AIP Advances

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Cite as: AIP Advances **11**, 055125 (2021); https://doi.org/10.1063/5.0051662
Submitted: 29 March 2021 . Accepted: 06 May 2021 . Published Online: 19 May 2021

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ABSTRACT

We present herein an experimental study on the ice-nucleation kinetics of two recently introduced aqueous supercooling modalities—oil-sealed isobaric supercooling and isochoric supercooling. A series of constant-cooling rate experiments compare the apparent nucleation temperatures of pure water supercooled under these modalities with conventional open-air isobaric supercooling, demonstrating that both methods significantly enhance the supercoolability of the system as compared to open-air supercooling. However, while the mean nucleation temperatures of the two methods are statistically comparable, isochoric supercooling displays approximately half the variability of isobaric oil-sealed supercooling, which may have important implications on the design of supercooling-based biopreservation protocols in which stability and reproducibility are paramount.

Safe multiday storage of biological materials is essential in biotechnology and medicine, with applications ranging from the preservation of reagents and vaccines to whole organs and complex tissues. During storage, biological matter deteriorates due to unavoidable biochemical reactions of varied nature, the reaction rates of which may be generally characterized as temperature-dependent and describable by the Arrhenius equation.[1] For humans, the rates of the sum of biochemical reactions, which comprise metabolism, are conservatively estimated to drop by a factor of 2–3 for every 10 °C reduction in temperature, and this arrest in the metabolism provides the guiding principle by which low-temperature biopreservation protocols are designed. While indefinite preservation of a biologic could theoretically be achieved by storage at absolute 0 [K], human biological matter is composed principally of water, which freezes at temperatures below 0 °C. Ice formation proves damning to most complex biological constructs, yielding levels of damage ranging from dehydration and osmotic shock to gross morphological disruption.[2] Accordingly, the avoidance of destructive ice formation at sub-0 °C temperatures represents one of the landmark goals of low-temperature biopreservation research.

To this end, promising recent efforts have sought leveraging the unique penchant of water to supercool, or remain in a metastable liquid state at temperatures beneath the equilibrium freezing point, in order to achieve ice-free biopreservation. While this supercooled state is not globally stable (i.e., does not reflect the lowest possible free-energy state of the system), various living organisms have found ways to survive subfreezing temperatures in a supercooled state, including various Arctic and Antarctic fish (which have developed specialized antifreeze proteins that non-colligatively enhance supercooling[3]) and insects (which can survive in a supercooled state at temperatures as low as – 54 °C for months on end[4]), which has inspired the application of supercooling to the preservation of medically relevant biological constructs.

Among contemporary supercooling efforts, three general approaches have been validated in biopreservation contexts. Antifreeze protein studies provided perhaps the earliest examples of supercooled storage of complex biologics (whole rat hearts and livers[5,6]), validating the core premise of ice-free metastable preservation. However, these proteins afforded stable supercooling on the order of only 1–1.5 °C, and thus, alternative methods of stabilizing supercooled water to deeper temperatures were sought.

Two new techniques suited to this purpose have recently been discovered: isobaric oil-sealed supercooling and isochoric supercooling. In the former technique, biological matter is supercooled in a dilute preservation solution under isobaric (constant-pressure) conditions at atmospheric pressure, and ice formation is averted by interposing low-density hydrophobic liquids (most frequently mineral oils) between the preservation solution and the surrounding air in order to eliminate air–water interfaces, which function as favorable sites for heterogeneous ice nucleation. This technique has achieved successful stable supercooling over a range of sub-0 °C temperatures, depending on the construct being preserved and the bulk volume of the system,[7–10] and was employed for the first supercooled preservation of a whole human organ.[9]

The latter technique, isochoric supercooling, employs the newly-developed thermodynamic principles of the aqueous isochoric process (constant-volume) in order to stabilize supercooling.[11,12] Thermodynamic and kinetic analyses have demonstrated that constant-volume systems, which are denied access to the atmospheric pressure reservoir and achieve stable high-pressure water–ice equilibria at sub-0 °C temperatures,[11] can enhance the kinetic barrier to ice nucleation[12] through a unique manifestation of Le Chatelier's principle and as a consequence of macroscopic confinement. Recent experimental work has also demonstrated the remarkable resistance of isochoric supercooling to destabilization from external perturbations,[14] driven largely by avoidance of cavitation-induced nucleation effects,[15] and isochoric supercooling was recently employed in the first multiday preservation of autonomously beating engineered human cardiac tissue.[13]

