Relating Metabolism Suppression and Nucleation Probability During Supercooled Biopreservation

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Aqueous supercooling provides a method by which to preserve biological matter at subfreezing temperatures without the deleterious effects of ice formation. The extended longevity of the preserved biologic is a direct result of a reduction in the rate of metabolism with decreasing temperature. However, because the nucleation of ice from a supercooled solution is a stochastic process, supercooled preservation carries the risk of random ice nucleation. Theoretical supercooled biopreservation research to date has largely treated these biological and thermophysical phenomena separately. Here, we apply a statistical model of stochastic ice nucleation to demonstrate how the possible reduction in metabolic rate is inherently related to supercooling stability (i.e., the likelihood of ice nucleation). We develop a quantitative approach by which to weigh supercooling stability versus potential metabolic reduction, and further show how the stability–metabolism relationship varies with system size for two assumed modes of nucleation. Ultimately, this study presents a generalizable framework for the informed design of supercooled biopreservation protocols that considers both phase transformation kinetics and biochemical or biophysical kinetics.
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Introduction

The challenge of preserving biological matter and organisms outside their native homeostatic environments is a wide-reaching problem with relevance to food preservation [1], organ transplantation [2], reproductive science [3], tissue engineering [4], and beyond. The myriad biochemical processes that comprise life rely on relatively controlled thermodynamic conditions (temperature, mass exchange, hydration, etc.) to proceed, and deviation from these conditions can drive spoilage in harvested foods, expiration of ex vivo donor organs, and potentially biological death for living organisms.

For centuries, humans have recognized that cold temperatures can stave off these negative effects (by reducing the overall rate of metabolism/biochemical reaction), leading to the widespread adoption of refrigeration. With the groundbreaking studies of Polge et al. [5], among others, this technique was extended into the realm of human biology in order to preserve organs and other constructs outside of the human body.

However, while refrigeration provides extended longevity, long-term preservation nigh-universally requires temperatures well below the freezing point of water, the principal material constituent of most biological matter of interest. The crystallization of ice at subfreezing temperatures can in turn cause irreversible mechanical, osmotic, and dehydrative damage, and thus much of the past half-century of cryobiology research has sought strategies by which to mitigate the damage caused by ice, implicitly accepting its presence [6].

Recently, however, the unique penchant of water to remain in a metastable liquid state below its equilibrium freezing temperature has received increasing attention as a potential method for ice-free biopreservation. This new modality, termed supercooling, has been leveraged to excellent effect in the preservation of rat livers [7], human livers [8], human cardiac microtissues [9], and various foods [10] at subfreezing temperatures yet in the absence of ice. However, the calculus required to design stable and predictable supercooling protocols, which operate at a continuous *metastable* equilibrium, is significantly different than that required to design conventional freezing protocols.

The rates of metabolism and other biological processes are temperature dependent [11,12] and these reactions typically slow at progressively lower temperatures. It may thus seem clear that indefinitely lower temperatures should be sought for increasingly robust preservation. While this logic is reasonable from a conventional cryopreservation standpoint, wherein ice growth is considered inevitable, it is incompatible with metastable supercooled preservation, because it ignores the physics of ice nucleation, which predict that the probability for ice nucleation (which is always nonzero for a supercooled liquid) increases with both the depth below the freezing point and with the time spent in a supercooled state.

A comprehensive analysis of supercooled biopreservation must thus necessarily consider both aspects of biochemical/biophysical kinetics and of ice nucleation kinetics. With this in mind, we have developed a new method of analysis that enables optimal design of cryopreservation protocols through a marriage of biology and physics.

In this study, we present a coupled analysis of metabolism and ice nucleation kinetics to describe the potential suppression of metabolic rate as a function of the probability for ice nucleation. We find that a compromise must be made between the competing design parameters (metabolic suppression, preservation duration, nucleation probability), and that this informed decision can only be made by following a multiphysics approach. Furthermore, it should be noted that while significant previous research has sought to characterize [14,15] and optimize [16] cryopreservation protocols via various modes of thermal transport analysis, the physics relevant to the stability of supercooling are not dominated by thermal transport, but by first-nucleus nucleation kinetics, offering a less-treaded path by which to approach the problem.

