Development of Natural Product-Derived Cationic Polymers for Biomaterial Applications

Moumita Sharmin Jui

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DEVELOPMENT OF NATURAL PRODUCT-DERIVED CATIONIC POLYMERS FOR BIOMATERIAL APPLICATIONS

by

Moumita Sharmin Jui

Bachelor of Science
Jahangirnagar University, 2014

Submitted in Partial Fulfillment of the Requirements
For the Degree of Master of Science in
Chemistry
College of Arts and Sciences
University of South Carolina
2020

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Cheryl L. Addy, Vice Provost and Dean of the Graduate School
DEDICATION

To my parents, for their love and support and guiding me to get the best education possible.

To Dr. Md Anisur Rahman, my loving husband, for his immense support and love. None of my accomplishments would be possible without him.

To my lovely daughter Anaisha Mahveen, for being there with her happy smile.
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor Dr. Chuanbing Tang. It has been a great honor for me to work with him in his research group. He has given an incredible amount of guidance and support not only in academia but also in my life. His passion for polymer science inspired me to work in this area. Without his guidance, encouragement and persistent help this thesis would not have been possible.

I would also like to thank Dr. Brian Benicewicz, for serving on my committee. I also want to express my appreciation to all my colleagues, Dr. Md Anisur Rahman, Dr. Parasmani Pageni, Dr. Tianyu Zhu, Dr. Meghan Lamm, and Yujin Cha. Working with you all has been a lot of fun and I want to thank you all for your advice and support.

Finally, I would like to thank all the funding supports from the University of South Carolina.
ABSTRACT

In this thesis, multicyclic natural products and metallocene-containing polymers were synthesized for biomedical applications.

Chapter 1 describes the overall background, the recent development of natural product-based antimicrobial biomaterial and metallocene containing polymer for biomedical application. Afterward, the primary research objectives of my research are illustrated.

In chapter 2, a multicyclic natural product derived guanidine-based facially amphiphilic homopolymers and copolymers were synthesized. Cholic acid is one of the bile acid derivatives used to prepare facially amphiphilic moieties. A neutral polyethylene glycol (PEG) component was used to prepare copolymers with cholic acid. Reversible-addition fragmentation chain transfer (RAFT) polymerization technique was used to prepare all the polymers. The self-assembly of all polymers was also investigated. Finally, the hemolysis activity of guanidine-based facial amphiphilic polymers was measured against red blood cells.

The synthesis of metal-containing copolymers was illustrated in chapter 3. The diblock copolymers were prepared from cationic cobaltocenium and neutral PEG moieties. PEG-based macro raft agent was used to make cobaltocenium containing diblock copolymer. These diblock block copolymers can be a good candidate for biomedical applications, especially for gene delivery.
Finally, a summary and future directions of this dissertation research are provided in chapter 4. In future work, some suggestions about developing new molecular biomass with true facial amphiphilicity for antimicrobial application and metallocene based polymers for gene delivery are given.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>AIBN</td>
<td>Azobisisobutyronitrile</td>
</tr>
<tr>
<td>AMP</td>
<td>Antimicrobial Peptide</td>
</tr>
<tr>
<td>CA</td>
<td>Cholic Acid</td>
</tr>
<tr>
<td>DCC</td>
<td>Dicyclohexyl carbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-(dimethylamino)pyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectrometry</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel Permeation Chromatography</td>
</tr>
<tr>
<td>HC</td>
<td>Hemolytic Concentration</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>PEGMA</td>
<td>Poly (ethylene glycol) methyl ether methacrylate</td>
</tr>
<tr>
<td>RAFT</td>
<td>Radical Addition-Fragmentation Chain Transfer</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>TBAACL</td>
<td>Tetrabutylammonium chloride</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
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</table>
### List of Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>$M_n$</td>
<td>Number average molecular weight</td>
</tr>
<tr>
<td>$D$</td>
<td>Dispersity</td>
</tr>
<tr>
<td>$D_h$</td>
<td>Hydrodynamic diameter</td>
</tr>
<tr>
<td>$\delta$</td>
<td>Chemical shift</td>
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CHAPTER 1

GENERAL INTRODUCTION
1.1 Bile Acid

Bile acids are amphiphilic steroid acids found in the mammals and serve as emulsifiers for the digestions of fat by the formation of micelles. Bile acids are produced from the cholesterol in the liver and stored in the gallbladder. They are classified into two groups within the human body based on their order of formation. Human liver cells produce primary bile acids such as cholic acid and chenodeoxycholic acid in a multi-step pathway via cytochrome P-450 enzyme-mediated oxidation of cholesterol. In the intestinal tract, primary bile acids are partly converted to secondary bile acids, deoxycholic and lithocholic acids by intestinal microorganism.

