Cryopreservation of a two-dimensional monolayer using a slow vitrification method with polyampholyte to inhibit ice crystal formation

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Abstract

Vitrification methods have been developed to improve the preservation of oocytes and embryos. However, successful vitrification and preservation typically requires very high cooling speeds. Here, we report a novel slow vitrification method for cryopreservation of two-dimensional (2D) cell constructs using a vitrification solution (VS) in PBS containing 6.5 M ethylene glycol, 0.5 M sucrose, and 10% w/w carboxylated poly-L-lysine (COOH-PLL), a novel polymeric cryoprotectant and stabilizing agent that is likely to inhibit ice crystallization. Stabilization of the glassy state and inhibition of devitrification was confirmed by thermal analysis using differential scanning calorimetry. The viability of cultured human mesenchymal stem cell (MSC) monolayers after freezing by our novel slow vitrification method at a rate of 4.9°C/min in VS with 10% COOH-PLL was significantly higher than that of cells frozen using our slow vitrification method in VS without COOH-PLL. Moreover, cells maintained the capacity for differentiation. We further confirmed that COOH-PLL improved the vitrification properties of the current vitrification system through inhibition of recrystallization properties. This novel, simple method for slow vitrification can be widely applicable for the preservation of tissue-engineered constructs and may facilitate the industrialization of regenerative medicine.

Keywords: Vitrification, ice recrystallization, polyampholyte, tissue-engineered construct, cryopreservation

Introduction

Low-temperature preservation, called cryopreservation, is used for long-term storage of biological materials containing cells. Two primary techniques of cryopreservation are slow freezing and vitrification, in which water transitions directly to the glassy state without crystallization[1,2]. Slow freezing methods, which utilize 10% dimethyl sulfoxide (DMSO) as a cryoprotectant, are effective for a variety of cell lines[3,4]. This method functions through dehydration of cells by freezing of outer membrane, leading to inhibition of intracellular ice crystal formation[5]. However, for the freezing of two- (2D) or three-dimensional (3D) cell constructs, dehydration of each cell causes cell shrinkage, which may damage and destroy cell-cell interactions.

Vitrification methods have been developed with the advent of preservation techniques for oocytes and embryos, primarily in the field of reproductive medicine[6,7]. Rapid cooling leads to the formation of amorphous ice. During the process of vitrification, water transforms directly to a glassy state, thereby preventing crystallization because of the rapid rate of freezing (e.g., 2000–20000°C/min)[8], which does not give the water molecules time to form ice crystals. Therefore, vitrification requires a high rate of cooling and high concentration of cryoprotectants (CPAs). Thus, there are several challenges that must be overcome for the successful vitrification of tissue-engineered constructs. First, the high concentrations of CPAs required to avoid ice crystallization may cause hypertonic toxicity[8].

The process of vitrification involves exposure to very high concentrations of CPAs and subsequent rapid cooling in liquid nitrogen. While reducing the concentration of the CPA may decrease toxicity, ice crystallization must still be inhibited. Additionally, rapid vitrification of tissue-engineered constructs causes mechanical stress during boiling of liquid nitrogen at the time of immersion, which may damage the thin, fragile structures of the tissue, resulting in the formation of cracks[9]. Finally, recrystallization during rewarming[10] may cause damage to the preserved tissues or cells. Therefore, inhibition of ice recrystallization is also needed for successful preservation.

In previous studies, we developed a polymeric cryoprotectant, carboxylated poly-L-lysine, which functioned by inhibiting ice recrystallization[11,12]. In addition, the best cryoprotective and ice recrystallization properties were found in polyampholyte, in which 65 mol% amino groups (approximately) were converted to carboxylated groups[13]. Indeed, we successfully preserved human induced pluripotent stem cells (iPS cells) in 65% carboxylated poly-L-lysine (COOH-PLL) in relatively large scale vitrification (200 μL)[14]. Similarly, Vorontsov et al. studied the inhibitory effects of COOH-PLL on the growth of ice crystals[15] in free-growth experiments of ice crystals in solutions at various COOH-PLL concentrations.

Clinical application of regenerative medicine requires the preparation of 2D and 3D tissue-engineered constructs. Cell sheet therapy involves covering a tissue lesion with a membranous sheet[16]. The success of tissue-engineering applications in regenerative medicine

requires further advances in low-temperature preservation. Preservation of tissues and tissue-engineering products is one of the most important techniques for the clinical and industrial application of tissue engineering. However, cryopreservation of regenerated tissues, including cell sheets and cell constructs, is not easy compared to the cryopreservation of cell suspensions.

Many studies have developed vitrification methods for various types of tissue-engineered constructs, such as bone[17], intestine[18], blood vessels[19], encapsulated cell constructs[20], cartilage[21], and cell sheets[22]. However, as described above, vitrification usually requires a fast cooling rate, which may damage cells. The use of COOH-PLL for stabilization of the glassy state during vitrification has been described[14]. In particular, cell sheets were coated with a viscous vitrification solution (VS) containing permeable and nonpermeable CPAs before vitrification in liquid nitrogen vapor, thereby preventing fracturing of the fragile cell sheet after vitrification and rewarming. Both the macro- and microstructures of the vitrified cell sheets were maintained without damage or loss of major components. However, no quantitative analysis of stabilization of the glassy state with changes in the cooling rate was conducted.

Therefore, in this study, we evaluated that the relationships between cooling rate and vitrification and between rewarming rate and recrystallization of various VSs in order to elucidate the effects of COOH-PLL on stabilization of the glassy state. From these analyses,

we propose a novel slow vitrification method based on the results of vitrification of mesenchymal stem cell (MSC) monolayers using COOH-PLL as a glassy state stabilizing additive.

