THE JOURNAL OF PHYSICAL CHEMISTRY LETTERS
A JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Letter

Cryoprotective Mechanism of DMSO Induced by the Inhibitory Effect on Eutectic NaCl Crystallization

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Cite This: J. Phys. Chem. Lett. 2022, 13, 11153−11159

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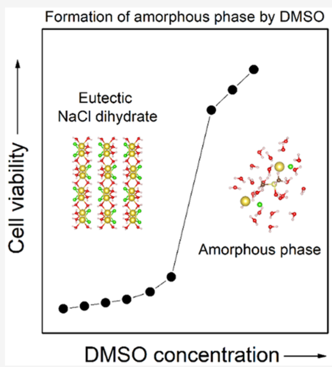
ABSTRACT: Cryopreservation is a critical procedure in autologous hematopoietic stem cell transplantation. Dimethyl sulfoxide (DMSO) is the cryoprotectant of choice. Optimization of the cryopreservation protocol in the past revealed a dramatic loss of cell viability associated with a reduction of the DMSO concentration below 2 vol % in the freezing medium. The cryoprotective mechanism of DMSO is usually ascribed to the ability to suppress ice formation and reduce the adverse effects of the freeze-concentrated solution. This work proposes an alternative hypothesis considering the detrimental impact of NaCl eutectic crystallization on cell viability. Thermoanalytical and microstructural analysis of the DMSO effect on eutectic phase transformation of cryoprotective mixtures revealed a correlation between the loss of cell viability and eutectic NaCl crystallization. DMSO inhibits the eutectic crystallization of NaCl and preserves cell viability. Thermodynamic description of the inhibitory action and possible mechanism of cryoinjury are provided.



Cryopreservation is a strategy applied to achieve degradation-free storage of biological material. It relies on the application of low subzero temperatures, which halt cellular biochemical processes and allow—in principle—indefinite preservation. However, freezing and thawing can be dangerous for biological material because cell viability is lost during these processes.[1] The addition of dimethyl sulfoxide into a cell freezing medium has been found to preserve cell viability and proper cell functionality.[2] In clinical applications, DMSO is often used as a cryoprotective additive.[3,4] Although it offers a cryoprotection, it introduces its toxicity.[3] The standard cryopreservation protocol in autologous hematopoietic stem cell transplantation relies on a slow freezing procedure (1 °C/min) with 10 vol % DMSO in the cell freezing medium.[5] To reduce the toxic effects of DMSO, an effort was made to optimize the cryopreservation protocol by reducing the DMSO concentration from the standardly used 10 to 4.5 vol % and then to 2.2 vol %.[6] It was found that even DMSO concentrations as low as 2.2 vol % provided sufficient cryoprotection in the cryopreservation of hematopoietic stem cells. The clinical study revealed that the application of 2.2 vol % DMSO was successful without any negative impact on transplant quality, as engraftment was rapid, without delayed hematologic recovery and without any toxic effects associated with the application of 2.2 vol % DMSO.[6,7] While 2.2 vol % DMSO provided sufficient cryoprotection, further reduction of DMSO concentration resulted in a dramatic loss of cell viability,[8] suggesting the existence of a critical concentration of DMSO required for the cryoprotective effect of DMSO. The critical concentration was determined to be 2 vol % DMSO. Other authors have later observed similar behavior.[9,10] Although many efforts have been made to explain the cryoprotective action of DMSO and other cryoprotectants, only partial success has been achieved, as evidenced by the ever-increasing number of studies on their cryoprotective action in the recent past focusing on solid−liquid transformations.[11−15]

During freezing, the viability of cells is threatened by ice formation. The intracellular ice formation appears the most problematic,[16−18] although there is evidence that interaction between extracellular ice crystals and the plasma membrane may also harm cell viability.[19−21] The mechanism of such cryoinjury is mainly attributed to mechanical forces acting on biological cells.[19] Besides the physical contact with ice, the formation of extracellular ice in combination with slow freezing leads to the freeze-concentration process, cell exposure to increased salt concentrations, and associated cell dehydration.[22,23] These factors represent additional sources of cryoinjury, also termed "solution effects" injury.[24] Cryoprotective agents such as DMSO are thought to suppress ice formation and reduce the adverse effects associated with a freeze-concentrated solution. Concerning the latter, the protective effect of cryoprotectants should be related to their