![Chemical structures of bile acid derivatives.](image)

**Figure 1.1** Chemical structures of bile acid derivatives.

Hydroxyl groups of bile acid molecules are positioned in the concave $\alpha$-face while the multicyclic hydrocarbon structure is constituted as the convex $\beta$-face, thereby providing the potential to achieve true facial amphiphilicity. Due to their unique structural features, these acids have been primarily used in biomedical applications such as drug delivery, prodrug formulation, and antimicrobial systems. They are abundant in nature and accessible as biomass-derived chemicals.
1.2 Antimicrobial Polymers

Antimicrobial resistance is now considered a major global challenge, compromising our ability to treat infectious diseases due to the lack of discovery of novel compounds from natural products or new classes of antimicrobials.4,5 Bacteria are capable of acquiring resistance against antibiotics through different mechanisms such as efflux pumps,3 chemical modification, genetic mutation, and gene transfer (Figure 1.2).6 The rise of drug-resistant Gram-negative bacteria is a great concern given the limited number of antimicrobial agents that can be used for such infections. Therefore, it is a crucial need to continuously develop antibiotics with novel modes of action to face this evolving resistance that can successfully treat bacterial infections.

Figure 1.2 (A) Mechanisms of antibiotic resistance. (B) Selective interactions between cell membranes and cationic antimicrobial polymers.3

Antimicrobial polymers are a class of hydrophilic cationic macromolecules, selectively destroy microorganisms such as bacteria or fungi with little or no cytotoxicity
to mammalian cells. They are a class of novel antimicrobial agents that is the combination of antimicrobial peptides (AMPs) and polymer disinfectants that have emerged as two distinct fields since the 1980s. Host-defense antimicrobial peptides (AMPs) are antimicrobials, act as a defense against invading pathogens including bacteria, protozoa, yeast and fungi. Natural antimicrobial peptides (AMPs) are amphiphilic, combining cationic charges and hydrophobic components, and are able to selectively bind to anionic bacterial membranes or other anionic targets over zwitterionic human cell membranes (Figure 1.2). AMPs form an α-helix structure with positive charges arrayed on one side and lipophilic groups aligned along the other side when comes to contact with bacteria. The global segregation of cationic and lipophilic side chains of these AMPs is also referred to as facial amphiphilicity (i.e., separate hydrophilic and hydrophobic faces). This feature (facial amphiphilicity) permits AMPs to strongly interact with biological membranes. However, the clinical implementation of AMPs is minimal due to their low bioavailability, low stability, as well as in many cases nonspecific toxicity to mammalian cells. Therefore, synthetic polymers with cationic charges, which mimic natural AMPs, have been investigated widely to combat bacteria.

1.3 Metal Containing Polymer

Metallopolymers, are a class of metal-containing polymer with metal center incorporated into the organic polymeric frameworks, have received significant attention over the past few decades. These polymers provide desirable mechanical and processing properties in polymeric frameworks with the combination of metal properties such as catalytic, magnetic, and electronic properties. Among metallopolymers, Cationic metallocenes (or metalloceniums) show great potential in optical, magnetic, catalytic,
electrochemical, and biomedical applications due to the physicochemical and processing advantages of organic polymers with the unique sandwich structures and multifunctionality of metalloccenes.\textsuperscript{17,18} There are two major states of a metalloccene: neutral metalloccene and cationic metalloccenium. Neutral metalloccene like ferrocene has 18 valence electrons and can easily undergo electrophilic aromatic substitution reactions.\textsuperscript{19} But 19-e cobaltocene can lose one electron easily to form the much more stable 18-e cobaltocenium cation, which is incapable of undergoing electrophilic aromatic substitution reactions.\textsuperscript{20} The isoelectronic cobaltocenium monomers and polymers show high solubility in water with the use of hydrophilic counterion, which could be more beneficial for applications in drug delivery.\textsuperscript{21}