Though we only explicitly consider simple models for the temperature dependence of metabolism and nucleation kinetics, the framework is entirely generalizable and can be supplemented with the development of more detailed models of biochemical/biophysical and ice nucleation kinetics.

Metabolic Rate Temperature Dependence

The temperature dependence of metabolism has been characterized by Gillooly et al. [11] for several classes of organisms using an Arrhenius function of the form

$$B(T) = B_0 e^{-E_i/k_B T} \tag{1}$$

where B_0 is a normalization constant with units $\mathrm{W \cdot kg^{-3/4}}$ (analogous to the classical Arrhenius frequency factor), E_i is a representative activation energy for the rate-limiting enzyme-catalyzed

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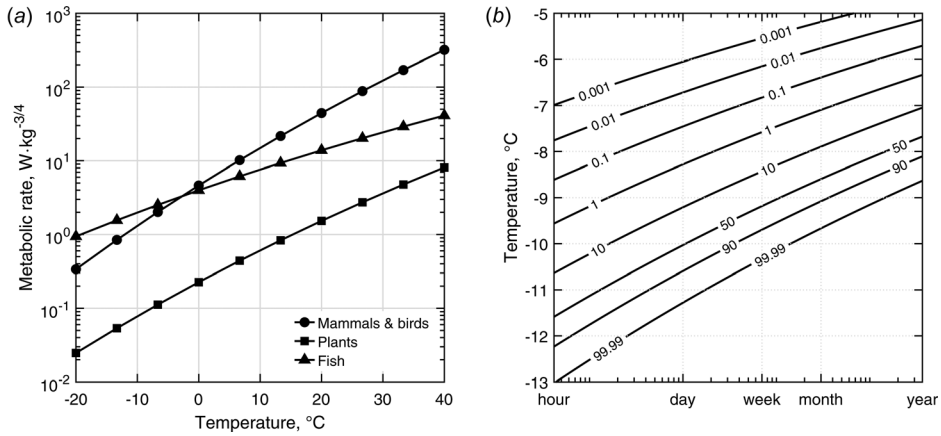

Fig. 1 (*a*) Temperature dependence of metabolic rate for mammals/birds, plants, and fish. Parameters from Gillooly et al. [11]. (*b*) Nucleation probability (%) as a function of temperature and supercooled duration for de-ionized water in a petrolatum-coated isochoric system [13].

biochemical reactions of metabolism, k_B is the Boltzmann constant, and T is the temperature in K.

This general model for mass-normalized resting metabolic rate was derived on the basis of allometry and biochemical reaction kinetics and fits metabolic data in a temperature range of 0 °C to 40 °C. In order to extend this model to supercooled temperatures (<0 °C), we risk a loss of quantitative accuracy, however the general trends should not be compromised.

Figure 1(*a*) depicts the metabolic temperature dependence determined by Gillooly et al. [11] for mammals/birds, plants, and fish. The respective metabolic rate parameters are provided in Table 1 in the Methods section

Nucleation Statistics

The stochastic process of solid phase crystallization from a supercooled liquid may be modeled by Poisson statistics [17–21]. For a liquid held at a constant subcooled temperature, the probability for nucleation, p, is given by the homogeneous Poisson distribution.

$$p(T,t) = 1 - e^{-J(T)\cdot t} \tag{2}$$

wherein J is the system nucleation rate, T is the temperature, and t is the time spent in a supercooled state. If nucleation is initiated in the bulk of a fluid, the total system nucleation rate is dependent on the fluid volume, V, and if nucleation is initiated on the surface encapsulating the fluid, then it is dependent on the total system surface area, A. If the temperature varies spatially or temporally, the nucleation process is considered a nonhomogeneous Poisson process, and the probability can be generalized as

$$p(T(t,V,A)) = 1 - \exp\left[-\int_t\int_V\int_A J(T(t,V,A))dtdVdA\right] \tag{3}$$

