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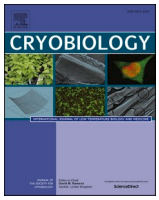

Disaccharide-assisted inkjet freezing for improved cell viability

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ABSTRACT

Non-permeable disaccharides are widely used as cryoprotectant agents due to their low cytotoxicity, but their protective effect is insufficient when the disaccharides are present only extracellularly. On the other hand, cryoprotectant agent (CPA)-free cryopreservation has been recently achieved by instantaneously inkjet-freezing cells as tiny droplets. However, CPA-free cryopreservation requires skilled handling operations due to instability of the vitreous water without the CPA. In this study, the effectiveness of separately adding two types of disaccharides in inkjet freezing of 3T3 cells was evaluated and the following results were obtained. First, trehalose showed the highest effect at 0.57 M, twice the plasma osmolarity, with a maximum cell viability of over 90 % when freezing 70 pL droplets. However, higher concentrations of trehalose decreased cell viability due to damage caused by dehydration. Similarly, sucrose gave cell viability close to 90 % at 0.57 M with 70 pL droplets, and higher concentrations decreased cell viability. Next, the relationship between minimum trehalose concentrations to prevent intracellular and extracellular ice crystal formation and droplet size was analyzed. The results indicated that trehalose of less than 0.57 M was able to inhibit intracellular ice crystal formation even in the largest droplet used in this study, 450 pL, while trehalose of nearly 0.57 M was required to inhibit extracellular ice crystal formation in the smallest droplet, 70 pL. In other words, the suppression of extracellular ice crystals by the addition of CPA was shown to be crucial in improving the viability of inkjet superflash freezing.

1. Introduction

Cryopreserved cells can be maintained semi-permanently by stopping their biological activities [20]. Freezing cells without taking specific care measures results in the formation of ice crystals around the cells, which can cause lethal damage due to injury to cell membranes and organelles [23]. Therefore, conventional cell cryopreservation methods use at least one type of cryoprotectant agent (CPA) such as dimethyl sulfoxide (DMSO) or ethylene glycol to inhibit ice crystal formation and induce vitrification [21]. However, CPAs are generally toxic to cells and living organisms [18]. Alternatively, rapid cooling has received a great deal of attention as a way to avoid the use of CPAs for cryopreservation [33]. Several groups have reported results using spraying or inkjet printing techniques that can confine cells within sub microliter or nanoliter droplets [10,24,34]. Although the rapid cooling reduced the necessary concentration of the CPAs, the addition of the highly toxic permeable CPAs [3] was still essential.

Recently, cryopreservation of cells without a CPA by superflash freezing (SFF) was reported using inkjet technology [1]. The SFF reduced the volume of inkjet droplets to a few tens of picoliters, and the cells in the droplet were instantaneously cooled on a liquid nitrogen-cooled substrate to achieve a much faster cooling rate than in previous studies. This cooling rate exceeded the estimated critical cooling rate (CCR) of 10^4 °C/s required for cell vitrification [2], thereby suppressing intracellular ice crystal formation (IIF). Due to the small difference between the CCR and the achievable cooling rate of inkjet freezing, skilled manipulation was required to obtain reproducible data. Any instability of the inkjet ejection would probably cause a decrease in ejection speed and misalignment of the ejection direction, resulting in a decrease in cooling rate and cell viability. In addition, the medium used for the freezing solution mainly consisted of inorganic salts, sugar, amino acids, and vitamins and had no CPA addition. The CCR of the medium would be considerably higher than the CCR of the cells because of the dilute solute concentration. The CCR of the medium has been estimated to be approximately 3×10^5 °C/s based on the effect of only the major salt, sodium chloride [13]. The reported SFF [1] would

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insufficiently suppress extracellular ice crystal formation (EIF) and complete suppression of EIF could further improve viability in CPA-free inkjet freezing.

To solve the problems in CPA-free SFF, this study proposes inkjet freezing assisted by addition of a disaccharide. Disaccharides are one of the most widely used non-permeable CPAs (npCPAs) due to their low influence on cells. Disaccharides are also recognized to be nontoxic CPAs compared to conventional permeable CPAs [28]. If the addition of a disaccharide sufficiently lowers the CCR of the extracellular solution and suppresses EIF, a further improvement in cell viability can be expected. The selected disaccharide, however, must be present both inside and outside the cell (in other words, on both sides of the cell membrane) [6]. Therefore, several methods have been investigated to introduce disaccharides impermeable to cells [5,11,22,25,29]. Another possible side effect of CPA addition is intracellular dehydration, leading to cytosolic solute concentration. Similar to the vitrification method for cryopreservation [12], when cells are immersed in a hypertonic disaccharide solution, the cells are dehydrated and shrink. At this time, the cytosolic solution is concentrated without leakage of cytosolic solutes due to the selective permeability of the cell membrane [14], which will decrease the CCR of the cell. Namely, the addition of the disaccharide improves the suppression of both EIF and IIF and compensates for the reduced cooling rate due to unstable inkjet ejection speed. In addition, the reduction in CCR allows for a larger inkjet droplet size. An increase in inkjet droplet size can increase inkjet printing efficiency, but it is accompanied by a decrease in CCR. The addition of the npCPA increases throughput and expands the applicable sample size.