Materials and Methods

Human MSC monolayer

Five independent human bone marrow MSC (hBMSC) lines (HMS0051, HMS0008, HMS0050, HMS0023, and HMS0024) established by Dr. Kato of Hiroshima University[23] were purchased from the RIKEN Cell Bank (RIKEN Bioresource Center, Ibaraki, Japan) in accordance with the regulations of the Life Science Committee of Japan Advanced Institute of Science and Technology. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS), 3 ng/mL basic fibroblast growth factor (bFGF; Wako Pure Chemical Industries Ltd., Osaka, Japan), 100 U/mL penicillin, and 100 μg/mL streptomycin. These hBMSCs were delivered in dry ice from the cell bank and stored in liquid nitrogen after arrival at our laboratory until experimental use. Cell culture was carried out at 37°C under 5% CO_2 in a humidified atmosphere. When the cells reached 80% confluence, they were detached using 0.25% (w/v) trypsin containing 0.02% (w/v) ethylenediaminetetraacetic acid in phosphate-buffered saline without calcium or magnesium [PBS(-)] and were seeded on a new tissue culture plate for subculture. The hBMSCs used in this study were between passages three and five.

Synthesis of COOH-PLL

We previously reported the synthesis of the polymeric cryoprotectant COOH-PLL[11]. To

synthesize COOH-PLL, 25% w/w ε-poly-L-lysine (PLL) aqueous solution (10 mL; JNC Corp., Tokyo, Japan) and succinic anhydride (1.3 g SA; Wako Pure Chem. Ind. Ltd., Osaka Japan) were mixed and reacted at 50°C for 1 h to convert 65% amino groups to carboxyl groups (Fig. S1). The number of amino groups was determined using the 2,4,6-trinitrobenzenesulfonate (TNBS) method[24]. Briefly, 0.3 mL of 250 μg/mL sample solution, 1 mL of 1.0 mg/mL TNBS solution, and 2 mL of 40 mg/mL sodium bicarbonate aqueous solution containing 10 mg/mL sodium dodecyl sulfate (pH 9.0) were mixed and incubated at 37°C for 2 h. After the mixture was cooled to 25°C, the absorbance was measured at 335 nm.

Preparation of VS

The VS developed for cryopreserving human iPS cells[14] was used after slight modifications (Table 1). We prepared a VS based on 6.5 M ethylene glycol (EG, Wako) and 0.75 M sucrose (Wako) in PBS. COOH-PLL was added to the solution at 10% w/v to evaluate the effects of the polymer (P-VS). A DAP213 solution (2 M DMSO, 1 M acetamide, and 3 M propylene glycol [all from Wako] in PBS) was used as a control[25]. An equilibration solution (ES) consisting of 15% (v/v) EG in PBS. A rewarming solution (RS) and dilution solution (DS) containing 1 and 0.5 M sucrose, respectively, were prepared.

Tables

Table 1. Composition of various vitrification solutions

Vitrification solution	DMSO / M	Acetamide / M	Propylene glycol /M	Ethylene glycol / M	COOH-PLL / wt%	Sucrose / M
DAP213	2	1	3	0	0	0
VS	0	0	0	6.5	0	0.5
P-VS	0	0	0	6.5	10	0.5

Slow vitrification procedure

First, 1.0 mL of ES was added to the MSC monolayer cultured in a 3.5-cm dish for pre-equilibration for 10 min. After equilibration, the ES was discarded, and 1.0 mL of three types of VSs was added to each MSC monolayer on ice. After 5 min, the VS was removed by aspiration, and the dish was placed on the stainless steel mesh and held various distances above the surface of liquid nitrogen. The temperature of the MSC monolayer was monitored with a thermocouple attached on the monolayer. After 20 min, the dish containing the vitrified MSC monolayer sheet was soaked in liquid nitrogen. An illustration of the practical procedure of slow vitrification is shown in Figs. S2 and S3.

The MSC monolayers were warmed on the same day by gently addition of prewarmed RS (2 mL). After 1 min, the RS was changed to DS. Three minutes later, the monolayer was washed with DMEM twice for 5 min each.

Evaluation of the survival of MSC monolayers

Two MSC monolayers in the dish were prepared for the same vitrification protocol. One was used for the evaluation of viability immediately after thawing, and the other was evaluated 1 day after thawing. Thawed MSC monolayers were stained by calcein AM and ethidium homodimers using a Live/Dead Assay Kit (Life Technologies, Carlsbad, CA, USA). The cells were then dispersed by trypsin solution, and the viability of the cell monolayers were determined using trypan blue staining.

Differential scanning calorimetry

To compare the stabilities of the glassy states among the VSs, thermal analysis was performed using a differential scanning calorimeter (DSC; Q2000; TA Instruments, New Castle, DE, UAS). Ten microliters of each solution was placed on the aluminum pan. The pan was then set on the DSC sample chamber, cooled to -170°C at 10°C/min, and then warmed to 0°C at 10°C/min. Calorimetric measurements were performed in the range from -170 to 0°C.

Induction of differentiation of MSC monolayers

Unfrozen and vitrified MSC monolayers were induced to differentiate into osteoblasts and adipocytes for 14 days in osteogenic and adipogenic media, respectively[26]. The osteogenic medium contained 0.1 mM dexamethasone, 10 mM β-glycerophosphate disodium salt, 0.07 mM L-ascorbic acid 2-phosphate magnesium salt n-hydrate (Sigma-Aldrich), 10% FBS, and 1% antibiotic/antimycotic in DMEM. The adipogenic medium contained 0.5 mM isobutyl methylxantine, 0.1 mg/mL insulin, 0.2 mM indomethacin, 1 mM dexamethasone, 10% FBS, and 1% antibiotic/antimycotic in DMEM. The culture medium was changed three times per week.

Evaluation of differentiation

Monolayer cultures treated with osteogenic differentiation medium were stained with 1% Alizarin Red S for 15 min after fixation with 4% neutral buffered formalin for mineralized matrix deposition. Monolayer cultures treated with adipogenic differentiation medium were stained with oil Red O solution to visualize the formation of fat vacuoles. Briefly, the cells were fixed with 4% neutral-buffered formalin and incubated for 20 min with 0.2% oil Red O isopropanol solution.

