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Role of Cryoprotectants in Freeze Concentration Technology for Gene Delivery Process

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There is an urgent need to develop highly effective techniques for successful and efficient gene delivery. In this study, we demonstrated the use of cryoprotectants in the newly developed freeze concentration technique in the gene delivery system. We found that at extremely low temperatures, gene transfection was effectively enhanced. Additionally, by comparing the effects of the commercially available cryoprotectant DMSO against a synthesized polyampholyte cryoprotectant, we were able to establish that the latter was more effective at increasing the rate of successful gene transfection. This particular feature could be attributed to the higher freeze concentration factor of polyampholytes in comparison with DMSO, under frozen conditions. Therefore, the study demonstrated the unique efficacy of polyampholytes as a cryoprotectant using the freeze concentration method for efficient *in vitro* gene delivery.

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INTRODUCTION

The advent of successful therapeutic gene delivery into target cells has witnessed a rising interest in gene delivery technology within the scientific community.[1)] The inability of nucleic acids to pass through cell membrane barriers by themselves[2)] fueled the development of gene transfer processes through viral and non-viral techniques.[3)] Although the high delivery efficiency of viral vectors resulted in their extensive use in gene therapy application,[4)] safety issues concerning the immunogenicity and oncogenicity of viral vectors[5)] led to the investigation of physical techniques as a means to a non-viral approach towards effective gene delivery. Gene delivery strategies like electroporation[6)] and ultrasonication[7)] were designed to increase the co-localization of DNA at target sites. However, these energy-based methods caused cell damage and high toxicity, limiting their application in gene delivery.[8)] As a result, researchers are currently attempting to develop new and efficient methods for safe gene delivery.

We previously found that the freeze concentration method produced outstanding results with protein cytoplasmic delivery.[9), 10)] Similar studies also determined that freeze concentration enhanced protein internalization to the cytosol of

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the cells.[11), 12)] Freeze concentration is a physical phenomenon where the water present within a system, under extreme, ultra-cold temperatures, is transformed into ice crystals leading to increased solute concentrations.

Freeze concentration technique has been successfully employed in the food industry to concentrate fruit juices by separating the water content from the mixture in the form of ice-crystals.[13)] The concentrate obtained through this process can later be used to make new products or simply mixed with water to produce fresh tasting juices. At low temperatures, the product quality was generally found to be high with the absence of a vapor-liquid interface. Freeze concentration has been particularly successful in concentrating citrus juices, several other aromatic beverages and other high-value extracts.[14)] Certain chemical reactions were also reported to be induced or accelerated at extremely low temperatures via freeze concentration by Pincock and co-workers.[15)]
In our work, we found that the freeze concentration procedure was a simple and affordable alternative that ensured low cellular damage during protein cytoplasmic delivery. Undoubtedly, cryoprotectants play a vital role in freeze concentration strategy for gene delivery applications. Cryoprotectants are able to limit freezing damage, improve cell viability, and significantly increase solute concentrations at a very low temperature. DMSO has been the most commonly used cryoprotective agent in terms of efficiency.[16)] However, DMSO has been reported to exhibit toxicity and negatively impact the differentiation potential of stem cells.[17)]

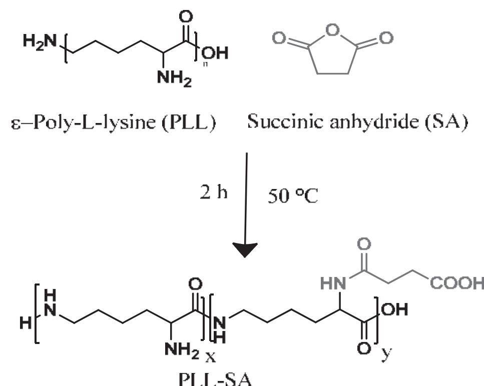
Recently, by changing the ratio of amino and carboxyl groups in poly-L-lysine (PLL), our group developed a unique polyampholyte-based cryoprotectant that exhibited high cryopreservation efficiency without the requirement for additional cryoprotectants.[18)] Even though efficient cell viability, after thawing, entirely depended on the ratio of amino groups to carboxyl groups, the cryoprotective nature of polyampholytes was compellingly established.