In this study, inkjet freezing is first performed with trehalose at different droplet sizes and concentrations, and the droplet cell viabilities are evaluated. Next, inkjet freezing with sucrose at the minimum droplet size is performed and evaluated at different concentrations. In addition, to take into account the effect of osmotic dehydration, cells are frozen in phosphate-buffered saline with different osmotic pressures. Then, the cooling rates for various droplet sizes are calculated numerically, and the estimated CCRs of EIF and IIF at each trehalose concentration are compared. Based on the comparison results, the relationship between droplet size and the minimum trehalose concentration required to inhibit ice crystal formation is obtained. Finally, appropriate disaccharide addition conditions are discussed by comparing experimentally determined cell viabilities with theoretically estimated freezing conditions.

2. Materials and methods

2.1. Inkjet freezing system

Details of the inkjet freezing system have been described elsewhere [1]. Briefly, inkjet droplets ejected from the inkjet head were deposited onto a glass substrate placed on an aluminum base cooled with liquid nitrogen and instantaneously frozen. Three sizes of inkjet heads to produce 70 pL, 200 pL, and 450 pL droplets (IJHD-100, 300, and 1000, Microjet) were used. The droplets were ejected to a 200 μm pitch grid using an automatic two-axis stage to avoid droplet overlap, which would slow the cooling rate. The cells in the frozen droplets were rapidly thawed using an automatic thawing device [32] which rotates using a hinge spring and the viability was evaluated.

2.2. Preparation of the freezing solutions

Trehalose (206–18455, Fuji Film Wako) and sucrose (196-00015, Fuji Film Wako) were used as disaccharides. The concentration of disaccharides was expressed as osmotic ratio, which is the concentration ratio to 0.285 M isotonic to plasma. For example, 0.5x, 1x, 1.5x, and 2x mean 0.143, 0.285, 0.428, and 0.570 M, respectively. Due to solubility limitations, disaccharide solutions greater than 4-fold could not be prepared. Disaccharide solutions were sterilized by filtration through a 0.2 μm membrane filter, and Dulbecco's phosphate-buffered saline (DPBS) was prepared by diluting 10x concentrated DPBS(−) (048–29805, Fuji Film Wako) with sterile reverse osmosis water to the desired concentration.

2.3. Cell preparation and evaluation

Throughout this study, 3T3 cells (RIKEN Cell Bank), cell lines of mouse embryonic fibroblasts, were used and cultured in culture medium (CM), high glucose DMEM (045–30285, Fuji Film Wako) with 10 % fetal bovine serum (FBS) and antibiotics. To eliminate the trehalose uptake due to slight cell permeabilization, the trypsin-recovered cells were frozen immediately after immersion in the freezing solution. Slow freezing was carried out in a deep freezer using a freezing vessel (BICELL, Nihon Freezer Co., Ltd.), and inkjet freezing was performed using the system mentioned in Section 2.1. After thawing, followed by 3 h of incubation, cell viability was evaluated based on the number of cells counted manually using calcein-AM and propidium iodide (PI) (Dojindo). These conditions were used for the following reasons. Esterase activity might remain in dead cells immediately after thawing, and the cells would be subjected to membrane damage by inkjet printing. Immediately after the inkjet printing, PI could permeate through the damaged membrane, resulting in red staining. It has been reported, though that after 3 h of incubation the membrane recovered and PI no longer permeated even 1.6 nm [8]. However, in this study, there were still cells that were stained both green and red and appeared yellow, probably due to the impact of inkjet printing plus freezing damage. Therefore, they were counted as yellow cells that may be alive despite membrane damage. The percentage of cells stained only green was evaluated as the percentage with intact viability, and the percentage of green cells plus yellow cells was evaluated as the percentage with damaged viability.

Cell size during dehydration with trehalose hypertonic solution was measured using a cell counter (TC-20, BioRad).

2.4. Cooling and warming rates calculation based on finite element simulation

The cooling and warming rates were calculated by solving the heat transfer equation for a two-dimensional axisymmetric model using finite element analysis software (COMSOL Multiphysics ver. 6.1). Calculation details have been described elsewhere [1]. The physical properties of the trehalose solutions (thermal conductivity, specific heat, and density) depend on temperature and concentration. The equations for these relationships were obtained from the literature [9,26]. The droplet volumes were 20 pL, 70 pL, 200 pL, and 450 pL. The obtained cooling and warming rates for each droplet size had hardly any effect from the trehalose concentration and the difference to pure water was less than 5 %. Therefore, the cooling rates with pure water were used.

2.5. Data analysis

Regression analysis and statistical processing were performed using Igor pro 7 (WaveMetrics). Statistical analysis was performed using the unpaired *t*-test with a significance level of $p < 0.05$. All the error bars in the figures show standard deviation (SD).