Received: October 1, 2022
Accepted: November 22, 2022

ability to decrease the salt concentration, reduce the extent of cell dehydration, or stabilize the plasma membrane against these stressors.[25−28]

In the presence of DMSO, liquid or amorphous freeze-concentrated solution can act as a protective layer surrounding the cells, minimizing contact with extracellular ice crystals.[29,30] However, a freeze-concentrated phase is also subject to solid−liquid phase transformation. Thus, an additional source of cryoinjury imposed by the eutectic crystallization of NaCl and ice might be expected. The basic cell medium is composed primarily of sodium chloride solution, whose phase behavior is well documented.[31−36] Eutectic phase transformation of this binary mixture occurs at −21.1 °C (252 K), resulting in crystalline NaCl dihydrate and ice formation. In the past, it was shown that eutectic crystallization of NaCl in cryoprotectant-free cell medium was associated with a direct cell injury and dramatic loss of cell viability.[37] The crystalline eutectic phase has been shown to surround the plasma membrane of frozen cells closely,[38,39] suggesting the possibility of cryoinjury similar to extracellular ice crystallization, at least because the eutectic phase transformation includes the formation of (pure) ice. Past phase behavior studies revealed that DMSO induces the formation of metastable glassy (amorphous) phase in mixtures with water (and NaCl).[8,40,41]

The presented knowledge allowed us to hypothesize that the dramatic loss of cell viability observed in hematopoietic stem cells cryopreservation could be related to the eutectic crystallization of NaCl—the primary component of cell freezing medium besides the cells and water. In this Letter, the protective action of DMSO is hypothesized to be associated with the induced formation of an amorphous freeze-concentrated phase, inhibiting the formation of eutectic crystalline phases. Therefore, this study aims to examine the effect of DMSO on the eutectic phase transformation of mixtures representing cell freezing media with cryobiologically relevant compositions. An experimental approach consisting of differential scanning calorimetry (DSC), electric conductivity measurements, positron annihilation lifetime spectroscopy (PALS), and Raman microspectroscopy is applied, allowing thermoanalytical and microstructural characterization of ternary mixtures in the relevant temperature range.

DSC analysis revealed that the eutectic phase transformation normally observed in the binary water−NaCl mixture is suppressed by DMSO (Figure 1). The eutectic temperature was gradually decreased with increasing DMSO concentration. The extent of eutectic crystallization was suppressed, as evidenced by lower enthalpy change (area under the endothermic peak), indicating the formation of an amorphous fraction of the freeze-concentrated phase. The most significant depression of eutectic transformation was observed for 1.5 vol % DMSO or 1.8 vol % DMSO when judged by temperature or peak intensity. For compositions of ternary mixture with DMSO concentration higher than 2 vol %, no eutectic crystallization was observed. Eutectic crystallization of NaCl in a binary mixture with water typically occurs from −36 to −38 °C (235−237 K) (see Figure S2 in the Supporting Information). The temperature of eutectic crystallization was also depressed by DMSO. For DMSO concentrations higher than 0.4 vol %, only cold eutectic crystallization was observed (for clarity, see Figures S2 and S3 in the Supporting Information), suggesting a kinetic aspect of the DMSO action on eutectic crystallization. DSC analysis also revealed an interesting observation of a disproportionately large freezing

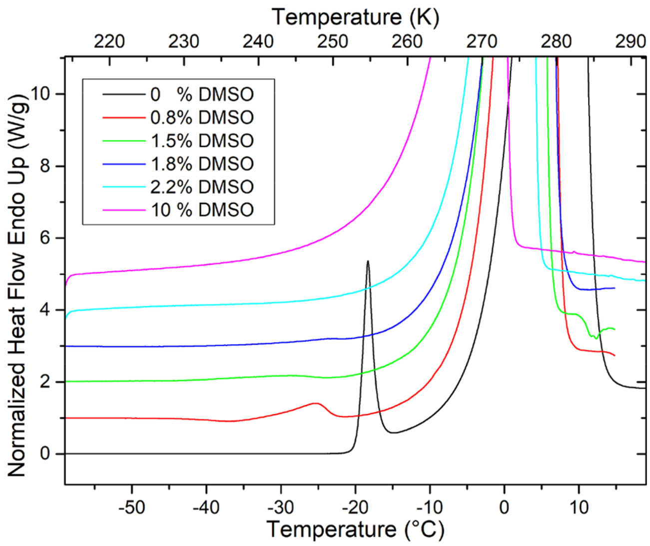


Figure 1. Heating DSC thermograms of ternary water−NaCl−DMSO mixtures. Heating scans are shown with emphasis on the eutectic melting. The figure highlights the inhibitory effect of DMSO on NaCl eutectic formation. 1.8 vol % shows signs of eutectic formation, and 2.2 and 10 vol % do not. Bigger peaks that are cut off in the picture are associated with ice melting. For detailed results see Figure S1 in the Supporting Information.