Lipofection is also another technique well suited for gene transfection via cationic lipids (structures that consist of a positively charged head group and one or two hydrocarbon chains).[19)] Therefore, in order to carry out a successful gene delivery procedure in this study, we complemented the use of lipofectamine as a carrier with the freeze concentration process and aimed to analyze the role of the cryoprotectant during the freeze concentration process and its impact on gene delivery. As a result, we determined that the polyampholyte cryoprotectant effectively enhanced the gene transfection process as compared to the commercially available cryoprotectant at -80˚C. Moreover, we further investigated the freeze concentration factor in order to quantify the role of cryoprotectants in gene transfection. This study demonstrated the distinct nature of polyampholyte as a cryoprotectant that induced and enhanced transfection efficiency.

MATERIALS AND METHODS

Synthesis of polyampholyte cryoprotectant

The polyampholyte cryoprotectant was prepared using a previously elucidated procedure.[18)] Briefly, an aqueous solution of 25% (w/w) PLL (3 mL; JNC Corp., Tokyo, Japan) and succinic anhydride (0.38 g; Wako Pure Chem. Ind. Ltd.



Scheme 1. Synthetic scheme for the preparation of polyampholyte as a cryoprotectant (PLL-SA).

Osaka, Japan) were reacted at 50˚C for 2 h to convert 65% of the amino groups to carboxyl groups (Scheme 1). The polyampholyte cryoprotectant is denoted as PLL-SA.

Transformation and purification of pDNA (plasmid DNA)

pAcGFP1-N2 plasmid, consisting of the green fluorescent protein (GFP) gene and pGL4.51[luc2/CMV/Neo] encoding for the luciferase gene, were transformed into *Escherichia coli* DH-5α competent cells, and the transformants were striped onto LB agar plates with the appropriate antibiotic. After overnight incubation at 37˚C, the resistant colonies were dissolved in Plusgrow liquid medium containing the appropriate antibiotic and cultured at 37˚C at 200 rpm overnight. The plasmids were isolated and purified with the help of bacterial cell culture using a Genopure plasmid maxi kit. The purified plasmids were then re-suspended in Tris-EDTA buffer and its concentration was determined using a Nanodrop1000. The plasmids were stored at -20˚C until further required.

Preparation of lipofectamine 3000 and pDNA complexes (lipoplexes)

To prepare the lipofectamine 3000-pDNA complexes, 1 μg of the reporter genes pAcGFP1-N2 (for GFP study) and pGL4.51 (for luciferase study) and 5 μL of P3000 reagent were dissolved in 125 μL of opti-MEM media. The commercially available transfecting carrier, lipofectamine 3000 (7.5 μL), was also dissolved in 125 μL of opti-MEM. The prepared stock solutions of lipofectamine 3000 and the pDNA solution were gently mixed, followed by incubation at room temperature for 15 min to form lipofectamine 3000-pDNA complex.

Cell culture

Human embryonic kidney cells (HEK-293T, American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM supplemented with 10% fetal bovine serum at 37˚C in the presence of 5% CO_2 humidified atmosphere. At 80% confluence, the cells were extracted using 0.25% (w/v) trypsin containing 0.02% (w/v) EDTA in PBS(-) and seeded into new tissue culture plates for subculture.

In vitro gene transfection via lipoplex and cryoprotectant aided cell freezing

To analyze the freeze concentration technique in the gene transfection process, the following protocol was used. HEK-293T cells were counted and re-suspended at a density of $1x10^6$ cells/mL in 10% PLL-SA or 10% DMSO cryoprotective solution. Re-suspended cells from each cryoprotectant solution (1 mL) were added to a 1.9mL cryovial (Nalgene, Rochester, NY) along with lipofectamine 3000-pDNA complex solution (50 μL, without fetal bovine serum) and cryopreserved overnight in a -80˚C deep freezer (Nihon freezer). After freezing overnight, the cryovials were thawed at 37˚C and the samples were washed three times with DMEM medium. To check the cell viability, we calculated cell viability with a hemocytometer using the tryptan blue staining method. Cell viability was determined as the number of viable cells divided by total number of cells. But for transfection studies, after thawing the cells were seeded into a glass bottom dish and incubated for 24 h. For unfrozen samples, an equal amount of lipofectamine 3000-pDNA complex solution was added to a glass bottom dish and incubated for 10 h. After the dish was washed three times with PBS(-), the GFP expression was observed using confocal microscope analysis (CLSM; FV-1000-D; Olympus, Tokyo, Japan).

