SELF-ASSEMBLY OF BIOACTIVE OR ELECTROCATALYTIC POLYASPARTAMIDE NANOPARTICLES

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemical Engineering in the Graduate College of the University of Illinois at Urbana-Champaign, 2014

Urbana, Illinois

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ABSTRACT

Submicrometer sized particles are being extensively studied because of their potentials to deliver various molecular drugs and imaging contrast agents to target diseased tissue and also support electrocatalytic activities of metallic particles. Successful use of nanoparticles in these biological and energy applications greatly depends on the ability to control the structural integrity and the surface functionality of nanoparticles. Innumerable methods to assemble bioactive or electrocatalytic nanocarriers were proposed to dates; however, most approaches accompany complex chemistry and multiple purification, thus reducing production yield while increasing costs. Therefore, the goal of this thesis research was to develop a simple but advanced method to control the lifetime and surface functionality of nanocarriers. Along this line, the thesis presents three different approaches: 1) To extend bioavailability of nanocarriers labeled with a near infrared (NIR) probe by decreasing the nanoparticle bilayer permeability and subsequently enhance the quality of tumor detection (Chapter 2); 2) To functionalize the nanocarrier surface with a wide array of antibodies in a simple yet elaborate bio-inspired approach (Chapter 3); 3) To improve electrocatalytic activities of polymeric micelles via surface coating with platinum nanocubes (Chapter 4). Overall, the results of this thesis study would greatly serve to allow us to engineer the function of nanocarriers in a simple and economic manner, and expedite their uses in a wide array of applications.
ACKNOWLEDGMENTS

First and foremost, I would like to show my sincere appreciation to Dr. Hyunjoon Kong and Dr. Jae Hyun Jeong. Dr. Hyunjoon Kong, my advisor of this dissertation who is extremely passionate in biomedical material research, has always been inspiring me academically by setting an example of diligence. He has contributed substantial suggestions and time with his immense academic knowledge to all the research projects I worked on at the University. Dr. Jae Hyun Jeong’s patient guidance, help and suggestions for the experiments, as well as the encouragement and heartwarming supports in life were indispensable to me, particularly in the first two years of my PhD life.

My committee members, Dr. Catherine Murphy, Dr. Charles Schroeder, and Dr. Huimin Zhao, contributed a lot of insightful suggestions for this dissertation during my preliminary and final exams. I am thankful for their precious time on improving the dissertation.

In the technical aspects, the former staff in Immunological Resource Center, Dr. Xiaoxia Wang and Dr. Liping Wang, kindly offered a lot of ideas and technological help in the surface plasmon resonance spectroscopy. Transmission electron microscopy and dynamic light scattering were carried out in Frederick Seitz Materials Research Laboratory Central Facilities, where I was fully trained on the instrument by Dr. Wacek Swiech and Ms. Lou Ann Miller. Nuclear magnetic resonance (NMR) and differential scanning calorimetry (DSC) were carried out in NMR Lab and Microanalysis Lab, School of Chemical Science; Dr. Dean Olson and Dr. Haijun Yao offered nice instructions on the NMR and DSC unit, respectively. I used the confocal microscope in The Imaging Technology Group, Beckman Institute for Advanced Science and Technology, where Dr. Dianwen Zhang trained and assisted me kindly. Additionally, my collaborators, Dr. Sangmin Lee and Dr. Kwangmeyung Kim in Korea Institute of Science and Technology, helped with the animal experiments in Chapter 2; Mr. Seonghwan Kim and Dr. Jeyong Yoon in Seoul National
University helped with the cyclic voltammetry and scanning electron microscope experiments in Chapter 4. The people mentioned above took a role in helping this dissertation, and my deepest appreciation goes to all of them.

Dr. Chaenyung Cha, Dr. Cathy Chu, Dr. John Schmidt, Dr. Ross DeVolder, and Dr. Youyun Liang assisted me in experiment-wise when I started in the Kong Lab. Cartney Smith contributed a lot of brilliant experiment suggestions and kind supports, and I have learned a lot from him since he joined the lab. Nick Clay, a thinker of science and humanity, often inspired and encouraged me in the process of completing the PhD. Ian (Kwanghyun) Baek offered technical help in lab, particularly the synthesis of platinum nanocubes in Chapter 4. All in all, to every Kong Group members have been listed or not, I value your presence to make my Illinois experience unique!

My appreciation also goes to my friends in Champaign. Matt, Kay, Kejia, and Shuyi supported me a lot in my starting years in US. Particularly, Kejia kindly offered a nice space for our studying the written qualifying exam in my first winter here. In the house of Cartney and Jess, I have so much memory depicting the fun moments with both of them, Grace, Nick, Jinrong, Rayna, Victor, Katie, Mike, Andy, Yu-Ching, and Jet! Dr. Robert Wilson is acknowledged for his friendship and legendary. I also would like to show my appreciation to those who attended my defense and the practice talk: Jinrong, Grace, Cartney, Ming-Hsu, Rayna, Jess, Nick, Ian, and Frank. They offered extremely helpful suggestions and supports for my defense.

My family members made my journey in the University special. I was glad to have them stayed in Champaign from time to time for the past five years to accompany me. I also appreciate for the support from many friends in Taiwan. It will be impossible to name all my friends who have been supportive throughout my PhD life in this note, so I keep this part private between those persons and myself. Lastly and most importantly, I feel extremely lucky to have Grace
Wan-Ting Chen around, who has demonstrated the greatest patience, companion, support, and love for the past five years, when some of the toughest times in my life came up.

The research projects in this dissertation are made possible by several funding programs, including National Institutes of Health (1R01 HL109192 and 1R21HL097314 to H.J.K.), American Heart Association (Scientist Development Grant to H.J.K), and Korean Institute of Industrial Technology. I appreciate the financial support from the Dow Chemical Company Graduate Fellowship for five semesters and the Study Abroad Scholarship from Ministry of Education of Republic of China (Taiwan) for six semesters (two academic years). Lastly, back to the beginning of the story, I am and always will be thankful for the admission letter from my department in the University. This makes everything possible in my life.

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Oct 08, 2014
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Chapter 1 Introduction

1.1 Motivation and Background

The targeted delivery of imaging contrast agents and drugs has been extensively studied to improve the quality of diagnoses and treatments of various chronic and malignant diseases. Specifically, nano-sized polymeric micelles and polymeric vesicles, often called polymersomes, have been increasingly studied for use in the targeted delivery of biomedical molecules.

Polymeric micelles, considerably more stable than surfactant micelles, were proposed as an exciting nanocarrier system in 1976. Polymeric micelles are usually formed by self-associating of amphiphilic polymers with distinguished hydrophobic and hydrophilic segments into core/shell structures in aqueous solution. By conjugating targeting ligand molecules to the micelle surface, numerous immunomicelles have been prepared and demonstrated high binding specificities and targetabilities as summarized in the review papers.

Polymersomes, a relatively new term which was coined in 1999, also formed a structure with aqueous solution in the core by the self-assembly of amphiphilic polymers. This unique architecture enables the hydrophilic lumen to load and protect sensitive biomedical agents, while the polymersome membrane provides a physical barrier for the encapsulated agent from external materials and may be modified with peptide or antibody to serve as the active-targeting surface.

Various amphiphilic polymers, including block copolymers and graft copolymers (or referred to as comb copolymer), have been studied for constructing polymeric micelles and polymersomes. Looking back at the history of nanomedicine, block copolymers, such as Pluronics, have been demonstrated as a promising material system for constructing nanocarriers. However, some limitations of nanocarriers made of block polymers remain to be overcome, including the limited functionality of the polymers and many purification steps for the polymer synthesis. Therefore, a
biocompatible graft copolymer, which features easy functionalizations, and a designed assembly strategy will allow us to overcome the limitations of current polymeric nanocarriers.

Polyaspartamide, a class of graft copolymers prepared by aminolysis of the starting polysuccinimide backbone with reactive grafts (either small molecules or polymers with amine functional groups), demonstrates the compositional flexibility and the relative simple polymer synthesis compared to block copolymers. Polyaspartamide has been explored by Giammona et. al. and Kim et. al., particularly for its applications as micellar drug carriers, micellar imaging probe carriers, and multi-functional micellar carriers. Increasing the hydrophobic graftings of polyaspartamide to form polyaspartamide polymersomes, though still at its early stage, has also been demonstrated experimentally and theoretically. In summary, this copolymer system can be altered and functionalized for various purposes and has more potential to be explored, considering its flexibility of the synthetic chemistry.

1.2 Research Overview

The goal of this dissertation research is to engineer an amphiphilic polyaspartamide to prepare nanocarriers for imaging contrast agents and catalytic platinum nanocubes, aiming to achieve the improved targetability and lifetime when the nanocarriers are used in the targeted delivery and to reach the enhanced catalytic performance when used as the catalyst support. Three research projects are presented in this dissertation accordingly.

In Chapter 2, decreasing the polymersome bilayer permeability will be demonstrated to extend the polymersome lifetime in blood stream and to improve the enhanced permeability and

\[ \text{1 The author will refer to polyaspartamide as graft “polymer” in the following chapters, instead of graft “copolymer”. This is because the grafted groups on polyaspartamide are not involved in the polymer chain growth in this dissertation.} \]
retention (EPR) effect for bioimaging in a murine inflammation model, when the polymersome coupled with a near infrared (NIR) probe. Self-assembled nanoparticles conjugated with various imaging contrast agents have been used for the detection and imaging of pathologic tissues. Inadvertently, these nanoparticles undergo fast, dilution-induced disintegration in circulation and quickly lose their capability to associate with and image the site of interest. In this chapter, poly(2-hydroxyethyl-co-octadecyl aspartamide), sequentially modified with methacrylate groups, will be used to build model polymersomes and to tackle this challenge.

In Chapter 3, a strategy of functionalizing the nanoparticle surface with a wide array of antibodies as the targeting motif via the modular self-assembly of antibodies and alkylated protein A with polyaspartamide micelles will be investigated. Delivering nanocarriers of functional molecules to the sites of interest is a common demand for various biomedical applications. The surfaces of nanocarriers are usually chemically modified with molecules that can specifically bind with the target site. However, this approach requires multiple chemical modification and purification steps, thus raising concerns on the production yield and cost. To resolve the challenge, poly(2-hydroxyethyl-co-octadecyl aspartamide) (PHEA-g-C_{18}) will be used to build the bacterium-mimicking nanoparticle surface, which is easily functionalized with different targeting antibodies, in this chapter.

In Chapter 4, the amphiphilic polyaspartamide will be applied to carry the catalytic platinum nanocubes by two different preparation methods. The effect of polymer interaction with the nanocubes on the catalytic performance of the nanoconstruct will be studied. Limited effective approaches of immobilizing Pt nanocubes on a solid support of interest have been studied, thus restraining the wide application of Pt nanocubes. To resolve the challenge, this chapter will demonstrate a method to associate the nanocubes with PHEA-g-C_{18} polymeric micelles, which
later bring the nanocubes to coat on the electrode, and will explore the distinct spatial organization of the Pt-PHEA-g-C\textsubscript{18} nanoconstructs and the resulting impact on the catalytic performance.

In Chapter 5, the main findings from the research projects in Chapter 2, Chapter 3, and Chapter 4 will be summarized. The experimental suggestions based on the three chapters will also be included for the future research on polyaspartamide designs and applications.

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Chapter 2 Tailoring Polymersome Bilayer Permeability Improves Enhanced Permeability and Retention Effect for Bioimaging

2.1 Introduction

Extensive efforts have been made to detect pathologic tissues (i.e., proinflammatory tissue, tumor) at an early stage, as treatment is more effective when they are found early.\(^1,2\) Common methods of diagnosis currently include biochemical screening of blood samples or whole body imaging.\(^3\) Specifically, whole body imaging offers several advantages, including the ability to locate the pathologic tissue and further assess therapeutic outcomes in a non-invasive manner. Biomedical imaging techniques that are used commonly in preclinical and clinical settings, including magnetic resonance imaging,\(^4\) optical coherence tomography,\(^5,6\) and near-infrared (NIR) fluorescence imaging,\(^7,8\) often employ molecules or nanoparticles that can provide enhanced image contrast.\(^4-8\) Furthermore, efforts are increasingly made to deliver these imaging contrast agents exclusively to the target tissue in order to pinpoint it using conventional imaging modalities.

One popular approach is to conjugate imaging contrast agents to nanoparticles that can diffuse through the leaky inflamed or tumor vasculature and accumulate in the extravascular tissue, termed as the enhanced permeation and retention (EPR) effect.\(^9,10\) Alternatively, nanoparticles conjugated with ligands can bind actively with receptors overexpressed by target pathologic cells.\(^4-6\) Integrating the EPR effect with the active targeting is also being explored in the design of advanced nanoparticles targeting tissues of interest. These efforts, however, are often plagued by the limited circulation time of the self-assembled nanoparticles in vivo.\(^11\) For

\(^2\) Reproduced with permission from *ACS Applied Materials & Interfaces* 2014, 6, 10821-10829 (DOI: dx.doi.org/10.1021/am502822n). Copyright 2014 American Chemical Society.
example, in mice and rats, nanoparticles should continue circulating for at least 6 hours to achieve the desired EPR effect.\textsuperscript{12} Accordingly, a variety of approaches have been developed to increase the circulation time of nanoparticles equipped with imaging contrast agents in the bloodstream. For instance, the nanoparticle surface is conjugated with poly(ethylene glycol) (PEG) to reduce the mononuclear phagocyte system-mediated clearance.\textsuperscript{13} Separately, the molecular weights of nanoparticle-forming molecules can be tailored to improve the structural integrity of nanoparticles: Nanoparticles formed from the self-assembly of high molecular weight polymers remained more stable than small molecular weight molecules, such as lipids and surfactants.\textsuperscript{14} These approaches are often combined together to attain synergistic improvements of the particle stability.

However, self-assembled polymeric micelles were still reported to disintegrate quickly in the circulation due to dilution effects. For example, poly(caprolactone)-b-poly(ethylene oxide) and poly($D,L$-lactide)-b-methoxypolyethylene glycol micelles were found to dissociate within one hour under biological conditions.\textsuperscript{15, 16} Similar to the polymeric micelle, it is highly plausible that polymeric vesicles, termed polymersomes, would also quickly dissociate in circulation before achieving an effective accumulation in target tissues. To resolve this challenge, we hypothesized that reducing the bilayer permeability of polymersomes would greatly enhance the particle structural stability in circulation. Accordingly, the particles with a reduced permeability would enhance the quality of image-based diagnosis of pathologic tissues.