1.4 Gene Delivery

Gene therapy has the ever-growing promise for the treatment of numerous inherited diseases such as genetic disorders, viral infections, and cancers.\textsuperscript{22} However, the clinical application of gene therapy for treating life-threatening illnesses remains largely unsuccessful due to various technical obstacles.\textsuperscript{23} The lack of safe and proper vectors is one of the main concerns. Viral and non-viral vectors have been used to deliver therapeutic genomes to the target cells.\textsuperscript{24-25} Despite the higher transfection efficiency of viral vectors, their biomedical applications are limited due to toxicity, immunogenicity and difficulty for large-scale production.\textsuperscript{26} A variety of non-viral or synthetic vectors including polymers, proteins peptides, liposomes, and exosomes have been developed over the last two decades. Among these non-viral vectors, cationic polymers gained significant attention due to their tunability, degradability, environmental stability, inexpensive synthesis and scalability.\textsuperscript{27} Cationic metalloccene with charged metal centers can interact with negatively charged
biomacromolecules like protein, DNA and enzymes. This bioconjugates exhibit active endocytosis and directed nuclear delivery, which are two key steps for gene release. 

1.5 Research Objectives

To address the current emergence of drug-resistant bacteria, the development of new antimicrobial agents with the potent ability to kill the MDR bacteria is a significant demand in our society. In this dissertation, new guanidine-based facial amphiphilic polymers were prepared from a multicyclic natural product such as bile acid. Guanidine-based polymers are potent anti-microbial materials because of their selective interactions with negatively charged bacterial cell membranes and due to the charge delocalization, the toxicity level is very lower. The clinical application of gene therapy for treating or preventing life-threatening illness remains largely unsuccessful due to various technical obstacles. The lack of safe and efficient vectors is the main objective to synthesize cationic metal-containing polymer for non-viral gene delivery. The new class of polymers as novel non-viral gene delivery vectors were developed based on cationic metal-containing polyelectrolytes. Metal containing polyelectrolytes with positively charged cobaltocenium can strongly bind with the anionic phosphodiester group of a nucleic acid through electrostatic interactions. Cobaltocenium-containing polyelectrolytes will exhibit minimal toxicity to mammalian cells while having a stronger binding ability to anionic macromolecules.
1.6 References


*Advances in Colloid and Interface Science* **2008**, *139*, 97-149.


CHAPTER 2

SYNTHESIS OF GUANIDINE FUNCTIONALIZED CHOLIC ACID-BASED FACIAL AMPHIPHILIC POLYMERS
2.1 Abstract

Guanidine-functionalized facial amphiphilic polymers are synthesized which can be used as a powerful antimicrobial agent. Guanidine-based polymers are potent antimicrobial materials because of their selective interactions with negatively charged bacterial cell membranes. We incorporated a guanidine functional group into facial amphiphilic cholic acid-based macromolecular architectures to prepare a potent antimicrobial agent that can interact preferentially with bacterial membranes. A multicyclic natural product such as cholic acid-based polymers is modified with guanidine derivatives. Interestingly, guanidine functionalized homopolymers form spheres and rods like nanostructures in water from the facial amphiphilic cationic polymers via supramolecular interactions. The hemolysis activity of homopolymers was also measured. To improve the hemolysis activity, polyethylene glycol was used to prepare the cholic acid-based guanidine functionalized amphiphilic diblock and random copolymers. Incorporation of polyethylene glycol exhibits better hemolysis activity.

2.2 Introduction

The increasing prevalence of antibiotic resistance is a serious emerging challenge. Antimicrobial resistance is an ever-increasing threat to public health due to the difficulty to treat them with conventional antibiotics.\textsuperscript{1-3} The lack of new molecule discovery over the last few decades has resulted in the rise of antibiotic-resistant pathogenic microorganisms.\textsuperscript{4} Among this multidrug-resistant (MDR) pathogens, Gram-negative bacteria pose more perilous threats to human life given the limited number of antimicrobial agents that can be used for such infections.\textsuperscript{5} The presence of dual membranes in Gram-negative bacteria acts
as an impermeable barrier to most antibiotics and this is the driving force for the development of new anti-microbial materials.\textsuperscript{6,7}

Antimicrobial agents such as antimicrobial peptides and antimicrobial polymers favor a facially amphiphilic orientation during their mechanism of action. Natural antimicrobial peptides (AMPs) are amphiphilic, shows broad-spectrum antimicrobial activities that typically involve a membrane-disruptive mechanism.\textsuperscript{8-11} These polymers/peptides form an $\alpha$-helix structure with positive charges arrayed on one side and lipophilic groups aligned along the other side in contact with bacterial membranes and are referred to as facial amphiphilicity (i.e. separate hydrophilic and hydrophobic faces).\textsuperscript{12-16}