3. Results and discussion

3.1. Cell damage from immersion in trehalose solutions

Cell viability after 1 h of immersion in trehalose solution is shown in Fig. 1; this time was selected for the observation as the inkjet freezing process takes approximately 30 min. Despite the absence of Na and K, which are necessary for the maintenance of intracellular potential, there was no major decrease in viability up to a 2x osmotic ratio. In particular,

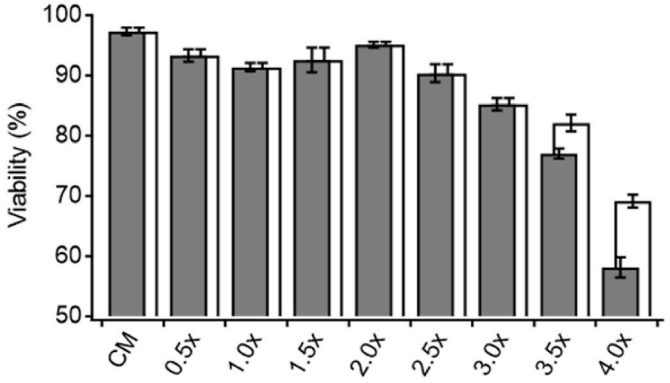

Fig. 1. Cell viability after 1 h immersion in trehalose solution. There were significant differences between the culture medium (CM) and all the trehalose solutions. The gray and white bars show intact and damaged viabilities, respectively. n = 5.

there was no difference between intact and damaged viability up to 3x, but at 3.5x and 4x osmotic ratios, yellow cells appeared which indicated the reduction of intact viability. All concentrations showed a slight but significant decrease in viability compared to CM as control. It was shown that the preferred trehalose concentrations were up to 2x, and they could minimize the cell damage caused by immersion.

3.2. Cell viability by inkjet freezing in trehalose solutions

The cryoprotective effect of trehalose was first evaluated by slow freezing at osmotic ratios ranging from 0.5x to 4.0x and the results are plotted in Fig. 2. For reference, the viability of slow freezing in 5 % DMSO is also shown, and the intact and damaged viabilities were 83 % and 90 %, respectively. Overall viability with trehalose was low, meaning that the protective effect of trehalose was insufficient when trehalose was present only externally [29]. Intact viability increased in a concentration-dependent manner, reaching a maximum of about 25 % at 2x, and it decreased at higher concentrations. This decrease was consistent with the cell damage caused by immersion in trehalose solution, which showed greater damage above 2.5x. Thus, dehydration damage seems to be more pronounced above 2.5x. Incomplete viability was almost constant at about 30 %. It was confirmed that trehalose alone was not sufficient for cryoprotection in slow freezing.

The results of inkjet freezing in 70 pL droplets with trehalose solution are shown in Fig. 3(a). In the CM without CPA, intact viability was

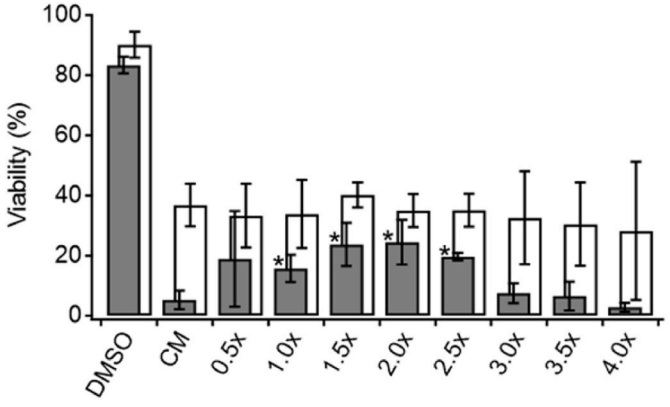

Fig. 2. Cell viability in the slow freezing method. The bars of the first group were obtained by the conventional slow freezing method with 5 % DMSO. The asterisks indicate intact viability results with statistically significant differences against the CM intact viability. The gray and white bars show intact and damaged viabilities, respectively. n = 5.