point depression of ice observed for 2 vol % DMSO equivalent (90 wt % water) compared to the 1.8 vol % DMSO (Supporting Information, Figure S4), associated with significantly reduced melting enthalpy, indicating the decreased amount of ice formed during freezing and thawing.

The results of the DSC analysis were confirmed by a complementary approach offered by temperature measurements of electric conductivity. A sudden change in electric conductivity is expected to occur during a first-order phase transformation of eutectic phases containing NaCl, resulting from loss/gain of ionic mobility.[42] This picture was confirmed for the binary water−NaCl mixture exhibiting a sudden change in electric conductivity at about −21 °C (252 K), a temperature corresponding to the eutectic point of a binary water−NaCl mixture (black line in Figure 2). The addition of DMSO into the mixture led to a gradual disappearance of the sharp transition. DMSO induced a more gradual change of electric conductivity with temperature. This behavior can be explained by the avoidance of NaCl eutectic crystallization and the formation of an amorphous freeze-concentrated phase. There is no apparent first-order eutectic phase transformation for mixtures with DMSO concentrations higher than 1.8 vol %. This result confirms the DSC measurements, which indicated the inhibition of the eutectic formation by DMSO. The gradual change in electric conductivity in the temperature range −100 to −35 °C (173−238 K) is consistent with the temperature behavior of electric conductivity of glass-forming liquids,[43] implying a second-order phase transition between the glassy and liquid state of the freeze-concentrated phase of the ternary mixtures.

The formation of NaCl dihydrate, which occurs during eutectic crystallization, is associated with sharp Raman peaks at 3405, 3420, and 3545 cm^{-1} (black line in Figure 3). Raman spectra of the 2.2 vol % DMSO equivalent (water content 80 wt %) showed no signs of NaCl dihydrate formation (green line in Figure 3), neither at −150 °C (123 K) nor −38 °C

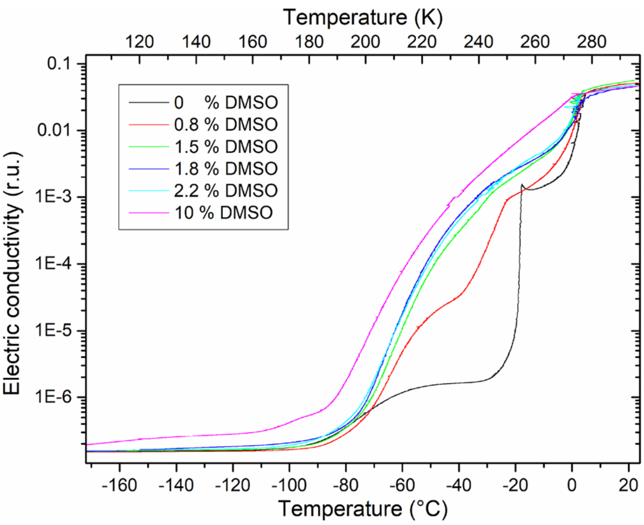


Figure 2. Electric conductivity of ternary mixtures during thawing measured with the applied alternating voltage with the frequency of 2 kHz. The cooling and heating rates were 20 and 10 °C/min, respectively.

(235 K) during the thawing. The spectra of 2.2 vol % DMSO equivalent are also characterized by strong DMSO peaks (680, 2920, and 3010 cm^{-1}) attributable to C−S and C−H stretching,[44] broad aqueous OH stretching peaks (3150–3700 cm^{-1}), and a decreased amount of ice (3100 cm^{-1}). A comparison of 2.2 vol % DMSO equivalent spectra at −38 °C (235 K) and 25 °C (298 K) highlights the liquid state of water in the freeze-concentrated phase, since the broad OH peaks associated with liquid water are practically the same for both temperatures (blue and magenta lines in Figure 3).