Equations for the nucleation rate are often sought through application of classical nucleation theory [22,23]; however, empirical relations may also be obtained by fitting parameterized equations to experimental data. A common form for an empirical nucleation rate equation is

$$J(\Delta T) = \gamma \Delta T^n \tag{4}$$

wherein $\Delta T = T_m - T$ is the depth below the equilibrium melting point, T_m, and γ and n are empirical fitting parameters [24,25]. With this system nucleation rate, the nucleation probability at constant temperature becomes

$$p(T,t) = 1 - e^{-\gamma(T_m - T)^n t} \tag{5}$$

A recent study characterized the nucleation of de-ionized water in an isochoric system ($V = 5.3$ mL, $A = 18.7$ cm^2) with petrolatum-coated walls [13], and average empirical nucleation parameters γ and n were evaluated as 2.20×10^{-22} and 22.06, respectively. The numerical results presented in the remainder of this study will use this data, as it is one of the few nucleation datasets available characterizing a bulk-volume nondroplet system (>1 mL). However, it should be emphasized that the methodology and equations presented herein are generalized and do not depend on specific values of γ or n, or on the specific form of the nucleation rate. Thus, the present analysis can be applied to any set of nucleation data.

Figure 1(*b*) depicts the nucleation probability (%) as a function of temperature and supercooled duration for the characterized system. This probability-temperature-time mapping gives the basest outline of the temperatures and preservation durations that are achievable with high stability (i.e., low ice nucleation probability). For example, nucleation is predicted to occur at a rate less than 0.1% for all temperatures above −6 °C and durations up to three months.

Qualitatively, we can see that for a given preservation period, the nucleation probability increases slowly up until 10%, after which the trend intensifies and reaches a nearly deterministic regime (>99.99%) in a span of only 2 °C. Therefore, while temperature can be varied relatively safely below and around the region of 10% nucleation probability, any incidental fluctuations thereafter carry significantly enhanced risk for nucleation.

Table 1 Metabolic rate parameters from Gillooly et al. [11]

	$\ln B_0$ (W·kg$^{-3/4}$)	E_i (eV)
Mammals and birds	34.67	0.78
Plants	26.55	0.66
Fish	19.65	0.43

Relating Metabolic Suppression and Nucleation Probability

By solving for temperature in Eq. (5) and substituting into Eq. (1), we can obtain the following relation for the metabolic rate as a function of supercooled duration and nucleation probability:

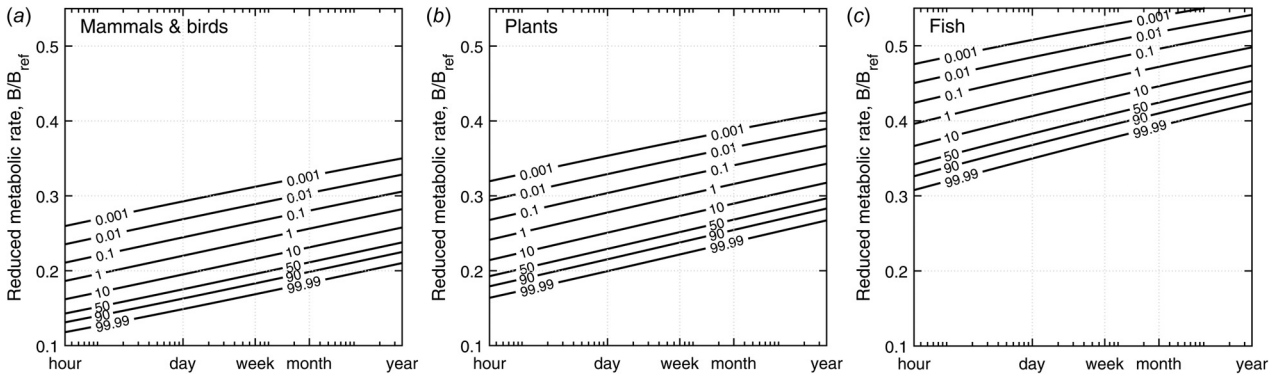

Fig. 2 Suppression of metabolic rate as a function of nucleation probability and supercooled duration for (*a*) mammals/birds, (*b*) plants, and (*c*) fish. Reduced metabolic rate calculated with reference to the rate at $T_{ref} = 4\,^{\circ}C$.