We examined this hypothesis by introducing a controlled number of methacrylate groups into the bilayer of polymersomes formed from the self-assembly of poly(2-hydroxyethyl-co-octadecyl aspartamide) (PHEA-C\textsubscript{18}), because methacrylate groups can associate with each other and increase the packing density of the bilayer (Figure 2.1). In addition, after particle assembly, the methacrylate groups were cross-linked to further decrease the bilayer permeability.
Afterward, the polymer was further modified with a NIR fluorescent probe, FPR-675, and the capability of the resulting polymersome in detecting and imaging pathologic tissues was evaluated by systemically injecting them into a mouse tumor model of squamous cell carcinoma. In summary, this study should greatly serve to expand the lifetime of polymersomes under physiological conditions, contribute to the early detection of pathologic tissues, and hence take image-based diagnostics to the next level.

2.2 Results

2.2.1 Preparation and characterizations of PHEA-C\textsubscript{18} and PHEA-C\textsubscript{18-MA}

Poly(2-hydroxyethyl-co-2-methacryloxyethyl-co-octadecyl aspartamide) (PHEA-C\textsubscript{18-MA}) was synthesized by modifying polysuccinimide (PSI) with octadecyl chains and methacrylate groups. PSI was prepared via the acid-catalyzed polycondensation of \textit{L}-aspartic acid. The successive reactions of PSI with the designated amounts of octadecylamine and 2-aminoethyl methacrylate hydrochloride resulted in PSI substituted with octadecyl and methacrylate groups (Figure 2.2, PSI-C\textsubscript{18-MA}). Subsequent addition of an excess of ethanolamine to the polymer solution led to the substitution of all remaining succinimide units with hydroxyl groups (Figure 2.2, PHEA-C\textsubscript{18-MA}), as confirmed with \textsuperscript{1}H NMR (Figure 2.3). Separately, PHEA-C\textsubscript{18-MA} without methacrylate groups (PHEA-C\textsubscript{18}) and unalkylated poly(2-hydroxyethyl aspartamide) (PHEA) were synthesized as control polymers.

The degree of substitution of octadecyl chains (\textit{DS}\textsubscript{C18}) of PHEA-C\textsubscript{18} and PHEA-C\textsubscript{18-MA} polymers, defined as the mole percent of succinimide units substituted with octadecyl chains, was tuned to the range of 36 to 37 mol\% according to the integrals of the characteristic NMR peaks at 0.85 to 0.95 ppm and 4.3 to 4.7 ppm (Figure 2.3 & Table 2.1). The peak at 0.85 to 0.95 ppm represents the protons of methyl groups at the ends of the substituted octadecyl chains, and
the peak at 4.3 to 4.7 ppm is due to the protons on the polymer backbone. Additionally, the degree of substitution of methacrylate ($DS_{MA}$) of PHEA-C$_{18}$-MA, defined as the mole percent of succinimide units reacted with 2-aminoethy methacrylate hydrochloride, was tuned to 2.7 and 4.8 mol%, as quantified by the integrals of the characteristic NMR peaks at 4.3 to 4.7, 5.7, and 6.1 ppm. The peaks at 5.7 and 6.1 ppm represent the two protons on the vinyl carbon of the substituted methacrylate, and the peak at 4.3 to 4.7 ppm represents the protons on the polymer backbone (Figure 2.3). For convenience, PHEA-C$_{18}$ and PHEA-C$_{18}$-MA polymers at $DS_{MA}$ of 2.7 and 4.8 mol% are termed as MA-0.0, MA-2.7, and MA-4.8, respectively.

The ability of MA-0.0, MA-2.7, and MA-4.8 to self-associate in aqueous media was analyzed by the emission intensity of pyrene incorporated into the hydrophobic domain of the polymeric assembly. It is common to assess the critical aggregation concentration (CAC) of amphiphilic molecules by measuring the increase in the pyrene emission intensity ratio ($I_3/I_1$) between 385 ($I_3$) and 373 ($I_1$) nm.$^{19}$ Pyrene mixed with unalkylated PHEA, formed by PSI substituted solely with hydroxyl groups, showed an insignificant increase in $I_3/I_1$ with the increasing polymer concentration, while the pyrene incorporated into a suspension of MA-0.0 showed an increase of $I_3/I_1$ at a CAC of $5\times10^{-2}$ mg/ml (Figure 2.4a). Furthermore, the CAC value was found to be inversely related to $DS_{MA}$ of the studied polymer (Figure 2.4b).

2.2.2 Preparation and analysis of PHEA-C$_{18}$ and PHEA-C$_{18}$-MA polymersomes

PHEA-based polymersomes were prepared by a solvent exchange process (Figure 2.5). Irgacure 2959 and polymers were first dissolved in dimethyl sulfoxide (DMSO) to provide chain mobility sufficient for intermolecular self-assembly in aqueous media. The subsequent introduction of this mixture into deionized water and the removal of DMSO by dialysis resulted in spherical polymersomes, as confirmed with transmission electron microscopy (TEM). The polymersome suspension was further exposed to ultraviolet (UV) light to activate the
cross-linking reaction within the bilayer of the polymersome. No significant difference of the particle morphology was found between the polymersome before (Figure 2.6a) and after (Figure 2.6b) the exposure to UV light.

The average radii of MA-0.0, MA-2.7, and MA-4.8 polymersomes were also measured with dynamic light scattering (DLS) (Table 2.1). No significant difference in the average hydrodynamic radius ($R_H$) was found between MA-0.0 or MA-2.7 polymersomes with or without a cross-linked bilayer. However, increasing the $DS_{MA}$ from 2.7 mol% to 4.8 mol% resulted in a decrease of $R_H$ from 102 to 90 nm and from 99 to 85 nm for the polymersome without cross-linked bilayers and those with, respectively.

We further analyzed how the $DS_{MA}$ and the subsequent cross-linking reaction influenced the bilayer permeability of polymersomes by measuring the amount of calcein released from the polymersome during the incubation in deionized water or 10 % (v/v) plasma solution. Calcein is a fluorescein derivative that self-quenches as its concentration exceeds 2-3 mM. Therefore, encapsulated calcein at high concentrations self-quenches within polymersomes, while that released into the media generates fluorescence emission. As such, the suspension of polymersomes with the lower bilayer permeability should show a slower recovery of calcein fluorescence than those with the higher bilayer permeability. As expected, MA-2.7 polymersome with the cross-linked bilayer showed a smaller increase of fluorescent intensity than MA-2.7 polymersome without the cross-linked bilayers, especially when the particles were incubated in 10 % (v/v) plasma aqueous solution (Figure 2.6c). In addition, increasing the $DS_{MA}$ from 2.7 to 4.8 mol% significantly limited the fluorescence recovery both in deionized water and in the 10 % plasma solution, even without cross-linking the bilayer. Interestingly, at the high $DS_{MA}$, chemical cross-linking of the bilayer did not make a difference in the fluorescence recovery rate (Figure 2.6c).
Biochemical stability of PHEA-based polymersomes was evaluated by monitoring changes of the particle radius while the polymersomes were incubated in PBS at 37 °C. MA-2.7 and MA-4.8 polymersomes displayed the better stability than MA-0.0 polymersomes, even without cross-linking the bilayer. The MA-0.0 polymersomes in PBS disappeared within 48 hours, following a two-fold increase of the average $R_H$ (Figure 2.7a). In contrast, MA-2.7 and MA-4.8 polymersomes without cross-linked bilayers retained their original size over 12 days (open squares in Figure 2.7b & 2.7c). Interestingly, increasing DS_{MA} significantly lessened the growth of $R_H$ over time. Specifically, the $R_H$ of MA-2.7 polymersomes increased from 108 to 214 nm within one day, followed by a gradual increase to 230 nm over the following 11 days (open squares in Figure 2.7b). In contrast, the $R_H$ of MA-4.8 polymersomes increased from 89 to 121 nm in the first day, followed by a minimal increase of $R_H$ over time (open squares in Figure 2.7c).

Further cross-linking of the polymersome bilayer reduced the degree of increase in $R_H$, depending on DS_{MA}. The $R_H$ of MA-2.7 polymersome with a cross-linked bilayer exhibited an increase from 100 to 200 nm within the first 24 hours and remained constant over the following 11 days (filled squares in Figure 2.7b), which showed a lessened expansion than the $R_H$ of the MA-2.7 polymersome without a cross-linked bilayer. In contrast, there was a minimal difference of the size change profile between the MA-4.8 polymersome with a cross-linked bilayer and that without a cross-linked bilayer (Figure 2.7c).

Incubating polymersomes at 90 °C, which is above the melting temperature of the polymer (Figure 2.8), has also demonstrated different structural disintegration rates between the particles with a cross-linked bilayer and those without. The MA-0.0 polymersome disappeared within one day (Figure 2.9a). In contrast, both MA-2.7 and MA-4.8 polymersomes with cross-linked bilayers showed limited size changes (Figure 2.9b & 2.9c), while MA-4.8 polymersome with a
cross-linked bilayer had the slightest change on its size distribution before and after being incubated at the evaluated temperature. Additionally, these polymersomes demonstrated little, if any, cellular toxicity according to an analysis of cellular metabolic activity using MTT reagent. Endothelial cells incubated with 0.25 mg/ml or 0.01 mg/ml of MA-2.7 or MA-4.8 polymersomes retained a level of metabolic activity similar to untreated cells (Figure 2.10).

2.2.3 Functionalization of polymersomes with a NIR fluorescent probe and in vivo assessment

The polymersomes of MA-2.7 and MA-4.8 were functionalized with a NIR fluorescent probe, FPR-675, in order to use the particles as a tool to detect and image pathologic tissue innervated by abnormal, leaky vasculature. The sulfonic acid group of FPR-675 was conjugated to the hydroxyl group of PHEA-C\textsubscript{18}-MA via esterification (Figure 2.11). Accordingly, the polymersome suspension displayed an absorbance peak at 675 nm (Figure 2.12a), as well as NIR fluorescence emission at 700 nm upon excitation at 675 nm (Figure 2.12b).

Next, the ability of FPR-675-labeled PHEA-C\textsubscript{18}-MA polymersomes to image tumor tissue was evaluated by systemically injecting them into circulation of a tumor-bearing mouse model and imaging the whole body with a real-time NIR fluorescence imaging system. The tumor was created by subcutaneously injecting squamous cell carcinoma (SCC7) into the back of a mouse. Injection of free FPR-675 solution via tail vein yielded a low level of positive fluorescence in tumor sites throughout 48 hours. The fluorescence intensity from the tumor site was comparable to the auto-fluorescence level of the neighboring tissue, thus making it difficult to distinguish tumor from the normal tissue (Figure 2.13a-I).

In contrast, FPR-675-labeled MA-2.7 polymersome without a cross-linked bilayer made a 1.4-fold increase of the fluorescent intensity compared to the free FPR-675 solution (Figure 2.13a & 2.13b). Although the fluorescence from the FPR-675-labeled MA-2.7 polymersome
without a cross-linked bilayer in the tumor site gradually decreased throughout 48 hours, it was kept at a higher level than that of a mouse injected with the free FPR-675 solution. In addition, the FRP-675-labeled MA-2.7 polymersome with a cross-linked bilayer generated 1.6 times higher fluorescent intensity at the tumor site than the MA-2.7 polymersome without cross-linking one hour after injection (Figure 2.13b). This represented the highest intensity among the three injections over 48 hours, making the tumor site clearly distinguished from the normal neighboring tissue (Figure 2.13a-III). However, further decreasing the permeability of the polymersome bilayer by increasing DS<sub>MA</sub> made minimal enhancements in elevating the fluorescence intensity in cancer sites over time (Figure 2.14).

Bio-distribution of free FPR-675 and FPR-675-labeled polymersomes were evaluated with NIR fluorescence images of multiple organs including liver, lung, spleen, kidney, heart, and tumor, all of which were collected 48 hours after the injection. Similar to the real time imaging, the highest NIR fluorescence of the ex vivo tumor was achieved with FPR-675-labeled MA-2.7 polymersome with a cross-linked bilayer (Figure 2.13c). Additionally, the difference in NIR fluorescence between the tumor tissue and other organs, with the exception of the kidney, was also largest with the FPR-675-labeled, bilayer cross-linked MA-2.7 polymersome. Interestingly, absence of the cross-linked structure in the polymersome bilayer caused the particles to accumulate more in the kidney than in tumor tissue.

2.3 Discussion

In summary, this study demonstrates an advanced method to improve the imaging of pathologic, tumor tissue using PHEA-C<sub>18</sub>-MA polymersomes engineered to present less permeable bilayers. The bilayer permeability was decreased by introducing more numbers of methacrylate groups into the bilayer of the polymersome without cross-linking treatment, and
also by further cross-linking the bilayer. We found that the polymersome with a larger $D_{\text{MA}}$ (MA-4.8 polymersome) was more stable in physiological media than that without methacrylate groups (MA-0.0 polymersome) and that with a smaller $D_{\text{MA}}$ (MA-2.7 polymersome), even without cross-linking treatment. Additionally, the cross-linking of the bilayer further decreased the permeability and enhanced the stability of the polymersome compared to its uncross-linked form, but only for the MA-2.7 polymersome. Lastly, the MA-2.7 polymersome that was modified to carry a NIR fluorescent probe and present a cross-linked bilayer significantly improved the imaging quality of tumor sites over 48 hours after systemic injection.