Ortho-positronium (*o*-Ps) lifetime measurements allowed the characterization of microstructural changes associated with solidifying and melting ternary mixtures. In the past, the *o*-Ps lifetime was found to be sensitive to phase transformations,[45]

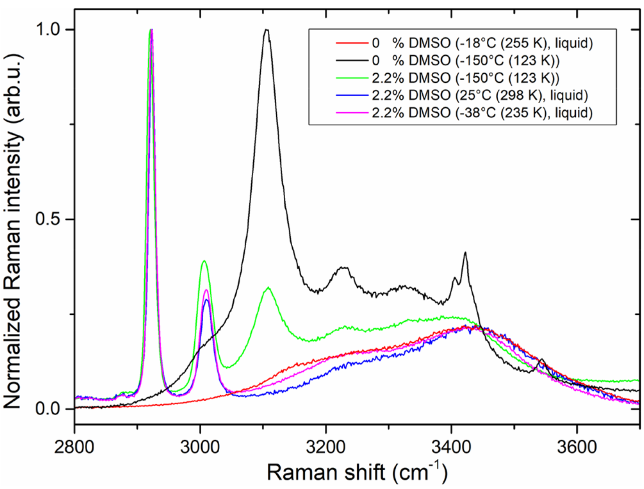


Figure 3. Raman spectra of the binary water−NaCl mixture (water content 80 wt %) and 2.2 vol % DMSO equivalent (water content 80 wt %) at various temperatures. For the exact composition of the mixtures, see Table 2 in the Supporting Information. All spectra except 0% DMSO (−18 °C) were normalized to the most intense peak. The spectrum of 0% DMSO was normalized so that the OH stretching region was comparable to other samples.

allowing its application in the phase behavior studies of various materials.[46−48] Our group applied this technique to study the phase behavior of binary water−DMSO mixtures in bulk state[8] or in the confinement of liposomes,[29] which represented a model of a biological cell. In both cases, the ability of the technique to detect eutectic crystallization was demonstrated, which was associated with the formation of DMSO trihydrate and ice.[49] In this study, a similar approach was used to study ternary mixtures, including NaCl. A significant hysteresis between heating and cooling measurements of *o*-Ps lifetimes was observed for 0.8 vol % DMSO equivalent (water content 80 wt %), as shown in Figure 4 (full symbols). The hysteresis is associated mainly with the eutectic phase transformation, which occurred during thawing (cold crystallization). The cold crystallization is confirmed by isothermal time measurements performed on this sample at −45 °C (228 K) (Supporting Information, Figure S6) and is consistent with the results of the DSC analysis.

PALS analysis of 2.2 vol % DMSO mixture equivalent (water content 80 wt %) revealed that hysteretic behavior is significantly reduced (hollow symbols in Figure 4). The *o*-Ps

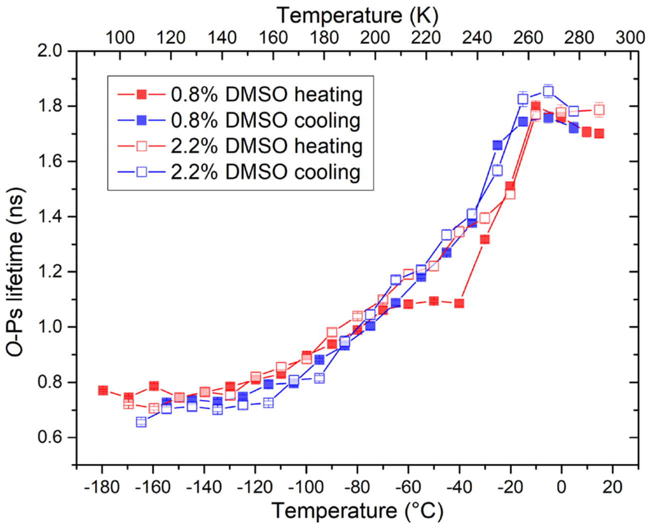


Figure 4. Temperature behavior of *ortho*-positronium lifetimes in 0.8 and 2.2 vol % DMSO equivalents (water content 80 wt %). For the exact composition of the mixtures, see Table 2 in the Supporting Information. The observed hysteresis is associated with cold eutectic crystallization of NaCl and, to some extent, supercooling of water during freezing. Note that error bars representing the standard deviation of *o*-Ps lifetimes are present in the graph but are of similar size as the data points.

