Enhanced Cytoplasmic Internalization of Biomacromolecules by Freeze Concentration

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Background

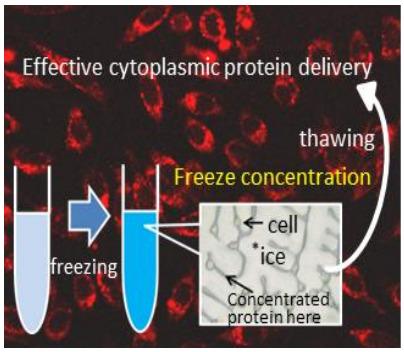
Over the past few years, targeting intracellular molecules remains challenging because most macromolecules cannot spontaneously cross the barrier imposed by the plasma membrane. A variety of methods such as microinjection, electroporation have been used to enhanced internalization and delivered macromolecules inside the cells. However, these physical strategies have found to be toxic and lead to cell damage. Hence, it is highly desirable to develop novel strategy for intracellular macromolecular delivery system for various therapeutic applications with low cell damage and high efficiency. Therefore, new strategies that can circumvent these limitations are of considerable interest. Here, I developed a new freeze concentration based approach for effective macromolecular delivery system. This thesis describes the development of novel and effective freeze concentration method for enhanced cytoplasmic delivery of macromolecules and addresses the feasibility of this method to be employed in gene therapy and immunotherapy applications.

Aim: (i) To develop freeze concentration based strategy for therapeutic applications

(ii) To develop new carriers for efficient and safe cytoplasmic delivery system

Results and Discussions:

In chapter 2, freeze concentration method was introduced for effective protein delivery system. At extreme ultra low temperature, the proteins were successfully adsorbed and transported to the cytoplasm of the cells comparing with non-frozen system. Moreover, I also developed safe and effective hydrophobic polyampholyte nanoparticles as a novel vehicle to carry proteins inside the cells. The surface charges of polyampholyte nanoparticles were easily manipulated and controlled by changing the introduction ratio of anionic functional group in polymeric backbone. In addition, polyampholyte nanoparticles have proven to be less toxic than cationic polymers. CLSM images demonstrated the effective delivery of proteins to the cytosol of the L929 cells by the combination of novel



Scheme-1 Freeze concentration induced cytoplasmic delivery of proteins

freeze concentration approach and hydrophobic polyampholyte nanoparticles. From the best of my knowledge, this was the first study of using novel, simple freeze concentration approach that can effective delivered proteins. In conclusion, freezing method was found to be effective and versatile for enhanced adsorption and internalization of protein *in vitro* (**Scheme 1**).

Chapter 3 shows the development of new polyampholyte-modified liposomes as a carrier by incorporating the polyampholytes in liposomes. The modified liposomes were found to be low toxic than bare polyampholytes. For delivery of proteins, combination of freezing and modified liposomes was utilized. The adsorption of proteins towards the cells was enhanced by 4 fold comparing with non-frozen conditions. Furthermore, the increased protein internalization was observed by using polyampholyte-modified liposomes comparing with unmodified liposomes. Additionally, modified liposomes exhibited high efficacy for promoting endosomal escape of proteins to the cytosol of the cells. From these evidences, we expect that this combination could be used for delivery of protein antigen into the cytoplasm for cancer treatment.

Chapter 4 extending the role of polyampholyte-modified liposomes and freeze concentration in immunotherapy applications. Freezing treated immune RAW 264.7 cells exhibited high uptake comparing with non-frozen system. Efficient delivery of OVA to the cytosol was shown to be partly due to the pH-dependence of the polyampholyte-modified liposomes. Cytosolic OVA delivery also resulted in significant up-regulation of the Major histocompatibility complex class I pathway through a process known as cross-stimulation, and well as an increase in the release of cytokines such as IL-1β, IL-6, and TNF-α. Administration of freeze concentration method treated cells is extremely effective for the induction of immunity. The combination of freeze concentration method and polyampholyte-modified liposomes can efficiently introduce antigen protein to MHC class I molecules for cancer immunotherapy applications.

In **Chapter 5**, the feasibility of freeze concentration in gene therapy was presented. I found a new freeze concentration-based gene transfection system that provides enhanced *in vitro* gene delivery compared to that provided by the commercially available systems. The system employs a facile freeze concentration step, whereby cells are simply frozen to very low temperatures in the presence of polymer-pDNA complexes. As part of system development, I also synthesized a low toxicity polyethyleneimine (PEI)-based polyampholyte prepared through succinylation with butylsuccinic anhydride. In aqueous solution, this modified

polyampholyte self-assembles to form small, positively charged, nanoparticles through a combination of hydrophobic and electrostatic interactions. Agarose gel electrophoresis analysis indicated that the polyampholyte nanoparticle was able to form a complex with pDNA that provided stability against nuclease degradation. Using transfection of HEK-293T cells, this study was demonstrated that using a combination of polyampholyte: pDNA, at an appropriate ratio, and the freeze concentration method resulted in significant enhancement of GFP (**Figure 1**) and luciferase expression compared to commercially available carriers. Endosomal escape of pDNA was also found to be increased when using the modified polyampholyte compared to branched PEI. This study suggests that the efficient combination of freeze concentration and the modified polyampholyte described here has great potential for *in vitro* gene therapy.

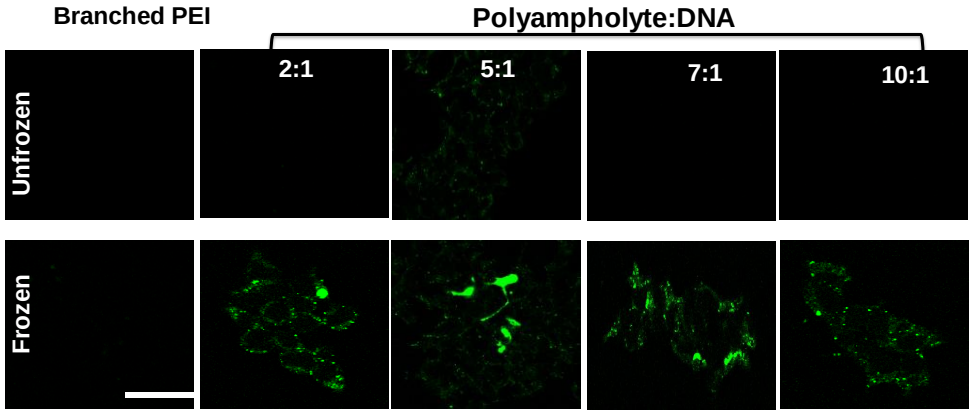


Figure 1 HEK-293T cells were either frozen in the presence of the different pDNA complexes along with 10% cryoprotectant (Frozen) or the pDNA complexes incubated with branched PEI, PEI-BSA: pDNA were added directly to the cells without freezing (Non-frozen) and were incubated for 10 h.

Conclusions

A novel and unique freeze concentration method have been developed for effective delivery of macromolecule to address number of challenges exist that most molecules face in their ability to be delivered effectively at target site. Freeze concentration based approach was found to be simple, reduces cell damage method and enhances the interaction between cell membrane and macromolecular complexes. This phenomenon was found to be a versatile method which has been shown to be efficient delivery of model proteins and genes. I believe I have been successfully able to develop new strategy which can have broad applications in nanomedicines.

Keywords: Freeze Concentration, Macromolecular delivery, Polyampholytes, Immunotherapy, Gene therapy