Statistical analysis

All data are expressed as the means ± standard deviations (SDs). Measurements of post-thaw viability were collected with three replicates for each sample. All experiments were conducted in triplicate. To compare data among more than three groups the Tukey-Kramer test was used. Differences with *P* values of less than 0.05 were considered statistically significant.

Results and Discussion

Evaluation of cooling speed

The cooling speed was highly affected by the distance between the dish and liquid nitrogen surface. Table 2 shows the cooling speed, which was recorded using a thermocouple attached to the MSC monolayer. The cooling speed was controlled between 4.9 and 34.5°C/min by changing the distance of the monolayer from the liquid nitrogen surface (Table 2). These cooling speeds were much slower than that usually used for vitrification[8,27]. When the dish containing the MSC monolayer was inserted directly into liquid nitrogen, the cooling speed was 3900°C/min.

Table 2. Cooling speed of slow vitrification of MSC monolayers controlled by the distance between the dish and liquid nitrogen.

Distance between the dish and liquid nitrogen surface / cm	Cooling speed / °C/min
0.1	34.5
0.5	20.4
1.0	10.8
6.0	4.9

Stabilization of the glassy state and inhibition of devitrification as evaluated by DSC

Thermal analysis of the three VSs was performed using DSC in order to evaluate the stabilization of the glassy state by COOH-PLL. Fig. 1 illustrates the heat flow curves of the three VSs. The samples were cooled from 20 to -150°C and warmed from -150 to 0°C at a rate of 10°C/min on the DSC sample holder. As shown in Fig. 1a, DAP213 crystallized during cooling at a rate of 10°C/min, while VS and P-VS showed no crystallization. DAP213 has been developed to be optimized for the vitrification of mouse oocytes and embryos[25]. The basic principle of the latest high-performance method of vitrifying embryos requires a very high cooling speed and very small amount of VS to avoid crystallization and devitrification[27,28]. Thus, DAP213 did not allow vitrification at a cooling rate of -10°C/min.

During heating, all vitrification solution showed glass transition at around -120°C, and even DAP213 achieved partial glass transition. VS and P-VS showed recrystallization (devitrification) during heating at the rate of 10°C/min. Interestingly, the recrystallization temperature of P-VS (-67.6°C) was higher than that of VS (-98.1°C), and the melting point of P-VS (-35.6°C) was lower than that of VS (-30.8°C; Fig. 1b). Although the actual rewarming speed was higher than 10°C/min, we selected this speed in order to compare the risk of devitrification. These data indicated that COOH-PLL stabilized the glassy state of VS, leading to a smaller temperature range of the crystalline state of P-VS (from -67.6 to -35.6°C) than that of VS (from -98.1 to -30.8°C), which may decrease the risk of recrystallization during thawing. This result was consistent with a previous report in which polyampholytes inhibited ice recrystallization[29].

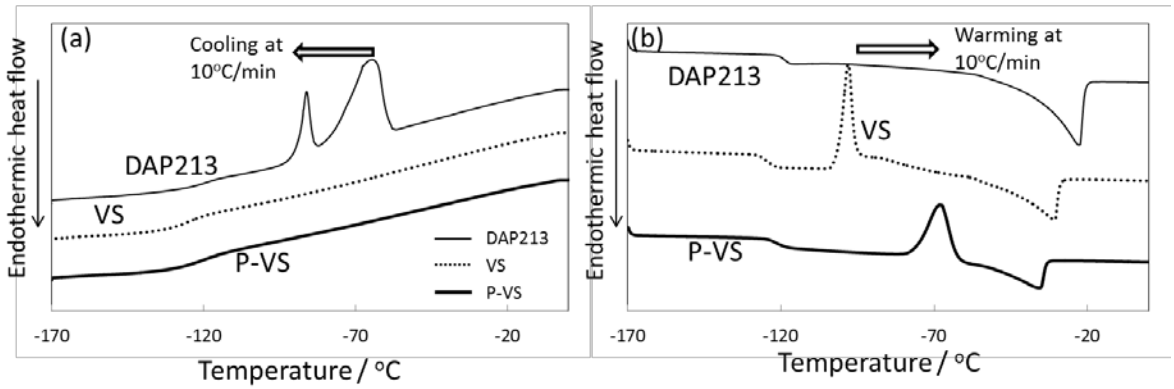

Fig. 1. DSC thermograms of various VSs for (a) cooling and (b) heating at 10° C/min.

Toxicity of VSs

MSC monolayers were immersed in DAP213, VS, or P-VS; 10 min later, the monolayers were washed with DS without cooling, and viability was evaluated. The results of Live/Dead

assays are shown in Fig. 2. Many dead cells stained in red were observed in DAP213, while almost all cells in VS and P-VS were alive. Quantitative analysis by trypan blue staining showed that the viabilities were 75%, 92%, and 96% for DAP213, VS, and P-VS, respectively, indicating that DAP213 exhibited the highest cytotoxic effects in MSC monolayers due to its high osmotic pressure and chemical cytotoxicity. The decrease in toxicity caused by the addition of COOH-PLL may be the result of improved cell membrane stability[14].

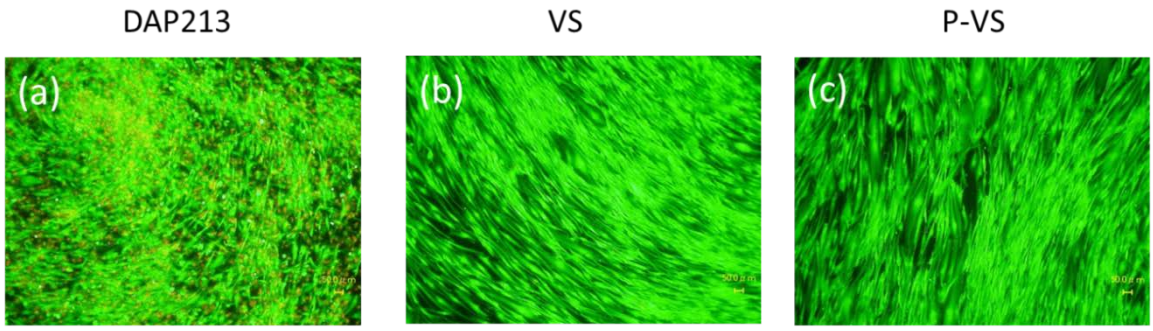

Fig. 2. Cell viability of MSC monolayers after 10 min treatment at room temperature with (a) DAP213, (b) VS, and (c) P-VS using a Live/Dead assay kit. Bar, 100 μm.