According to previous studies, polymersomes should disassemble more slowly than liposomes of a similar CAC when the concentration falls below the CAC. However, the complete disappearance of PHEA-C$_{18}$ polymersomes in PBS within 48 hours implies that increasing the molecular weight of self-assembling molecules does not perfectly circumvent the structural disassembly on a long-term basis. We propose that methacrylate groups linked to the PHEA-C$_{18}$ polymer backbone hydrophobically associate with each other in the confined bilayer together with octadecyl chains, due to the mismatch of Hildebrand solubility parameter between ethyl methacrylate ($8.61 \text{ (cal/cm}^3\text{)^{1/2}}$) and water ($23.5 \text{ (cal/cm}^3\text{)^{1/2}}$). Additionally, it is unlikely that these methacrylate groups impairs the vesicular structure of PHEA-C$_{18}$-MA, as confirmed with TEM micrographs and supported by the theoretical calculation (Table 2.2). We suggest that both the hydrophobic association and the pH-independent cross-linking bonds between methacrylate groups greatly serve to improve particle stability and subsequent targeted imaging quality, whereas other cross-linking bonds such as hydrazine and disulfide bonds may disintegrate in tissue with pH deviating from neutral. Therefore, methacrylate groups incorporated into the bilayer likely played a similar role as cholesterol in phospholipid liposome bilayers. It is well agreed that cholesterol reduces the free space in bilayers, and
subsequently enhances liposome stability.\textsuperscript{33} Similarly, increasing the packing density should decrease the bilayer permeability and further decelerate the structural disintegration of polymersomes caused by dilution effects, as manifested with the slower calcein release from MA-4.8 than MA-2.7 polymersomes without cross-linking and the smallest size increase of MA-4.8 polymersomes. We therefore interpret that the stability achieved by increasing $DS_{MA}$ is due to the increase of packing density in the bilayer. Additionally, the decrease of $R_H$ with increasing $DS_{MA}$ should be attributed to an increase of the hydrophobic association between polymers and the subsequent formation of a more compact bilayer.

Additionally, cross-linking of the bilayer has further enhanced the MA-2.7 polymersome stability exclusively. The slower recovery of fluorescence from calcein in the MA-2.7 polymersome with a cross-linked bilayer can be attributed to the decreased bilayer permeability, while the limited size increase of MA-2.7 polymersomes with a cross-linked bilayer over time indicates that the particle has a greater resistance to structural disintegration. In contrast, the independence of the bilayer permeability and particle size change on the cross-linking of the MA-4.8 polymersome bilayer is likely due to the high packing density of the bilayer negating the effect of cross-linking.

Taken together, we propose that the control of bilayer permeability and particle stability serve to improve the quality of NIR fluorescence imaging of cancerous tissues. Prior studies on the EPR effect suggested that particle diameter should be smaller than 400 nm.\textsuperscript{34,35} The size of PHEA-C\textsubscript{18}-MA polymersomes used in this study fit within this particle size range. However, it is likely that lipids in blood serum accelerate the expansion or disassembly of MA-2.7 polymersomes without cross-linked bilayers,\textsuperscript{15,16,36} thus limiting the accumulation of particles in the tumor site via the EPR effect as well as accelerating the renal clearance of disassembled monomers due to the low molecular weight.\textsuperscript{37} Additionally, the accumulated particles in the
tumor site can be degraded gradually and hence the fluorescence of the area of interest decreased. In contrast, the MA-2.7 polymersome with a cross-linked bilayer should remain stable in circulation over an extended time period to extravasate through leaky tumor vasculature, and the accumulated particles in the tumor site, while still degradable, were degraded slower than the polymersome without cross-linked bilayers.

Previously, some studies demonstrated that cross-linking the bilayer of a micro-sized gigantic polymersome results in increasing the surface elastic modulus, wall stress, and resistance to surfactant-induced disassembly than traditional liposomes.\textsuperscript{38, 39} However, no efforts have been made to improve detection and imaging of cancer tissue by translating these findings into the assembly of nano-sized polymersomes to date. Therefore, we envisage that our finding will be very useful to designing nanoparticles used for diagnostics and also treatment of various acute and malignant diseases, including cancer and cardiovascular diseases.\textsuperscript{40, 41} Further extending the lifetime of polymersomes in circulation, as well as the time window for cancer imaging with a single dose, could potentially be achieved by conjugating PEG chains to the PHEA-C\textsubscript{18}-MA polymersome. The quality of image-guided diagnostics will be further improved by conjugating peptides or antibodies that can specifically bind with target cells to the polymersomes developed in this study. On a separate note, though PHEA-C\textsubscript{18}-MA polymersomes have been shown to demonstrate limited toxicity to the mouse endothelial cells, further investigation is needed to evaluate performance in humans.

\section*{2.4 Conclusion}

In summary, this study demonstrated a nanoparticle platform engineered to present a reduced permeability which is useful for significantly improving the detection and imaging of pathologic tissue. The methacrylate groups attached to a self-assembling polyaspartamide
polymer served to reduce the bilayer permeability, likely because they associated with other hydrophobic alkyl chains and increased the packing density. Cross-linking of the methacrylate groups post-assembly of the nanoparticles further reduced the bilayer permeability, thus leading to another increase of the particle stability in physiological media, exclusively at intermediate degree of substitution for methacrylate groups to the polymer. Therefore, the polymersome conjugated with a NIR fluorescent probe and also tailored to display the reduced permeability significantly improved the imaging quality of tumor sites over 48 hours after systemic injection. These results certify the importance of extending the lifetime of nanoparticles in improving their function in detecting and imaging pathologic tissues of interest. Finally, the polymersomes with a tailored permeability will be useful in future studies to modulate the release rate of various therapeutic molecules and ultimately improve the quality of diagnosis and treatment of diverse diseases.

2.5 Experimental Section

Materials were purchased from Sigma-Aldrich (USA) and used without further purification unless otherwise specified.

2.5.1 Synthesis of poly(2-hydroxyethyl-co-octadecyl aspartamide) (PHEA-C\textsubscript{18}) and poly(2-hydroxyethyl-co-2-methacryloxyethyl-co-octadecyl aspartamide) (PHEA-C\textsubscript{18}-MA)

Polysuccinimide (PSI) was synthesized by thermal condensation of L-aspartic acid (30 g) suspended in sulfolane (150 ml) at 170°C under a nitrogen atmosphere for 14 hours with phosphoric acid (0.612 mL, Fisher Scientific) as a catalyst.\textsuperscript{18} PSI was precipitated in excess methanol and successively washed with deionized water until the pH of the suspension reached neutral. The precipitate was dried by lyophilization. The molecular weight of PSI was determined by gel permeation chromatography (Breeze 2 GPC, Waters), with Styrage\textregistered HT
column (Waters). \(N,N\)-dimethylformamide (DMF) containing 20 mM LiBr was used as the eluent, with the elution rate of 1 mL min\(^{-1}\). Polystyrene standards were used for calibration. \(M_n = 13,600 \text{ g mol}^{-1}\) with PDI = 1.4.

Purified PSI (79 mg) was dissolved in DMF (5 ml, Fisher Scientific), and octadecylamine (79 mg for MA-0.0, MA-2.7, and MA-4.8, respectively) was added to the reaction mixture. The reaction was stirred at 70 °C for 24 hours. Next, a designated amount of 2-aminoethyl methacrylate hydrochloride (AEMA) (0 µL for MA-0.0, 195 µL for MA-2.7, and 391 µL for MA-4.8) and triethylamine (TEA, Fisher Scientific) (0 µL for MA-0.0, 10.6 µL for MA-2.7, and 21.2 µL for MA-4.8) were added to the reaction mixture, and the solution was stirred at 45 °C for 24 hours. The molar ratio of AEMA to TEA was 1:3. Lastly, an excess amount of ethanolamine was added (96.8 µL for MA-0.0, 82.3 µL for MA-2.7, and 75.4 µL for MA-4.8) and the reaction was further stirred at 45 °C overnight. Following completion of the chemical reactions, the products were dialyzed (MWCO 3500 Da, Fisher Scientific) extensively in deionized water at room temperature for two days and then lyophilized to yield dry powders.

2.5.2 Characterization of PHEA-C\(_{18}\) and PHEA-C\(_{18}\)-MA polymers

\(^1\text{H NMR (Varian Unity 500 MHz) was applied to analyze the polymer dissolved in DMSO-}d_6\) (Cambridge Isotope Laboratories, Inc.). The integrals of characteristic peaks were used to quantify the degree of substitution of octadecyl chains (\(DS_{C18}\)) and the degree of substitution of methacrylate groups (\(DS_{MA}\)) in each sample by Eq. (1) and (2):

\[
DS_{C18} \text{ (mol\%)} = \frac{\text{The integral of the peak in 0.85 – 0.95 ppm/3}}{\text{The integral of the peak in 4.3 – 4.7 ppm}} \times 100 \% \quad \cdots \cdots \cdot (1)
\]

\[
DS_{MA} \text{ (mol\%)} = \frac{\text{The sum of the integrals of the peak at 5.7 ppm and at 6.1 ppm/2}}{\text{The integral of the peak in 4.3 – 4.7 ppm}} \times 100 \% \quad \cdots \cdots \cdot (2)
\]
2.5.3 Measurement of the critical aggregation concentration (CAC)

Pyrene molecules (Acros) were suspended in the polymer solution at a concentration of $10^{-4}$ mg ml$^{-1}$. The fluorescent spectra of the suspensions with varying polymer concentrations were collected using a FluoroMax®-4 spectrometer (HORIBA Jobin Yvon). The excitation wavelength was set at 330 nm and the resulting emission between 350 and 450 nm was collected; the slit widths for excitation and emission were both set as 1 nm. The CAC was determined by the polymer concentration at the point where the emission intensity ratio ($I_3/I_1$) between the third vibronic peak at 385 nm ($I_3$) and the first vibronic peak at 373 nm ($I_1$) was significantly increased.$^{19}$

2.5.4 Synthesis of PHEA-$C_{18}$-MA labeled with FPR-675

5 µmol FPR-675 (BioActs) was dissolved in 500 µl of DMSO (Merck) and added dropwise to PHEA-$C_{18}$-MA dissolved in DMSO (10 mg in 2 mL). The reaction mixture was stirred overnight at room temperature in the dark. The products were dialyzed extensively in deionized water with light protection at room temperature to remove the free dye and DMSO, and then lyophilized to yield dry powders. The resulting polymers were analyzed with a UV/Vis spectrophotometer Lambda 7 (Perkin-Elmer).

2.5.5 Preparations of PHEA-$C_{18}$ and PHEA-$C_{18}$-MA polymersomes

PHEA-$C_{18}$-MA polymers and Irgacure 2959 (Ciba Specialty Chemicals) were dissolved in DMSO at a concentration 10 and 100 mg mL$^{-1}$, respectively. 100 µL polymer in DMSO was mixed with 20 µL initiator in DMSO, and the mixture was dropped into 1 mL deionized water. The solution was sonicated for 10-15 seconds and then dialyzed in deionized water extensively to remove DMSO and excess initiator overnight. During dialysis, the suspension was covered with aluminum foil to prevent exposure to light. The purified suspension was finally exposed to UV light (Model 20 CHIPhERASER, Jelight Co. Inc.) for 5 min to cross-link the bilayer of the
polymersome. Separately, in preparation of PHEA-C_{18} or PHEA-C_{18}-MA polymersomes without cross-linked bilayers, Irgacure 2959 was not mixed with the polymer solution; nor was the polymersome suspension exposed to UV light. In the stability analysis, 10× phosphate buffered saline (PBS) was added to adjust the ionic strength of the polymersome suspension to 1× PBS after the particle preparation.

2.5.6 Cytotoxicity evaluation of PHEA-C_{18}-MA polymersomes

C166 mouse endothelial cells (ATCC) were seeded on a 96-well plate at 10^4 cells per well. Cells were incubated for 24 hours with MA-2.7 polymersomes or with MA-4.8 polymersomes. MTT reagent ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, ATCC) was then added to the cell culture media. Metabolically active cells convert the MTT reagent to formazan dye, which can be quantified by measuring its absorbance at 570 nm (Tecan Infinite 200 PRO, Tecan AG). Cellular metabolic activity is presented as a percent of a control, i.e. cells cultured without the presence of polymersome. Error bars are shown as the standard deviation of three replicates.

2.5.7 Characterization of PHEA-C_{18} and PHEA-C_{18}-MA polymersomes

The morphology of self-assembled PHEA-C_{18}-MA polymersomes was observed using TEM (JEOL 2100 with LaB_6 emitter) operating at an accelerating voltage of 120 keV. The polymersome suspension was dropped onto a 300-mesh copper grid coated with carbon and formvar (SPI Supplies), and the sample was stained with 2 % (w/v) phosphotungstic acid solution (adjusted to pH 7.4 with NaOH). The size of PHEA-C_{18}-MA polymersomes was measured using Malvern Zetasizer Nano ZS (Malvern, 4 mW He-Ne laser operating at a wavelength of 633 nm) with 173° backscattering. The hydrodynamic radius was determined using Cumulant analysis (International Organization for Standardization 13321:1996). All measurements were carried out in triplicate and performed at 25°C.
2.5.8 Calcein release study

PHEA-C<sub>18</sub>-MA polymersomes were prepared by the solvent exchange process. Then, 50 mM calcein (Fisher Scientific) aqueous solution was added slowly into the polymersome suspension to a final concentration of 25 mM for diffusional loading. The suspension was incubated at room temperature with stirring for one day for calcein to reach equilibrium, and exposed to UV light to activate the cross-linking reaction in the bilayer of polymersomes. Certain polymersome suspensions were not exposed to UV light for a control experiment. The excess calcein which was not incorporated into the polymersomes was removed by Amicon® Ultra-0.5 Centrifugal Filter Devices (MWCO 100k Da, Millipore Corporation). The polymersomes were incubated within deionized water or deionized water supplemented by 10% (v/v) human plasma (Equitech-Bio Inc.). After incubation for 30 minutes at room temperature, the amount of calcein released from the polymersome was measured with the emission intensity at 535 (± 20) nm at the excitation of 485 (± 20) nm using a microplate reader (Infinite 200Pro, Tecan Group Ltd.).

