almost equivalent to an "infinite" height of nucleation barrier. Because the nucleation barrier affects the nucleation rate exponentially (compare Equation 1), such a high $\Delta G^* / kT$ makes the nucleation of ice in Regime III practically an impossible event at low supercoolings. Therefore, the initiation of freezing can only be possible when entering the particle-mediated nucleation regime (Regime I). If the transition between Regime I and Regime III can be approximately characterized by $\langle \Delta T \rangle_{\text{mid}}$, the freezing temperature can be estimated from $\langle \Delta T \rangle_{\text{mid}}$, which is to a large extent determined by the local roughness or the nano structure of foreign particles and their structural match with ice nuclei. In other words, $\langle \Delta T \rangle_{\text{mid}}$ is the threshold temperature for freezing. According to Equations 3 and 5 and Table I, a small change in $R'$ will cause a significant change in $\langle \Delta T \rangle_{\text{mid}}$ (or the freezing temperature), meaning that the freezing temperature of water is determined by the effective local curvature of a foreign body.

To let us compare the difference in freezing behavior between the DI water filtered by a 20-nm filter and a 200-nm filter. The change from the 200-nm filter to the 20-nm filter will eliminate some types of particles, which gives rise to a slight increase in the structural mismatch (40% increase in $f(m)$) shown by Fig. 3B. In addition, the total number of foreign particles, $N_o$, is reduced. Nevertheless, these changes bear only a minute impact upon the total freezing behavior of water. According to Equation 1, the changes in $N_o$ and $f(m)$ will then lead to a shift of the ln($\nu$) $\sim 1/T(\Delta T)^2$ plot along the $y$ axis, as shown by Fig. 2. The most important impact is brought about by the change in the size of particles, which as a consequence causes the change in $R'$. As shown by Table I, a reduction of 10% in $R'$ gives rise to an increase of 5 K in $\langle \Delta T \rangle_{\text{mid}}$. This implies that the change in the roughness of foreign particles exerts a more profound impact upon the freezing temperature than other factors.

Is $-40^\circ C$ the Homogeneous Nucleation-mediated Freezing Temperature?—It is worth noting that it is commonly believed that $-40^\circ C$ is the homogeneous nucleation-controlled freezing temperature (21–23). One of the key arguments is that the $-40^\circ C$ temperature is commonly found to be the temperature required for freezing in the atmosphere and can be readily produced in the lab (22). From the biological point of view, it is also believed (21–23) that the freezing temperature of all organisms could drop to $-40^\circ C$ if biological ice nucleators, such as ice nucleation protein, were removed.

In practice, any condensation in the atmosphere is impossible to be 100% particle-free. As mentioned at the beginning, minute foreign particles exert an inevitable and crucial impact on water freezing. If we use our micro DI water droplet filtered by a 20-nm filter to mimic the freezing of condensed micro-water droplets in the atmosphere, it can be seen from Table I that $\langle \Delta T \rangle_{\text{mid}}$ for such a system is $-3.9^\circ C$, which is close to $-40^\circ C$, as reported (21–23). This result implies that the spontaneous freezing temperature at $-40^\circ C$ or below is $\langle \Delta T \rangle_{\text{mid}}$, the transition temperature between normal heterogeneous nucleation and the inverse homogeneous-like nucleation for a very clean and pure (but not particle-free) water droplet.

Implications for the Solid-Liquid Interfacial Tension and the Critical Radius—One of the key applications for nucleation experiments is to measure the solid-fluid interfacial free energy $\gamma_{sl}$ (24, 25) (compare Equations 1 and 4). For this purpose, $f(m, R') = 1$ (i.e. the effect of foreign particles on the nucleation barrier is eliminated completely) should be attained, or the

\[^{2}\text{X.-Y. Liu and N. Du, unpublished data.}\]
measurement of $\gamma_{cf}$ will never be accurate. This remains an extremely challenging task, as genuine homogeneous nucleation is difficult to achieve (20, 24). The conventional approach for removing foreign particles, by filtrating the liquids or solutions to obtain “homogenous nucleation,” is, according to the above analyses, not workable. But it is possible to attain $f(m, R') = 1$ by taking advantage of the inverse homogeneous-like nucleation. For the above DI water (filtrated by a 20-nm filter), one obtains from segment 2 of curve A in Fig. 2 $\gamma_{cf} = 47.8$ (mJ/m$^2$) (taking $\Omega = 32.531 \times 10^{-30}$ m$^3$ (25), compare Table II). On the other hand, for the nucleation based on segment 1 of curve A, in which the nucleation barrier of ice is strongly affected by minute foreign particles, one obtains $\gamma_{cf} = 7.11$ (mJ/m$^2$), which is an $-750\%$ deviation from the correct value of $\gamma_{cf}$.

