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Novel Concentration-Based Freezing Method for Efficient Protein Delivery

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We developed a novel, well-controlled slow-freezing concentration-based strategy for effective protein delivery using polyampholyte NPs in the absence of a cryoprotectant. Here, we demonstrate the feasibility of this freeze concentration method, which is exceptionally favorable for cytoplasmic protein delivery, and focus on a simple slow freeze-thaw method using slow freezing program. We used confocal laser scanning microscopy to investigate the adsorption and internalization of lysozyme proteins at variable freezing temperatures. Quantification of fluorescence intensity revealed that proteins were more easily internalized by gradually decreasing the temperature. We believe that application of this concentration-based freezing technique could be extended to gene therapy and cancer immunotherapy.

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INTRODUCTION

Recently, substantial efforts have been made towards developing new approaches for the delivery of therapeutics such as proteins and drugs.[1)] Several techniques, such as electroporation and ultrasonication, have been used to deliver proteins and drugs to cells.[2)] However, there are still drawbacks associated with these methods, such as cytotoxicity, cell death, and low affinity between the proteins and the cell membrane, which would not be beneficial to cytoplasmic delivery.[3)] There is therefore some urgency to eliminate these drawbacks associated with therapeutic delivery.

Previously, we developed a novel freeze concentration method for enhancing the cytoplasmic delivery of proteins.[4)] During freezing, water is transformed into ice crystals at extreme low temperatures. Eventually, these ice crystals will separate the solute molecules that are present in the solution. This unfrozen solution leads to an enhanced concentration, known as the freeze concentration.[5)]. When the solution is extremely cooled, the unfrozen solution gets present near the cells located in residual solution. This phenomenon can enhance the interaction between cell membrane and protein based drugs. In addition, freeze concentration offers so many advantages such as simple, low cost and without any toxicity. However, this comes with a high risk of intracellular or extracellular ice formation, which can cause severe damage to cells and tissues.[6)]

In our previous research, we developed a polyampholyte nanocarrier for delivery of proteins. We also used a low toxicity polyampholyte

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cryoprotectant to protect our system from freeze damage at extreme low temperatures.[7] The cryoprotectant was used to obtain high cell viability by preventing the formation of ice crystals and to maintain osmotic balance at -80 °C, thus preserving the cells for protein delivery. To further optimize the freeze concentration system for protein delivery, it is important to avoid adding any external additives such as cryoprotectants.

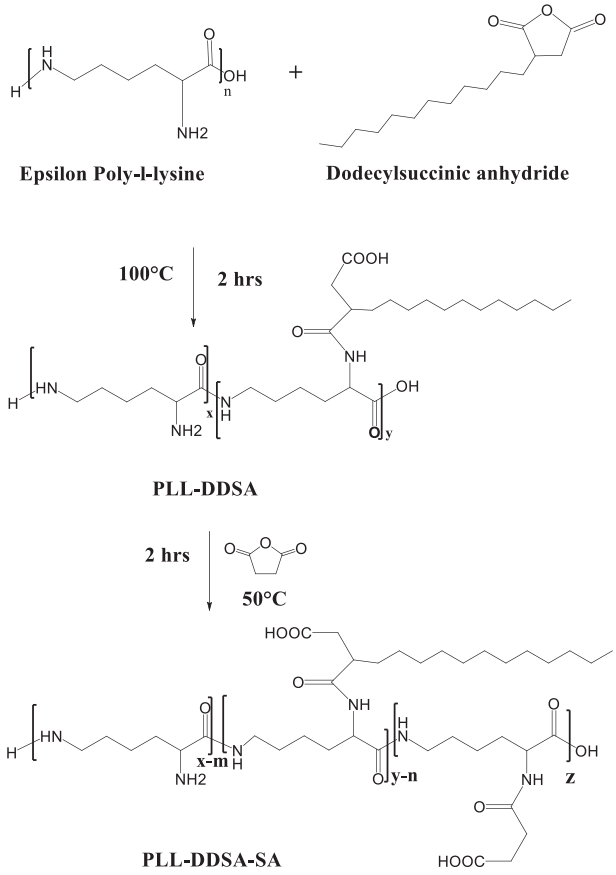
In the present study, we developed a slow-freezing-based protein delivery system that does not require a cryoprotectant. This method offers a simple, inexpensive, and very effective method for protein delivery, simply by controlling the freezing rate. Rapid and slow cooling rates have been shown to have different effects on the long-term viability of cells. A fast cooling rate is more likely to result in the formation of a large number of small-size intracellular ice crystals. On the other hand, slow freezing favors the formation of larger size ice crystals, specifically in an extracellular location.[8] In addition, slow freezing results in a maximum displacement of water, which enhances the concentration of unfrozen fractions outside the cells.[9]