2.5.9 In vivo non-invasive NIR fluorescence imaging of polymersomes in tumor-bearing mice

All animal experiments were performed in compliance with the guidelines approved by the institutional ethics committee for animal care of Korea Institute of Science and Technology (KIST). To generate a tumor-bearing mice model, squamous cell carcinoma (SCC7) tumors were induced into 5-week-old male athymic nude mice (Institute of Medical Science, Tokyo) by subcutaneous injection of 1.0×10<sup>6</sup> SCC7 cells. When the tumor diameter grew to approximately 10 mm, the test samples (5 mg kg<sup>-1</sup>) were injected into the tumor-bearing mice through the tail vein, and the whole body images were taken at 1, 3, 6, 12, 24, and 48 hours after the injection, using a time-domain fluorescence imager eXplore Optix (ART Advanced Research Technologies.
Laser power and count time settings were optimized at 9 μW and 0.3 second per point. Excitation and emission spots were raster-scanned in 1 mm steps over the selected region of interest to generate emission wavelength scans. A 675 nm pulsed laser diode was used to excite FPR-675 molecules. NIR fluorescence emission at 700 nm was collected and detected with a fast photomultiplier tube (Hamamatsu) and a time-correlated single photon counting system (Becker and Hickl GmbH). NIR fluorescence intensities of all samples were adjusted to the same values based on the data obtained by a 12-bit CCD camera (Kodak Imaging Station 4000 MM, New Haven) equipped with a special C-mount lens and a Cy5.5 bandpass emission filter set (680 to 720 nm, Omega Optical). Furthermore, the accumulation level of samples in the tumors was evaluated by measuring the NIR fluorescence intensity at the cancer site. All data were processed using the region of interest (ROI) function of the Analysis Workstation software (ART Advanced Research Technologies Inc.).

2.5.10 Ex vivo organ analysis

After intravenous injection of FPR-675 labeled polymersomes, major organs and tumors were dissected from mice 48 hours after the injection. NIR fluorescence images of dissected organs including liver, lung, spleen, kidney, heart and tumors were obtained with Kodak Image Station 4000 MM. The image station was equipped with a 12-bit CCD camera, halogen lamp (150 W), and excitation/emission filter sets for FPR-675 (600-700 nm; Omega Optical). The biodistribution of polymersomes was evaluated by quantifying the NIR fluorescence intensity of the organ and tumor images processed with Kodak molecular imaging software.

2.6 References


2.7 Figures

**Figure 2.1.** Schematic description of the structure of a poly(2-hydroxyethyl-co-2-methacryloxyethyl-co-octadecyl aspartamide) (PHEA-C$_{18}$-MA) polymersome, in which the bilayer became less permeable due to the increased packing density resulted from the higher degree of substitution of methacrylate ($DS_{MA}$) and also from the cross-linking of methacrylate groups.
Figure 2.2. Synthesis of PHEA-C\textsubscript{18}-MA, where the polymer backbone was substituted with octadecyl chains and a varying degree of substitution of methacrylate ($DS_{\text{MA}}$, denoted as ‘z’ in the scheme; $z = 0$ for PHEA-C\textsubscript{18}). ‘x’ and ‘y’ in the reaction scheme represent the degree of substitution of hydroxyl groups and the degree of substitution of octadecyl chains ($DS_{\text{C}18}$), respectively.
Figure 2.3. $^1$H NMR (DMSO-$d_6$, 500 MHz) spectra of (a) PHEA-C$_{18}$ without methacrylate groups (MA-0.0), (b) PHEA-C$_{18}$-MA at $D_{SM}$ of 2.7 mol% (MA-2.7), and (c) PHEA-C$_{18}$-MA at $D_{SM}$ of 4.8 mol% (MA-4.8).
Figure 2.4. Pyrene-based fluorescence analysis of polyaspartamide polymersomes. (a) The fluorescence spectrum while the pyrene was incorporated into a suspension of MA-0.0, MA-2.7, MA-4.8, and PHEA in phosphate buffered saline (PBS), respectively. (b) The critical aggregation concentration (CAC), marked by the turning point of $I_3/I_1$ values in (a), of MA-0.0, MA-2.7, and MA-4.8 in PBS.
Figure 2.5. Schematic description of the solvent exchange process to prepare PHEA-C$_{18}$-MA polymersomes with cross-linked bilayers.
Figure 2.6. Morphology characterization and bilayer permeability analysis of polyaspartamide polymersomes. (a) TEM micrographs of the self-assembled polymersomes of (I) MA-0.0, (II) MA-2.7, and (III) MA-4.8, all of which the bilayers were not cross-linked. (b) TEM micrographs of the self-assembled polymersomes of (I) MA-2.7 and (II) MA-4.8. The bilayers of MA-2.7 and MA-4.8 polymersomes were cross-linked. All the scale bar in (a) and (b) represent 200 nm. (c) Calcein-based fluorescence analysis of MA-2.7 and MA-4.8 polymersomes, either with cross-linked (CL) or without cross-linked (NCL) bilayers. The filled and open bars represent polymersomes incubated in deionized water and in deionized water supplemented with plasma (10%, v/v), respectively.
Figure 2.7. Stability analysis of polyaspartamide polymersomes with dynamic light scattering (DLS). The changes in the hydrodynamic radius ($R_H$) of the polymersomes of (a) MA-0.0, (b) MA-2.7, and (c) MA-4.8. The polymersomes were incubated in PBS at 37 °C. The filled and open squares represent the polymersomes with bilayers in which methacrylate groups were cross-linked and not cross-linked, respectively. *After two days, $R_H$ of MA-0.0 polymersomes could not be measured with DLS.
**Figure 2.8.** Differential scanning calorimetry (DSC) thermogram of PHEA and PHEA-C$_{18}$. DSC measurements were performed under a nitrogen atmosphere at a heating rate of 10 °C/min using Perkin Elmer Diamond DSC, and the heat flow of the polymer during the heating process was measured.

**Figure 2.9.** Effects of $DS_{MA}$ on the size distribution of the polymersomes of (a) MA-0.0, (b) MA-2.7, and (c) MA-4.8. Bilayers of MA-2.7 and MA-4.8 polymersomes were cross-linked. The filled and open circles represent the polymersomes suspended in PBS before and after incubation at 90 °C for 24 h, respectively. *The majority of MA-0.0 polymersomes disappeared after incubation at 90 °C.
Figure 2.10. Cellular metabolic activity of C166 mouse endothelial cells analyzed with an MTT reagent.

![Graph showing cellular metabolic activity](image)

Figure 2.11. PHEA-C\textsubscript{18}-MA functionalized with a near-infrared (NIR) fluorescent probe, FPR-675.
Figure 2.12. Characterization of PHEA-C$_{18}$-MA polymersomes modified with the NIR probe, FPR-675. (a) UV-visible spectrum of MA-2.7 conjugated with FPR-675. (b) The NIR fluorescence image of (I) free FPR-675 solution, (II) FPR-675-labeled MA-2.7 polymersome suspension, and (III) FPR-675-labeled MA-4.8 polymersome suspension. The methacrylate groups in the bilayers of polymersomes were cross-linked.
Figure 2.13. In vivo evaluation of PHEA-C\textsubscript{18}-MA polymersomes in targeting and imaging tumors. (a) NIR fluorescence images of tumor-bearing mice after the intravenous injection of (I) free FPR-675 probes, (II) FPR-675-labeled MA-2.7 polymersomes without cross-linked bilayers, and (III) FPR-675-labeled MA-2.7 polymersomes with cross-linked bilayers. Arrows mark the tumor site. (b) NIR fluorescent intensity changes over 48 hours in the tumor tissues of the corresponding mouse shown in (a). (■□■) represents free FPR-675 dye, (●●●) represents FPR-675-labeled MA-2.7 polymersomes without cross-linked bilayers, and (▲▲▲) represents FPR-675-labeled MA-2.7 polymersomes with cross-linked bilayers. (c) NIR fluorescence intensities of the ex vivo organ images from the tumor-bearing mice 48 hours after the injection. The black, red, and blue bars represent free FPR-675 dye, FPR-675-labeled MA-2.7 polymersomes without cross-linked bilayers, and FPR-675-labeled MA-2.7 polymersomes with cross-linked bilayers, respectively.
Figure 2.14. In vivo evaluation of PHEA-C_{18}-MA polymersomes in targeting and imaging tumors. (a) NIR fluorescence images of tumor-bearing mice after the intravenous injection of (I) free FPR-675 probes, (II) FPR-675-labeled MA-2.7 polymersomes with cross-linked bilayers, and (III) FPR-675-labeled MA-4.8 polymersomes with cross-linked bilayers. Arrows mark the tumor site. (b) NIR fluorescence intensity changes over 48 h in the tumor tissues of the corresponding mice shown in (a). (■) represents free FPR-675 dye, (○) represents FPR-675-labeled MA-2.7 polymersomes with cross-linked bilayers, and (▲) represents FPR-675-labeled MA-4.8 polymersomes with cross-linked bilayers.
### 2.8 Tables

**Table 2.1.** Molecular analysis of PHEA-C<sub>18</sub> and PHEA-C<sub>18</sub>-MA with controlled DS<sub>MA</sub>.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DS&lt;sub&gt;C18&lt;/sub&gt; [mol%]&lt;sup&gt;a)&lt;/sup&gt;</th>
<th>DS&lt;sub&gt;MA&lt;/sub&gt; [mol%]&lt;sup&gt;a)&lt;/sup&gt;</th>
<th>Hydrodynamic radius (R&lt;sub&gt;H&lt;/sub&gt;, nm)&lt;sup&gt;b)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Not cross-linked</td>
</tr>
<tr>
<td>MA-0.0</td>
<td>36.7</td>
<td>0.0</td>
<td>102 ± 2</td>
</tr>
<tr>
<td>MA-2.7</td>
<td>36.4</td>
<td>2.7</td>
<td>102 ± 8</td>
</tr>
<tr>
<td>MA-4.8</td>
<td>36.9</td>
<td>4.8</td>
<td>90 ± 5</td>
</tr>
</tbody>
</table>

<sup>a)</sup> Determined with the <sup>1</sup>H NMR spectra of the polymers;  
<sup>b)</sup> Determined by dynamic light scattering (DLS).  
The particles were suspended in deionized water.  
Data represented are the average values followed by the standard deviation from three independent experiments.
Table 2.2. Molecular analysis of PHEA-C\textsubscript{18} and PHEA-C\textsubscript{18}-MA with controlled DS\textsubscript{MA}.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DS\textsubscript{C18} [mol%]\textsuperscript{a)}</th>
<th>DS\textsubscript{MA} [mol%]\textsuperscript{a)}</th>
<th>mass percentage of the hydrophilic grafting to the total macromolecule\textsuperscript{b)}</th>
<th>Morphology\textsuperscript{c)}</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA-0.0</td>
<td>36.7</td>
<td>0.0</td>
<td>44.1</td>
<td>polymersome</td>
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<td>2.7</td>
<td>42.2</td>
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</tr>
<tr>
<td>MA-4.8</td>
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<td>4.8</td>
<td>40.0</td>
<td>polymersome</td>
</tr>
</tbody>
</table>

\textsuperscript{a)} Determined with the \textsuperscript{1}H NMR spectra of the polymers; \textsuperscript{b)} Calculated by 
\[
\frac{(100 - DS\textsubscript{C18} - DS\textsubscript{MA}) \times \text{mass of PHEA unit substituted with a hydroxyl group}}{\text{averaged unit molecular weight}} \times 100%,
\]

where the average unit molecular weight is calculated by averaging the mass of the PHEA unit substituted with a hydroxyl group (174 g mol\textsuperscript{-1}), substituted with an octadecyl group (381 g mol\textsuperscript{-1}), and substituted with a methacrylate group (242 g mol\textsuperscript{-1}) by the degree of substitution; \textsuperscript{c)} The amphiphilic PHEA-C\textsubscript{18} substituted with a mass percentage of the hydrophilic grafting to the total macromolecule in the range of 25-45 % have been shown to favor the polymersome formation in aqueous solution.\textsuperscript{345}


Chapter 3  Bacterium-Mimicking Nanoparticle Surface Functionalization with Targeting Motifs

3.1 Introduction

Nanoparticles formed from intermolecular self-assembly, such as micelles and vesicles, have been extensively studied as carriers of various molecules used in a wide array of applications, including sensing,\textsuperscript{1, 2} bioimaging,\textsuperscript{3-5} and drug delivery.\textsuperscript{6, 7} Amphiphilic molecules constituted with hydrophobic and hydrophilic segments are being commonly used as a building block of the self-assembled nanoparticles, because of the association between hydrophobic segments in aqueous media.\textsuperscript{8} The size and the morphology of resulting nanoparticles are significantly dependent on the molecular weight and the packing parameter of amphiphilic molecules.\textsuperscript{9, 10}

These nanoparticle surfaces are often functionalized with varied bioactive molecules that can specifically bind with surfaces of interest, in order to deliver molecular cargos to a target site and subsequently elevate their desired performance. It is common to chemically conjugate targeting biomolecules to nanoparticles before or post the self-assembly.\textsuperscript{11} Alternatively, the nanoparticle surface and the targeting ligand are connected by conjugating biotin and avidin or streptavidin to each pair.\textsuperscript{11} These processes, however, require multi-step chemical modification and effortful purification, which inadvertently lead to a low production yield and a large production cost. Additionally chemical conjugation of targeting biomolecules to amphiphilic molecules prior to nanoparticle assembly can encounter loss of bioactivity of biomolecules as well as localization into the nanoparticle core.

Apart from these conventional methods, a living organism offers a simple way to present a wide array of biomolecules on its surface. For example, the bacterium \textit{Staphylococcus aureus}
expresses protein A (SpA), a surface protein that can bind with the heavy chain in the Fc-region of immunoglobulins. SpA associates with the membrane of *Staphylococcus aureus* and inhibits opsonophagocytosis by binding with the host antibody, so it aids the survival of the bacterium. Inspired by this process, we hypothesized that insertion of alkylated SpA into the surface layer of a self-assembled nanoparticle will allow us to readily modify the nanoparticle surface with various antibodies of interest that can function as targeting motifs (Figure 3.1). We examined this hypothesis by using SpA modified with palmitic acid (SpA-PA) and directing self-association between SpA-PA and a micelle of poly(2-hydroxyethyl aspartamide) substituted with octadecyl chains, termed PHEA-g-C$_{18}$. The micelle coupled with SpA-PA was coated by antibodies to vascular cell adhesion molecules-1 (VCAM-1) or integrin $\alpha_v\beta_3$ via simple mixing of pre-made micelle with antibodies. The biological association between the SpA-PA of the micelle and the antibody was monitored by analyzing the fluorescence resonance energy transfer (FRET) between SpA-PA and the antibody, each of which was labeled with FRET donor and acceptor, respectively. The targetability of resulting micelles toward a substrate coated with VCAM-1 or integrin $\alpha_v\beta_3$ was evaluated with surface plasmon resonance (SPR) spectroscopy and a blood vessel-mimicking flow chamber. Taken together, this study will greatly serve to advance the surface functionalization process via the bio-inspired modular self-assembly and potentially elevate the performance level of a wide array of nanoparticles.