Slow vitrification

Next, we examined the effects of cooling rate on cell viability. Fig. 3 shows images from the Live/Dead assay at 1 day after thawing of MSC monolayers frozen with the three types of VSs with various cooling speeds ranging from 4.9 to 34.5°C/min. At cooling speeds of 34.5, 20.4, and 10.8°C/min, almost all cells frozen with P-VS were alive. Some dead cells were observed in monolayers frozen in VS. However, in contrast, very few live cells were observed in cells frozen in DAP213. At the cooling speeds of 10.8 and 20.4°C/min, black

space that was not stained green or red could be observed, particularly in MSC monolayers frozen with VS. This may be explained by desquamation of the monolayer from the dish due to the mechanical stress of ice crystal formation during thawing, leading to decreased cell

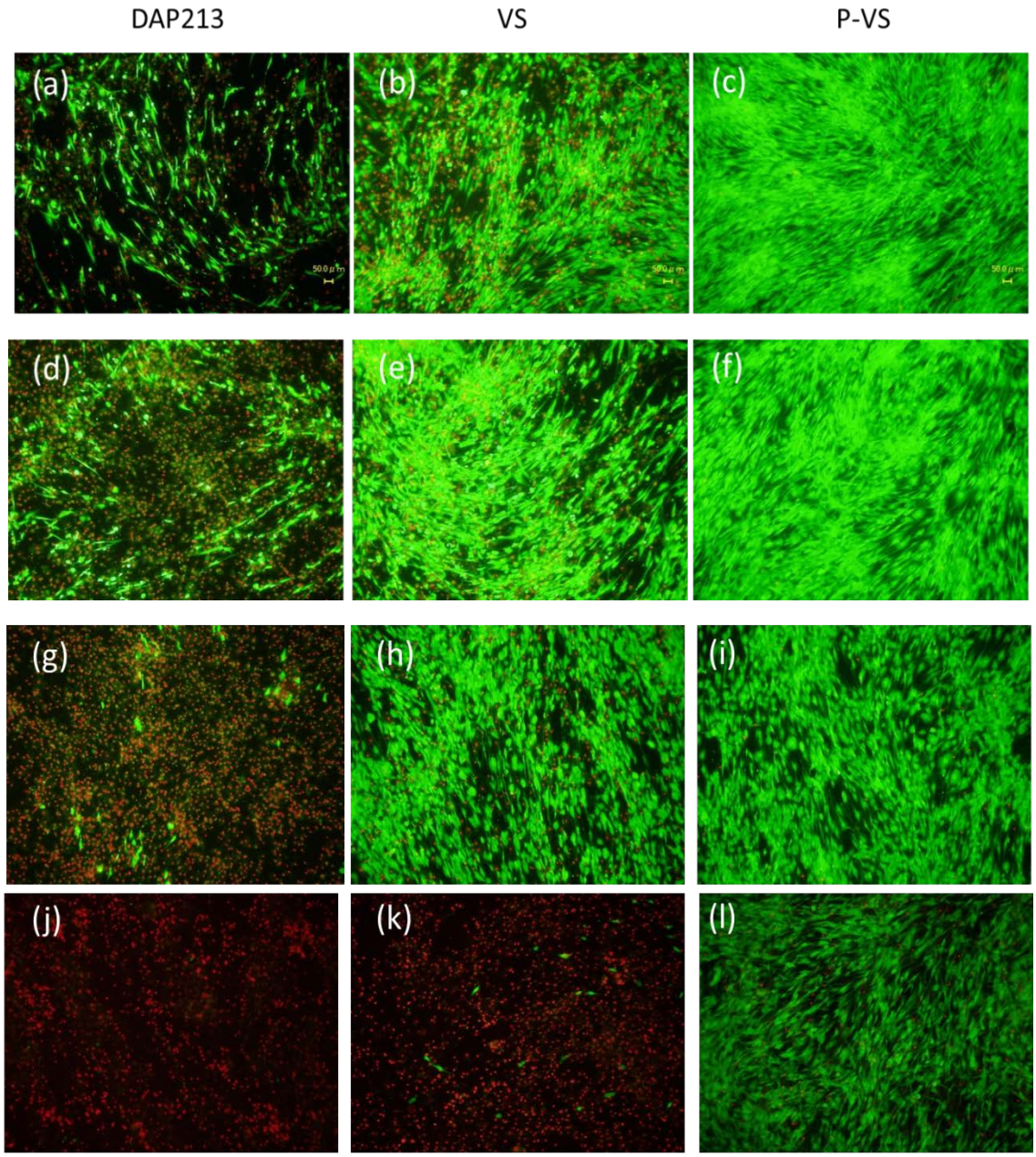

Fig. 3. Cell viability of MSC monolayers after slow vitrification with DAP213 (a, d, g, and j), VS (b, e, h, and k), and P-VS (c, f, i, and l). During slow vitrification, the cooling speed was controlled at 34.5 (a–c), 20.4 (d–f) 10.8 (g–i), and 4.9 (j–l). Bars, 100 µm.

viability. Interestingly, when the cooling speed was decreased to 4.9°C/min, with the distance between liquid nitrogen and the dish set at 6.0 cm, live cells were observed only when frozen with P-VS. Fig. 4 shows the quantitative analysis of MSC viability just after thawing (Fig. 4a) and after 1 day of culture (Fig. 4b). The viability of MSCs was higher just after thawing than at 1 day after thawing. In particular, the viability of cells frozen at a rate of 4.9°C/min in VS was the lowest due to detachment of a part of the monolayer during thawing. However, it should be noted that viability by trypan blue staining may be overestimated as some damaged cells may be considered alive[11]. These weakened cells may not be able to attach the dish to grow; thus, viability may be reduced after 1 day of culture. Quantification of viability after 1 day of culture was consistent with the results of the Live/Dead assays (Figs. 3 and 4b). At all cooling rates, viability with P-VS was significantly higher than those with DAP213 and VS, particularly for the rate of 4.9°C/min, at which P-VS showed 80% viability.