$$B(t,p) = B_0 \exp\left(-\frac{E_i/k_B}{T_m - \left(\frac{-\ln(1-p)}{\gamma t}\right)^{1/n}}\right) \tag{6}$$

The results of this analysis are shown in Figures 2(*a*)–2(*c*) relative to the metabolic rate at 4 °C, which is the current clinical standard for organ preservation [2] and is further commonly encountered in food refrigeration. As it may be expected, because the relative reduction in metabolic rate trends strongly with the temperature dependence (or equivalently the ice nucleation probability, as shown in the figure), organisms with larger characteristic activation energies, E_i, experience larger reductions in metabolism.

Additionally, a similar trend with nucleation probability at constant duration is observed with metabolism as with temperature. A large change in metabolism does not significantly affect the nucleation probability in the region below 10%; however, the trend quickly steepens, and for nucleation probabilities greater than 10%, further change in metabolic suppression is accompanied by large changes in probability and swift entering of a quasi-deterministic regime.

Scaling With System Size

The experimental data obtained by Consiglio et al. [13] and used in this study characterized the nucleation rate of a specific 5.3 mL isochoric system, and further investigation is required to identify the mode(s) of nucleation and determine how the nucleation rate scales with volume and container surface area. Despite this gap in our current knowledge, however, we can still conject how the nucleation phenomena (probability-time-temperature relation) would scale by assuming that only surface-mediated *or* volumetric nucleation will dominate.

Assuming this independent scaling (i.e., that only one nucleation mode is applicable), the nucleation rate would become

$$J(V,\Delta T) = \frac{V}{V_0}\gamma\Delta T^n \quad \text{or} \quad J(A,\Delta T) = \frac{A}{A_0}\gamma\Delta T^n \tag{7}$$

wherein V_0 is the volume (5.3 mL) and A_0 the surface area (18.7 cm^2) of the chamber from Consiglio et al. [13], and V and A are the volume and surface area of a hypothetically scaled system.

The relation between temperature, supercooled duration, and system size for two nucleation probabilities (0.001% and 10%) is shown in Fig. 3(*a*). The indicated volumes correspond to the volumetric scaling assumption, and conversely, the indicated surface areas correspond to the surface area scaling assumption.

Toward the Design of Supercooled Biopreservation Protocols

We may now seek to consider specific applications using these new design tools, and one of the most promising applications is full organ supercooling for transplantation purposes. By applying the simple scaling analysis above, we can estimate the metabolic suppression possible in a petrolatum-coated isochoric system of arbitrary size. Shown in Fig. 3(*b*) is the dependence of mammalian metabolism on nucleation probability and supercooled duration for a 4 L system (volume scaling assumed). This volume is

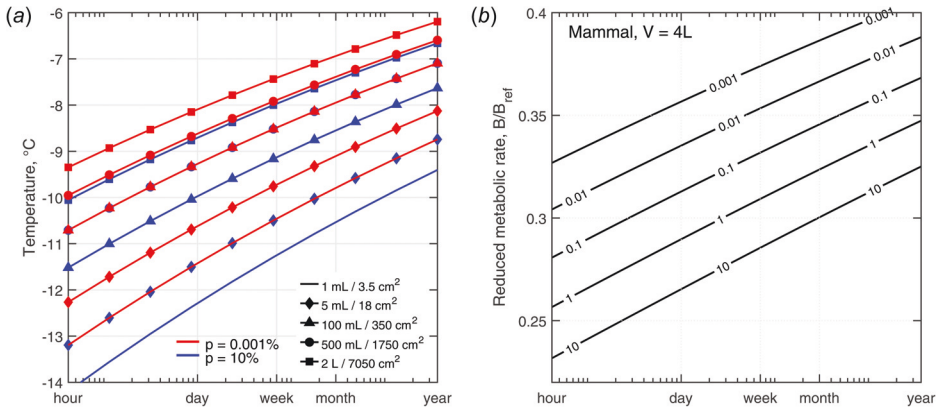

Fig. 3 (*a*) Scaled temperature versus time dependence for nucleation probabilities of 0.001% (red) and 10% (blue). Indicated volumes correspond to volume scaling assumption. Indicated areas correspond to surface area scaling assumption. (*b*) Dependence of mammalian metabolic rate on supercooled duration and nucleation probability for a hypothetical 4 L system applicable to human organ preservation (volumetric scaling assumed).

expected to be able to contain any mammalian organ, excluding perhaps the lungs. We can see that a two-thirds reduction in the metabolic rate ($B/B_{ref} \approx 1/3$) may be achieved compared to preservation at 4 °C for a preservation period of one week and nucleation probability of only 0.01%.