Notice that DI water filtrated by different pore-sized filters (200 nm, 100 nm, 20 nm) should give rise to the identical $\gamma_{cf}$, because under the conditions for inverse homogenous nucleation, the presence of foreign particle nucleation should not affect the nucleation barrier. This hypothesis was confirmed by our experiments: as shown in Table II, the ice-water interfacial
energies $\gamma_d$ for DI water filtrated by different pore-sized filters (200 nm, 100 nm, 20 nm) turn out to be almost the same.

In the presence of AFPIII, the interfacial tension between water and ice should decrease. As mentioned in our recent publication (26), to change the nucleation kinetics of ice, the AFP molecules should be able to adsorb onto either the surface of foreign particles or the surface of ice nuclei (compare with Fig. 4B). According to the Gibbs equation (27)

$$\Gamma_{add} = \frac{1}{RT} \frac{d\gamma}{d\ln a_{add}}$$  \hspace{1cm} (Eq. 9)

(where $R$ is the gas constant, $a_{add}$ is the activity of AFP in the aqueous solutions, and $\Gamma_{add}$ is the surface excess of AFP in the chosen dividing surface), the accumulation of AFPIII on the surface of ice should then lead to a lowering of the interfacial tension $\gamma_d$. This hypothesis is confirmed by the results shown in Table II.

To estimate the critical radius of ice nuclei $r_c$ at different supercoolings is very important for freezing and antifreeze in general. Because of the fact that ($\Delta T^\text{mid}$ corresponds to the supercooling at $R^c/r_c \approx 1$, the relationship between $r_c$ and $\Delta T$ will allow us to estimate the effective radius $R^c$ of the local curvature of foreign bodies and to predict some key features of antifreeze and nucleation promotion agents (see below). This relationship can be obtained on the basis of Equation 3, given that a reliable $\gamma_d$ is available. Let us take the DI water (filtrated by 20-nm filters) system as an example. Substituting $\gamma_d$, $\Omega$, $\Delta S_m$ (compare Tables I and II) into Equation 3, the correlation between $r_c$ and $\Delta T$ is obtained as

$$r_c \text{ (nm)} = 84.5/\Delta T$$  \hspace{1cm} (Eq. 10)

Plotting $r_c$ versus $T_f$ (freezing temperature) based on Equation 10 gives rise to a graph (shown in Fig. 6). As can be seen from this graph and Equation 10, within the range of $T_f = -30$ to $-60^\circ$C, $r_c$ changes only from 3 to 1.4 nm. Within the framework of freezing/antifreeze, this implies that in this regime, a slight change in the effective radius of the local curvature of foreign bodies (or substrates) will lead to a drastic decrease in freezing temperature. On the other hand, at temperatures close to 0, in particular $T_f > -5^\circ$C, $r_c$ increases drastically (from 17 nm upward) as $T_f$ approaches 0. This means that, to promote ice nucleation at temperatures close to the melting temperature, the surface foreign bodies/substrates should be essentially flat.

We notice that some estimated $r_s$ with temperature were reported by various investigators (22). Nevertheless, because of the confusion between heterogeneous and homogeneous nucleation mentioned before, $r_s$ have been severely underestimated because of much too low $\gamma_d$.

Implications for Biological Ice Nucleation Promotion and Antifreeze—The phenomenon of ice nucleation has both intrigued and demanded the attention of biologists from a wide range of disciplines. Plant physiologists and crop scientists found it relevant because of the agricultural importance of frost-sensitivity. Applications suggested (28) that ice nucleators, being a small group of epiphytic bacteria, play a very important role from snowmaking, to food technology, to the use of inca genes as reporters of transcription and transduction. Moreover, bacterial epiphytes were discovered to be an important source of ice nuclei in the atmosphere.

The ice nucleation promotion effect can be mainly attributed to ice nucleation proteins (INPs), which are located at the outer membrane of ice nucleator bacteria and, indeed, have a flat ice bonding surface ($R^c \rightarrow \infty$) with a large surface area (29). The INPs aggregate into membrane-bound arrays to enlarge the effective surface.

It has been found (30) that biological ice nucleators are capable of inducing freezing at about $-1.6 \pm 2^\circ$C. According to Equation 10 and Fig. 6, the radius of the curvature of the ice nucleator surface should be larger than 44 nm. At the molecular scale, this implies that the surface of INPs should be essentially flat, which, in fact, agrees well with the reported results (29). Given that the surface of ice nucleators is considered to be flat, the activity of ice nucleators will then be determined by $m$ ($= \cos \theta$) and the surface area of INPs or INP aggregates. As suggested (31), INP is plate-like. Assuming INP to be a round disc, the dependence of the activity of ice nucleation promotion on $r_s$ and the minimal radius $r_e$ of INP can be estimated based on the diagram shown in Fig. 7A.