In order to safely apply supercooling to the preservation of biological matter, and because ice nucleation is an inherently stochastic process, it is essential to characterize the stability of a given supercooled system, i.e., the probability of ice nucleation under a given set of conditions (most probably at a given temperature). Previous studies have examined thermo-kinetic aspects of stability at constant sub-freezing temperatures by recording the frequency with which nucleation occurred within a given period of time[10,14] and mechano-kinetic aspects of stability by recording the frequency with which nucleation occurred upon exposure to various external perturbations.[14]

In this study, we evaluate the effective kinetic ice nucleation temperature of pure water under isobaric oil-sealed, isochoric, and conventional open-air isobaric conditions by using constant-cooling rate temperature ramping,[15,16] and we use the variability in the ice nucleation temperature as a metric of relative stability between techniques.

A difficulty (and opportunity) presented by aqueous isochoric systems is that upon nucleation and ice formation, the pressure in the system can increase to over 220 MPa.[11] Therefore, for these experiments, we employ a stainless-steel isochoric chamber that can withstand these pressures and incorporate digital pressure transduction that enables detection of pressure as an additional indicator of ice nucleation under isochoric conditions. A photograph of the chamber, which was used for all experiments (in slightly varying configurations), is shown in Fig. 1(a), and the system is described in detail in the Methods section.

The three thermodynamic conditions examined were enforced as follows: for isochoric conditions, the pictured chamber was filled with pure deionized (DI) water and sealed completely, with careful care taken to avoid the introduction of bulk air bubbles (which can corrupt isochoric conditions due to their high compressibility); for isobaric oil-sealed conditions, an identical chamber was filled with DI water, but instead of capping the chamber with a pressure transducer, the chamber was capped with an ~2mm-high layer of partially synthetic oil (see Methods for more detail), exposing the water within to the atmospheric pressure reservoir but eliminating the air–water interface; finally, conventional open-air isobaric conditions were achieved by filling a third identical chamber and leaving the uppermost liquid surface unsealed and exposed to the environment. More detail on the materials and assembly processes may be found in Methods.

After assembly, the three chambers were uniformly insulated with rock wool and introduced vertically into a low-temperature freezer (Vestfrost VT407) [Fig. 1(b)] cooled to −40 °C. The insulation thickness was chosen so as to yield a uniform cooling rate at subfreezing temperatures of ~8.5 °C/h [Fig. 1(c)], at which the temperature of nucleation is presumed to be independent of the cooling rate.[15] The chambers each were instrumented with thermocouples on the outer surface at the same location, as shown in Fig. 1(a). The onset of nucleation is marked by the recalescence (a sudden spike in temperature) that accompanies crystallization in supercooled liquids, which was monitored via the thermocouple for all chambers. In addition, because isochoric freezing yields only partial transition of the system to ice,[12] thus producing a weaker recalescence signal, nucleation under isochoric conditions was verified by detection of a sudden increase in pressure. Figures 1(d) and 1(e) show typical temperature and pressure traces observed in our experiments, with arrows marking the precise onset of nucleation. For consistency, after verifying the agreement in time between the apparent onset of nucleation based on temperature and based on pressure, we used temperature traces from the thermocouples mounted on the external surfaces of the chambers [as shown in Fig. 1(e)] for subsequent comparison and analysis.

The results of this study are shown in Fig. 2. Figure 2(a) gives the nucleation temperature measured in each of the three systems across 12 experiments. Statistical analysis of these data to compare the effects of thermodynamic conditions on nucleation temperature was done by one-way ANOVA, with Bonferroni's method used for the multiple comparisons test, and box plots are shown in Fig. 2(b). Statistical analysis demonstrates that both oil-sealed isobaric and isochoric supercooling suppress the probability of kinetic ice nucleation as compared to conventional open-air isobaric supercooling ($p < 10^{-5}$ for each) and that these two techniques provide statistically similar enhancement of supercooling (i.e., suppression of the observed nucleation temperature) ($p = 0.67$).