In the case of full organ preservation, given the extreme value of transplantable donor organs (the average heart transplant cost more than $1 M in 2017 [2]), minimization of nucleation probability must be prioritized since any formation of ice and freezing of tissue risks lethal and irreversible damage.

On the other hand, for cell suspensions divided into a multitude of individual vials, a smaller margin of error may be viable since 100% survival is often not necessary and enhanced metabolic suppression may be of greater value. The 5 mL isochoric system from Consiglio et al. [13] may be a well-suited volume for cell preservation. In this case, we can reference Fig. 2(*c*) directly and observe that nearly a three-quarter reduction in the metabolic rate compared to 4 °C may be obtained for preservation of one week and nucleation probability of 0.1%.

The preservation of food may likewise not demand total assurance of the avoidance of ice nucleation if a certain loss is acceptable. Future scenario-specific analyses may seek an optimal protocol that balances nucleation frequency with extension of shelf life. For example, if it is currently impossible to preserve sashimi-grade tuna for longer than 72 h, a protocol that enables longer preservation times but with a 50% chance of ice nucleation may still be viable and valuable, generating 50% high-quality survival of a yield that otherwise may be entirely lost.

For each of these scenarios, marriage of the modeling methodology herein to experimental validations in applied biopreservation should be sought in future work, and could be leveraged to develop standards for acceptable thresholds of nucleation probability suited to each application.

Conclusions

Although this study has only related the temperature dependence of nucleation kinetics with metabolism as whole, the methodology can be extended to any temperature-dependent biochemical or biophysical process of interest. For example, the relation provided in Eq. (6) can be used to characterize any first-order chemical reaction with known pre-exponential factor and activation energy. In addition to metabolic rate, the temperature dependence of membrane processes, enzymatic activity, adenosine triphosphate depletion, and other processes related to hypothermia and ischemia-induced damage would be of interest for low-temperature biopreservation. These processes might possess temperature dependencies ill-described by the Arrhenius function of Eq. (1), and in this case an alternative temperature-dependent rate equation such as the Eyring equation from transition state theory [12] may be substituted in its place.

Furthermore, as discussed in previous sections, the nucleation data used in this study is of limited scope. In order to rigorously quantify nucleation probabilities applicable to real applications, further investigations should seek to characterize the volumetric and surface scaling, and nucleation data reflecting the precise preservation medium to be employed should be used (as opposed to pure water data).

Future work should also seek to experimentally distill the metabolism reduction described herein into more actionable temperature-dependent metrics, such as gross organ, tissue, or cell viability, degree of food expiration, etc., and thereby validate directly the results of this modeling approach.

In conclusion, we have shown that, armed with knowledge of the temperature dependence of the metabolic rate and rate of ice nucleation, the safety (against ice nucleation) and effectiveness (reduction of metabolism) of a proposed supercooled biopreservation protocol can be quantitatively characterized. We find that a compromise must be made amongst the various competing design parameters since metabolism decreases monotonically with temperature and conversely the probability for ice nucleation from a supercooled liquid increases monotonically with temperature and supercooled duration.

Methods

The metabolic rate parameters B_0 and E_i, summarized in Table 1, are computed from data in Fig. 1 of Gillooly et al. [11].

Nucleation rate parameters were computed by parametric inference of data from constant cooling rate nucleation experiments [13]. The survivor curve representation of this data (unfrozen fraction versus temperature) corresponds to the cumulative distribution of a nonhomogeneous Poisson process, the rate parameter of which is the nucleation rate. By assuming a form for the nucleation rate (such as in Eq. (4)), a distribution may be fitted to the data to obtain the empirical parameters.