In this study, we have utilized a slow-freezing technique for enhanced protein delivery. We used lysozyme as a model protein containing polyampholyte nanocarrier and investigated protein adsorption and internalization into fibroblast L929 cells by means of a slow freeze-thaw process. These findings reveal the importance of low-temperature-based cellular uptake of proteins without the addition of external additives such as cryoprotectants.

MATERIALS AND METHODS

Preparation of polyampholyte nanoparticles

Hydrophobic polyampholyte nanoparticles (NPs) were prepared as previously described.[4] Briefly, a 25% w/w aqueous solution of poly-L-lysine was mixed with dodecylsuccinic anhydride (5% molar ratio, $COOH/NH_2$) and stirred at 100 °C



Scheme 1. Preparation of the hydrophobically modified polyampholyte nanocarrier.

for 2 h. Succinic anhydride (65% molar ratio, $COOH/NH_2$) was added and reacted at 50 °C for 2 h (Scheme 1). The degree of substitution was determined using a ^1H NMR and TNBS (2,4,6-trinitrobenzenesulfonate) assay.

Texas Red (TR) labeling of lysozyme protein

Lysozyme proteins were dissolved in chilled buffer (sodium bicarbonate, 0.1 M), and then TR sulfonyl chloride solution (1 mg in 50 μL acetonitrile) was added. The reaction was desalted using a desalting column (3 K), and equilibrated with PBS.

Adsorption of protein-loaded polyampholyte NPs

TR-labeled lysozyme proteins (2 mg) were incubated with 1% polyampholyte NPs for 2 h. Unloaded protein was discarded using a centrifugal

filter (MWCO: 50kDa) at 13,200 rpm for 15 min. After centrifugation, the encapsulated protein-loaded polyampholyte NPs were obtained.

Cell freezing with protein-loaded polyampholyte NPs

Protein-loaded polyampholyte NPs were prepared in Dulbecco's modified Eagle medium (DMEM) without fetal bovine serum and sterilized using a 0.22 μm syringe filter. Protein-loaded polyampholyte NPs were added to L929 fibroblast cells, at a density of $1x10^4$ cells/mL. Cells containing protein-loaded polyampholyte NPs were transferred to cryo-straws using a freeze controller cryobath at 0.20 °C/min and frozen from -1 °C to -15 °C. At each temperature, ice seeding (induction of ice crystal formation) was done for 5 min and the straw was withdrawn from the cryobath. Ice seeding was done manually using cold tweezers (pre-chilled with liquid nitrogen). The straw solution was thawed by immersing in water at 37 °C. Solutions were centrifuged at 1000 rpm for 4 min and replaced with fresh medium. Cell viability was calculated using a trypan blue assay as the number of viable cells divided by the total number of cells. Adsorption of proteins was observed with a confocal laser scanning microscope (CLSM).

