博士論文（要約）

Exploring Membrane Properties of Polyion Complex Vesicles (PICsomes) by Tuning their Constituent Polymers and Cross-linking Degree

（ポリイオンコンプレックス型ベシクル（PICsomes）の膜特性制御に関する研究）

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1. Introduction

Interests in submicrometer-sized self-assembled hollow structures continuously expand due to their high potential in various fields, especially in delivery of cargoes and biologically active molecules inside the body[1]. Among several systems, polymer-based hollow architecture, particularly polymersomes, offer more flexibility and tunability of their properties compared to their counterparts[2].

Among polymersome systems, a special kind of system was reported, called polyion complex vesicles (PICsome), whose formation is driven by electrostatic interaction of oppositely charged polypeptides in aqueous medium[3]. Potential utility of PICsomes with sub-100 nm size (Nano-PICsomes) have been proved for drug delivery applications, due to their simple preparation method, unilamellar and semi-permeable vesicle membrane, easy loading of water-soluble macromolecules, and long blood circulation in mice after crosslinking[3,4].

Nevertheless, considering the tunability of the PICsomes, still further and careful studies are necessary for better understanding of their features, and practical applications have been sought. One of the most important feature to be intensively studied is membrane property of PICsomes, since they determine most of their behaviors, such as burst/sustained release of cargoes, mechanical or rheological properties, stability under the physiological conditions, interactions with cells, their biodistribution and so on[5].

In this research, my focus is to provide deeper understanding of PIC membrane and explore its tunability by (1) introducing hierarchical assembly from secondary structures to PIC compartment, (2) investigating crosslinkable nature of polyion complex (PIC) membrane, and (3) influence of hydrophobicity of PIC forming polycation on PICsome features with special interest in semipermeability of macromolecules and mechanical properties of PICsomes. Through this research, tuning strategy for PIC membrane properties would be established.

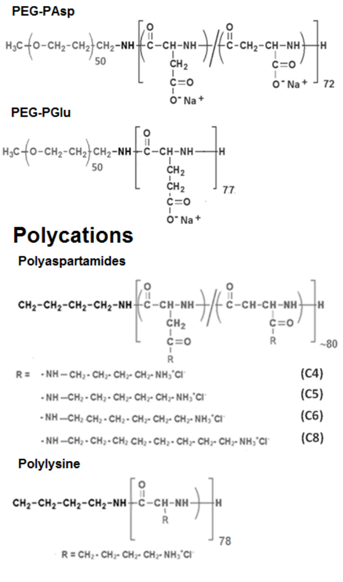
2. Experimental

Poly(ethylene glycol)-*block*-polyaspartate (PEG-PAsp) and poly(ethylene glycol)-*block*-poly(L-glutamate) (PEG-PGlu) with degree of polymerization (DP) of 72 and 77, respectively, were used as an anionic block copolymers, and a series of polyaspartamide-based homo-polymers with aliphatic chain of butane, pentane, hexane and octane (PAsp-C*n*, where *n* is number of carbons in aliphatic chain) with DP around 80 and poly(L-lysine) (PLys) with DP of 78 were used for PIC preparation (Fig.1). PEG-PAsp and Homo-P(Asp-C*n*) were separately dissolved in a 10 mM phosphate buffer (pH 7.4), whereas PEG-PGlu and PLys were dissolved in deionized (DI) water and mixed by vortex mixing at the charge ratio of unity, as previously reported[6]. For crosslinking PIC layer, carbodiimide based crosslinker (EDC, MW: 191) which crosslinks carboxylic and amine groups of the polymer side chain was used with 0.5, 1, 3, 5 and 10 equivalent of carboxylic groups (EDC/COO^-). Crosslinking efficiencies of samples were measured by FT-IR spectroscopy.

3. Results and Discussion

3.1 Influence of secondary structure

Increasing the order of the polymeric systems through hierarchical assemblies such as α-helix and β-sheet has been a great tool to tune their mechanical properties[6]. In order to exploit this strategy to tune membrane properties of PICsomes, homochiral polypeptides, PEG-PGlu and PLys were used for PICsome formation. Although CD analysis revealed the successful β-sheet formation, assembly morphologies were found to be micellar (PIC micelle) instead of vesicular structure (PICsome). This result suggested that employing homochiral polypeptides and forming higher order secondary structures in PIC systems may not be effective for tuning of PICsome properties, although it determines the morphology of the formed



assemblies.