In our group, we have developed several antimicrobial macromolecules utilizing natural rosin with impressive activities.\textsuperscript{17,18} Recently, we developed a new class of cationic polymers with a strong antimicrobial activity using cholic acid, a derivative of bile acid against Gram-negative bacteria, where quaternary ammonium has been used as a cationic group.\textsuperscript{19,20} However, the weak interaction between the quaternary ammonium and the phosphate groups of phospholipids within cell membranes and the poor alkaline stability are a matter of concern.\textsuperscript{21}

**Figure 2.1** Facially amphiphilic structure of cholic acid.
In recent years, guanidine-based polymers have attracted significant attention as selective antimicrobial materials due to the selective interaction between guanidine groups and negatively charged bacterial cell-membrane instead of electrically neutral mammalian cell membranes, and nontoxic antimicrobial materials.\(^9\) The positive charges of the guanidine group are delocalized over three nitrogen atoms. When guanidine is ionized it shows a strong interaction with phosphate groups by forming hydrogen-bonded ion pairs.\(^{22}\) The bidentate binding between guanidine groups and phosphate groups is much stronger than between amine groups and phosphate groups, resulting in a high bacterial rate at a low antimicrobial concentration of guanidine-based polymers.\(^{21}\) Moreover, the toxicity of this charge-delocalized guanidine is much lower than that of the charge-localized linear amine head group. Recently, Yang and coworkers reported guanidium-functionalized non-degradable poly-norbornenes and polymethacrylates are more potent against bacteria in vitro than their amine-functionalized counterparts.\(^9\) In order to reduce the toxicity level by increasing the selectivity of antimicrobial polymers toward bacteria, we have synthesized a series of guanidium-functionalized cholic-acid based cationic copolymers which can possess intrinsic local facial amphiphilicity clustered together via a flexible macromolecular chain.

Cholic acid is composed of large cross-sectional hydrophobic multicyclic hydrocarbons on the convex \(\beta\)-face and multiple cationic charges on the concave \(\alpha\)-face, which provides facial amphiphilicity (Figure 2.1).\(^{19, 23, 24}\) Antimicrobial activity of synthetic polymers is largely dictated by the balance of hydrophilic to hydrophobic moieties.\(^{25}\) Amphiphilic copolymers comprising hydrophilic and hydrophobic segments can self-assemble in water to form a wide variety of aggregates such as spheres, rods, and
vesicles.\textsuperscript{26, 27} We prepared polymer nanoparticles to study the effect of cationic and hydrophobic functionalities on antimicrobial polymers over their selectivity between bacteria and a mammalian cell. Cholic acid and neutral polyethylene glycol (PEG) components were chosen to make amphiphilic copolymers that can form nanosized particles.\textsuperscript{20} We have synthesized different polymer architectures such as diblock and random copolymers using guanidium-functionalized cholic acid-based facial amphiphilic copolymers with a tunable PEG component to make them attractive as antimicrobial nano-objects.

Herein, we showed three synthetic strategies to fabricate the Guanidine functionalized cholic acid-based cationic polymers.

\textbf{2.3 Experimental Section}

\textbf{Materials.}

All chemicals were purchased from commercial sources and used as received unless otherwise stated. Cholic acid (CA, \(\geq 98\%\)), 2-hydroxyethyl methacrylate (HEMA, 97\%), 1,1,3,3-tetramethyl guanidine, 1-bromo ethane, 4-dimethylamino pyridine (DMAP, 99\%), Poly(ethylene glycol) methyl ether (Mn = 2000 Da) and poly(ethylene glycol) methyl ether methacrylate (PEGMA, Mn = 500 Da) were purchased from Sigma-Aldrich and used without further purification. 1-(3-Dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC-HCl, 98\%) was purchased from TCI and used without further purification. 6-Bromohexanoyl chloride (97\%) and 1-bromo ethane were purchased from Alfa-Aesar. 4-Cyano-4-(thiobenzylthio)pentanoic acid (CTP, 97\%) was purchased from Strem Chemicals. Azobisisobutyronitrile (AIBN, Sigma, 98\%) and solvents such as hexane, anhydrous N, N-dimethylformamide (DMF, 99.9\%), tetrahydrofuran (THF),
dichloromethane (DCM) were purified by standard procedures. Monomers (2-methacyrloyloxy)ethyl cholate (MAECA) was synthesized following our work reported earlier.\textsuperscript{19} CDCl\textsubscript{3} (99.9\% D), D\textsubscript{2}O (99.9\% D) and DMSO-d\textsubscript{6} (99.9\% D) were purchased from Cambridge Isotope Laboratories.