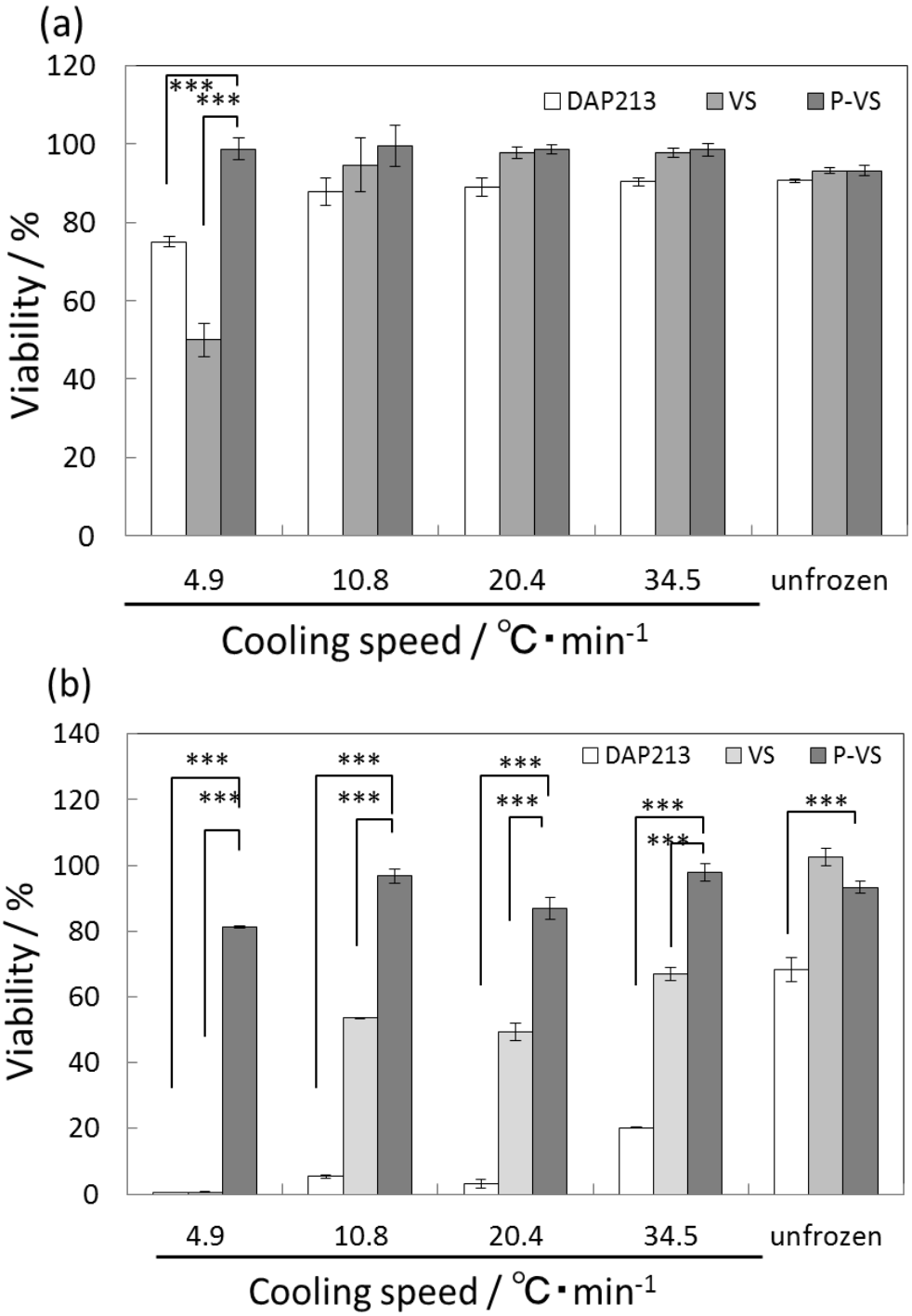

Fig. 4. Quantitative results of viability of MSCs after slow vitrification with various VSs with different cooling speeds (a) immediately after thawing and (b) after 1 day of culture. ***$P < 0.001$.

Ice crystallization was inhibited in VS and P-VS at 10°C/min, as confirmed by DSC (Fig. 1). In fact, although the vitrification conditions different for DSC and MSC monolayers in

culture dishes in terms of VS volume, the stabilizing effect of COOH-PLL on the glassy state was clearly observed in that monolayer sheets frozen with DAP213 or VS turned white when rewarming. Effective vitrification has been well studied; however, most investigations have focused on how to obtain rapid cooling using a variety of methods, such as the open pulled straw method[30], cryotop method[2,27], and hollow fiber vitrification[28]. We succeeded in establishing a new method for obtaining highly viable MSC monolayers via slow (4.9°C/min) vitrification by adding COOH-PLL to the vitrification solution. This slow vitrification method was realized for the first time by addition of COOH-PLL; this material had low toxicity and was effective at stabilizing the vitreous status of the solution during vitrification and rewarming, thereby resulting in a high rate of survival of MSCs.

