Title	DEVELOPMENT OF SYNTHETIC POLYAMPHOLYTES VIA RAFT POLYMERIZATION AND THEIR BIOMATERIAL APPLICATIONS LIKE CELL CRYOPROTECTION AND PROTEIN AGGREGATION INHIBITION
Author(s)	ROBIN, RAJAN
Citation	
Issue Date	2016-06
Type	Thesis or Dissertation
Text version	ETD
URL	http://hdl.handle.net/10119/13722
Rights	
Description	Supervisor:松村　和明，マテリアルサイエンス研究科，博士

氏　　名	ROBIN RAJAN
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 403 号
学位授与年月日	平成 28 年 6 月 24 日
論文題目	DEVELOPMENT OF SYNTHETIC POLYAMPHOLYTES VIA RAFT POLYMERIZATION AND THEIR BIOMATERIAL APPLICATIONS LIKE CELL CRYOPROTECTION AND PROTEIN AGGREGATION INHIBITION （RAFT 重合による合成両性電解質高分子の開発とその細胞凍結保護剤およびタンパク質凝集抑制剤としてのバイオマテリアル応用）
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 高木　昌宏　同　教授 金子　達雄　同　教授 長尾　祐樹　同　准教授 加藤　功一　広島大学　教授

論文の内容の要旨

Introduction

Polyampholytes are those polymers which encompass both the positive as well as negative charges. A sub-class of polyampholytes are zwitterionic polymers, which have the same number of anionic and cationic groups within a single repeating unit. They are very versatile systems and have therefore found applications in a plethora of fields including catalysis, water desalination, and a wide range of other applications. They have also been used in a variety of biomedical applications. Recently, our group developed a non-penetrating polyampholyte-based cryoprotective agent (CPA), carboxylated poly-l-lysine (COOH-PLL). It was found to be an excellent CPA with various cell types. This was an important development, because the CPAs which are used in clinical research presently (dimethyl sulfoxide (DMSO) and glycerol) have various shortcomings, but are still used due to the absence of any efficient alternative, hence it was imperative to develop newer CPA. However, the mechanism was not clear and also it was not known whether synthetic polyampholytes can also display similar efficiency. Hence, I prepared synthetic polyampholyte with the dual aim of developing newer CPA and to elucidate mechanism, because it is easier to modify various parameters in synthetic polyampholytes in order to explain the various characteristics required for imparting high cryopreservation property. I also developed zwitterionic polymers and compared it with a polyampholyte copolymer to investigate the importance of polymer structure.

Also, the polymers were employed for protein aggregation inhibition studies. Protein instability is an

ongoing challenge in the field of biopharmaceutics. With physical and chemical deterioration, protein aggregation is one of the foremost causes of protein instability. Over the years, large number of organic compounds and protein engineering techniques has been employed for thermal inhibition of lysozyme aggregation with relatively good success but very high efficiencies have not been achieved yet. Polymers, especially synthetic polymers, have not been employed for such work with high efficiencies. Hence, with this this study, I unveiled the significance of synthetic polymers in the field of protein therapeutics. I also transformed these polymers into core-shell nanogels in order to further enhance the efficiency of synthetic polymers for protein aggregation inhibition.

Results and Discussion

Firstly in **Chapter 2**, I developed synthetic polyampholyte, poly-(MAA-DMAEMA), via RAFT polymerization. The polymer displayed all the characteristics of living polymerization, with narrow molecular mass distribution, first-order kinetics and control over molecular weight, composition, etc. The neutralized random polyampholyte, which had an equal composition ratio of monomers, showed high cryoprotective properties in mammalian cells. Introduction of a small amount of hydrophobic monomer enhanced cell viability after cryopreservation, indicating the importance of hydrophobicity. Leakage experiments confirmed that these polyampholytes protected the cell membrane during cryopreservation. Due to low cytotoxicity, this polyampholyte has the potential to replace the conventional cryoprotective agent DMSO. This study was the first to show that it is possible design a polymeric cryoprotectant that will protect the cell membrane during freezing using appropriate polymerization techniques.

In **Chapter 3**, I developed two more zwitterionic polymers (poly-SPB and poly-CMB) in addition to the polyampholyte synthetized in Chapter 2. This was done in order to ascertain whether all polymers containing both the charges possess excellent cryoprotective property or not. Cryopreservation results revealed that poly-(MAA-DMAEMA) shows excellent cryoprotective property. On the other hand, poly-SPB showed only intermediate property and poly-CMB showed no cryoprotective property (Fig. 1). These data suggested that the polymer structure strongly influences cryoprotection, providing an impetus to elucidate the molecular mechanism of cryopreservation. I investigated the mechanism by studying the interaction of polymers with cell membrane, which allowed to identify the interactions responsible for imparting different properties. Results unambiguously demonstrated that polyampholytes cryopreserve cells by strongly interacting with cell membrane, with hydrophobicity increasing the affinity for membrane interaction, which enable it to protect the membrane from various freezing induced damages. Additionally cryoprotective polymers, especially their hydrophobic derivatives, inhibit the recrystallization of ice, thus averting cell death. Hence, the results provide an important insight into the complex mechanism of cryopreservation, which might facilitate the rational design of polymeric CPAs with improved efficiency.