**Characterization**

Monomer and compound purity, and polymer conversion were monitored by 300 MHz proton nuclear magnetic resonance (1H NMR) spectroscopy using Bruker Avance III HD 300 spectrometer. Spectra were recorded in deuterated chloroform, deuterium oxide or dimethylsulfoxide solvent in ppm (δ) with tetramethylsilane as an internal standard. Molecular weight and molecular weight distribution of polymers were measured by gel permeation chromatography (GPC) equipped with a Waters 1525 Binary Pump, three Styragel columns, and a Waters 2414 Refractive Index (RI) detector. HPLC-grade THF solvent was used as an eluent at 35 °C with a flow rate of 1.0 mL/min. A series of narrowly-dispersed polystyrene standards obtained from Polymer Laboratories were used to calibrate the GPC system. GPC samples were prepared by dissolving polymers in HPLC-grade THF at a concentration of 5-10 mg/mL and filtered by PTFE micro-filters with an average pore size of 0.2 μm.

**Dynamic light scattering (DLS)**

A Zetasizer Nanoseries ZEN3690 (Malvern Instruments, Malvern, UK) instrument was used to measure hydrodynamic diameter (Z-average) and Zeta potential of polymer aggregates. The samples were prepared by dissolving copolymers in filtered (0.2 μm GHP membrane filter) deionized water with a concentration of 1 mg/mL. The solutions were at
pH 7.0 and the measurements were carried out at 25 °C. The data processing was done using a general-purpose algorithm provided in the Zetasizer Software. Sample measurements were acquired in triplicate and reported as an average value.

**Atomic Force Microscopy (AFM)**

AFM imaging was carried out using a Multimode Nanoscope V system (Bruker, Santa Barbara, CA). Tapping mode AFM was used to map surface topography by tapping the surface using an oscillating tip. The measurements were achieved using commercial Si cantilevers with a nominal spring constant and resonance frequency at 20–80 N m–1 and 230–410 kHz, respectively (TESP, Bruker AFM Probes, Santa Barbara, CA).

**Synthesis of cholic acid-based homopolymer**

![Synthetic route of Cholic acid homopolymer](image)

**Figure 2.2** Synthetic route of Cholic acid homopolymer.

(2-methacryloyloxy)ethyl cholate (MAECA) monomer was polymerized using a typical RAFT polymerization technique. For example, MAECA (0.70 g, 1.35 mmol), 4-Cyano-4-(thiobenzylthio)pentanoic acid (CTP) (6.27 mg, 0.0224 mmol), and azobisisobutyronitrile (AIBN) (0.74 mg, 4.487 µmol) were placed in a 10 mL Schlenk flask and dissolved in N, N-dimethylformamide (DMF) (2 mL). The mixture was performed
with three freeze-pump-thaw cycles protected under nitrogen and immersed into a preheated oil bath set at 70 °C. After a certain period of time, the polymerization was quenched by exposure to air and cooled under an ice water bath. The reaction mixture was precipitated twice into a mixture of hexane and DCM (50:50) and finally dissolved in THF and precipitated into hexane. The polymer was dried under vacuum. The final product was characterized by $^1$H NMR (Figure 2.3).

Synthesis of bromoalkyl-containing cholic acid Homopolymer

CA homopolymer (300 mg) was placed in a 25 mL round bottom flask and dissolved in anhydrous DMF (3 mL). Excess 6-bromohexanoyl chloride (3 mL) or 4-bromobutanoyl chloride (3 mL) or bromoacetyl bromide (3 mL) was added to the polymer solution dropwise at room temperature. The reaction mixture was allowed to stir at 55 °C
for 48 h and precipitated into methanol. The product was redissolved in DCM (2 mL), precipitated in methanol twice, and dried under vacuum. Final product was characterized by $^1$H NMR (**Figure 2.5**).