3.2 Study on Factors for Permeability Tuning

Crosslinking process conjugates adjacent constituent polymers in the PIC layer, and it is expected to have direct influence on semipermeability of PICsomes. Also, hydrophobicity of PIC layer assumed to be correlated with permeability. Here, effect of these two factors on permeability was carefully examined.

3.2.1 Effect of Cross-linking Degree on Permeability

Initially, in order to reveal extent of the relation between crosslinking degree and permeability of PICsome, aliphatic side chain length of polycation was fixed to five carbon (C5), and fluorescein-conjugated PEG (f-PEG) with MWs of 6, 12, 20 and 42 kDa was used as model cargo molecules. Cross-linking degree (CLD) of PIC membranes, which were treated with different amounts of EDC, was determined by liquid state FT-IR (Fig.3a). Then release amounts of cargo molecules were monitored at different temperatures (4, 25, 37 and 50 °C) by size exclusion chromatography (SEC). Based on the results, influence of the cross-linking was successfully exploited to control semipermeability. Nonetheless, it should be noted that this influence varied greatly on MW of cargo molecules. For lowest MW cargo (PEG 6k), crosslinking was only effective at higher CLD PICsomes Fig.3b). The influence was best observed for the release of PEG 12k and it decreased with higher MW cargo molecules (Fig. 4)

Figure 1: Block polyanion and homo polycation polymers used in PIC preparation

Furthermore, the effect of CLD on permeability became more visible, when the PICsomes were incubated at higher temperatures. Low CLD PICsomes (32%, 48%, and 68%) are more vulnerable to temperature increase, and release amount of f-PEG showed great difference between 4 and 50 °C samples, while high CLD (78%, 89%) were less affected by temperature fluctuations.

3.2.2 Effect of Length of Aliphatic Side Chains of Homo-polycations on Permeability

Formation of PICsomes by employing polycations with different aliphatic side chains was previously reported by our group[7,8]. Since this could be another parameter of tuning permeability, they were further studied and compared with earlier work.

In order to compare results meaningfully with C5 PICsomes, the CLD of PIC membranes were fixed at two values: ~40% (low cross-linked) and ~80% (high cross-linked). The shortest aliphatic side chain length PICsome (C4), systems release considerably high amount of cargo to medium (Fig.5) especially for low MW cargo molecules and higher temperatures.

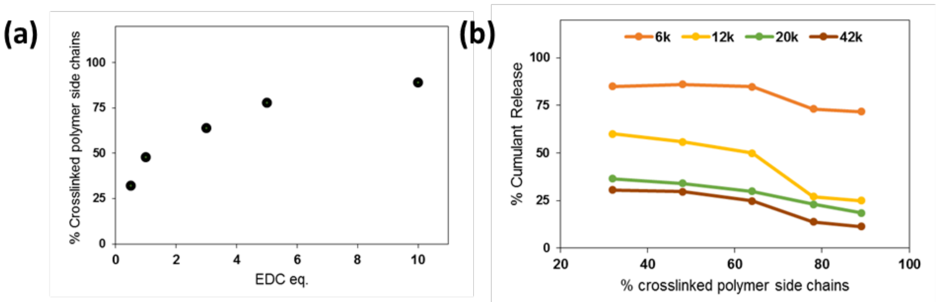


Figure 3. (a) Cross-linking amount of C5 PICsomes calculated by FT-IR, **(b) r**elease amount of PEG cargo molecules based on CLD C5 PICsomes after one week at 37°C

In the case of C6 PICsome system, it can be seen that release suppression is very clear compared to C5 PICsomes in both low and high degree crosslinked system (Fig.4). Moreover, C6 PICsomes are much less susceptible to temperature fluctuations, even at low crosslinking degree case. Finally, C8 PICsomes showed least amount of cargo diffusion, which remained less than 7% for all samples, and effect of temperature-increase on diffusion was negligible compared to other PICsomes.

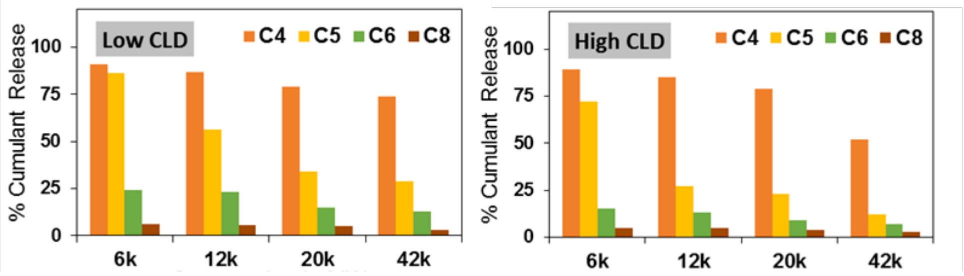


Figure 4. Release amounts of cargo molecules from PICsomes with different polycation side chain lengths at at 37°C