Quantification of the *in vitro* gene transfection process by luciferase activity

In this investigation, we also compared the

efficacy of the two different cryoprotectants, PLL-SA and DMSO, in gene delivery by quantifying the luciferase activity during the gene transfection process. To observe this, pGL4.51[luc2/CMV/Neo] plasmid was used which contained the luciferase reporter gene. For frozen samples, the same procedure detailed earlier for cell suspensions was applied. After preparation of the samples, the cell suspensions were seeded to 12-well plates. For non-frozen samples, the lipofectamine 3000-pDNA complex solution was added directly to the HEK-293T cells in 12-well plates. All the plates were incubated for 24 h. At the time of observation, the cells were washed three times with PBS(-) and extracted from the plates by scraping using lysis reagent (200 μL/well; 25 mM Tris phosphate, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100; pH-7.4). The cells were then transferred into micro-centrifuge tubes and stored on ice for 5 min, followed by centrifugation at 12,000 *g* for 2 min. The supernatant was withdrawn and transferred to new centrifuge tubes. To measure luciferase expression, the luciferase assay kit reagent (100 μL, Promega) and the cell culture supernatant was added to a luminometer tube and the mixture was vortexed. Luciferase activity was recorded using a luminometer (Berthold Technology, Lumat^3 LB 9508), which was programmed to perform a 2-s measurement delay followed by a 10-s measurement and the values obtained were expressed as relative light units (RLU). All experiments were done in triplicate.

Determination of freezing points of cryoprotectants

The freezing point of various cryoprotectants such as DMSO, polyampholytes, and protein loaded polyampholyte nanoparticles were determined to calculate the respective freeze concentration factors based on a procedure established in an earlier study.[20] Briefly, the samples (3 mL) were dispensed into plastic tubes (15 mm in diameter) and were kept at -20˚C in a low temperature bath (NCB-3200, Eyela, Tokyo) with a thermistor (0.01˚C in accuracy: D641, Takara thermistor, Yokohama) until the samples were completely frozen. Next, the samples were warmed and melted at room temperature by stirring with a vortex mixer. The change in the temperature was documented by a recorder (PRR-5021, Toa DKK, Tokyo). Finally, the freezing point was determined by plotting a melting curve.

RESULTS AND DISCUSSION

Gene transfection of lipofectamine 3000-pDNA complexes by using cryoprotectants for freeze concentration technique

To evaluate the role of cryoprotectants using freeze concentration technology in gene delivery, we cryopreserved the lipofectamine 3000-pDNA complexes in 10% PLL-SA or 10% DMSO for 24 h.

But before evaluating transfection, we have investigated cell viability after thawing. Interestingly, we found that with the presence of 10% PLL-SA has higher cell viability as compared to 10% DMSO (Fig. 1).

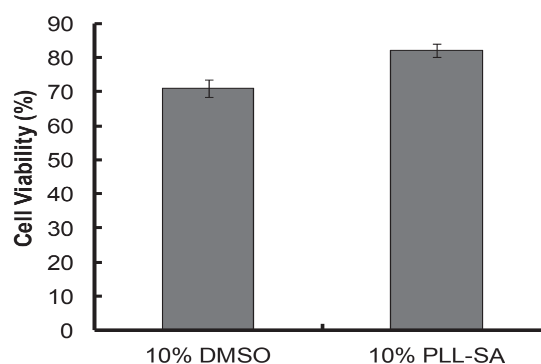


Fig. 1. Cell viability after being frozen at -80 ˚C for one day containing Lipofectamine3000:pDNA complexes. Cells were frozen in the presence of cryoprotectant with either 10% PLL-SA or 10% DMSO. Data is expressed as mean±SD.

Next step is to explore the use of cryoprotectant in gene transfection studies. For transfection studies, after freezing, the cells were thawed, seeded into glass bottom dishes, and further incubated for an additional 24 h to observe the expression of GFP, under a confocal laser scanning microscope (CLSM).

In order to increase the points of comparison, non-frozen samples were also prepared in which the lipofectamine 3000-pDNA complexes were added directly to the cells and the resulting cell suspension was seeded to glass bottom dishes and incubated for 24 h.