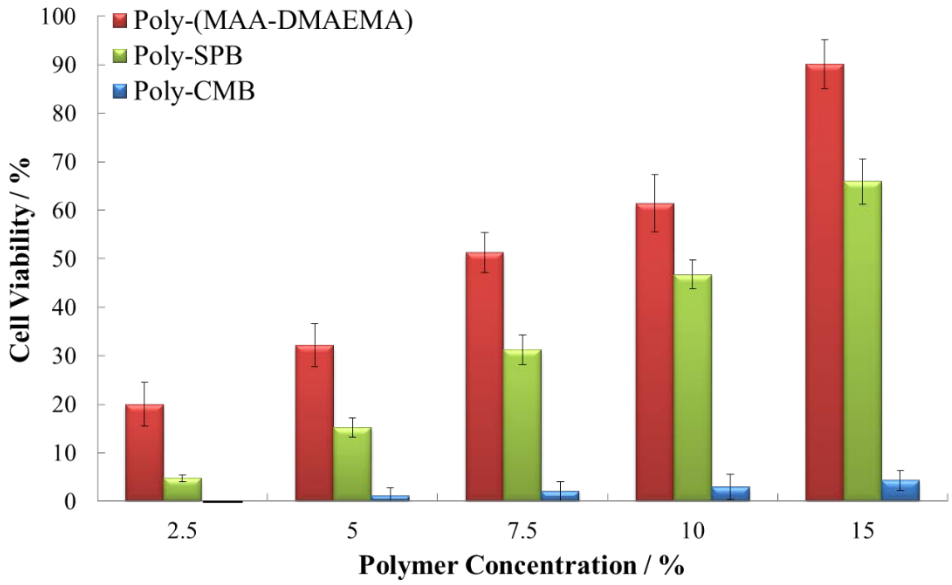

Figure 1. Cryoprotective properties of poly-(MAA-DMAEMA), poly-SPB, and poly-CMB (a) at various polymer concentration

In **Chapter 4**, the propensity of polyampholytes for protection of biological systems was utilized by employing them for protein aggregation inhibition. It is well known that proteins under extreme conditions undergo aggregation to form fibrils. This causes many neurodegenerative diseases and is also a principle deterrent in the field of protein biopharmaceutics. Lysozyme was employed as a model protein for this study and it is known that lysozyme undergoes aggregation when heated to high temperatures. When poly-SPB was mixed with lysozyme and heated, no aggregation was observed and fibril formation was also suppressed. The polymer was found to be more efficient than previously described inhibitors of protein aggregation (Fig. 2). Conformational studies revealed that lysozyme when heated without any additive loses its secondary structure and transforms into a random coil conformation. Presence of the polymer facilitates retention of partial higher order structures and lysozyme solubility at higher temperatures. The high efficiency of the polyampholyte was ascribed to its ability to prevent collisions between aggregating species by acting as a molecular shield.

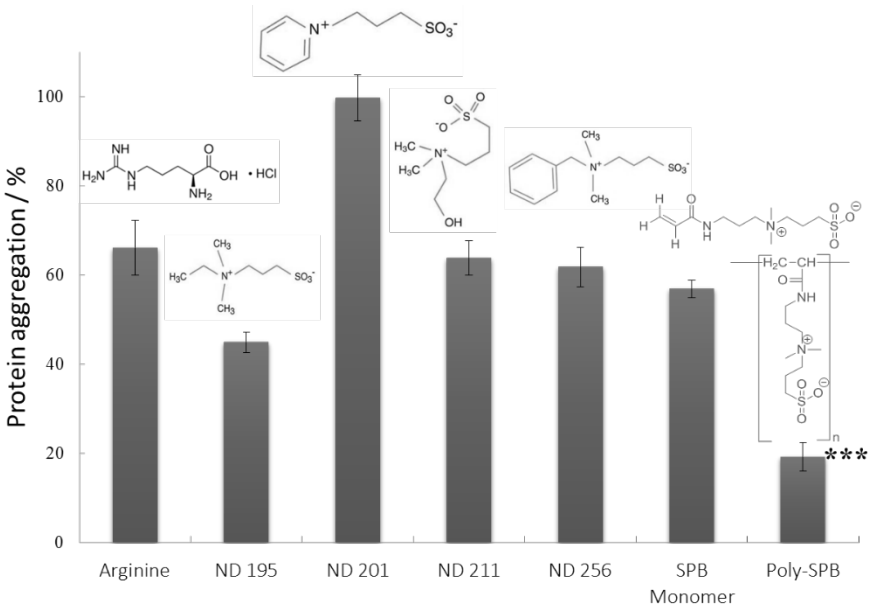

Figure 2. Protein aggregation of lysozyme (0.5 mg/mL) when heated to 90°C for 30 minutes in the presence of various reagents (5% w/v).