3.3 Study on factors for deformability tuning

Previously, it was reported that CLD has direct influence on cellular uptake of PICsomes, where low CLD PICsomes showed higher cellular uptake and it decreases sharply with high CLD[9]. In addition, PICsomes with longer aliphatic side chain on polycations exhibited enhanced stability against salt and temperature. In both cases, increased stability can be linked to some mechanical properties, for example, less deformability or higher rigidity of PICsomes, which was my further interest to compare stability of PICsomes by their deformability.

3.3.1 Effect of Cross-linking Degree on Deformability

For this purpose, atomic force microscopy (AFM) with liquid mode was utilized to obtain spreading PICsome diameter on mica substrates under the constant force (R_f). Then, R_f values were compared to their hydrodynamic diameters (R_h, from light scattering) to discuss relative change in deformability. In this study, deformability was evaluated by R_f/R_h. For the low CLD PICsomes (32% and 48%) the calculated size from AFM images were quite larger than their hydrodynamic radius, ($R_f/R_h > 1.5$) while high CLD PICsomes showed similar values to their hydrodynamic radius ($R_f/R_h \cong 1$), which suggests significant increase of rigidity with higher CLD. This assumption was supported by further force curve analyses of samples.

3.3.2 Effect of Length of Aliphatic Side Chains of Homo-polycations on Deformability

Further study of PICsomes with same CLD (~40%) via AFM exposed that C4 and C5 PICsomes have softer membrane (higher spreading on mica surface, $R_f/R_h > 1.5$) whereas C6 and C8 PICsomes show more rigid structure ($R_f/R_h \cong 1$) (Fig.5b). These results show high correlation with previously reported stability data.

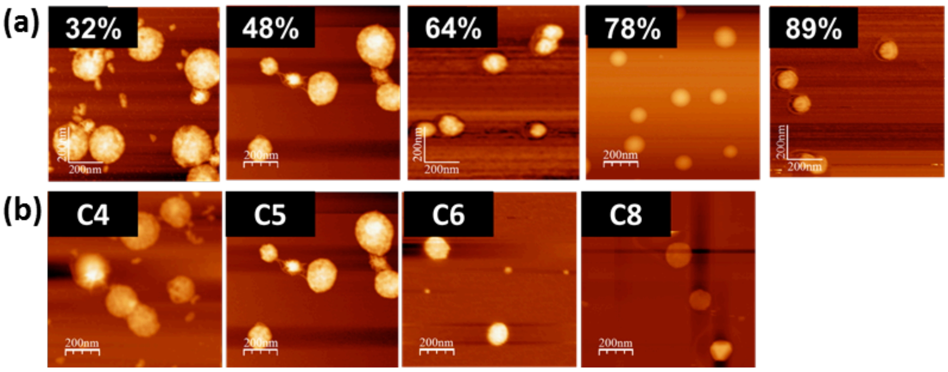


Figure 5. AFM analysis **of (a)** C5 PICsome morphologies on mica surface (scale bars: 200nm) and **(b)** spreading of different aliphatic side chain length PICsomes on mica substrate (scale bars: 200nm)

4. Conclusion

Due to unique features of PICsomes, carriers with wide range of properties were analyzed in this study. Although increasing the order of polypeptides in PIC compartment via secondary structure formation (β-sheet) did not yield PICsomes and could not be utilized as control factor for membrane property tuning, by taking advantage of cross-linkable layer, ability to incorporate of polycations with different aliphatic side chain length for PEG-PAsp and P(Asp-C*n*) combination, semipermeability and deformability of the PICsomes were deeply investigated. Results suggested that semipermeability and deformability are controlled by abovementioned two factors to great extent. In the case of semipermeability, additional influence of temperature and MW of the cargo molecules were further observed.

5. Future Outlook

Since EDC crosslinking of PICsomes forms highly stable structures with very low deformability, environment sensitive crosslinking agents can be utilized to provide PICsomes ability to recognize changes in their environment and respond to it. Besides, further and quantitative studies are necessary to investigate the relation between PICsome deformability and their behavior in biological systems. Therefore, my next goals will be introducing environment sensitive PICsomes and biological evaluation of PICsomes with different aliphatic side chain lengths.

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