This empirical fitting method may be applied to arbitrary nucleation experiments reporting unfrozen fraction versus temperature survivor curves, and the application of the analysis presented herein to the design of specific supercooling protocols should use nucleation data that represents the precise system of interest, in order to capture surface-mediated nucleation effects specific to that system.

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Conflict of Interest

The authors declare no competing interest.

Data Availability Statement

All data are available from the authors upon reasonable request.

References

[1] Mercier, S., Villeneuve, S., Mondor, M., and Uysal, I., 2017, "Time–Temperature Management Along the Food Cold Chain: A Review of Recent Developments," Compr. Rev. Food Sci. Food Saf., **16**(4), pp. 647–667.

[2] Giwa, S., Lewis, J. K., Alvarez, L., Langer, R., Roth, A. E., Church, G. M., Markmann, J. F., Sachs, D. H., Chandraker, A., Wertheim, J. A., Rothblatt, M., Boyden, E. S., Eidbo, E., Lee, W. P. A., Pomahac, B., Brandacher, G., Weinstock, D. M., Elliott, G., Nelson, D., Acker, J. P., Uygun, K., Schmalz, B., Weegman, B. P., Tocchio, A., Fahy, G. M., Storey, K. B., Rubinsky, B., Bischof, J., Elliott, J. A. W., Woodruff, T. K., Morris, G. J., Demirci, U., Brockbank, K. G. M., Woods, E. J., Ben, R. N., Baust, J. G., Gao, D., Fuller, B., Rabin, Y., Kravitz, D. C., Taylor, M. J., and Toner, M., 2017, "The Promise of Organ and Tissue Preservation to Transform Medicine," Nat. Biotechnol., **35**(6), pp. 530–542.

[3] Dou, M. J., Lu, C. N., Rao, W., Dou, M., Lu, C., and Rao, W., 2022, "Bioinspired Materials and Technology for Advanced Cryopreservation," Trends Biotechnol., **40**(1), pp. 93–106.

[4] Arutyunyan, I., Elchaninov, A., Fatkhudinov, T., Arutyunyan, I., Elchaninov, A., Sukhikh, G., and Fatkhudinov, T., 2021, "Cryopreservation of Tissue-Engineered Scaffold-Based Constructs: From Concept to Reality," Stem Cell Rev. Rep.

[5] Polge, C., Smith, A. U., and Parkes, A. S., 1949, "Revival of Spermatozoa After Vitrification and Dehydration at Low Temperatures," Nat., **164**(4172), pp. 666–666.

[6] Mazur, P., 1984, "Freezing of Living Cells: Mechanisms and Implications," Am. J. Physiol. Physiol., **247**(3), pp. C125–C142.

[7] Berendsen, T. A., Bruinsma, B. G., Puts, C. F., Saeidi, N., Usta, O. B., Uygun, B. E., Izamis, M.-L., Toner, M., Yarmush, M. L., and Uygun, K., 2014, "Supercooling Enables Long-Term Transplantation Survival Following 4 Days of Liver Preservation," Nat. Med., **20**(7), pp. 790–793.

[8] de Vries, R. J., Tessier, S. N., Banik, P. D., Nagpal, S., Cronin, S. E. J., Ozer, S., Hafiz, E. O. A., van Gulik, T. M., Yarmush, M. L., Markmann, J. F., Toner,