In **Chapter 5**, I prepared core-shell nanogels from the zwitterionic polymer poly-SPB. The nanogels were prepared by using end functionalized poly-SPB (prepared by RAFT polymerization) as the macro chain transfer agent and by using a chemical cross-linker. The nanogels were prepared with and without an additional hydrophobic monomer in the core. The nanogels exhibited much higher efficiency than the corresponding linear polymer, poly-SPB. The nanogels enabled lysozyme to retain higher enzymatic activity at a much lower concentration of nanogels when compared to poly-SPB. Efficiency was further enhanced by introducing small amount of hydrophobicity in the core of the nanogel. The higher activity of nanogels can be ascribed to its ability to act as artificial molecular chaperones which protects proteins under severe conditions.

Conclusion

I believe I have been successfully able to develop synthetic polyampholytes which can have various biomaterial applications. I have demonstrated that it is very easy to develop synthetic polyampholytes via RAFT polymerization and the molecular weight and the functionality of the polymers can also be controlled efficiently. Due to the synthetic nature of these polymers, they offer a set a set of advantages over peptide (or amino acid) based polyampholytes because its synthetic nature facilitates modification of surface hydrophobicity and hydrophilicity, as well as easy control over molecular weight and polydispersity, which may lead to higher efficiency, depending on the desired application. The studies in Chapter 2 and 3 which identified the cryoprotective property of polyampholytes and its possible mechanism of cryopreservation can lead to the development of numerous polymer based CPAs in the

future which can then be employed for preserving 2D and 3D cell containing constructs. On the other hand, development of polymer based systems for protein aggregation inhibition studies can revolutionize the field of protein biopharmaceutics where more polymers, particularly zwitterionic polymers can be successfully employed.

Keywords: Polyampholytes, Zwitterionic Polymers, Living Polymerization, Cryopreservation, Protein Aggregation Inhibition

論文審査の結果の要旨

本論文は、合成した両性電解質高分子による細胞の凍結保護作用およびタンパク質凝集抑制効果を明らかとし、その構造との相関に関して詳細に検討したものである。両性電解質高分子は、その分子内にプラスとマイナスの両方の電荷を持ち、pH や塩により電荷を調整できる事などから近年、スマートマテリアルとしての応用に期待されている。バイオマテリアル分野においては、細胞やタンパク質との低い相互作用が報告されており、タンパク質低付着表面や血液適合表面への応用が期待されている。今回は、既に報告されているポリリジン由来の両性電解質高分子に比べ、分子量の調節や分子設計を容易にするために、ビニル重合により両性電解質高分子を合成し、その凍結保護活性およびタンパク質凝集抑制活性を評価した。可逆的付加開裂連鎖移動（RAFT）重合の手法により、分子量および分子量分布を調節した両性電解質高分子が、凍結保護作用を持つ事を確認し、その作用のメカニズムについて、細胞およびリポソームを用いて検討した結果、氷晶の形成抑制作用に伴う細胞膜の保護作用が重要であることを示した。また、一分子中にプラスとマイナスの双方の電荷を持つという意味では両性電解質高分子の一種であるが、一つの側鎖に双方の電荷を持つ双性イオン高分子も合成し、その凍結保護効果を調べたところ、プラスの電荷を持つモノマーとマイナスの電荷を持つモノマーを共重合した両性電解質高分子に比べて低い活性であった。これにより、構造により効果が異なる事が分かった。ブチル基やオクチル基などの疎水性モノマーを共重合させる事で、凍結保護活性のある高分子に関しては、凍結保護活性を向上させることに成功した。これは疎水性基の導入による氷晶形成抑制効果および膜との相互作用の向上によるものであり、元から細胞膜保護効果の無い双性イオン高分子では効果の向上がみられないことが確認された。続いて、タンパク質の加熱凝集およびフィブリル形成の抑制に、双性イオン高分子であるポリスルホベタインが有効であることを示した。これはポリスルホベタインのタンパク質との弱い相互作用により、加熱時のタンパク質間に働く疎水性相互作用を阻害するモレキュラーシールド効果によるものであることを明らかとした。これらの一連の研究は、氷の結晶やタンパク質のフィブリル形成に共通する核形成抑制作用が、高分子化合物の電荷および電荷の分布状態に関連していることを示した初めての報告である。

以上、本論文は、両性電解質高分子の細胞凍結保護作用及びタンパク質凝集抑制作用のメカニズムの一部を解明し、それを用いて機能の向上に成功した点において、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。