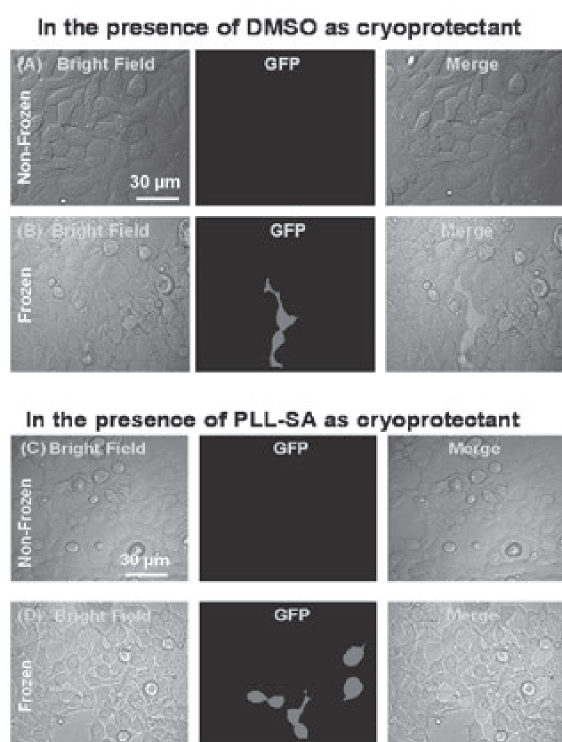


Fig. 2. Confocal images of HEK-293T cells treated with lipofectamine 3000-pDNA complexes. For non-frozen samples, lipofectamine 3000-pDNA complexes were added directly to the cells and incubated for 24 h. (A) 10% DMSO (C) 10% PLL-SA. For frozen samples, HEK-293T cells were frozen with different cryoprotectants in the presence of lipofectamine 3000-pDNA complexes. After thawing, the cells were seeded and incubated for 24 h. (B) 10% DMSO (D) 10% PLL-SA. Scale bar: 30 µm

expression demonstrated in the frozen samples when compared to the unfrozen samples (Fig. 2. A and C). We also found that 10% DMSO exhibited relatively lower transfection capability than 10% PLL-SA (Fig. 2. B and D) after freezing. Furthermore, quantifying the results by microscope validated the microscopic images that indicated that 10% PLL-SA visibly increased gene transfer to the nucleus in frozen samples (Fig. 3).

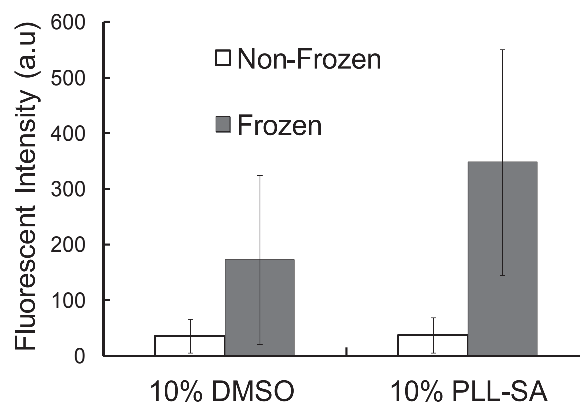


Fig. 3. Quantitative analysis of GFP expression by using confocal microscopy to compare between the two different cryoprotectants: 10% DMSO and 10% PLL-SA. HEK-293T cells were frozen in the presence of either 10% PLL-SA or 10% DMSO containing lipofectamine 3000-pDNA complexes. For non-frozen samples, the lipofectamine 3000-pDNA complexes were added directly to the HEK-293T cells. White bar: non-frozen samples; Grey bar: frozen samples. Data is expressed as mean±SD.

Quantification of transfection efficacy by luciferase activity

In order to further confirm the transfection capability for gene delivery platform by the freeze concentration method, we evaluated luciferase activity by using luciferase pDNA pGL4.51 as a reporter gene. The transfection efficacy was quantitatively analyzed by measuring the luciferase activity after 24 h.

As shown in Fig. 4, it was confirmed that luciferase activity was much greater in the presence of 10% PLL-SA when compared to 10% DMSO. The gene transfection was higher in the presence of polyampholyte as a cryoprotectant, possibly owing

to the high capability of transferring more pDNA into cells.

This result was consistent with our earlier result indicating that 10% PLL-SA increased transfection capability more effectively than 10% DMSO (Fig. 2 and Fig. 3). The increased gene concentration caused by the polyampholyte cryoprotectant may also be the reason for greater gene internalization.