Evaluation of differentiation

Fig. 5 shows the results of differentiation of MSCs after slow vitrification with P-VS. Histological evaluation revealed that MSC monolayers were well differentiated into osteoblasts (Fig. 5c) and adipocytes (Fig. 5d), similar to nonfrozen cells (Fig. 5a and b). MSCs have a multipotent capability to differentiate into various functional cell types of mesodermal tissues[31,32]. In this study, MSC monolayers were vitrified with our novel method of slow vitrification with COOH-PLL as a stabilizer of the glassy state, without any other CPAs and or animal-derived proteins. The results showed that the novel vitrification method did not alter the phenotype characteristics of MSCs, preserving their viability and

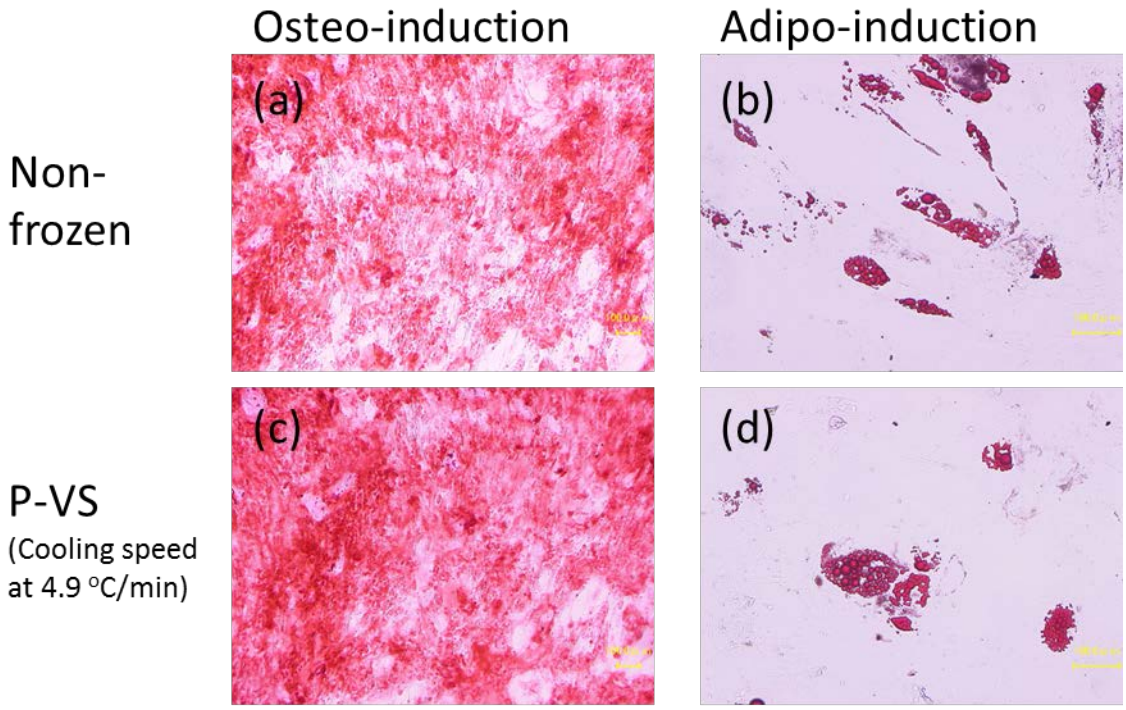

Fig. 5. Histological evaluation of differentiation of MSC monolayers after slow vitrification. (a, b) Unfrozen control, (c, d) slow vitrification with P-VS. Bars, 100 µm. (a, c) Alizarin Red staining for osteoblast differentiation, (b, d) oil Red O staining for adipocyte differentiation.

differentiation capacity after thawing. In our previous reports, also we found that MSC differentiation was maintained after slow freezing using COOH-PLL[11,12]. Thus, these results confirmed that COOH-PLL did not affect MSC differentiation during preservation.

Conclusion

In this study, we propose a novel slow vitrification method for the cryopreservation of tissue constructs. Addition of COOH-PLL markedly stabilized the glassy state of EG-based

vitrification solution and inhibited recrystallization during rewarming. MSC monolayers could be cryopreserved, even at a cooling rate of -4.9°C/min, without decreasing cell viability using P-VS. This novel concept of slow vitrification can be widely applicable for the simple preservation of tissue-engineered constructs without the requirement for high technical expertise. Thus, this may facilitate the industrialization of tissue engineering applications by allowing long-term storage of tissue-engineered constructs.

Supporting Information Available

The following files are available free of charge:

Supplemental synthetic procedures and ^{1}H-NMR of COOH-PLL, schematic illustration of the slow vitrification apparatus and procedures.

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Figure Legends

Fig. 1. DSC thermograms of various VSs for (a) cooling and (b) heating at 10°C/min.

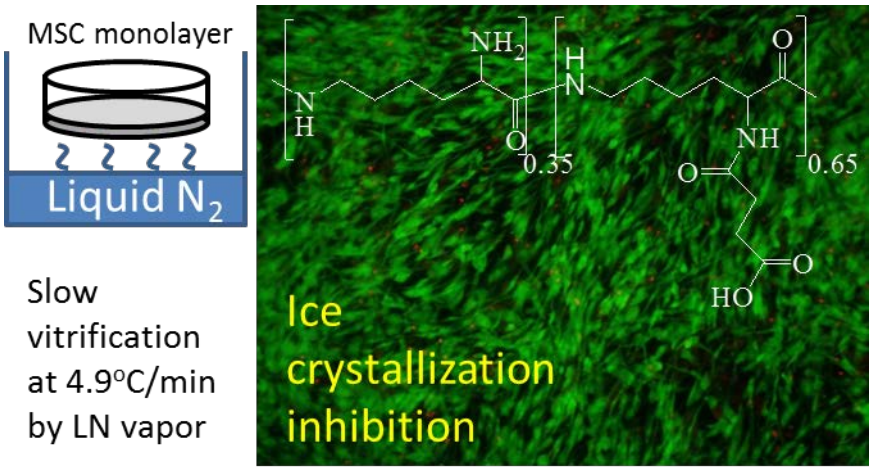
Fig. 2. Cell viability of MSC monolayers after 10 min treatment at room temperature with (a) DAP213, (b) VS, and (c) P-VS using a Live/Dead assay kit. Bar, 100 μm.