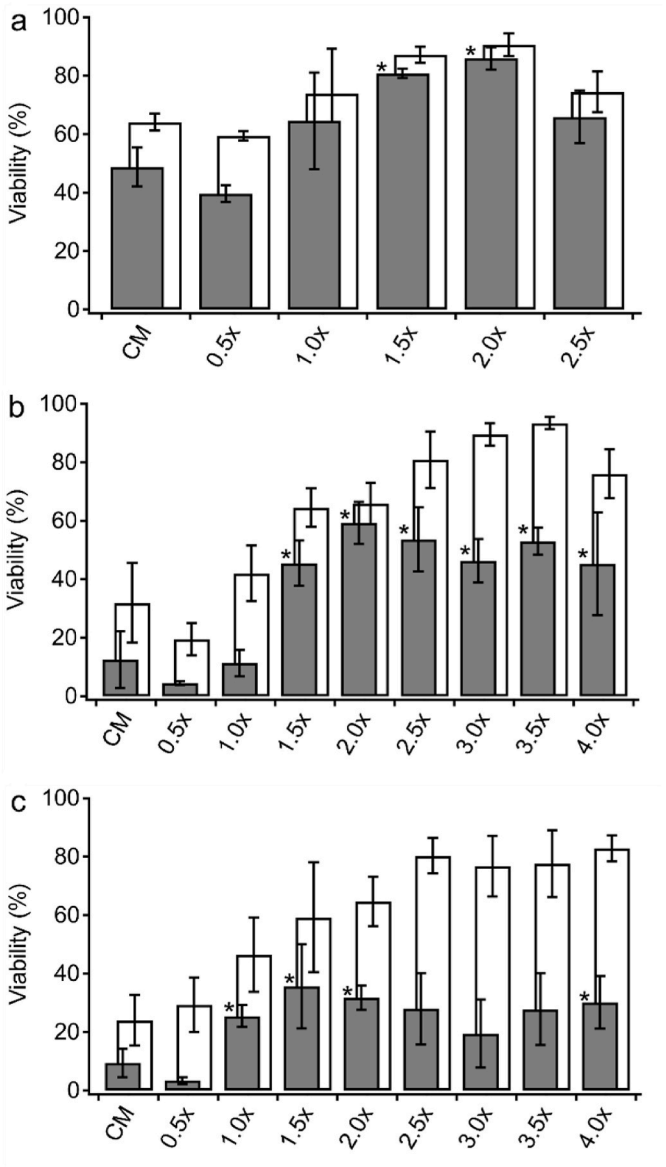

Fig. 3. Cell viability with or without trehalose by the inkjet freezing with (a) 70 pL droplets, (b) 200 pL droplets, and (c) 450 pL droplets. The gray and white bars show intact and damaged viabilities, respectively. Statistically significant differences in intact viability were detected between the asterisked bars and the CM. n = 3 for (a) and n = 5 for (b) and (c).

approximately 50 % but increased at concentrations greater than 1x. Viability increased with increasing trehalose concentration, with a maximum of 86 % intact viability and damaged viability over 90 % at 2x. This is fully comparable to the slow freezing method using 5 % DMSO. The viability after trypsinization and centrifugation was about 95 % as shown in Fig. 1 and there was added damage caused by a pretreatment such as filling the inkjet head. Taking these findings into account, the damage caused by freezing was considered to have disappeared and it would be difficult to further improve the viability. The viability at 2.5x decreased the same as in the case of slow freezing, and inkjet dispensing at 3.0x could not be performed due to high viscosity. Thus, even with inkjet freezing, high osmotic pressures above 2x were found to cause damage due to excessive dehydration.

Next, viability was evaluated in 200 pL and 450 pL droplets (Fig. 3(b) and (c)). The increase in droplet size allowed both droplets to be discharged stably up to 4x. For both droplet sizes, intact viability at CM was about 10 %, indicating that CPA-free cryopreservation was difficult. In

200 pL droplets, the addition of trehalose above 1.5x increased viability to nearly 50 % at all concentrations. As with slow-freezing and 70 pL droplets, the highest viability was obtained at 2.0x and decreased at higher concentrations. In 450 pL droplets, the addition of trehalose from 1x to 2x improved the maximum intact viability to over 45 %. There was a slight decrease at higher concentrations and a trend toward an increase at 3.5x and 4x. At both 200 and 450 pL, viability was not significantly improved by trehalose concentrations higher than 2.5x. This invalidity would be derived from canceling out the cryoprotective effect of trehalose and the dehydration damage caused by exosmosis. On the other hand, the damage viability increased to 3x for both droplet sizes as the concentration increased, probably due to the dominant protective effect of trehalose, and then remained roughly constant.

3.3. Cryoprotective effect of sucrose in inkjet freezing

The effect of sucrose, another disaccharide commonly used as a CPA [27], was evaluated in inkjet freezing with 70 pL droplets. First, viability after 1 h of immersion was examined, and the results are shown in Fig. 4 (a). Damage was found to be slightly more extensive than for trehalose. In particular, the intact viabilities at 2x and 3x were below 90 % and 75 %, respectively. Next, the viability of inkjet-frozen cells in 70 pL droplets is shown in Fig. 4(b). As with trehalose, the highest intact viability was obtained at 1.5x and 2x with only a few yellow cells being observed. In particular, both intact and damaged viabilities were comparable to those of trehalose. These results showed that sucrose, like trehalose, was sufficiently effective as the CPA for inkjet freezing.

3.4. Cell viability 24 h after freeze-thaw

The viability of inkjet freezing of 70 pL droplets without and with disaccharide at 2x and the conventional method 24 h after thawing was also examined. This was done to account for cryopreservation-induced, delayed-onset cell death (CIDOCD), which is reported to occur hours to days after thawing [4]. The result together with viability at 3 h is shown in Fig. 5(a). No significant differences in viability between 3 h and 24 h were observed for both inkjet freezing and slow freezing methods. It should be noted that in fluorescence microscopic observations, all cells remained round and no cells were found to be elongated after thawing for 3 h, but some cells were found to be adherent and elongated in all conditions as shown in Fig. 5(b). In particular, a higher percentage of cells with elongated pseudopodia were observed in inkjet freezing with trehalose and sucrose than in the other conditions. This quicker start of cell activity after thawing suggests that inkjet freezing with disaccharide addition would result in better intracellular freezing. In addition, an increasing trend was observed for inkjet freezing without disaccharide. This is possibly because the cells were frozen at a physiological osmotic pressure without dehydration, so that CIDOCD did not occur very often. Alternatively, the cells might start to divide relatively quickly after thawing, resulting in a higher apparent viability. Further studies are needed to clarify these causes.