lifetimes of 2.2 vol % DMSO are higher than the lifetimes of the 0.8 vol % DMSO equivalent through the temperature range, demonstrating the hysteretic behavior. Higher values indicate the presence of a higher fraction of the liquid (amorphous) phase. The PALS experiments thus confirmed the hypothesis that the extent of eutectic crystallization in the studied ternary mixtures is suppressed by increasing the DMSO concentration relative to NaCl, since no sharp changes of an *o*-Ps lifetime in the critical temperature range were observed.

From the perspective of the formulated hypothesis about the origin of the dramatic loss of cell viability seen during cryopreservation of hematopoietic stem cells, the inhibitory action of DMSO upon eutectic crystallization is considered a

https://doi.org/10.1021/acs.jpclett.2c03003
J. Phys. Chem. Lett. 2022, 13, 11153−11159

beneficial effect. The presented results indicate that eutectic crystallization is suppressed thermodynamically and kinetically in the presence of DMSO. The eutectic crystallization of NaCl is wholly suppressed by about 2 vol % DMSO in isotonic NaCl solution (0.9 wt %). It correlates with the strongly concentration-dependent cryoprotective effect of DMSO. This striking correlation supports the hypothesis that the cryoprotection of low DMSO concentrations (>2 vol %) originates from the inhibition of NaCl eutectic crystallization.

The kinetic aspect of the DMSO inhibitory effect was manifested by the appearance of cold eutectic crystallization (crystallization occurring during the thawing procedure) observed by DSC and PALS measurements. The kinetic aspect may indicate an increased nucleation barrier associated with eutectic crystallization or reduced diffusion of NaCl and water. However, it still holds that even if the amorphous freeze-concentrated phase is formed during freezing, it might crystallize upon thawing. DMSO concentrations higher than 2 vol % offered complete protection against eutectic crystallization.

Since the effect of low DMSO concentrations (<2 vol %) on the eutectic phase transformation of the binary water−NaCl mixture resembles the classic freezing point depression effect, a thermodynamic description of such ternary mixtures is provided. Considering that at DMSO concentrations lower than 2 vol %, NaCl is the dominant solute (see Table 1 in the Supporting Information), it is reasonable to model the effect of DMSO by considering the DMSO to be a simple diluent while keeping the full nonideality of the binary water−NaCl mixture. Within this model, the effect of DMSO is purely entropic, as it dilutes the water and NaCl in a liquid mixture, increasing the entropy of the liquid mixture and thus decreasing the chemical potential of water and dissolved NaCl. The chemical potentials of the liquid and solid (eutectic) phases are equalized at a lower temperature, resulting in a eutectic point depression. In this model, where DMSO is considered as a diluent (pseudoideal mixture), the freezing point or temperature of the eutectic reaction (T) can be determined by solving the Schroeder equation[50] while setting the activity coefficient γ equal to 1 (the ideality assumption):

$$\ln \gamma x = \frac{\Delta H}{R T_0}\left(1 - \frac{T_0}{T}\right) \quad (1)$$

where T_0 is −21.1 °C (252 K), the standard freezing point of a binary water−NaCl mixture of eutectic composition, R is the gas constant, x is the mole fraction, and ΔH is the heat of fusion of eutectic reaction, 233 J/g.[51] In this description, the reasonable definition of a reference state is given by the total chemical potential of 23.3 wt % NaCl in water at −21.1 °C (252 K) (the eutectic composition and eutectic temperature of a binary water−NaCl mixture). The mole fraction x in the logarithm represents the concentration of this binary water−NaCl mixture, which is decreased by the addition of DMSO into the mixture and can be calculated as $x = n_{NaCl}/(n_{NaCl} + n_{DMSO})$, where n refers to the number of individual species. This model predicts the depression of eutectic temperature induced by DMSO. During the eutectic reaction, NaCl dihydrate and ice are formed, and DMSO is further concentrated to NaCl, leading to further depression of eutectic temperature. The model agrees with the experimentally observed phase behavior of these ternary mixtures, as is depicted in Figure 5, where the eutectic temperature is plotted against the (nominal) concentration of DMSO in the ternary mixture. There is a good agreement between the pseudoideal model and the actual temperature of the eutectic reaction up to 1 vol % DMSO. A minor negative deviation from the model develops at higher DMSO concentrations. The most significant eutectic point depression was observed for the 1.5 vol % DMSO, while the lowest enthalpic change was associated with the 1.8 vol % DMSO (Figures 1 and 5). This observation and the deviation of the eutectic temperature of the 1.8 vol % DMSO from the pseudoideal mixture model indicate the existence of a ternary eutectic point at about 1.5 vol % DMSO concentration.

