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Title	DEVELOPMENT OF SYNTHETIC POLYAMPHOLYTES VIA RAFT POLYMERIZATION AND THEIR BIOMATERIAL APPLICATIONS LIKE CELL CRYOPROTECTION AND PROTEIN AGGREGATION INHIBITION
Author(s)	ROBIN, RAJAN
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Development of synthetic polyampholytes via RAFT polymerization and their biomaterial applications like cell cryoprotection and protein aggregation inhibition

Robin Rajan

Kazuaki Matsumura Laboratory, School of Materials Science, JAIST

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.

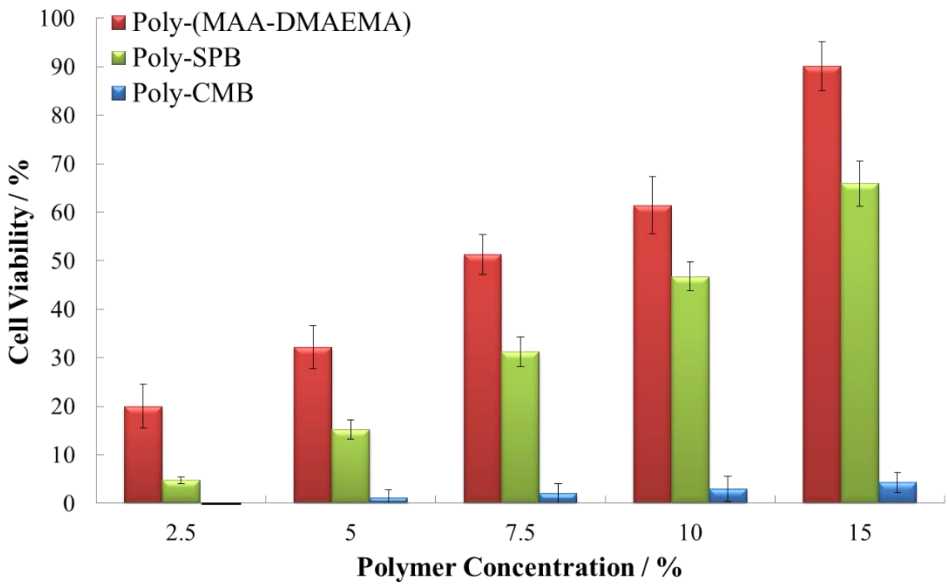

Figure 1. Cryoprotective properties of poly-(MAA-DMAEMA), poly-SPB, and poly-CMB (a) at various polymer concentration

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.

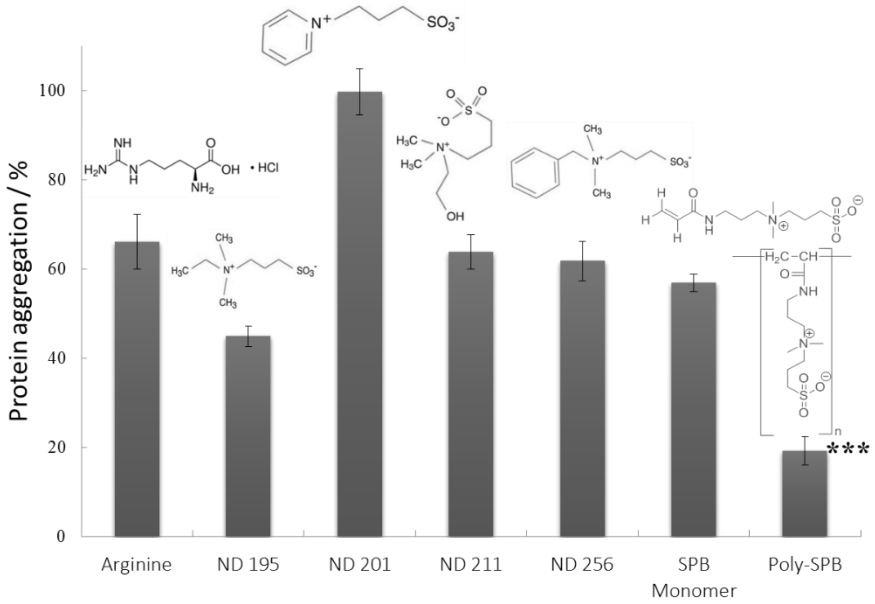

Figure 2. Protein aggregation of lysozyme (0.5 mg/mL) when heated to 90°C for 30 minutes in the presence of various reagents (5% w/v).

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