M., Yeh, H., and Uygun, K., 2019, "Supercooling Extends Preservation Time of Human Livers," Nat. Biotechnol., **37**(10), pp. 1131–1136.
[9] Powell-Palm, M. J., Charwat, V., Charrez, B., Siemons, B., Healy, K. E., and Rubinsky, B., 2021, "Isochoric Supercooled Preservation and Revival of Human Cardiac Microtissues," Commun. Biol., **4**(1), pp. 1–7.
[10] Park, D. H., Lee, S., Hong, G. P., Park, D., Hyeon, Lee, S., Lee, Jiseon, Kim, E. J., Jo, Y.-J., Kim, Honggyun, Choi, M.-J., and Hong, G.-P., 2021, "Stepwise Cooling Mediated Feasible Supercooling Preservation to Extend Freshness of Mackerel Fillets," LWT-Food Sci. Technol., **152**, p. 112389.
[11] Gillooly, J. F., Brown, J. H., West, G. B., Savage, V. M., and Charnov, E. L., 2001, "Effects of Size and Temperature on Metabolic Rate," Science (80-.), **293**(5538), pp. 2248–2251.
[12] Arcus, V. L., Prentice, E. J., Hobbs, J. K., Mulholland, A. J., Van Der Kamp, M. W., Pudney, C. R., Parker, E. J., and Schipper, L. A., 2016, "On the Temperature Dependence of Enzyme-Catalyzed Rates," Biochemistry, **55**(12), pp. 1681–1688.
[13] Consiglio, A., Lilley, D., Prasher, R., Rubinsky, B., and Powell-Palm, M. J., 2021, "Methods to Stabilize Aqueous Supercooling Identified by Use of an Isochoric Nucleation Detection (INDe) Device," Cryobiology.
[14] Devireddy, R. V., Smith, D. J., and Bischof, J. C., 2002, "Effect of Microscale Mass Transport and Phase Change on Numerical Prediction of Freezing in Biological Tissues," ASME J. Heat Transfer-Trans. ASME, **124**(2), pp. 365–374.
[15] Acharya, T., and Devireddy, R. V., 2010, "Cryomicroscopic Investigations of Freezing Processes in Cell Suspensions," Open Biotechnol. J., **4**(1), pp. 26–35.
[16] Goh, B. C., Thirumala, S., Kilroy, G., Devireddy, R. V., and Gimble, J. M., 2007, "Cryopreservation Characteristics of Adipose-Derived Stem Cells: Maintenance of Differentiation Potential and Viability," J. Tissue Eng. Regen. Med., **1**(4), pp. 322–324.
[17] Turnbull, D., 1952, "Kinetics of Solidification of Supercooled Liquid Mercury Droplets," J. Chem. Phys., **20**(3), pp. 411–424.
[18] Skripov, V. P., Koverda, V. P., and Butorin, G. T., 1979, "Crystal Nucleation Kinetics in Small Volumes," Рост Кристаллов/*Rost Kristallov*/*Growth of Crystals*, A. A. Chernov, ed., Vol. 11, Springer US, Boston, MA, pp. 22–25.
[19] Morton, C. W., Hofmeister, W. H., Bayuzick, R. J., and Robinson, M. B., 1994, "A Statistical Approach to Understanding Nucleation Phenomena," Mater. Sci. Eng. A, **178**(1–2), pp. 209–215.
[20] Filipponi, A., Di Cicco, A., and Principi, E., 2012, "Crystalline Nucleation in Undercooled Liquids: A Bayesian Data-Analysis Approach for a Nonhomogeneous Poisson Process," Phys. Rev. E Stat. Nonlinear Soft Matter Phys., **86**(6), p. 066701.
[21] Koop, T., Luo, B., Biermann, U. M., Crutzen, P. J., and Peter, T., 1997, "Freezing of $HNO_3/H_2SO_4/H_2O$ Solutions at Stratospheric Temperatures: Nucleation Statistics and Experiments," J. Phys. Chem. A, **101**(6), pp. 1117–1133.
[22] Kalikmanov, V. I., 2013, "Classical Nucleation Theory," *Nucleation Theory*, Springer, Dordrecht, The Netherlands, pp. 17–41.
[23] Kelton, K. F., and Greer, A. L., 2010, "Chapter 2—The Classical Theory," *Nucleation in Condensed Matter*, K. F. Kelton, and A. L. Greer, eds., Pergamon, UK, pp. 19–54.
[24] Lilley, D., Lau, J., Dames, C., Kaur, S., and Prasher, R., 2021, "Impact of Size and Thermal Gradient on Supercooling of Phase Change Materials for Thermal Energy Storage," Appl. Energy, **290**, p. 116635.
[25] Kubota, N., 2015, "Analysis of the Effect of Volume on Induction Time and Metastable Zone Width Using a Stochastic Model," J. Cryst. Growth, **418**, pp. 15–24.