Fig. 3. Cell viability of MSC monolayers after slow vitrification with DAP213 (a, d, g, and j), VS (b, e, h, and k), and P-VS (c, f, i, and l). During slow vitrification, the cooling speed was controlled at 34.5 (a–c), 20.4 (d–f) 10.8 (g–i), and 4.9 (j–l). Bars, 100 μm.

Fig. 4. Quantitative results of viability of MSCs after slow vitrification with various VSs with different cooling speeds (a) immediately after thawing and (b) after 1 day of culture. ***$P <$ 0.001.

Fig. 5. Histological evaluation of differentiation of MSC monolayers after slow vitrification. (a, b) Unfrozen control, (c, d) slow vitrification with P-VS. Bars, 100 μm. (a, c) Alizarin Red staining for osteoblast differentiation, (b, d) oil Red O staining for adipocyte differentiation.

TOC figure


MSC monolayer
Liquid N_2
Slow vitrification at 4.9°C/min by LN vapor
Ice crystallization inhibition

Supporting Information

Cryopreservation of a two-dimensional monolayer using a slow vitrification method with polyampholyte to inhibit ice crystal formation

Kazuaki Matsumura*, Keiko Kawamoto, Masahiro Takeuchi, Shigehiro Yoshimura, Daisuke Tanaka, Suong-Hyu Hyon

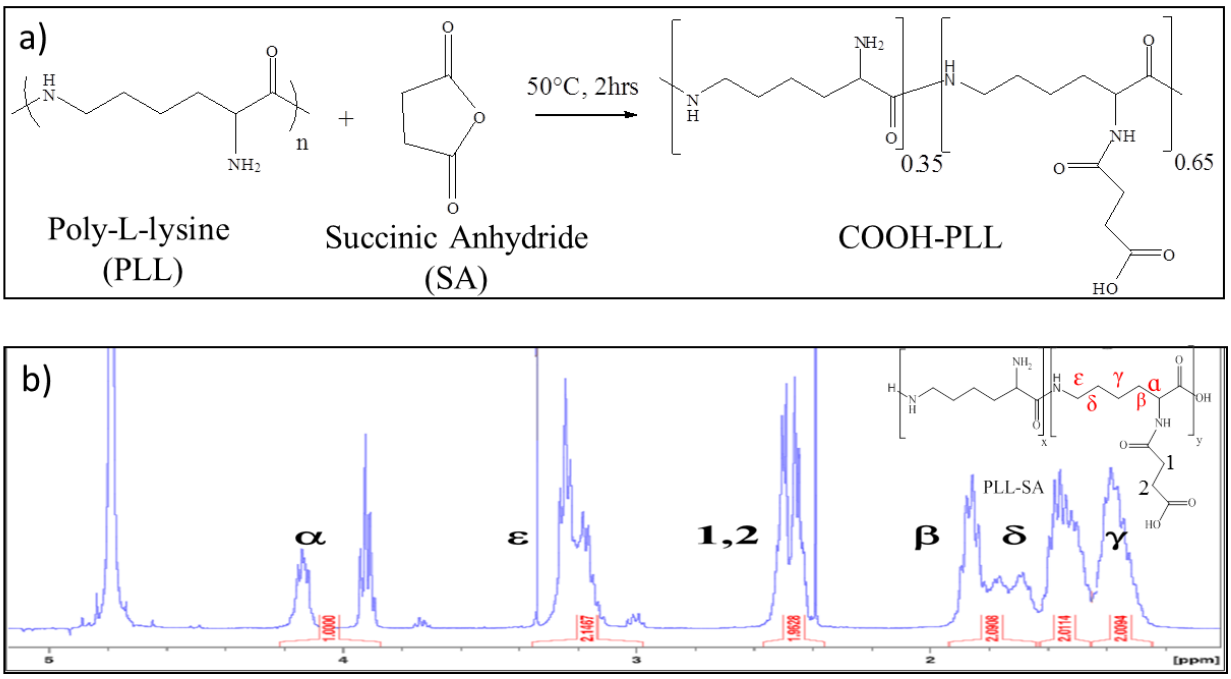


Fig. S1. Synthesis of carboxylated poly-L-lysine. (a) Synthetic scheme and (b) ^{1}H-NMR chart of COOH-PLL obtained with a Bruker AVANCE III 400 MHz spectrometer (Bruker Biospin, Switzerland).