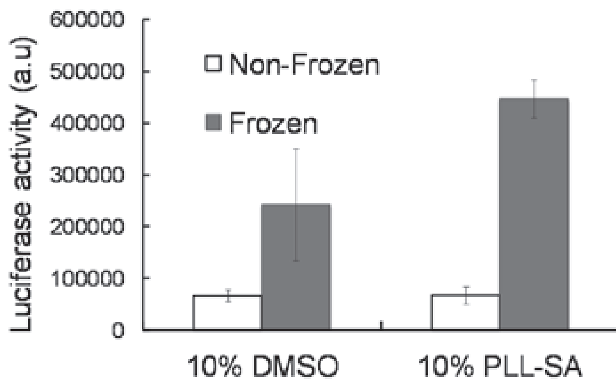


Fig. 4. Quantification of the *in vitro* transfection capability of lipofectamine 3000-pDNA complexes, containing the luciferase gene, in HEK-293T cells. The cells were frozen in the presence of either 10% PLL-SA or 10% DMSO containing lipofectamine 3000-pDNA complexes. For non-frozen samples, the lipofectamine 3000-pDNA complexes were added directly to the HEK-293T cells. Luciferase activity of samples was measured 24 h after transfection without the change of medium. White bar: Non-frozen; Grey bar: Frozen

During the freezing process, spontaneous ice nucleation occurs between -5˚C to -45˚C resulting in ice growth in all directions while simultaneously increasing the solute concentration in the remaining unfrozen content of the system. In our earlier report, we investigated the sodium ion concentration in the presence of polyampholyte and DMSO cryoprotectant using solid state NMR.[9] The sodium ion concentration in the presence of DMSO cryoprotectant at -40˚C was found to be 7 times higher than at normal temperature. On the other hand, in the presence of polyampholyte cryoprotectant, the sodium ion concentration was more than 10 times concentrated at -40˚C when compared with room temperature. It was found that polyampholyte cryoprotectants notably increased concentration in frozen samples. In our current study, we also found that polyampholyte cryoprotectants increased the chance of introducing genetic materials inside the cells after freezing. The mechanism of increased concentration of polyampholytes during freezing are still needs to be investigated.

Determination of freeze concentration factor of cryoprotectants

It is important to quantify the freeze concentration factor of cryoprotectants at -80˚C. Miyawaki and colleagues found a simple method to quantify freeze concentration factor (α) at low temperatures.[20] They explained that the driving force behind the increased solute concentration was ice crystal formation. Theoretically, α related to freezing point depression and was estimated from the analysis on the fraction of frozen water. They simplified information from referenced literature and calculated the direct relationship with freezing point depression.[20]

$$\alpha = T/T_f \qquad \ldots\ldots\ldots\ldots\ldots (1)$$

where, T (˚C) is the temperature of interest and T_f (˚C) is the freezing point.

Therefore, as stated in equation (1), the freeze concentration factor can be estimated by the analysis of the fraction of frozen water. We calculated the freeze concentration factor of cryoprotectants by estimating the freezing point (T_f) values after freezing completely at -80˚C. The T_f values of samples in the presence of DMSO were found around -4.475. In the case of 10% PLL-SA, T_f value was at -0.683. So, based on the T_f values, the freeze concentration factor was calculated as

stated in the equation 1. We found that only 10% PLL-SA demonstrated an approximate high value of 117.1, whereas 10% DMSO exhibited a significantly lower value of 17.9. These results clearly indicated that 10% PLL-SA exhibited high freeze concentration factor at freezing temperatures when compared to 10% DMSO and also that PLL-SA played an important role for gene delivery.

In this study, we presented a novel strategy of gene delivery combined with freeze concentration process that provided a new perspective for the design of a future gene delivery process. We also found the efficient use of polyampholyte cryoprotectant which showed promising gene transfection capabilities in comparison to DMSO. Moreover, the enhanced freeze concentration factor in the case of polyampholyte cryoprotectant was found to be the possible reason for inducing gene delivery to the cytoplasm of the cells. These results suggest that the use of polyampholyte cryoprotectant during the freeze concentration process could serve as a versatile and efficient combination for gene therapy.

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凍結濃縮法を利用した遺伝子導入技術における凍結保護物質の役割 ：Sana Ahmed，宮脇　長人，松村　和明　（北陸先端科学技術大学院大学先端科学技術研究科，東京海洋大学食品生産科学部門）

［キーワード：凍結濃縮，遺伝子導入，凍結保護剤］