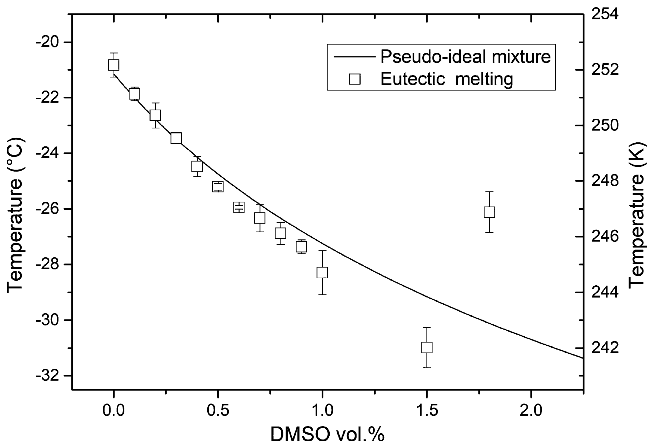


Figure 5. Depression of eutectic temperature by DMSO. The solid line represents the pseudoideal mixture model as described by eq 1. The eutectic temperature is given as a mean value of three measurements with error bars representing standard deviations. Note that in the cases of 1.5 and 1.8 vol % DMSO, the eutectic crystallization was observed in two (of three) measurements.

Although such a ternary eutectic reaction was not observed in our experiments, published studies demonstrated a phase transformation at −35 °C (238 K), which could be interpreted as the ternary eutectic reaction. This phase transformation was—in some cases—observed in ternary mixtures of composition equivalent to 0.4, 0.8, 1, and possibly 1.9 vol % DMSO but never at concentrations higher than or equal to 2.45 vol % DMSO.[31,42,52,53] The ice freezing point data from the cited studies indicate another ternary eutectic point at about −70 °C (203 K) and a higher DMSO/NaCl concentration ratio than the ratio of 2 vol % DMSO mixture, although this ternary eutectic reaction was probably never observed. A transition between these two ternary eutectic points in the ternary phase diagram is relatively sharp for the DMSO concentration change. In the case of 1.9 vol % DMSO, the ternary eutectic point is at −35 °C (238 K),[53] while in the case of 4.1 vol % DMSO,[31] it is equal to or lower than −65 °C (208 K) (in the latter case, no eutectic reaction occurred; the temperature is estimated from the freezing point of ice). The transition between these two ternary eutectic points may be related to the inhibition of eutectic crystallization by DMSO and the dramatic onset of its cryoprotection. It may also explain the observation of a disproportionately large freezing point depression of ice observed for 2 vol % DMSO equivalent (90 wt % water) compared to the 1.8 vol % DMSO (Supporting Information, Figure S4).

It might be hypothesized that the lower ternary eutectic point is associated with the eutectic formation of different phases. An interesting perspective is offered by considering the

https://doi.org/10.1021/acs.jpclett.2c03003
J. Phys. Chem. Lett. 2022, 13, 11153−11159

crystallization of pure NaCl crystal. Although the NaCl dihydrate is thermodynamically more stable phase at temperatures lower than 0.1 °C (273.25 K) in the binary water−NaCl mixture, the situation may be different in mixtures containing DMSO, especially when poor solubility of NaCl in pure DMSO is considered,[54] indicating unfavorable intermolecular interactions between DMSO and NaCl. This picture is also supported by molecular dynamics simulation, which revealed that DMSO and sodium ions are primarily solvated by water in ternary mixtures.[55] Interactions between DMSO and water molecules were also found to be stronger than DMSO−DMSO or water−water interactions in binary mixtures.[56] The solubility of pure NaCl might be affected differently by DMSO than the solubility of NaCl dihydrate, indicating the possibility of pure NaCl being a more stable phase under some conditions (higher DMSO content). This speculation is supported by a comparison of the pure NaCl solubility obtained at 25 °C (298 K),[57] which is decreased with increasing DMSO concentration, as shown in Figure S7 in the Supporting Information. It is reduced below the eutectic concentration of NaCl in the binary water−NaCl mixture (5.2 molal). The eutectic concentration of NaCl, which may be expressed from the published phase diagrams, also depends on the DMSO concentration and is decreased by DMSO (Figure S7). However, the solubility of pure NaCl falls more quickly. Above some DMSO concentrations, it is lower than the eutectic concentration of NaCl attained during freeze-concentration, implying that pure NaCl might be the thermodynamically more favorable phase for mixtures with higher DMSO content.