### 3.2 Results and Discussion

#### 3.2.1 Preparations and characterizations of PHEA-g-C$_{18}$ micelles

First, PHEA-g-C$_{18}$ was synthesized through nucleophilic substitution of designated amounts of octadecylamine and excessive amount of ethanolamine to poly(succinimide) (PSI) (Figure 3.2a), as characterized with the $^1$H NMR spectra (Figure 3.3). According to integrals of NMR
peaks at 0.85 to 0.95 ppm and 4.3 to 4.7 ppm, the degree of substitution for octadecyl chains ($DSC_{18}$) to the polymer, defined as the mole percentage of succinimide units substituted with octadecyl chains, was varied from 2.4 to 6.4 and 14.8 mol % (Table 3.1). The peak at 0.85 to 0.95 ppm in the NMR spectrum represents protons of methyl groups at the ends of the substituted octadecyl chains, and the peak at 4.3 to 4.7 ppm represents protons on the polyaspartamide backbone (Figure 3.3). For convenience, PHEA-g-C$_{18}$ at $DSC_{18}$ of 2.4, 6.4, and 14.8 mol % were termed as DS-2.4, DS-6.4, and DS-14.8, respectively.

Addition of PHEA-g-C$_{18}$ into phosphate buffer saline (PBS) and subsequent sonication created polymeric micelles via *in-situ* self-assembly. Increasing $DSC_{18}$ of PHEA-g-C$_{18}$ has dramatically decreased the corresponding critical micelle concentration (CMC), which was marked by the significant increase in the emission intensity ratio ($I_3/I_1$) between 385 ($I_3$) and 373 ($I_1$) nm of pyrene added in the aqueous micelle suspension$^{15}$ (Table 3.1 & Figure 3.4). The $z$-average hydrodynamic radii of the micelles, measured with the dynamic light scattering (DLS) unit, became larger by increasing $DSC_{18}$ of PHEA-g-C$_{18}$ (Table 3.1). Micrographs acquired with the transmission electron microscope (TEM) confirmed the spherical morphology of PHEA-g-C$_{18}$ micelles (Figure 3.2b). The zeta-potential of micelles were in the range of -10 to -12 mV independent of $DSC_{18}$ (Figure 3.2c).

### 3.2.2 Surface modification of PHEA-g-C$_{18}$ micelles with palmitic acid-conjugated *Staphylococcus aureus* protein A (SpA)

Chemical reaction of palmitic acid N-hydroxysuccinimide ester with amine groups of SpA resulted in the SpA conjugated with palmitic acid, termed SpA-PA (Figure 3.5a-1). The resulting SpA-PA coupled with PHEA-g-C$_{18}$ micelles via a simple mixing process at room temperature (Figure 3.5a-2), as monitored with the SpA-PA labeled with fluorescein. In contrast, the unmodified SpA-PA minimally associated with PHEA-g-C$_{18}$ micelles (data not shown). The
percentage of SpA-PA associated with the polymeric micelles increased from 9 (± 0.5 %) to 38 % (± 6 %) by increasing $DS_{C18}$ of PHEA-g-C$_{18}$ from 2.4 to 14.8 %, as quantified with the fluorescence intensity from the micelle following centrifugation-based purification (Figure 3.5b).

The underlying mechanism by which the palmitic acid enhanced the association of SpA-PA with the PHEA-g-C$_{18}$ micelles was examined by quantifying changes in the Gibbs free energy ($\Delta G$), enthalpy ($\Delta H$), and entropy ($\Delta S$) of this self-assembly process. $\Delta G$ calculated using Eq. (1) became a more positive value at a given temperature ($T$) as $DS_{C18}$ decreased from 14.8 to 2.4 mol % (Figure 3.5c).

$$\Delta G = -RT \ln K_{eq}$$

Eq. (1)

Where $K_{eq} = \frac{[\text{Micelle-coupled SpA-PA}]}{[\text{Free SpA-PA}][\text{Micelles}]}$

$\Delta H$ calculated from the slope of Van’t Hoff plot (Figure 3.5d based on Eq. (2)) was 11.4 kJ/mol for the DS-14.8 coupled with SpA-PA, while $\Delta H$ for DS-2.4 was -6.3 kJ/mol (Figure 3.5e). In addition, $\Delta S$ calculated from the intercept of Van’t Hoff plot presented a larger, positive number for the association of SpA-PA with DS-14.8 micelles than that with DS-2.4 micelles (Figure 3.5e).

$$\ln K_{eq} = \left(\frac{-\Delta H}{R}\right) \left(\frac{1}{T}\right) + \frac{\Delta S}{R}$$

Eq. (2)

These analytical results suggest that the association of SpA-PA with DS-2.4 was thermodynamically unfavorable, as characterized with the negative $\Delta S$ and the positive $\Delta G$ of the process. In contrast, increasing $DS_{C18}$ led to thermodynamically favorable association between SpA-PA and DS-14.8 as confirmed with the positive $\Delta S$ and the negative $\Delta G$. The positive $\Delta S$ of
the assembly of SpA-PA with DS-14.8 was likely due to the release of ions and water molecules from the insertion interface between SpA-PA and DS-14.8 micelle surface.\textsuperscript{4} In contrast, the negative $\Delta S$ of SpA-PA coupled with DS-2.4 is due to the entropic penalty of assembling SpA-PA-coupled DS-2.4 micelles was more, in magnitude, than the entropy increase resulting from the desolvation happened in the interface between SpA-PA and DS-2.4 micelle surface. In sum, decreasing $DSC_{18}$ of PHEA-g-C\textsubscript{18} lessened this desolvation effect and hence conferred the negative $\Delta S$ for SpA-PA-coupled DS-2.4 micelles. Additionally, the assembly of DS-2.4 with SpA-PA, driven by $\Delta H$ while penalized entropically, can be referred to as the “non-classical” hydrophobic effect.\textsuperscript{16-18}

3.2.3 FRET analysis of the assembly of antibody-coated PHEA-g-C\textsubscript{18} micelles

The PHEA-g-C\textsubscript{18} micelle coated by antibodies to VCAM-1 and integrin $\alpha_v$ was created by simply mixing the SpA-PA-coupled DS-2.4 or DS-14.8 micelles with antibodies at 4 °C for 60 minutes. The FRET assay was conducted to confirm the binding between the antibody and SpA-PA on the micelle surface (Figure 3.6a). Interestingly, mixing of micelles coated by the fluorescein (i.e., FRET donor)-conjugated SpA-PA with the rhodamine (i.e., FRET acceptor)-conjugated VCAM-1 antibodies resulted in a significant decrease of the fluorescein emission intensity maximized at the wavelength of 520 nm and an increase of the rhodamine emission intensity maximized at the wavelength of 580 nm (Figure 3.6b). These results confirmed the specific association between SpA-PA and antibodies. The degree of FRET ($D_{\text{FRET}}$) between the SpA-PA and the antibody was quantified with the emission intensity of fluorescein in the absence ($I_{\text{fluorescein},0}$) and presence ($I_{\text{fluorescein}}$) of rhodamine-labeled antibody following Eq. (3):\textsuperscript{19}

$$D_{\text{FRET}} = 1 - \frac{I_{\text{fluorescein}}}{I_{\text{fluorescein},0}}$$

Eq. (3)
Note that $D_{\text{FRET}}$ increases proportional to the number of antibodies bound to SpA-PA coupled on the micelle surface. During the incubation of antibodies with SpA-PA-coupled micelles in PBS, $D_{\text{FRET}}$ with DS-14.8 ones remained at approximately 0.9, while $D_{\text{FRET}}$ with DS-2.4 ones significantly decreased (Figure 3.6c). The reduced $D_{\text{FRET}}$ indicated the active separation of antibodies from the surface of SpA-PA-coupled DS-2.4 micelles.

3.2.4 Analysis of the adhesion of antibody-coated PHEA-g-C$_{18}$ micelles onto a target substrate

The targeting capability of antibody-coated PHEA-g-C$_{18}$ micelles was evaluated with a surface plasmon resonance (SPR) spectroscopy. VCAM-1 and integrin $\alpha_v\beta_3$ were chosen as the target proteins. It is well known that inflamed endothelium has higher levels of VCAM-1 and integrin $\alpha_v\beta_3$ expression than healthy endothelium. The targeting efficiency of the antibody-coated SpA-PA-micelles was evaluated by measuring the binding response unit (RU) of the micelle with a model cell membrane, which was built by conjugating VCAM-1 or integrin $\alpha_v\beta_3$ to a monolayer of 11-mercaptoundecanoic acid (MUA) on the SPR chip (Figure 3.7a).

RU after the injection (RU$_{\text{disso}}$) of SpA-PA-coupled DS-14.8 micelles coated by antibodies to VCAM-1 (solid line in Figure 3.7b-1) was 6-fold higher than that of SpA-PA-coupled DS-14.8 micelles free of antibodies (dashed line in Figure 3.7b-1) (Figure 3.7d-1). Note that the binding response of the sensogram represents the mass density of the micelles on the model cell membrane due to the association and dissociation of micelles. Likewise, RU$_{\text{disso}}$ of SpA-PA-coupled DS-14.8 micelles coated by antibodies to integrin $\alpha_v$ (solid line in Figure 3.7b-2) was more than 2-fold higher than that of SpA-PA-coupled DS-14.8 micelles free of antibodies (dashed line in Figure 3.7b-2) (Figure 3.7d-1). In contrast, SpA-PA-coupled DS-2.4 micelles coated by antibodies exhibited a relatively small increase in RU$_{\text{disso}}$ compared with that of the SpA-PA-coupled DS-2.4 micelles free of antibodies, because of the smaller loading of
antibodies characterized with FRET analysis. RU_{disso} of SpA-PA-coupled DS-2.4 micelles coated by antibodies to VCAM-1 and integrin α_v (solid line in Figure 3.7c) was only 3.3-fold and 1.3-fold, respectively, higher than that of the antibody-free SpA-PA-coupled DS-14.8 micelles (dashed line in Figure 3.7c) (Figure 3.7d-2). For another control experiment, DS-14.8 micelles free of SpA-PA were mixed with antibodies to VCAM-1. RU_{disso} of the control was almost 2-fold smaller than that of SpA-PA-coupled DS-14.8 micelles coated by antibodies (Figure 3.8).

Finally, the targeted adhesion of SpA-PA-coupled DS-14.8 micelles coated by antibodies to integrin α_v was evaluated by injecting them into a flow chamber that presented a substrate coated with integrin α_vβ_3 (Figure 3.9a). To simulate in vivo circulation, the average bulk flow velocity of the media was set as 1 mm/s. DS-14.8 polymers were labeled with rhodamine in order to locate SpA-PA-coupled DS-14.8 micelles bound to the substrate with a confocal microscope. The SpA-PA-coupled DS-14.8 micelles coated by antibodies adhered to the substrate more actively than ones without antibodies, as confirmed with a significantly larger fluorescence area (Figure 3.9b & 3.9c). In contrast, no significant difference was found between antibody-coated SpA-PA-coupled DS-14.8 micelles and antibody-free ones with a control substrate free of integrin α_vβ_3 (results not shown).

Overall, this study demonstrates that SpA-PA can act as a linker to bridge antibodies of interest to PHEA-g-C_18 micelle surfaces. It is suggested that SpA-PA bind with the Fc domain of the antibody without affecting specific interaction between antibodies and target proteins on surfaces. Previous studies have covalently conjugated SpA or protein G to the particle surface, and then incubated the particle with the antibody of interest to confer particles the specific targetability. Our modular coupling of alkylated SpA to micelles via thermodynamically driven self-assembly should be distinguished from previous approaches that should undergo multi-steps of chemical modification and purification. We envisage that such simple yet
elaborate antibody immobilization strategy would be useful to functionalize a wide array of nanoparticles assembled via self-assembly between lipids and amphiphilic copolymers.

3.3 Conclusion

In conclusion, this study successfully demonstrates that alkylated SpA (SpA-PA) act as a linker to immobilize antibodies of interest on PHEA-g-C\textsubscript{18} micelle surfaces assembled in advance. The entropy-driven self-association between SpA-PA and micelles was tuned by increasing the number of alkyl chains (DS\textsubscript{C18}) conjugated to PHEA-g-C\textsubscript{18}. Thus, SpA-PA coupled with micelles assembled by PHEA-g-C\textsubscript{18} with a higher DS\textsubscript{C18} resulted in stable immobilization of antibodies on micelle surfaces, as confirmed with FRET analysis. The micelles functionalized with antibodies to VCAM-1 or integrin $\alpha_v$ via this sequential, biological coupling displayed a larger binding affinity to target substrates than the micelles without antibodies. Likewise, under circulation-mimicking flow, the antibody-coated micelles adhered to the target substrate more favorably than the antibody-free ones. In sum, this simple yet elaborate bioinspired protein immobilization would be broadly useful to surface functionalization of nanoparticles with a wide array of proteins of interest. Ultimately, the results of this study will greatly serve to improve the quality of nanoparticles used in various applications including sensing, diagnosis, and clinical treatments.

3.4 Experimental Section

Materials were purchased from Sigma-Aldrich (USA) and used without further purification unless otherwise specified.
3.4.1 Synthesis of poly(2-hydroxyethyl-co-octadecyl aspartamide) (PHEA-g-C_{18})

Poly(succinimide) (PSI) was synthesized by thermal condensation of L-aspartic acid suspended in sulfolane at 170°C under nitrogen atmosphere for 14 hours with the phosphoric acid (Fisher Scientific) as a catalyst.\textsuperscript{26} PSI was precipitated in excess methanol and successively washed with deionized water until the pH of the suspension reached neutral. The precipitate was dried by lyophilization. The molecular weight of PSI was determined by gel permeation chromatography (Breeze 2 GPC, Waters), with Styrage® HT column (Waters). N,N-dimethylformamide (DMF) containing 20 mM LiBr was used as the eluent, with the elution rate of 1 ml min\textsuperscript{-1}. Polystyrene standards were used for calibration. $M_n = 13,600$ g mol\textsuperscript{-1} with PDI = 1.4.