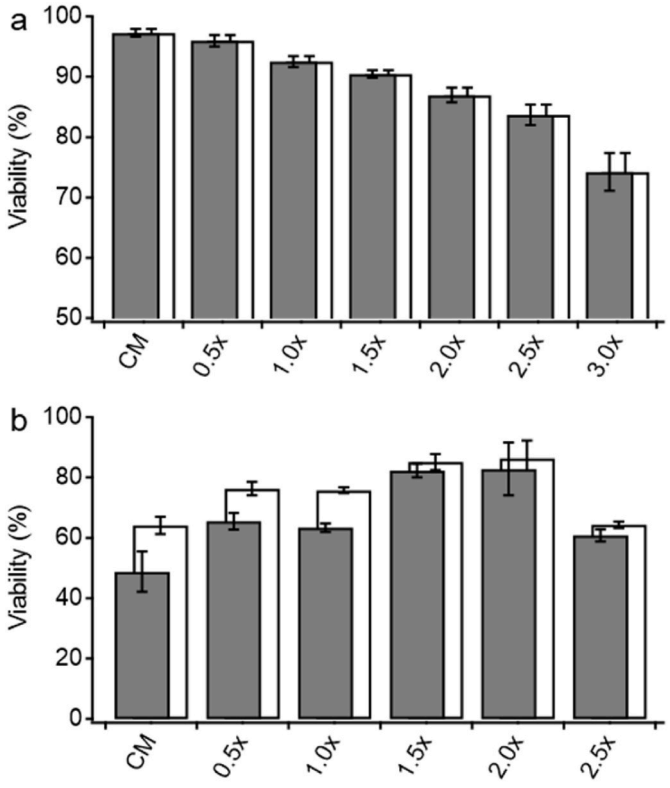

Fig. 4. Cell viability after (a) immersing for 1 h and (b) freezing in sucrose solution. The gray and white bars show intact and damaged viabilities, respectively. There were statistically significant differences between the CM and all the sucrose solutions in both graphs. n = 5 for (a) and n = 3 for (b).

3.5. Comparison to freezing in high osmotic buffer saline

Both the disaccharides, trehalose and sucrose, were effective in improving viability in inkjet flash freezing. To confirm that this effect was not solely due to concentration by dehydration, inkjet freezing was performed with 70 pL droplets of various osmotic concentrations of DPBS and the results are shown in Fig. 6. As with the disaccharides, intact viability was highest at 2x, but remained at 60 % and then decreased at higher concentrations of DPBS. At all concentrations of DPBS, these viabilities were lower than those of the disaccharide solution at the same osmotic ratio. The decrease showed that an effect other than the concentration by dehydration contributed to the improved viability of the disaccharide. One possible factor would be the protective effect of disaccharides, and another would be the prevention of EIF by lowering the CCR of the extracellular solution due to the addition of disaccharides.

Trehalose could have a protective effect on cell membranes. The 70 pL droplets required no trehalose to suppress IIF and above 1.8x trehalose to suppress EIF, indicating that EIF occurred (discussed in section 3.6). However, Fig. 3a and 4b show that 1.5x disaccharide solutions increased viability to as high as almost 2x and nearly completely suppressed freeze damage. It has previously been reported that trehalose must be present on both sides of the cell membrane to exert its cryoprotective effect [6,7], and that trehalose and sucrose are barely taken up by cells in the short term [17]. Considering these studies, it was concluded that trehalose could exert its cryoprotective effect when it was present only on the side where ice crystals formed. Considering that disaccharides were present only on the extracellular side having cryoprotective properties, this suggested that they had a protective effect on the cell membrane, which was the boundary separating the inside of the cell from its outside.

It is known that FBS has a cryoprotective effect [13], whereas the effect of increasing the concentration of inorganic salts in DPBS on CCR is negligible and small. Therefore, the higher viability of CM than 1x DPBS would be due to the cryoprotective effect of FBS.

3.6. Critical concentrations to inhibit EIF and IIF

In the slow freezing method, cell dehydration due to exosmosis during freezing based on chemical potential must be considered [19,30]. In principle, if the solution is cooled at a cooling rate that exceeds the CCR of the solution, the solution will be vitrified without ice crystal formation. In other words, if the solute concentration exceeds the minimum concentration required for vitrification (critical concentration) at any droplet size, the droplet will be vitrified. In this study, water migration by exosmosis during freezing was negligible because tiny