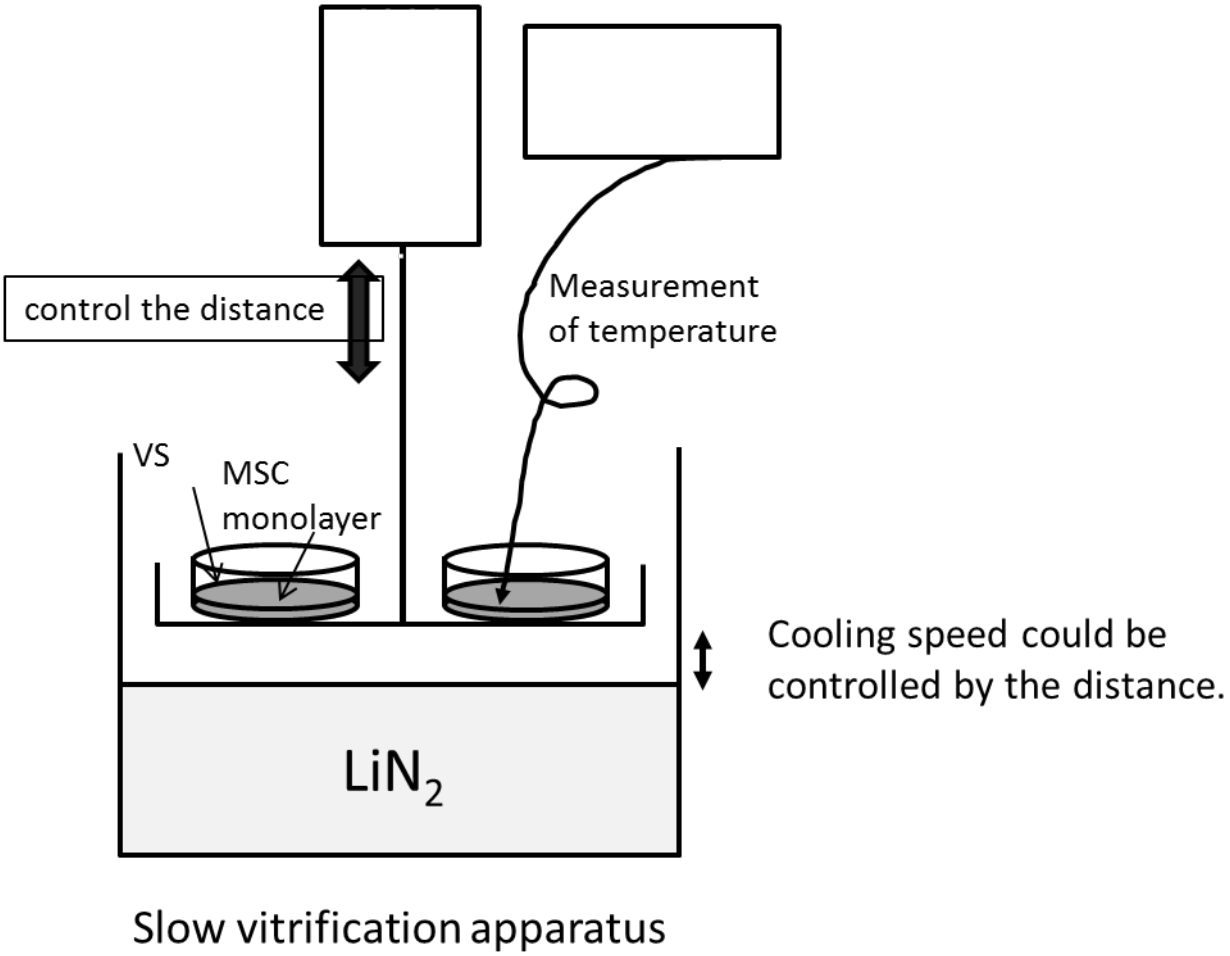


Fig. S2. Schematic illustration of the slow vitrification apparatus for controlling the distance between the dish and liquid nitrogen.

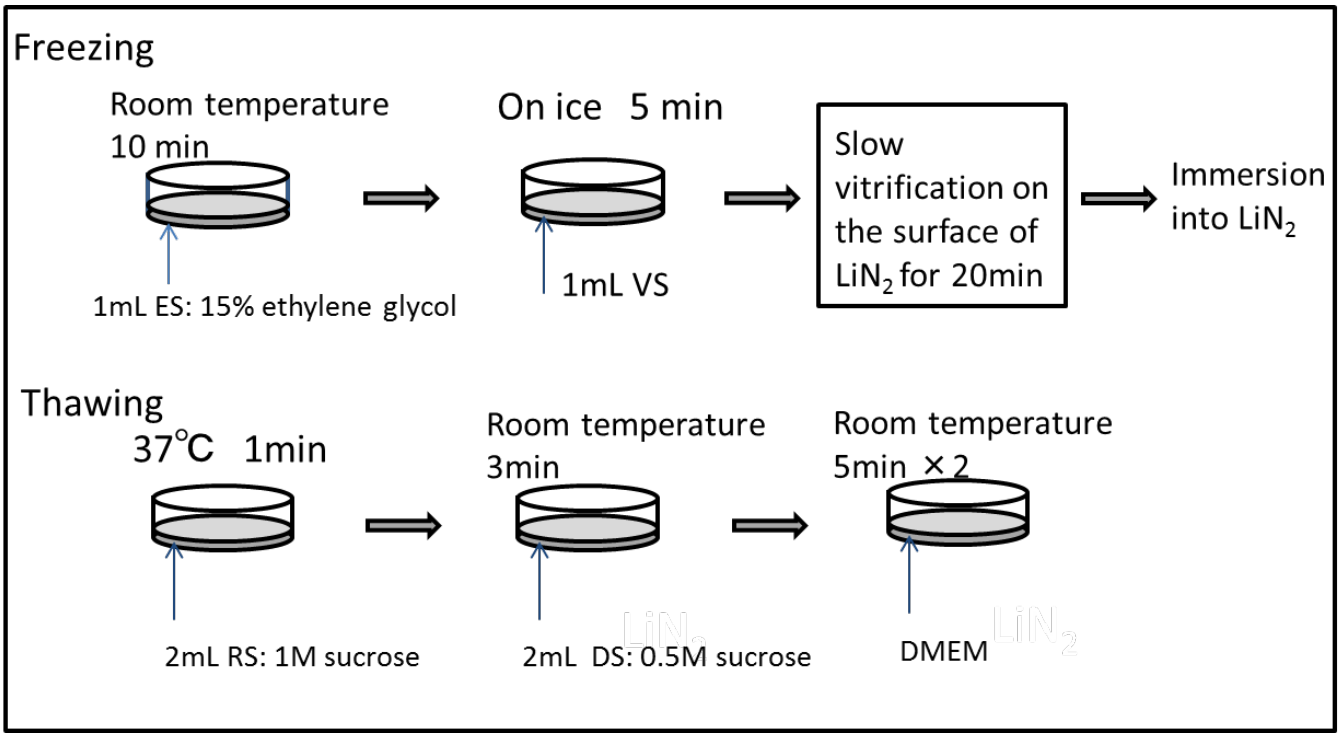


Fig. S3. Protocol of slow vitrification.