149 mg purified PSI was dissolved in 8 ml N,N-dimethylformamide (DMF, Fisher Scientific), and octadecylamine (6, 22, and 44 mg for DS-2.4, DS-6.4, and DS-14.8, respectively) was added to the reaction mixture. The reaction was stirred at 70 °C for 24 hours. Next, excess amount of ethanolamine (272, 261, 247 µL for DS-2.4, DS-6.4, and DS-14.8, respectively) was added and the reactant was further stirred at 45 °C overnight. Following completion of the chemical reactions, the products were dialyzed (MWCO 3500 Da, Fisher Scientific) extensively in deionized water at room temperature for two days and then lyophilized to yield dry powders.

3.4.2 Characterizations of PHEA-C_{18} polymers

$^1$H NMR (Varian Unity 500 MHz) was applied to analyze the polymer dissolved in DMSO-$d_6$ (Cambridge Isotope Laboratories, Inc.). The integrals of characteristic peaks were used to quantify the degree of substitution of octadecyl chains (DS_{C_{18}}) in each sample by Eq. (4)

$$DS_{C_{18}} \text{ (mol\%)} = \frac{\text{The integral of the peak in 0.85–0.95 ppm/3}}{\text{The integral of the peak in 4.3–4.7 ppm}} \times 100 \% \quad \text{Eq. (4)}$$
3.4.3 Measurement of the critical micelle concentration (CMC)

Pyrene molecules (Acros) were suspended in the polymer solution at a concentration of $10^{-4}$ mg/ml. The fluorescent spectra of the suspensions with varying polymer concentrations were collected using a FluoroMax®-4 spectrometer (HORIBA Jobin Yvon). The excitation wavelength was set at 330 nm and the resulting emission between 350 and 450 nm was collected; the slit widths for excitation and emission were both set as 1 nm. The CMC was determined by the polymer concentration at the point where the emission intensity ratio ($I_3/I_1$) between the third vibronic peak at 385 nm ($I_3$) and the first vibronic peak at 373 nm ($I_1$) was significantly increased.\textsuperscript{15}

3.4.4 Preparation of PHEA-g-C\textsubscript{18} micelles

The PHEA-g-C\textsubscript{18} polymer was suspended in PBS at a concentration of 0.1 mg/ml and sonicated briefly to make a homogeneous colloidal solution. PHEA-g-C\textsubscript{18} micelles formed spontaneously after the sonication.

3.4.5 Morphological and size analysis of PHEA-g-C\textsubscript{18} micelles

The micelle suspension was dropped onto a 300-mesh copper grid coated with carbon and formvar (SPI Supplies), and the sample was stained with 2 % (w/v) phosphotungstic acid solution (adjusted to pH 7.4 with NaOH). Then the morphology of self-assembled PHEA-g-C\textsubscript{18} micelles was observed using TEM (JEOL 2100 with LaB\textsubscript{6} emitter) operating at an accelerating voltage of 120 keV. Separately, the diameter of PHEA-g-C\textsubscript{18} micelles was measured using a Malvern Zetasizer Nano ZS instrument (Malvern, 4 mW He-Ne laser operating at a wavelength of 633 nm) with 173° backscattering. The hydrodynamic radius was determined using Cumulant analysis.\textsuperscript{27} All measurements were carried out in triplicate and performed at 25°C.
3.4.6 Synthesis of *Staphylococcus aureus* protein A coupled with palmitic acid (SpA-PA)

The chemical conjugation of palmitic acid to SpA has been reported previously. In short, SpA was suspended at 1 mg/ml in PBS containing 0.3 % sodium deoxycholate (DOC), 0.1 % sodium bicarbonate, and 0.1 % sodium azide. *N*-hydroxysuccinimide ester of palmitic acid (NHS-PA) was dissolved in ethanol at 10 mg/ml and added to the protein solution to yield a final concentration of 0.1 mg NHS-PA per ml. This mixture was incubated at room temperature for 24 h with constant mixing, and then purified with a centrifugal filter (Amicon® Ultra 3K device, Millipore Corporation). The product was adjusted to 1 mg/ml in PBS with 0.1 % DOC.

3.4.7 SpA-PA labeled with fluorescein

400 µl SpA-PA was suspended in PBS at 2.73 mg/ml, and 12.96 µl FITC solution (10 mg/ml in DMSO) was added into the SpA-PA suspension. The mixture was incubated at room temperature in the dark for 40 minutes, while keep stirring it. Afterward, the solution was dialyzed in a dialysis cassette (Thermo Scientific, MWCO 3000) against PBS at 4 °C for two days. Finally, the product was concentrated with a centrifugal filter (Amicon® Ultra 3K device, Millipore Corporation) and re-suspended in PBS containing 0.1% NaN₃ at a concentration of 1.26 mg/ml.

3.4.8 Antibody labeled with rhodamine

150 µL VCAM-1 antibody solution (CD106) at 500 µg/ml PBS was mixed with 30.15 µL rhodamine B isothiocyanate at 5 mg/ml DMSO, and the mixture was incubated at room temperature in the dark for 2 hours. The excess free rhodamine was removed by dialyzing the raw product against PBS at 4 °C for 2 days, and then the sample was concentrated with a centrifugal filter (Amicon® Ultra 3K device, Millipore Corporation). The concentrate was re-suspended with PBS equal to the antibody concentration at 500 µg/ml.
3.4.9 Coupling of PHEA-g-C18 micelles with SpA-PA, association percentage of SpA-PA, and FRET analysis

500 µl of PHEA-g-C18 solution (0.1 mg/ml PBS) and 10 µL of SpA-PA labeled with fluorescein were mixed and sonicated briefly. Then the mixture was incubated at a designated temperature (277, 294, or 310 K; 294 K if not specified) for 10 min, and purified with a centrifugal filter (Amicon® Ultra 100K device, Millipore Corporation). The concentrate was added with PBS equal to the weight spun off during the ultracentrifugation to make the final sample.

For studying the association percentage of SpA-PA, part of the sample was taken out as the control before the ultracentrifugal purification. Both the control and the final sample were measured with the fluorescence emission intensity at 535 (± 20) nm at the excitation of 485 (± 20) nm, using a microplate reader (Infinite 200Pro, Tecan Group Ltd.). The association percentage of SpA-PA, defined as the ratio of the amount of SpA-PA associated with the micelle to the amount of SpA-PA added in prior the assembly process, was calculated from the ratio of fluorescence of the final sample to the control. For FRET analysis, 250 µl of the final sample was mixed with 12 µL VCAM-1 antibodies labeled with rhodamine or mixed with 12 µL PBS as the control. Micelle samples mixed with antibodies and those without were measured with the fluorescence emission intensity in the rage of 500 to 700 nm at the excitation of 490 nm using FluoroMax®-4 spectrometer (HORIBA Jobin Yvon).

3.4.10 Measurements of micelles bound to target proteins using surface plasmon resonance (SPR) spectroscopy

A gold sensor chip (GE Healthcare, USA) was modified to present a 11-mercaptopoundecanoic acid (MUA) monolayer by imersing the chip in 1 mM MUA solution overnight to form a self-assembly monolayer. The carboxylic groups of the MUA monolayer
were then activated by injecting the mixture of 0.4 M 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Thermo Scientific) and 0.1 M N-hydroxysuccinimide (NHS) (1:1, v/v) into the flow cell in a Biacore 3000 (GE Healthcare) for five minutes at 5 µl/min. After activation, recombinant human VCAM-1 (R&D Systems) or recombinant human integrin αvβ3 (R&D Systems) was chemically linked to the MUA layer by flowing the solution at 2 µl/min until the response unit was saturated. The remaining NHS-ester groups on the MUA surface were blocked by injecting 1.0 M ethanolamine into the flow cell at 5 µl/min.

SpA-PA-coupled micelles coated with or without antibodies at a concentration of 0.1 mg/ml PBS were injected into the flow cell to examine the association and dissociation rates of the micelles with the gold sensor chip modified with the target protein on MUA. The media flow rate was kept constant at 5.0 µL/min. RU_{disso} was defined as the RU reading at the third minute after the sample injection stopped. The kinetic data from SPR sensorgrams were obtained with the assistance of BIAevaluation version 4.1.

3.4.11 PHEA-g-C18 micelles labeled with rhodamine

1 ml PHEA-g-C18 dissolved in 10 mg/ml dimethyl sulfoxide (DMSO) was mixed with 0.135 ml rhodamine B isothiocyanate dissolved in 10 mg/ml DMSO. The mixture was incubated at room temperature in the dark for 24 hours. The excess rhodamine was removed by dialyzing the raw product against water for 3 days. Then, the sample was lyophilized to get a power product.

3.4.12 Analysis of the micelle adhesion onto a protein-coated substrate in a flow chamber

Integrin αvβ3 was coated onto the bottom of a 35 mm petri-dish, which was then assembled with the gasket (GlycoTech) and exposed the coated substrate to a flow at an average velocity of 1 mm/s. The suspension of PHEA-g-C18 micelles labeled with rhodamine was injected into the
chamber via a syringe pump (KDS 100 Infusion Pump, KD Scientific), and the micelles were circulated for 10 min followed by an injection of PBS to remove unbounded micelles. The adhesion of micelles to the substrate was examined by capturing fluorescence from the substrate using a confocal microscope (Leica SP2). Finally, the fluorescence was quantified with ImageJ (NIH). Five different areas of the substrate were analyzed with at least three different samples per condition.

3.5 References


3.6 Figures

Figure 3.1. Schematic description of a strategy to immobilize antibodies on the surface of PHEA-g-C_{18} micelle using the alkylated *Staphylococcus aureus* protein A (SpA-PA), termed SpA-PA.
Figure 3.2. Assembly of nano-sized micelles using PHEA-g-C_{18} with controlled $DSC_{18}$ (a)

The reaction scheme of synthesizing PHEA-g-C_{18}. (b) TEM micrographs of DS-2.4 (b-1), DS-6.4 (b-2), and DS-14.8 micelles (b-3). Scale bar is 200 nm. DS-2.4, DS-6.4, and DS-14.8 represent PHEA-g-C_{18} at $DSC_{18}$ of 2.4, 6.4, and 14.8 mol%. (c) The zeta-potential of the micelles with varied $DSC_{18}$ in PBS.
Figure 3.3. $^1$H NMR spectra of PHEA-g-C$_{18}$ polymers with $DS_{C18}$ of 2.4 mol % (a), $DS_{C18}$ of 6.4 mol % (b), and $DS_{C18}$ of 14.8 mol % (c) in DMSO-$d_6$. In the spectra, every characterization peak was assigned according to the polymer chemical structure.
Figure 3.4. Fluorescent analysis to determine critical micelle concentrations (CMCs) of PHEA-g-C$_{18}$ polymers with $DSC_{18}$ of 2.4 mol % (a), $DSC_{18}$ of 6.4 mol % (b), and $DSC_{18}$ of 14.8 mol % (c) in PBS. The pyrene molecules were incorporated into the mixture of PHEA-g-C$_{18}$ and water to determine CMCs marked by the onset of increasing $I_3/I_1$, where $I_3$ and $I_1$ represent the pyrene emission intensity measured at wavelengths of 385 and 373 nm, respectively.
Figure 3.5. Assembly of PHEA-g-C_{18} micelles coupled with SpA-PA. (a) (a-1) Reaction scheme of conjugating palmitic acid to SpA. (a-2) Hydrophobic interaction-induced self-assembly between SpA-PA and PHEA-g-C_{18}. (b) Fluorescence analysis to evaluate effects of $D_{S_{C_{18}}}$ of PHEA-g-C_{18} micelles on the percentage of SpA-PA associated with micelles suspended in PBS.
(Continued from the previous page)

For this analysis, SpA-PA was labeled with fluorescein. (c) $\Delta G$ underlying the self-assembly between SpA-PA with DS-2.4 (filled triangles) or DS-14.8 (open squares) in PBS. $\Delta G$ was calculated from $K_{eq}$ using Eq. (1), and $K_{eq}$ was quantified from the association percentage of SpA-PA presented in (b) using Eq. (1). (d) Van’t Hoff plot for self-assembly between SpA-PA and DS-2.4 (filled triangles) and DS-14.8 (open squares) micelles in PBS. (e) $\Delta S$ (open bars) and $\Delta H$ (filled bars) underlying the self-assembly process of SpA-PA with DS-2.4 and DS-14.8 micelles in PBS. $\Delta S$ and $\Delta H$ were calculated from (d) and Eq. (2).
Figure 3.6. FRET analysis of the biological coupling of antibody to PHEA-g-C_{18}. (a) Schematic description of the FRET assay designed to characterize the binding between the fluorescein-conjugated SpA-PA and the rhodamine-labeled antibody on the micelle surface. (b) Fluorescence emission spectra of SpA-coupled micelles mixed with VCAM-1 antibodies right after mixing. (b-1) Mixture of SpA-PA-coupled DS-2.4 micelles with antibodies (red curve) and without antibodies (black curve). (b-2) Mixture of SpA-PA-coupled DS-14.8 micelles with antibodies (red curve) and without antibodies (black curve). (c) The degree of FRET ($D_{\text{FRET}}$) for
the mixture of antibodies with SpA-PA-coupled DS-2.4 micelles (filled bars) or with SpA-PA-coupled DS-14.8 micelles (open bars) right after mixing (0 min) and after incubation in PBS for 60 min, respectively.
Figure 3.7. SPR analyses of PHEA-g-C_{18} micelles coated with or without antibodies to VCAM-1 or integrin α_{4}β_{3}. (a) Schematic SPR configuration for studying the binding between VCAM-1 and integrin α_{4}β_{3}.
antibody-coated PHEA-g-C\textsubscript{18} micelles and the MUA substrate coupled with target proteins. (b) SPR sensorgrams for binding of SpA-PA-coupled DS-14.8 micelles coated with corresponding antibodies (solid line) and without antibodies (dashed line) to the substrate coupled with VCAM-1 (b-1) and integrin $\alpha_v\beta_3$ (b-2). (c) SPR sensorgrams for binding of SpA-PA-coupled DS-2.4 micelles coated with corresponding antibodies (solid line) and without antibodies (dashed line) to the substrate coupled with VCAM-1 (c-1) and integrin $\alpha_v\beta_3$ (c-2). (d) RU after the injection (RU\textsubscript{disso}) for SpA-PA-coupled DS-14.8 micelles (d-1) and DS-2.4 micelles (d-2) coated with antibodies (filled bars) and without antibodies (open bars) to the substrate coupled by VCAM-1 and integrin $\alpha_v\beta_3$ in (b) and (c).
Figure 3.8. SPR sensorgrams for binding of SpA-PA-coupled DS-14.8 micelles coated by antibodies to VCAM-1 (black line) and DS-14.8 micelles simply mixed with antibodies (red line) with the substrate conjugated with VCAM-1.
Figure 3.9. *In vitro* analysis of the targeted adhesion of SpA-PA-coupled DS-14.8 micelles in a flow chamber. (a) Schematic of the flow chamber. (b) Confocal micrographs of integrin α₃β₃-coated substrate in the flow chamber following exposure to SpA-PA-coupled micelles coated by antibodies to α₃ (b-1) and micelles free of antibodies (b-2). (c) Quantitative analysis of the number of SpA-PA-coupled DS-14.8 micelles adhered to integrin α₃β₃-coated substrate. The differences of the fluoresce area percentages between the two conditions were statistically significant (p* < 0.05). Each value and error bar in the plot represents the mean and standard deviation from at least four different areas of the substrate in three different samples per condition.
3.7 Tables