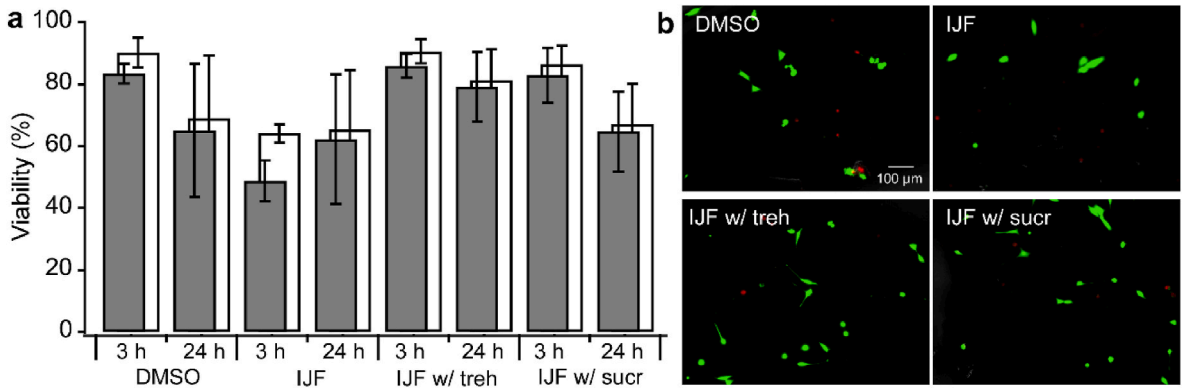

Fig. 5. Evaluation of cell viability 24 h after thawing under various freezing conditions. (a) Comparison of viability 24 h after thawing with that 3 h after thawing. DMSO indicates the slow freezing with 5 % DMSO. IJF indicates inkjet freezing of 70 pL droplets. (b) Fluorescent microscopic images of cells 24 h after thawing. Live and dead cells are stained with green and red, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

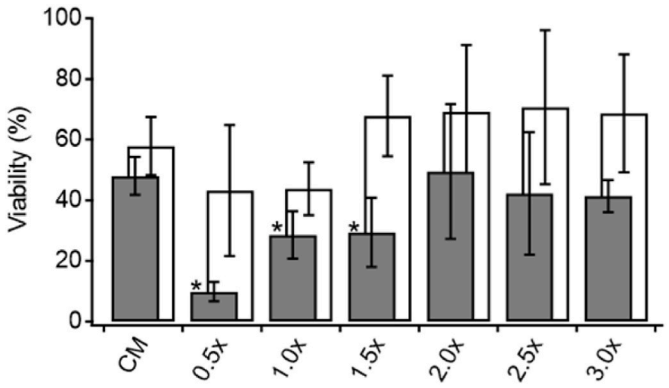

Fig. 6. Cell viability by inkjet freezing in various concentrations of DPBS. The gray and white bars show intact and damaged viabilities, respectively. The asterisks indicate intact viability results with statistically significant differences from the CM intact viability. n = 5.

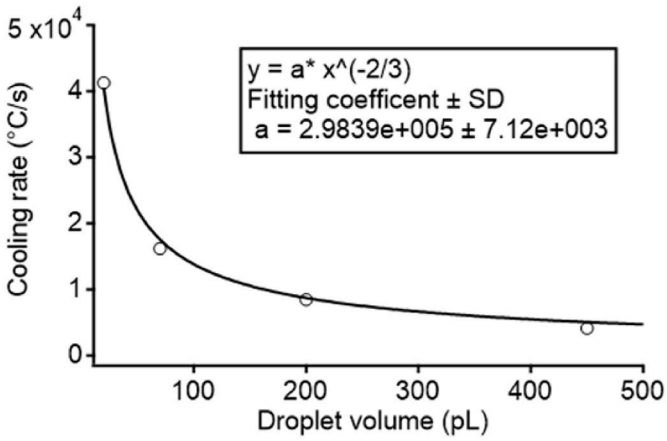

Fig. 7. Relationship between droplet size and cooling rate obtained by finite element simulation.

droplets were frozen instantaneously by the inkjet freezing. Therefore, the formation of ice crystals can be treated separately as extracellular and intracellular: EIF is suppressed by the decrease in CCR of the extracellular solution due to the addition of trehalose; and IIF is suppressed by the decrease in CCR of the intracellular solution due to exosmosis by hypertonic trehalose solution. Therefore, the relationships between the critical concentrations for suppression of EIF and IIF and droplet size were determined and evaluated.

First, the relationship between the critical concentration of trehalose and droplet size required to suppress EIF was determined. Since the freezing of inkjet droplets involves a large temperature difference between the surface and the interior of the droplet, the cooling time will be proportional to the square of the representative length of the droplet. Therefore, the cooling rate obtained by the finite element simulation was used and a regression analysis was performed on a curve showing that the cooling rate v [°C/s] was proportional to the −2/3 power of the volume V [pL] (Fig. 7). As a result, the following relational equation was obtained.

$$v = 2.9809 \times 10^5 \left(V^{-\frac{2}{3}} \right) \tag{1}$$

According to the literature [31], the CCR of a trehalose solution v_c [°C/s] is expressed using the concentration c [w/v%] as follows.

$$v_c = 3.0 \times 10^5 \exp(-0.16193\, c) \tag{2}$$

From equations (2) and (3), the critical concentration of trehalose to suppress ice crystal formation with respect to EIF c_{c_EIF} [w/v%] was expressed by the following equation.

$$c_{c\_EIF} = \ln\left(\frac{3.0}{2.9809} V^{\frac{2}{3}} \right) \Big/ 0.16193 \tag{3}$$

The addition of trehalose at a concentration higher than this c_{c_EIF} will suppress EIF.