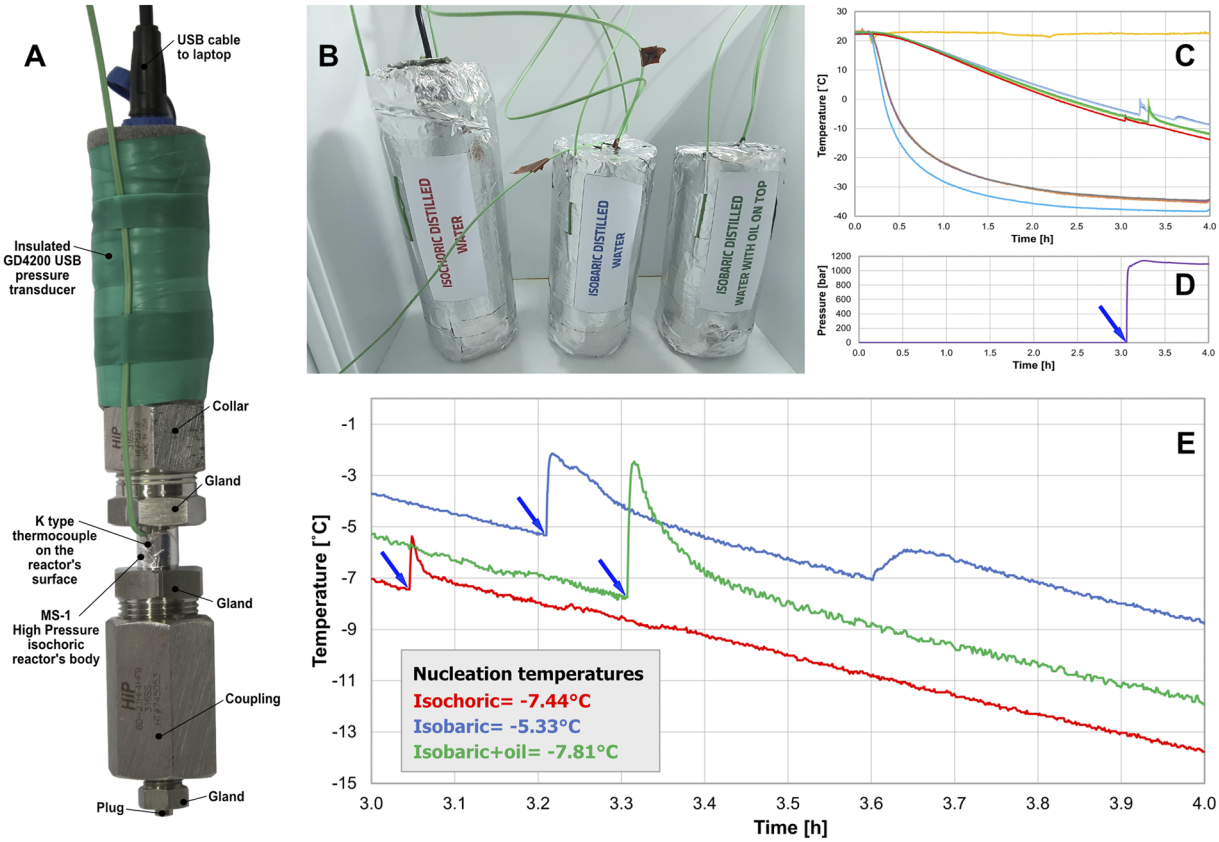
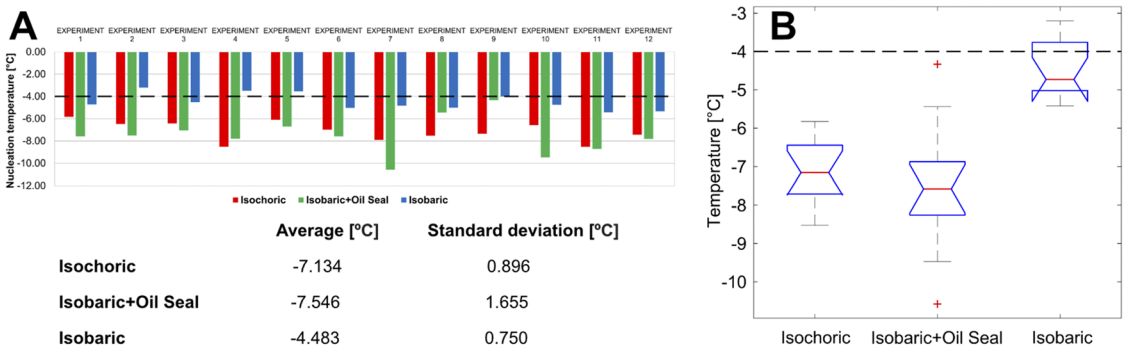


FIG. 1. (a) Photograph of the isochoric system with the main components; (b) a photograph of the three reactors inside the low temperature freezer; (c) a typical plot for all measured temperatures; (d) a typical pressure plot with the arrow pointing to the nucleation event; (e) typical temperatures on the outer surface of the cylinders with the arrow pointing to the nucleation events.