Table 3.1. Analysis of the chemical structure and the critical micelle concentration (CMC) of PHEA-g-C$_{18}$, and the hydrodynamic radius of PHEA-g-C$_{18}$ micelles

<table>
<thead>
<tr>
<th>Sample</th>
<th>$DS_{C18}$ (mol %)$^a$</th>
<th>CMC (mg/ml)$^b$</th>
<th>Hydrodynamic radius of micelles (nm)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS-2.4</td>
<td>2.4</td>
<td>2.0×10$^{-2}$</td>
<td>103</td>
</tr>
<tr>
<td>DS-6.4</td>
<td>6.4</td>
<td>1.0×10$^{-2}$</td>
<td>116</td>
</tr>
<tr>
<td>DS-14.8</td>
<td>14.8</td>
<td>0.8×10$^{-2}$</td>
<td>138</td>
</tr>
</tbody>
</table>

$^a$ Determined based on $^1$H NMR spectra of the polymer

$^b$ Determined based on fluorescent emission spectra of pyrene in PBS

$^c$ Determined with a dynamic light scattering unit
Chapter 4  Improved Electrocatalytic Performance of Platinum Nanocubes by Different Preparation Approaches of Polymer-Nanocubes Nanoconstructs

4.1 Introduction

Platinum (Pt) is the most effective catalyst known to increase the rate of hydrogen oxidation and oxygen reduction to date, and Pt nanoparticles supported on porous carbon (Pt/C) is considered as the best electrocatalyst in proton exchange membrane fuel cells and phosphoric acid fuel cells.\textsuperscript{1} Achieving a higher catalytic activity than the standard Pt/C catalyst used in current fuel cells is one of the keys to enable the fuel cells feasible for commercialization. Pt/C catalyst can be prepared by impregnation method,\textsuperscript{1, 2} microemulsion method,\textsuperscript{2, 3} and colloidal method.\textsuperscript{1, 2} Specifically, the colloidal method, preparing the Pt colloid followed by adsorption of Pt on the carbon support, has the advantage of providing a precise size control of Pt nanoparticles.\textsuperscript{1, 4}

Compared to the commercial Pt catalyst, Pt nanocubes have been shown to exhibit an enhanced electrocatalytic activity and over 2-fold specific activity for oxygen reduction.\textsuperscript{5, 6} Therefore, it is intriguing to find an effective approach to immobilize Pt nanocubes on a solid support of interest. Several conventional methods have been proposed to immobilize colloidal Pt nanoparticles from the solution onto the surface of solid supports by chemical conjugation,\textsuperscript{7, 8} hydrogen bonding interaction,\textsuperscript{9, 10} electrostatic adsorption,\textsuperscript{11} and ligand coordination.\textsuperscript{12} However, these methods have not been proved applicable to Pt nanocubes immobilization, and potential challenges are anticipated: a suitable capping material is needed for the successful synthesis of homogeneous colloidal Pt nanocubes and for serving as the binding interface of immobilizing the nanocubes on a solid support at the same time. This is challenging because how the precursors,
capping molecules, or additives operate mechanistically in the shape formation has not been well understood.\textsuperscript{6}

To resolve these challenges, we proposed that different preparation approaches of the nanoconstructs composed of polymeric micelles and Pt nanocubes will affect the electrocatalytic performance of Pt by controlling the nanocubes distribution in the polymer matrix (Figure 4.1). We examined this hypothesis by 1) associating Pt nanocubes with 200 nm particles of poly(2-hydroxyethyl aspartamide) substituted with octadecyl chains, termed PHEA-g-C\textsubscript{18}, by a slow-evaporation method, and 2) stabilizing the Pt nanocube clusters with PHEA-g-C\textsubscript{18} in aqueous by emulsification. The Pt nanocubes associated with or on the PHEA-g-C\textsubscript{18} micelles were dominated by the removal rate of hexane, which is a good solvent for Pt nanocubes but a selective solvent for PHEA-g-C\textsubscript{18}. The electrocatalytic performance of Pt-PHEA-g-C\textsubscript{18} samples was evaluated with cyclic voltammetry (CV), and the sample coated on the electrode was further analyzed with scanning electrode microscopy (SEM) to elucidate the underlying mechanism of the improved electrocatalytic performance from the slow-evaporation sample. Taken together, the results of this study will greatly serve to advance the understanding that how preparation methods can control the Pt nanocubes distribution in polymer matrix and can further affect the distribution of Pt nanocubes on the carbon support, which ultimately lead to improving the electrocatalytic efficiency of current Pt catalyst on the carbon support from a different point of view.

4.2 Results

4.2.1 Synthesis and characterization of PHEA-g-C\textsubscript{18} and platinum (Pt) nanocubes

As mentioned in Chapter 3.2.1, PHEA-g-C\textsubscript{18} was synthesized through nucleophilic substitution of the designated amount of octadecylamine and excessive amount of ethanolamine
to poly(succinimide), and its structure was characterized with $^1$H NMR spectroscopy. The degree of substitution for octadecyl chains ($D_{SC_{18}}$) to the polymer, defined as the mole percentage of succinimide units substituted with octadecyl chains, was determined to be 14.8 mol % in this study. For convenience, PHEA-g-C$_{18}$ at $D_{SC_{18}}$ of 14.8 mol % was termed as DS-14.8. On a separate note, Pt nanocubes were synthesized by a modified approach based on the previous study, and the structure of Pt nanocubes was characterized by transmission electron microscopy (TEM) (Figure 4.2).

4.2.2 Slow-evaporation method and emulsification for preparing Pt-PHEA-g-C$_{18}$

Pt-PHEA-g-C$_{18}$ nanoconstructs were prepared by two different approaches (Figure 4.1). In the slow-evaporation method, 200 nm sized particles of PHEA-g-C$_{18}$ was first self-assembled in aqueous media. Then Pt nanocubes solution was added into the micelle solution (Figure 4.3a), and this mixture was stirred vigorously to make a vortex which continuously brought the Pt nanocubes to mix with the PHEA-g-C$_{18}$ micelle (Figure 4.3b). The mixing proceeded until all the hexane evaporated out. In the emulsification method, PHEA-g-C$_{18}$ in aqueous media mixed with Pt in hexane, at the same ratio as used in the slow-evaporation approach, were sonicated to form a homogenous cloudy emulsion. Then the hexane was quickly removed out of the mixture by a rotary evaporator (Figure 4.1b). The Pt-PHEA-g-C$_{18}$ samples prepared by both methods had a brownish color (Figure 4.3c).

TEM micrographs confirmed the morphology of Pt-PHEA-g-C$_{18}$ nanoconstructs prepared by two different methods. The nanoconstruct prepared by the slow-evaporation method had a dominating morphology of Pt nanocube clusters, of which the shape was either spherical or close to spherical and the observed packing density of Pt nanocubes was dense (Figure 4.4a). In contrast, Pt-PHEA-g-C$_{18}$ nanoconstructs prepared by emulsification had loosely spreading of Pt nanocubes in different domains of PHEA-g-C$_{18}$ polymers (Figure 4.4b).
4.2.3 Electrocatalytic performance of Pt nanocubes examined with cyclic voltammetry (CV)

Cyclic voltammetry (CV) was used to study the electrocatalytic properties of Pt nanocubes on the electrode, prepared by drop casting of the Pt-PHEA-g-C\textsubscript{18} samples on the carbon support surface. The cyclic voltammograms of the samples prepared by two approaches were found to be very different in terms of the shape and the current intensity of the measured curves of currents versus scan rates (Figure 4.5a). In oxidation reaction, no peak currents and increased current densities were presented in the Pt-PHEA-g-C\textsubscript{18} sample prepared by the slow-evaporation method, while peak currents were observed in the emulsification sample. The higher current density from the slow-evaporation sample signified more electrocatalytic activities than that from the emulsification sample. Furthermore, Randles-Sevcik equation was applied to analyze the actual active area by peak current in the oxidation current (\(i_p\) of the slow-evaporation sample was assumed as the highest current in the voltage range from 0 to 0.6 V):

\[
i_p = (2.69 \times 10^5)n^{1.5}AD^{0.5}Cv^{0.5}
\]

where \(n\) is the number of electrons transferred in the redox event, \(A\) is the electrode surface area or actual active area (cm\(^2\)), \(D\) and \(C\) is the diffusion coefficient (cm\(^2\)/s) and the concentration of the electroactive species in the bulk solution (mol/cm\(^3\)), respectively, and \(v\) is the scan rate (V/s).

\(i_p\) was proportional to the square root of \(v\). From the slope of \(i_p\) versus \(v^{0.5}\) plots (Figure 4.5b), given that \(n\), \(D\), and \(C\) were constant as all the experiments were conducted under the identical conditions, the actual active area of the slow-evaporation sample was about 2.5-folds of the emulsification sample as measured by CV.

4.2.4 Electrode surface study with SEM

The macroscopic distribution of Pt nanocubes on the carbon substrate coated with Pt-PHEA-g-C\textsubscript{18} samples prepared by two methods was examined with SEM. Two distinguished
domains, the carbon and the Pt-PHEA-g-C\textsubscript{18} coated carbon, were observed on the electrode surface coated with the slow-evaporation sample. SEM-EDX confirmed the bright region (Area 1, \textbf{Figure 4.6}) contained a higher percentage of Pt nanocubes than the dark region (Area 2, \textbf{Figure 4.6}) in the low-magnification micrograph of the electrode surface. The local brightness on the SEM micrograph clearly indicated the carbon surface were coated by platinum nanocubes or not, even without further EDX analysis.

By zooming in the bright region of the low-magnification micrograph of the slow-evaporation sample (1\textsuperscript{st} row in \textbf{Figure 4.7a}), the Pt nanocubes were observed to be wide-spreading on the carbon support of the electrode. Pt nanocubes formed small islands scattering on the carbon support in the dark region of the low-magnification micrograph of the slow-evaporation sample (2\textsuperscript{nd} row in \textbf{Figure 4.7a}). In contrast, neither huge coverage nor small islands of Pt nanocubes was found on the electrode surface coated with the emulsification sample (\textbf{Figure 4.7b}).

\textbf{4.3 Discussion}

Overall, this study demonstrates a novel method to improve the electrocatalytic activity of Pt nanocubes using PHEA-g-C\textsubscript{18} as the matrix, which was then coated on the carbon support. The electrocatalytic activity of the Pt-PHEA-g-C\textsubscript{18} was improved by decreasing the hexane removal rate when the sample was prepared with the two phase mixture, composed of Pt nanocubes in hexane and PHEA-g-C\textsubscript{18} in water. We found that the sample prepared by a slow-evaporation method led to higher measured current densities and no peak current in CV measurements than that prepared by the emulsification method, where a rotator evaporator was used to quickly remove the hexane. A higher Pt nanocubes coverage on the electrode coated with slow-evaporation sample was observed with SEM than that with the emulsification sample. The
well-dispersed Pt nanocubes on the electrode supported the improved electrocatalytic performance of the Pt-PHEA-g-C\textsubscript{18} prepared by the slow-evaporation method.

PHEA-g-C\textsubscript{18} is not expected to self-assemble into micelles with a perfect hydrophilic exterior in aqueous media, due to its randomly grafted polymer architecture. Randomly modified polymers have been proposed to associate in a manner that hydrophobic and hydrophilic parts of the polymer are entangled together which results in the possible contact between the core and the aqueous media.\textsuperscript{13} Therefore, Pt nanocubes with a hydrophobic coating layer can start to associate spontaneously with the exposing hydrophobic domain of PHEA-g-C\textsubscript{18} micelles during the hexane-removal process. Additionally, the polymeric micelle integrity was minimally disrupted by hexane during the slow-evaporation process considering two factors. First, hexane is not a good solvent for PHEA-g-C\textsubscript{18} hydrophilic polymer backbones, which help resist the hexane to solvate the polymeric micelles. Secondly, hexane has a high air/water partition coefficient,\textsuperscript{14} which indicates the great tendency of hexane to evaporate out compared to stay in aqueous phase and solvate the micelles.

Though it is not straightforward to observe Pt nanocubes coated on the outside of PHEA-g-C\textsubscript{18} micelles prepared by the slow-evaporation method from TEM micrographs, different Pt distribution patterns in PHEA-g-C\textsubscript{18} were observed for the two PHEA-g-C\textsubscript{18} nanoconstructs. For emulsification method, PHEA-g-C\textsubscript{18} started to self-assemble after the energy input of sonication was applied to form the stable emulsion. Hexane was removed quickly to make PHEA-g-C\textsubscript{18} nanoconstructs before the emulsion became unstable. However, this quick method yielded a less dense clustering of Pt nanocubes, as shown in the TEM micrographs, compared to the slow-evaporation method.