Next, the cooling rate of the intracellular solution to suppress IIF was estimated. The cells were immersed in trehalose solution of each osmolarity for 20 min and then their size was measured. Based on the diameters obtained, the volume ratio of the cells R to the CM culture was calculated with the assumption that the cells were spheres (Fig. 8). For the trehalose concentration ratio to 0.285 M x, regression analysis yielded the following relationship.

$$R = 0.5673 + 1.5296 \exp(-1.2599\, x) \tag{4}$$

With the assumptions that the intracellular solution was concentrated as the volume was reduced due to dehydration and that the CCR of the cell in the isotonic solution was 10^4 °C/s, the CCR of the cell v_{c_cell} was expressed by the following formula.

$$v_{c\_cell} = 3 \times 10^5 \exp(-3.401197/R) \tag{5}$$

Finally, equations (2), (4) and (5) yielded equation (6), which expresses the critical concentration ratio with respect to IIF x_{c_IIF}.

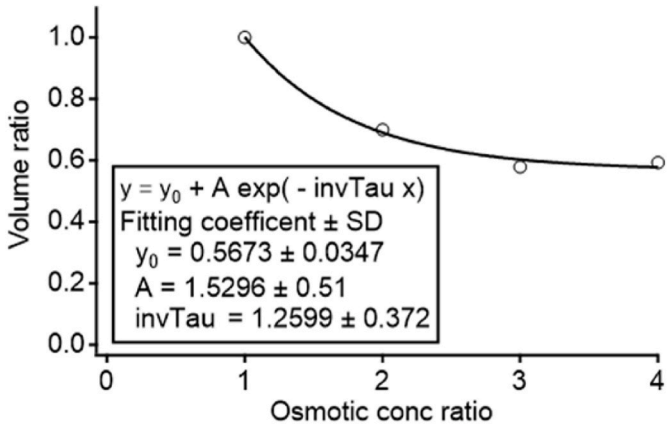

Fig. 8. Volume ratio of cells at each trehalose concentration to that in the CM.

$$x_{c\_IIF} = \ln\left(\left(\left(-\frac{3.401197}{\ln\left(\frac{2.9809}{3}V^{-\frac{2}{3}}\right)}-0.5673\right)\Big/1.5296\right)\Big/(-1.2599)\right)$$
$$= -\ln\left(\left(\frac{2.2236}{\frac{2}{3}\ln V+0.006387}-0.3709\right)\Big/1.2599\right) \quad (6)$$

Equations (3) and (6) relating droplet size to critical concentration in EIF and IIF are visualized in Fig. 9. For IIF, the cooling rate even at 450 pL, the largest droplet size, exceeded the CCR of the 1.5x trehalose solution. This meant that IIF was suppressed by disaccharide addition at relatively low concentrations. In contrast, EIF exceeded 2x at even 200 pL, like 1.8x at 70 pL did, 2.2x at 200 pL did, and 2.6x at 450 pL did. This meant that vitrification of extracellular solutions would require high concentrations of trehalose at a level capable of causing significant cell damage, which could result in reduced viability.

3.7. Calculated results of warming rate

The small droplet size would also contribute to rapid thawing in the warming process. The warming rates obtained by finite element simulation were regressed on a curve proportional to −2/3 of the volume V [pL] using the same procedure as for the cooling rate (Fig. 10). The result showed that the fitting was as good as for the cooling rate, and was as follows.

$$v_w = 7.1652 \times 10^5 \left(V^{-\frac{2}{3}}\right) \quad (7)$$

Comparison of this equation with equation (1) indicates that the warming rate was 2.4 times faster than the cooling rate for the same droplet size. However, this value should be insufficient since the critical warming rate (CWR) is generally considered to be an order of magnitude faster than CCR. In particular, the CWR for a 50 w/v% sucrose solution is about 10^7 °C/s [16], which was two orders of magnitude higher compared to the calculated warming rates. Therefore, improving the warming rate would also undoubtedly improve viability. It should be noted that the actual warming rate might be higher since fluid flow was neglected to simplify the model in the simulation. In addition, the relationship between CCR and CWR is discussed in the literature [15], but there are no data on sugar solutions at such high rates comparable to rates of inkjet freezing. Precise understanding of inkjet droplet thawing requires more data on CWR at rapid warming.

3.8. Guidelines for CPA addition to inkjet freezing

Experimental results indicated that the preferred guideline for the trehalose addition in inkjet freezing was the 2x osmotic concentration (0.57 M), which had sufficiently small adverse effects on cells. Concentrations higher than 2x could cause damage due to cell dehydration. Therefore, even if EIF was suppressed by the addition of higher concentrations, cell viability would not be improved. Similarly, sucrose would be basically preferable at a 2x osmotic concentration. Since the molecular weight of sucrose is the same as that of trehalose and the effect of reducing CCR should be considered to be close [16], the same guidelines as for trehalose would be applicable.