The basic assumption is that an INP disc should be large enough to accommodate the critically sized ice nucleus on its surface. The (minimal) size (the diameter of the disc) can be given according to Fig. 7A, by

$$2r_e = 2r_s \sin \theta = 2r_s (1 - m^2)^{1/2}$$  \hspace{1cm} (Eq. 11)

As indicated by Equation 3, $r_s$ is directly proportional to $\gamma_d$. Currently, $\gamma_d$ in water with the presence of INP is unavailable. Nevertheless, because of the fact that the basic molecular structure of INP is similar to AFP and, in biological systems, INPs always coexist with AFPs (23), we will then (in our estimation) use $\gamma_d$ of the DI water with 2.5 mg/ml AFPIII. Substituting $\gamma_d$, $\Omega$, and $\Delta S_m$ to Equation 3 and combining it with Equation 11 yields

$$2r_s \text{ (nm)} = 2r_s \sin \theta = 114.6(1 - m^2)^{1/2}/\Delta T$$  \hspace{1cm} (Eq. 12)

Fig. 7B shows that $2r_s$ decreases drastically as $m$ approaches 1 for a given freezing temperature ($-2^\circ$C) according to Equation 12. This result implies that, as the interaction and the
interfacial structure match between ice nuclei and the ice nucleator surface is improved, the minimal size of ice nucleators will significantly decrease. On the other hand, for a given ice nucleator, the required minimal size of the effective surface drastically increases as the freezing temperature approaches to zero (Fig. 7C).

Because INPs are essentially flat and large (in particular, the “wetting” between INPs and ice is very good), it is not surprising that such INPs can promote ice nucleation quite efficiently. Apart from this, the aggregation of INPs at the surface of the cell membrane is an effective way to enlarge the effective area of ice nucleators.

Ice nucleation also governs the winter survival of some ectothermic animals. Many freeze-intolerant insects avoid lethal freezing by depressing the temperature at which internal ice nucleation can occur. Similarly, some freeze-tolerant forms synthesize ice-nucleating proteins to ensure that freezing happens at high subzero temperatures.

For the antifreeze mechanism of antifreeze protein, there has been much confusion for more than two decades (3). As ice nucleation is the initial and one of the most crucial steps in freezing, how ice nucleation occurs is both critical and crucial for freezing. Unfortunately, almost all analyses are based upon the unanalyzed assumption of homogeneous nucleation and the interaction between AFP and ice nuclei, ignoring the critical role of foreign nano-particles in freezing (1–4, 33, 34). Our results (compare Figs. 2, 3B, and 5) have shown that AFP will change the interaction/structural matching between foreign particles and ice nuclei; $R^*$, therefore, alters $(\Delta T)_{\text{mid}}$ (compare Figs. 2 and 3B, Table I). This exerts a critical impact on the freezing temperature. Based on this result, the antifreeze mechanism of AFP can be analyzed quantitatively for the first time (26).

Finally, the zero-sized effect of foreign particles and the inverse homogeneous-like nucleation should also occur in other crystallization systems. Our recent study has shown that similar phenomena were observed in CaCO$_3$ nucleation experiments.3

In conclusion, genuine homogeneous nucleation of ice never occurs even in ultra-clean micro-sized water droplets because of the pre-existence of minute foreign particles. When the critical size of nuclei is much larger than the radius of the local curvature of foreign particles, the pre-existing foreign nanoparticles are incapable of lowering the nucleation barrier of ice nucleation, as if they had physically vanished. This will then cause a sharp increase in the nucleation barrier at low supercoolings, which prevents the occurrence of nucleation in this so-called inverse homogeneous-like nucleation regime. The freezing temperature of water corresponds to the transition temperature from the inverse homogeneous-like nucleation regime to foreign particle-mediated heterogeneous nucleation; it is mainly determined by the surface roughness of nucleators at large supercoolings, the interaction and structural match between nucleating ice and the substrate, and the size of the effective surface of nucleators at low supercoolings. We propose that $-40 \, ^\circ\text{C}$ corresponds to such a transition for very clean and pure water rather than a temperature for genuine homogeneous nucleation-mediated freezing. The interfacial tension can only be measured precisely based on nucleation when inverse homogeneous-like nucleation occurs. Freezing promotion requires ice nucleators to be flat and large and have strong interactions and an optimal structural match with ice nuclei, whereas antifreeze should be focused on the enhancement of the surface roughness of pre-existing foreign particles rather than other factors.

Acknowledgments—We thank Prof. C. L. Hew for providing AFPIII for the experiments, and Dr. C. Strom for valuable discussion and suggestions.

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