The mechanism of cell cryoinjury imposed by eutectic crystallization has not yet been elucidated. A detrimental mechanical aspect of crystallization has been suggested as a likely cause of injury.[37] Elucidation of the detailed mechanism of this kind of cryoinjury is beyond the scope of this study. However, some insight may be gained by considering a possible explanation provided by authors of a tensodilatometric study of cryobiologically relevant mixtures,[58] in which volumetric variations associated with solidification and melting were observed. Authors argued that the volume expansion associated with ice crystallization in closed fluid inclusions (patches of unfrozen freeze-concentrated phase between individual ice crystals that are not interconnected) could lead to the development of a surplus hydrostatic pressure, which might be relaxed by the movement of ice crystals in the surrounding ice matrix. The authors considered this effect a source of mechanical injury to biological cells. Similar to ice, a relative 5.2% volumetric expansion is associated with the eutectic crystallization of NaCl,[59] suggesting a possible mechanism of cryoinjury.

In summary, the presented results support the formulated hypothesis considering the eutectic NaCl crystallization to be the cause of cryoinjury observed in cryopreservation of hematopoietic stem cells with DMSO concentrations lower than 2 vol %. This conclusion is justified by (i) the presented correlation between the minimal DMSO concentration needed to inhibit the eutectic crystallization and the concentration securing the cell viability and (ii) the fact that eutectic crystallization of NaCl was shown in the past to cause direct cell cryoinjury and dramatic loss of cell viability.[37] A possible mechanism of cryoinjury imposed by eutectic crystallization was provided by considering the volumetric expansion of the crystalline eutectic phase. The cryoprotective mechanism of DMSO was found to be related to the ability to inhibit the eutectic crystallization. The central aspect of the inhibitory effect of DMSO upon the eutectic crystallization of NaCl appears to be related to the suppression of the eutectic phase transformation kinetically and thermodynamically. Thermodynamic considerations suggest competition between DMSO and NaCl for interaction with water molecules, favoring the formation of eutectic phases that are more difficult to precipitate. This result represents an advance in understanding cryobiologically relevant processes, emphasizing the role of eutectic phase transformation. These findings enable a targeted optimization of cryopreservation protocols, especially regarding the composition of the cell freezing medium and the associated cryoprotectant content. In addition, it brings another criterion that novel cryoprotectants should meet—the ability to suppress eutectic crystallization during freezing and thus protect the viability of biological material. The physicochemical description of the ternary mixture may also offer insight into cryoprotection provided by deep eutectic solvents, characterized by dramatic freezing point depression, which are currently being evaluated and applied as novel cryoprotectants.[60]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jpclett.2c03003.

> Materials: Composition of mixtures, Methods: Differential scanning calorimetry; Electric conductivity measurements; Raman microspectroscopy; Positron annihilation lifetime spectroscopy, Detailed results: Differential scanning calorimetry—detailed results, cold crystallization, and disproportionally large freezing point depression of ice; Raman microspectroscopy; Positron annihilation lifetime spectroscopy—isothermal time measurements; Solubility of NaCl in ternary water−NaCl−DMSO mixtures, eutectic concentrations of NaCl during freezing (PDF)
>
> Transparent Peer Review report available (PDF)

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https://doi.org/10.1021/acs.jpclett.2c03003
J. Phys. Chem. Lett. 2022, 13, 11153−11159

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The research was funded by the APVV agency (project no. APVV-16-0369, SK-BY-RD-19-0019, and APVV-21-0335), VEGA agency (project no. 2/0134/21 and 2/0166/22), KEGA agency (project no. 041UK-4/2020), and Foundation for Cell Transplantation.

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