氏　　名	SANA AHMED			
学位の種類	博士(マテリアルサイエンス)			
学位記番号	博材第 427 号			
学位授与年月日	平成 29 年 6 月 23 日			
論文題目	Enhanced Cytoplasmic Internalization of Biomacromolecules by Freeze Concentration (凍結濃縮法による細胞内への生体高分子の効率的導入法)			
論文審査委員	主査	松村　和明	北陸先端科学技術大学院大学	准教授
		前之園　信也	同	教授
		芳坂　貴弘	同	教授
		濱田　　勉	同	准教授
		岩﨑　泰彦	関西大学	教授

論文の内容の要旨

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.

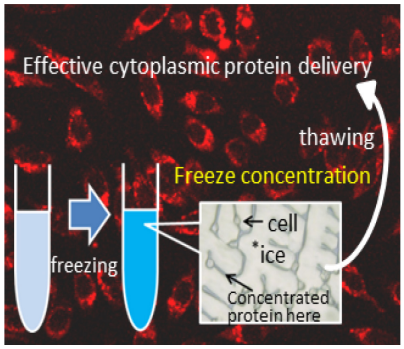
Aim: (i) To develop freeze concentration based strategy for therapeutic applications

(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 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**).



Scheme-1 Freeze concentration induced cytoplasmic delivery of proteins

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.

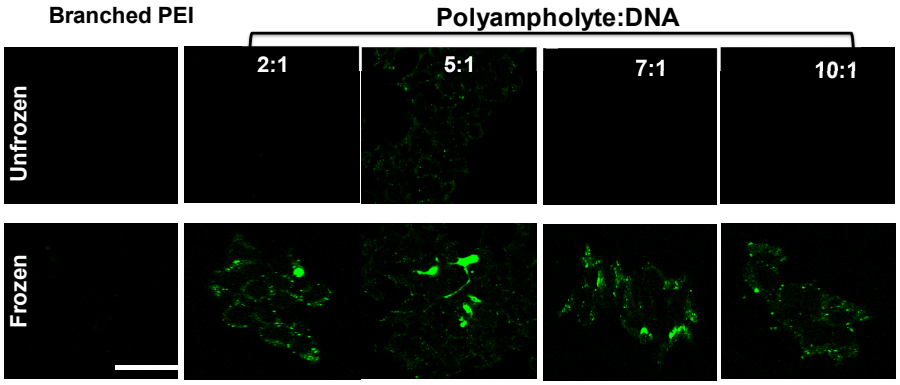


Figure 1 HEK-293T cells were either frozen in the presence of the different pDNA complexes along with 10% cryoprotectant (Frozen) or the pDNA complexes incubated with branched PEI, PEI-BSA: pDNA were added directly to the cells without freezing (Non-frozen) and were incubated for 10 h.

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

論文審査の結果の要旨

本論文は、凍結濃縮という手法と細胞親和性の高分子ナノキャリアを組み合わせることにより、これまでの手法に比べて細胞への傷害性を低く保ったまま、細胞質内への生理活性物質輸送の効率化を達成したものである。細胞質内へタンパク質や遺伝子を送達することは、細胞治療や細胞の活性化など多くの研究や応用において重要な問題であるが、細胞膜を通過させるためには様々な工夫が必要となる。そこで、本研究では、凍結濃縮という現象を用いてタンパク質を細胞膜周囲に集積し、膜に吸着させることを試みた。凍結濃縮とは、凍結時にできる氷結晶が、溶質を排除しながら成長するため、残存水溶液の溶質が濃縮される現象のことで、果汁の濃縮などに利用される技術である。1℃/min 程度の降温速度における緩慢な条件での凍結の際、細胞は未凍結の溶液成分に存在し、凍結濃縮により細胞膜周囲に溶質が濃縮される。その結果、溶質中のタンパク質の細胞膜への吸着が促進されることがわかった。このとき、吸着したタンパク質を解凍後に速やかに細胞内に取り込ませるため、細胞膜親和性のナノキャリアと複合化させた。ナノキャリアは、プラスに帯電したポリリジンのアミノ基の一部を無水コハク酸でカルボキシル基に変換し、さらにドデシル無水コハク酸を一部反応させた、疎水性両性電解質高分子を用いた。このナノキャリアの電荷を調整することで、種々のタンパク質と静電的相互作用により安定なナノ複合体を形成することが可能であった。次に、静電的相互作用のみによらず、より多くの種類の機能性物質を担持できる様に、リポソームをナノキャリアとして選び、内部にタンパク質を担持した後、凍結濃縮法により細胞内への送達を試みた。このとき、リポソームに pH 依存性の高分子を被覆した。それにより、エンドサイトーシスによって細胞内に取り込まれた複合体が、エンドソームで消化されてしまう前に、細胞質内に脱出することが可能であることを確認した。これは、エンドソーム中の低 pH（約 5.5）条件下で高分子がプロトン化し、凝集を引き起こすことでエンドソーム膜を不安定化させるためであると考えられる。この作用により、細胞質内への物質送達が達成されることになる。この技術を用い、抗原をマクロファージ内に送達することで、主要組織適合抗原を介した抗原提示を誘導することが可能となり、免疫機能を強化させることで、免疫治療への応用が期待される成果を得た。また、遺伝子を担持させたナノキャリア存在下で細胞を凍結解凍することで、効率的な遺伝子導入に成功した。以上、本論文は、凍結という手法と新規細胞親和性ナノキャリアを組み合わせた独自の細胞内物質送達技術を開発することに成功し、様々な細胞治療への応用への道を開く結果となった。また、細胞内への取り込み過程の詳細を明らかにするなど学術的に貢献するところが大きい。よって博士（マテリアルサイエンス）の学位論文として十分価値あるものと認めた。