Importantly, however, there are notable differences in the consistency with which these techniques produce this enhanced supercooling, as indicated by the spread of the data. While the arithmetic means of the nucleation temperatures produced by isobaric oil-sealed and isochoric supercooling are statistically equivalent, the standard deviation of these nucleation temperatures is nearly twice as large under isobaric oil-sealed conditions (standard deviation = 1.66 °C) as under isochoric conditions (standard deviation = 0.90 °C), indicating higher stability of kinetic supercooling under isochoric conditions than under isobaric oil-sealed conditions.



	Average [°C]	Standard deviation [°C]
Isochoric	-7.134	0.896
Isobaric+Oil Seal	-7.546	1.655
Isobaric	-4.483	0.750

FIG. 2. (a) Nucleation temperature measured in 12 repeats for each of the three systems. (b) The boxplots for isochoric, isobaric oil-sealed, and conventional open-air isobaric supercooling.

These results confirm in a dynamic context the findings of previous steady-state isochoric supercooling and the isobaric oil-sealing studies.[10,14] As found in both previous studies, both techniques improve supercooling stability relative to conventional supercooling; however, consistent with previous findings that isochoric conditions offer superior stability in the face of various mechanical perturbations, our data herein demonstrates that they also confer a superior degree of stability against kinetic nucleation during the cooling process, providing more predictable behavior and higher certainty of nucleation avoidance at lower degrees of supercooling.

The supercooling afforded by these systems (up to ~7–7.5 °C) is substantially larger than that afforded by antifreeze proteins (1–1.5 °C) and is not contingent on the introduction of foreign non-physiological species. It should be emphasized furthermore that these experiments were performed with pure water and thus reflect a lower limit on the supercooling that might be observed during practical biopreservation. Conventional human biopreservation solutions such as the classic University of Wisconsin formulation will lower the equilibrium freezing point by an additional 0.56 °C, which should hence lower the temperature threshold of stable supercooling by at least this amount.

Finally, taking an applied biopreservation standpoint, it is useful not only to consider the temperatures at which supercooled water *will certainly* nucleate ice (as is reported directly by the data) but also to estimate the likely temperatures at which supercooled water *will not* nucleate ice. Based on the standard deviation of the nucleation temperatures reported herein, we may estimate the threshold after which kinetic ice nucleation (i.e., ice nucleation which occurs upon cooling) becomes negligibly likely: for isochoric supercooling, the likelihood of nucleation is 0.3% at ~−4.5 °C, and for isobaric oil-sealed supercooling, the likelihood of nucleation is 0.3% at ~−2.5 °C.

This result suggests that it may be practical and safe to preserve biological matter in isochoric chambers at temperatures down to ~−4 °C without altering the composition of the preservation medium and with limited concern over kinetic ice nucleation due to exterior perturbations.[14] Accordingly, it should be noted that conventional preservation of organs and tissues in a clinical context is typically conducted at +4 °C and that by reducing this preservation temperature from +4 to −4 °C, the rate of metabolism may be reduced by a factor of ~2, thereby extending the preservation time by at least the same factor, all without the requirement of chemical additives or complex technological intervention.

To conclude, we note that this study represents one addition to the understanding of the relative stabilities of isochoric and isobaric oil-sealed supercooling. Future studies should examine the effective nucleation temperatures and variabilities in systems of increasingly large volume and the induction times of nucleation at steady state as a function of temperature in order to complete the picture and clarify the practical limits of supercooled stability.

Details on the design of the experiments can also be found in the supplementary material.

The loan for the low temperature freezer (Vestfrost VT407) from Chr. Hansen SRL, Braşov, for this study is gratefully acknowledged. We are grateful to SC Criomec SA, Galaśi, for the continuous motivation and support offered to our research group and for the financial aid provided for the scientific research through Contract No. 1290/01.23.2018.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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