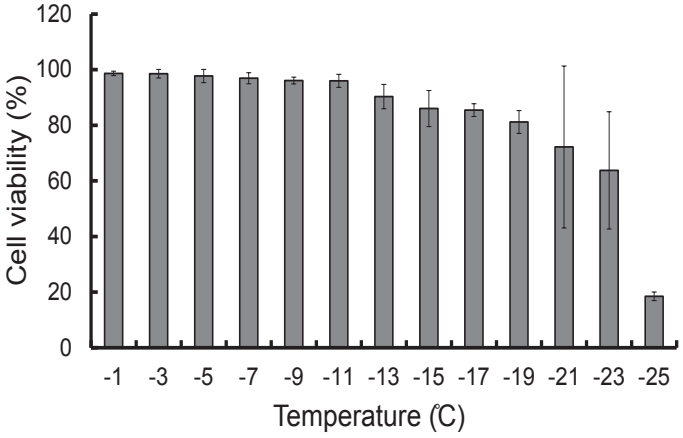


Fig. 1. Cell viability of lysozyme protein-loaded polyampholyte nanocarriers after thawing. Cells were incubated at different temperatures by using a slow programmable freezer. Data are expressed as mean ± SD for 3 independent experiments.

Investigation of protein internalization after thawing from different frozen temperatures

After freezing at different temperatures, cells containing the protein-loaded polyampholytes were thawed at 37 °C. Cells were washed in medium, seeded onto a glass-bottom dish, and incubated for 2 days. Attached cells were washed in PBS and observed by CLSM. All experiments were conducted in triplicate.

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RESULTS AND DISCUSSION

Preparation of self-assembled polyampholyte nanocarriers

To protect therapeutic proteins from degradation, we developed polyampholytes as a carrier to enhance protein stability during systematic delivery.[4] Hydrophobic polyampholyte is denoted as PLL-DDSA (5)–SA (65) to indicate that 5% and 65% amino groups have been converted to a carboxyl group (Scheme 1) with dodecylsuccinic anhydride and succinic anhydride, respectively. These polyampholyte nanocarriers were reported to be very small and stable for over a week. They also showed high adsorption efficiency with cationic charged lysozyme proteins. In the current study, we used protein-loaded polyampholyte NP complexes to establish a new method for protein delivery.

Cell survival of protein-loaded polyampholyte nanocarriers by slow freezing program

A freeze control programmer was used to allow slow freezing of the protein nanocarrier complex and cells. L929 cells, at a density of $1x10^4$ cells/mL, were frozen with protein-loaded polyampholytes at a rate of 0.20 °C/min. Freezing started from 25 °C and continued to -25°C at a rate of 0.20 °C/min. Cells were rapid-thawed at 37 °C. We thawed cells rapidly because there are many reports which suggested that rapid thawing increases survival compared to slow thawing[10, 11]

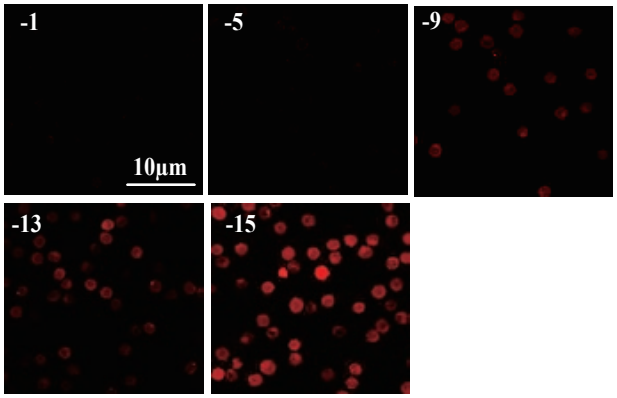

Fig. 2. CLSM images of TR-labeled lysozyme protein-loaded polyampholyte after thawing from different temperatures from -1 to -15°C, followed by ice seeding. Scale bar- 10 μm.

because it inhibits the recrystallization process. First, we measured cell viability using a trypan blue assay (Fig. 1). These results indicate that cells were retained at viability between 80-90% until -19 °C. However, cells were drastically lost at lower temperatures. At -25 °C, cell survival was less than 20%, indicating an intolerance to freezing below a certain temperature.

Slow and gradual freezing for too long can cause cell death due to dehydration or intracellular ice crystallization. Therefore, we employed temperatures down to -15 °C to ensure high cell viability during protein delivery.