The experimental results for disaccharides indicated that a substance that lowered the CCR and inhibited the increase in the osmotic pressure as much as possible was desirable as the npCPA used in inkjet freezing. This would avoid cell damage due to osmotic pressure, even if the CPA was added at high concentrations to prevent damage due to EIF. For example, the cooling rate of 450 pL is 4100 °C/s, which allows for intracellular vitrification when dehydrated at 1.5x osmotic concentration. If EIF can be suppressed at this cooling rate up to 2x osmotic pressure (0.57 M), high viability is achieved. It has also been shown that IIF can be suppressed up to 1600 pL when the intracellular solution is concentrated to 2x. Therefore, development of a CPA that sets the CCR at 0.57 M to be below the cooling rate of 2180 °C/s for this 1600 pL droplet

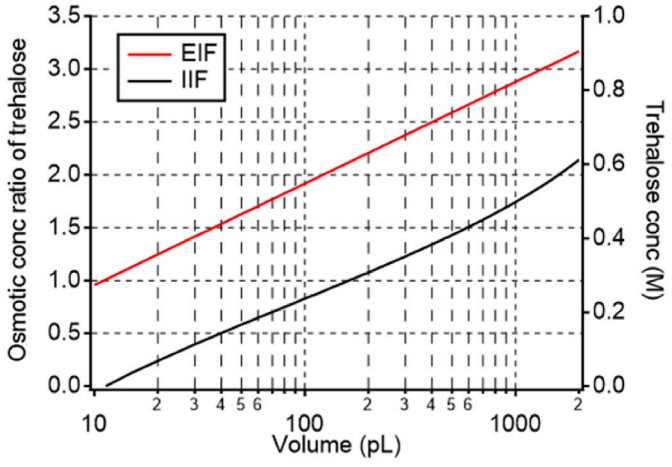

Fig. 9. Critical trehalose concentrations of EIF and IIF as a function of droplet volume. The vertical axes on the left and right show the trehalose concentrations as osmotic ratio to plasma and in moles, respectively.

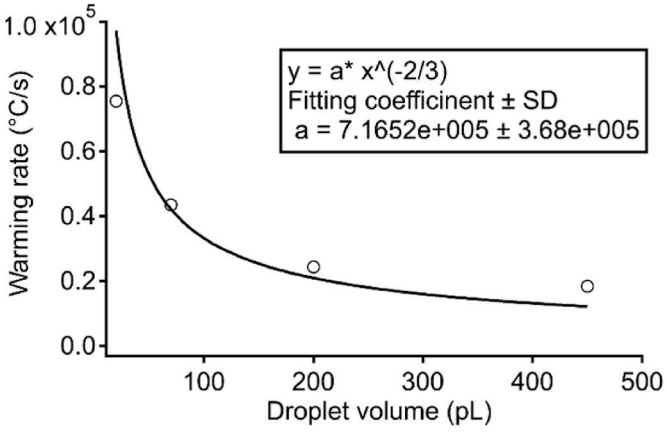

Fig. 10. Relationship between droplet size and warming rate obtained by finite element simulation.

should lead to cryopreservation of cells in large droplets with sufficient viability.

As the viabilities for disaccharides and the medium including FBS were higher than for DPBS when compared at the same osmolarity, the viability could be improved by adding an additional substance with a cryoprotective effect. Experimental results with trehalose showed that the damage by EIF could be suppressed somewhat by the membrane-protective effect of trehalose. In addition, DPBS 1x decreased viability relative to the medium containing FBS. When the cooling rates to suppress EIF and IIF are not achievable, in addition to CPA such as disaccharides, additives such as FBS and antifreeze proteins, should be effective in improving viability in inkjet freezing.

4. Conclusions

In this study, the suitability of disaccharides as npCPAs in inkjet freezing was investigated using 3T3 cells. Trehalose was generally most effective when added at a concentration of 2x osmotic concentration (0.57 M), and viability decreased at higher concentrations due to dehydration damage. In particular, 70 pL droplets with 2x concentration of trehalose almost completely suppressed freeze damage. Similarly, sucrose at 2x osmotic concentration effectively suppressed the damage. Thus, disaccharides were confirmed to be highly effective as CPAs for inkjet freezing. However, it was also seen that npCPA at high concentrations reduced viability due to dehydration, so a 2x concentration would be preferable.

The viability of cells frozen in disaccharide solution was higher than in DPBS at the same osmolarity, indicating that disaccharides not only inhibited IIF by concentrating cytoplasmic solutes, but they also provided cryoprotection. In particular, disaccharides would have a cryoprotective effect on the cell membrane, since disaccharides present only in extracellular solution inhibited some damage by EIF. In addition, FBS in the medium improved viability, suggesting that it would be useful as an additive. Therefore, if the addition of npCPA, such as disaccharides, alone was not sufficient, additives such as FBS or antifreeze proteins would also be expected to be effective in improving viability in inkjet freezing.

CRediT authorship contribution statement

Tomona Takigawa: Investigation, Visualization, Data curation, Formal analysis. **Hiroki Watanabe:** Conceptualization, Investigation, Methodology. **Yoshitake Akiyama:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Software, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing interest

The authors declare that they have no conflict of interest.

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