The nanostructure feature of the slow-evaporation sample led to improved electrocatalytic performances, including higher current densities and no peak current in CV measurements. The
CV results indicated no mass-transfer limit of the electroactive species and three-dimensional diffusion happened in measuring the slow-evaporation sample, both of which are typical characteristics of nano-sized materials on the electrode. It suggested the exposure of the Pt nanocubes on the electrode surface. The macroscopic distribution of Pt nanocubes on the carbon surface of the electrode was further examined with SEM to study the electrocatalytic performance improvement of the slow-evaporation sample. The high coverage of immobilized Pt nanocubes formed on the electrode surface coated with the slow-evaporation sample, because PHEA-g-C$_{18}$ helped carry the nanocubes to spread on the porous carbon effectively by serving as nanocarriers to prevent the instability of the nanocubes during the preparation process. Even though we expected PHEA-g-C$_{18}$ in the emulsification sample played a similar role, the Pt nanocubes distributed inside of the nanoconstructs compromised the electrocatalytic performances.

4.4 Conclusion

This study describes a novel concept that preparing Pt nanocubes associated with and loaded in polymeric micelles can lead to the different electrocatalytic performance of Pt, due to the changed distribution of Pt nanocubes on the CV electrode surface. The removal rate of hexane, in which colloidal Pt nanocubes was originally suspended, of the preparation methods determined the effectiveness of this polymer-assisted strategy for the nanocube coating on the electrode. Pt nanocubes mixed with PHEA-g-C$_{18}$ was studied as the model system. The nanoconstructs prepared by the slow-evaporation method has led to a distinct spatial organization of the Pt nanocubes coated on the polymeric micelles, resulted in 2.5-folds higher electrocatalytic activity as examined with CV, and showed a higher surface coverage of the Pt nanocubes on the electrode surface than those prepared by emulsification. Overall, we envisage
this study will advance the understanding that how the interaction between Pt nanocubes and the supporting polymeric micelles can improve the electrocatalytic efficiency of Pt catalyst on the carbon support of electrodes. This study is important for immobilizing heterogeneous catalysts on a substrate of interest using colloidal method.

4.5 Experimental Section

All chemicals and materials were purchased from Sigma-Aldrich (USA/Korea) and used without further purification unless otherwise specified.

4.5.1 Synthesis of and characterization of poly(2-hydroxyethyl-co-octadecyl aspartamide) (PHEA-g-C$_{18}$)

Refer to Experimental Sections of Chapter 3 (Section 3.5.1).

4.5.2 Preparation of PHEA-g-C$_{18}$ micelles

The PHEA-g-C$_{18}$ polymer was suspended in cell-culture grade water at a concentration of 2 mg/ml and sonicated briefly to make a homogeneous colloidal solution. PHEA-g-C$_{18}$ micelles formed spontaneously after the sonication.

4.5.3 Synthesis of platinum (Pt) nanocubes

Pt(acac)$_2$ (0.1 g), octadecene (10 ml), oleic acid (OA) (1 ml), and oleylamine (OAm) (1 ml) were mixed under N$_2$ and magnetic stirring. The mixture was then heated to 120 °C and kept in 30 minutes. A solution of Fe(CO)$_5$ in hexane (0.1 ml, prepared by adding 0.1 ml Fe(CO)$_5$ in 1 ml hexane) was injected into the solution. The solution was further heated to 200 °C and kept at this temperature for 1 hour before it was cooled down to room temperature (heating rate: 240 °C/h). 40 ml of isopropanol was added and then the suspension was centrifuged (8000 rpm, 10 min) to separate the nanoparticles. The particles were dispersed in 10 ml hexane and precipitated out by
adding ethanol, then the suspension was centrifuged again (8000 rpm, 10 min). The final product (50 mg) was dispersed in 10 ml of hexane for further use.

4.5.4 Slow evaporation method for preparing Pt-PHEA-g-C18 nanoconstructs

9 ml platinum nanocubes (1.8 mg/ml hexane) were mixed with 8 ml PHEA-g-C18 micelle solution (2 mg/ml), and this mixture was stirred vigorously in the hood over 30 hours to ensure all the hexane evaporated out. The sample was a brownish solution, and then lyophilized to yield dry powders for the following experiments.

4.5.5 Traditional emulsification method for preparing Pt-PHEA-g-C18 nanoconstructs

PHEA-g-C18 in aqueous was mixed with Pt nanocubes in hexane at the same ratio as described in the slow-evaporation approach. The mixture was sonicated to form a homogenous cloudy emulsion, and then the hexane was quickly removed out of the mixture by a rotary evaporator. The sample was also a brownish solution, and then lyophilized to yield dry powders for the following experiments.

4.5.6 Morphological analysis of Pt nanocubes and Pt-PHEA-g-C18 nanoconstructs

The sample suspension was dropped onto a 300-mesh copper grid coated with formvar (Electron Microscopy Sciences) and air-dried. Then the morphologies of Pt nanocubes and Pt-PHEA-g-C18 nanoconstructs were observed using TEM (JEOL 2100 with LaB6 emitter) at an accelerating voltage of 200 keV.

4.5.7 Electrode preparation and procedure of cyclic voltammetry (CV)

Pt-PHEA-g-C18 sample was dissolved in ethanol (99 %) at 3.5 mg/ml with a mild sonication (Lab companion, Korea, 40 kHz) for 20 seconds. A drop of 50 µL sample was coated on the working electrode (surface area of 2.54 cm²), composed of titanium foil (0.25 mm thickness) and covered by a carbon paste (ELCOAT CX-12, CANS, JAPAN), and then was air-dried at 70 °C for 1 hour.
The electrochemical property of the coated film was investigated by a (PARSTAT 2273A, Princeton Applied Research, USA) with 10 mmol K$_3$Fe(CN)$_6$ and 100 mmol KNO$_3$ solution as the electrolyte. Platinum mesh and Ag/AgCl, KCl (sat’d.) were used as the auxiliary and the reference electrode, respectively. Then, the cyclic voltammetry was examined by the three-electrode cell at a voltage scan rate of 10-200 mV/s at room temperature. The electrode with only carbon paste without sample coating was tested under the same conditions as the background signal, which was subtracted from all the other sample measurements. Four times of pre-scans were run at a scan rate of 100 mV/s before the measurement. CV measurement was repeated for the same sample on the 1st, 2nd, and 9th day after the electrode was coated. The electrocatalytic stability was well maintained during this period as the measured curves remained similar (data not shown).

4.5.8 Surface analysis of electrodes with SEM

The surface morphology of Pt-PHEA-g-C$_{18}$ nanoconstructs on the electrode, after CV measurements, was observed using FEI Helios 650 Nanolab FEI/SEM (FEI, Eindhoven, Netherlands) operating at an accelerating voltage of 5 keV to seek the area of interest. The element analysis was performed using Energy dispersive X-ray spectroscopy with same equipment.

4.6 References


4.7 Figures

(a) Slow evaporation method

(b) Emulsification

Figure 4.1. Schematic description of two disparate strategies to load Pt nanocubes on or in the PHEA-g-C_{18} micelles. (a) Slow-evaporation method enabling the localization of Pt nanocubes on the micelle surface. (b) Emulsification to load Pt nanocubes within the micelle.
Figure 4.2. Characterization of Pt nanocubes. (a) TEM image of the Pt nanocubes. (b) High resolution TEM image of Pt nanocubes. The scale bar in (a) and (b) represents 20 nm and 5 nm, respectively.

Figure 4.3. The slow-evaporation method and the resulting samples. (a) A photograph of the Pt solution mixed with the PHEA-g-C_{18} micelle solution without stirring. (b) A photograph and a scheme showing the two components in (a) were mixed continuously by vigorously stirring. (c) A photograph of the colloidal samples of Pt-PHEA-g-C_{18} prepared by emulsification (left) and the slow-evaporation method (right).
Figure 4.4. Morphological analysis of the spatial organization of Pt nanoparticles on or in PHEA-g-C$_{18}$ micelles with TEM. Pt-PHEA-g-C$_{18}$ nanoconstructs prepared by the slow-evaporation method (a) and emulsification (b). The scale bar represents 100 nm.
Figure 4.5. Analysis of electrocatalytic activities for Pt nanocubes loaded on or in PHEA-g-C\textsubscript{18} micelles. (a) Cyclic voltammograms of the Pt-PHEA-g-C\textsubscript{18} samples prepared by the slow-evaporation method (a-1) and emulsification (a-2) measured at different scanning rates. (b) Pick current analysis of the samples prepared by the slow-evaporation method (red reverse triangles) and the traditional emulsification (black dots). Lines in (b) represent the linear fitting curves.
Figure 4.6. SEM-EDX for the surface elemental analysis of the bright region (Area 1) and the dark region (Area 2) on the SEM micrograph of the electrode coated with Pt-PHEA-g-C\textsubscript{18} nanoconstructs which were prepared by the slow-evaporation method. Scale bars represent 2 µm.
Figure 4.7. Surface analysis of electrodes deposited by PHEA-g-C$_{18}$ micelles with the controlled spatial organization of Pt nanocubes (a) SEM micrographs of the electrode coated with Pt-PHEA-g-C$_{18}$ nanoconstructs prepared by the slow-evaporation method. The scale bar on the 1$^{st}$ column represents 2 µm, and scale bars on the images displayed on the 2$^{nd}$ and 3$^{rd}$ columns represent 100 nm. Yellow circles on the center micrograph of the 2$^{nd}$ columns represent Pt islands. (b) SEM micrographs of the electrode coated with Pt-PHEA-g-C$_{18}$ nanoconstructs prepared by emulsification. The scale bar on the image of the 1$^{st}$ column represents 20 µm, and the scale bar on the image of the 2$^{nd}$ column represents 100 nm.
Chapter 5 Conclusion and Future Work

The goal of this dissertation research is engineering an amphiphilic polyaspartamide to prepare nanocarriers of 1) imaging contrast agents and 2) catalytic Pt nanocubes to achieve the improved targetability and lifetime and the enhanced Pt electrocatalytic performance, respectively. These projects inherently take advantage of the polyaspartamide characteristics: the easy functionalization of the starting polysuccinimide by grafting different side groups of interest onto the backbone via aminolysis, and the low expense of preparing polyaspartamide (neither vacuum nor catalyst needed), compared to block copolymers.

In chapter 2, we hypothesized that decreasing the bilayer permeability of PHEA-C_{18}-MA polymersomes can stabilize their structure, extend their lifetime in circulation, and hence improve the quality of bioimaging when the polymersome was coupled with an imaging probe. The bilayer permeability of the polymersome was decreased by increasing the packing density of the bilayer with methacrylate groups, and was further decreased by inducing chemical cross-linking reactions between the methacrylate groups. The polymersome with the decreased bilayer permeability demonstrated greater particle stability in physiological media, and ultimately can better highlight tumors in mice over two days compared to those with a higher bilayer permeability after labeling with a near-infrared (NIR) fluorescent probe.

In chapter 3, a simple method was proposed and evaluated to immobilize antibodies that can act as targeting motifs on surfaces of nanocarriers by recapitulating a process that bacterium adopt for immobilization of the host’s antibodies. We hypothesized that alkylated \textit{Staphylococcus aureus} protein A (SpA-PA) would self-assemble with PHEA-g-C_{18} micelles and subsequently induce stable coupling of antibodies of interest to the micelles. The self-assembly between micelles and SpA-PA, confirmed with a fluorescent tag to SpA-PA, was more
thermodynamically favorable by increasing the degree of substitution of octadecyl chains to PHEA-g-C$_{18}$ due to a positive entropy change. The final anchorage of antibodies to SpA-PA coupled micelles was also corroborated with the fluorescence resonance energy transfer (FRET) technique. The micelles coated with corresponding antibodies using SpA-PA as the linker displayed the higher binding affinity to a substrate coated by VCAM-1 and integrin $\alpha_\text{v}\beta_3$ than antibody-free micelles, as observed with surface plasmon resonance (SPR) spectroscopy. Similar results were found with micelles coated with antibodies to integrin $\alpha_\text{v}$ for a substrate coated by integrin $\alpha_\text{v}\beta_3$ in a circulation-mimicking flow chamber.

In chapter 4, a method that associating the nanocubes with polyaspartamide micelles, which would bring the nanocubes to coat on the electrode, was examined to enable the effective immobilization of Pt nanocubes on the carbon solid support. We hypothesized that the removal rate of hexane, in which colloidal Pt nanocubes were suspended, determined the effectiveness of this PHEA-g-C$_{18}$ polymer-assisted strategy for the nanocube coating on the electrode. Two preparation methods, which have significantly different hexane removal rates, led to distinct spatial organizations of the Pt nanocubes with the polymer micelles. The sample prepared by the slow evaporation method resulted in 2.5-folds higher electrocatalytic activity than that prepared by emulsification, as examined with cyclic voltammetry. Scanning electron micrographs (SEM) showed a higher surface coverage of Pt nanocubes on the electrode coated with Pt-PHEA-g-C$_{18}$ nanoconstructs prepared by the slow evaporation method than by emulsification, and this explained the improved electrocatalytic performance.

Overall, the results of these projects greatly serve to allow us to better understand the design principles of the biocompatible polyaspartamide-derived nanocarriers, and also offer the insight about the effect of amphiphilic polyaspartamide interaction with Pt nanocubes on the catalytic performance of Pt.
Future work based on the research projects in this dissertation is listed as follows:

Chapter 2

- Further study the mechanism of polyaspartamide (graft polymer) self-assembly into nano-sized polymersomes in the solvent exchange process with theoretical simulation.
- Functionlize the polymersome surface with targeting motifs to further advance in its targetability or/and load therapeutic drugs for the tumor treatment.

Chapter 3

- In vivo animal experiment for evaluating the effectiveness of this targeting strategy.

Chapter 4

- Further activate the catalyst and remove the polymer matrix after Pt-PHEA-g-C_{18} coated on the electrode; then confirm the catalyst distribution on the electrode and the electrocatalytic activity after the activation.
- Test other amphiphilic materials (e.x. block copolymers) to see if the two preparation approaches still generate distinct Pt-PHEA-g-C_{18} nanoconstructs.
- Evaluate the degree of hydrophobiclity of PHEA-g-C_{18} micelle surfaces to further optimize the Pt loading capacity and the coating performance on the electrode.