Adsorption of TR-labeled protein loaded polyampholyte NPs at 0.20 °C/min

For investigation of the adsorption of protein-loaded polyampholyte nanocarriers, the same procedure was followed as described previously[4]. Freeze-thawed solutions were observed using CLSM. Lysozyme proteins labeled with TR were progressively adsorbed at gradually lower temperatures (Fig. 2). As cooling continued, the concentration of unfrozen protein-loaded polyampholyte across the cell membrane was increased after thawing because of freeze concentration. These results showed that with gradual freezing, a low concentration of protein accumulated in the peripheral cells, because cells must be located in the residual concentrated water due to the freezing concentration. Thus, concentration could play an important role in the slow-freezing process to enhance the interaction between lysozyme proteins and the cell membrane.

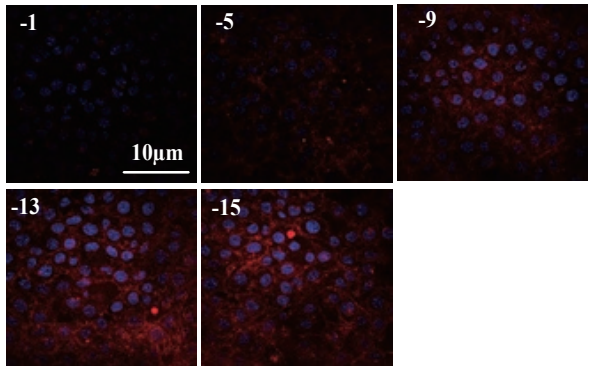

Fig. 3. CLSM images of internalized lysozyme protein in L929 cells. Lysozyme protein was labeled with TR red; nucleus was stained with Hoechst 33258 (blue). Scale bar-10 μm.

Internalization of lysozyme proteins after thawing

After thawing, cells were seeded onto culture dishes for internalization of the protein. As shown in Fig. 3, proteins were labeled with TR dye (red) and the nucleus was labeled with Hoechst (blue). Interestingly, these data showed that with decreasing temperatures the protein was efficiently internalized to the cytosol of the cells. At -15°C, high fluorescence indicated that the lysozyme protein was effectively delivered at a lower

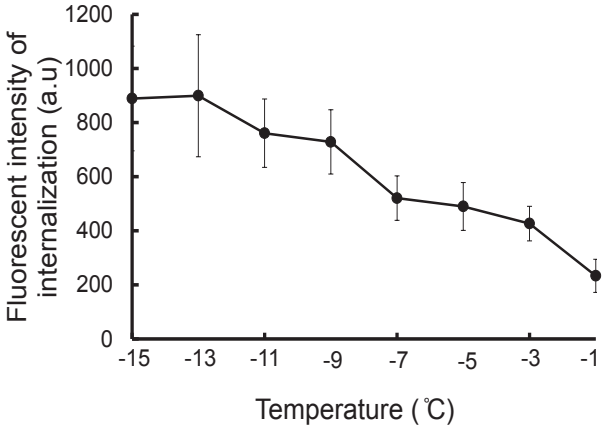

Fig. 4. Quantification of fluorescence intensity of internalized lysozyme proteins observed by CLSM. Data are expressed as mean ± SD for 3 independent experiments.

temperature. We also quantified fluorescence intensity using CLSM (Fig. 4). These results confirmed our previous findings. Fluorescence intensity of the internalized protein at -15 °C was 4 times higher than at -1 °C. This could be due to the fact that after thawing proteins can effectively diffuse into, rather than out of, the cells. From these results we can conclude that the freeze concentration method of slow freezing enhances the concentration of protein-loaded polyampholyte complexes at subzero temperatures, allowing efficient internalization of proteins.

Our findings confirm the use of cell freezing for cytoplasmic delivery of proteins. These results hold great promise for the use of an enhanced freeze concentration-based protein delivery method by slow freezing in cancer immunotherapy and gene-based therapies.

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凍結濃縮法を利用したタンパク質デリバリー： Sana Ahmed、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）［キーワード：タンパク質デリバリー、凍結濃縮］