**Figure 2.4** Synthetic route of Bromo hexyl functionalized Cholic acid homopolymer.

**Figure 2.5** $^1$H NMR of Bromo hexyl functionalized Cholic acid homopolymer.

**Synthesis of Cholic acid-based Guanidine-functionalized homopolymer**

6-bromohexyl-modified CA homopolymer (200 mg) was dissolved in DMF (4 mL). Then, Excess 1,1,3,3-tetramethyl guanidine (1 mL) was added to the reaction mixture and
stirred for 24 h at 55 °C. After cooling and concentrating the reaction mixture, the resulting solution was precipitated in THF and centrifuged to collect the product. The product was washed with THF and dried under vacuum. Final product was characterized by $^1$H NMR (Figure 2.7).

Figure 2.6 Synthetic route of Guanidine functionalized Cholic acid homopolymer.

Figure 2.7 $^1$H NMR of Guanidine functionalized Cholic acid homopolymer.

**Quaternization of Cholic acid-based Guanidine-functionalized homopolymer**

Guanidine containing CA polymer (200 mg) was dissolved in DMF (4 mL). Then, excess 1-bromo ethane (3 mL) was added to the reaction mixture and stirred for 24 h at
55 °C. After cooling and concentrating the reaction mixture, the resulting solution was precipitated in THF and centrifuged to collect the product. The product was washed with THF and dried under vacuum. Finally, the product was further purified by dialysis against DI water (1 L × 3) for 24 h. The solution in the dialysis bag was collected and freeze-dried to obtain a white product. The final product was characterized by ¹H NMR (Figure 2.9).

Figure 2.8 Quaternization of Guanidine functionalized CA homopolymer.
Figure 2.9 $^1$H NMR of quaternization of Guanidine functionalized CA homopolymer.

Synthesis of PEGylated Raft agent

4-Cyano-4-(thiobenzylthio)pentanoic acid (CTP) (100 mg, 0.358 mmol), Poly(ethylene glycol) methyl ether (644 mg, 0.322 mmol) and DMAP (8.73 mg, 0.07 mmol) were dissolved in dry DCM (4 mL) under nitrogen atmosphere. To this solution was added DCC (81.29 mg, 0.39 mmol) in 3 mL dry DCM drop-wise under nitrogen. Then, the reaction mixture was stirred for 3 days at room temperature. The resulting solution was then filtered and precipitated in Diethyl Ether. The product was washed with Diethyl Ether and dried under vacuum. The final product was characterized by $^1$H NMR (Figure 2.11).

Figure 2.10 Synthesis of PEGylated RAFT agent.
Synthesis of Cholic acid and PEG-based Diblock copolymer

(2-methacryloyloxy)ethyl cholate (MAECA) monomer were polymerized using PEG-based RAFT agent. MAECA (0.40 g, 0.76 mmol), PEG-CTP (88 mg, 0.0385 mmol), and azobisisobutyronitrile (AIBN) (1.26 mg, 0.0076 mmol) were placed in a 10 mL Schlenk flask and dissolved in N,N-dimethylformamide (DMF) (2 mL). The mixture was performed with three freeze-pump-thaw cycles protected under nitrogen and immersed into a preheated oil bath set at 70 °C. After a certain period of time, the polymerization was quenched by exposure to air and cooled under an ice water bath. The reaction mixture was precipitated twice into hexane. The polymer was dried under vacuum. Final product was characterized by $^1$H NMR (Figure 2.13).
Figure 2.12 Synthesis of CA-PEG diblock copolymer.

Figure 2.13 $^1$H NMR of CA-PEG diblock copolymer.

Synthesis of Bromine Functionalized Cholic acid and PEG-based diblock copolymer

Post-polymerization modification was carried out by following a previously reported method. Above CA-PEG Diblock copolymer (300mg) was placed in a 25 mL
round bottom flask and dissolved in anhydrous DMF (2 mL). An excess amount of 6-bromohexanoyl chloride (3 mL) was added to the polymer solution dropwise at room temperature. The reaction mixture was allowed to stir at 55 °C for 48 hrs. After the completion of reaction, the reaction mixture was precipitated into methanol. The product was redissolved in DCM (2 mL), precipitated in methanol twice, and dried under high vacuum. Final product was characterized by \(^1\)H NMR (Figure 2.15).

Figure 2.14 Bromination of CA-PEG Diblock copolymer.
Synthesis of Guanidine Functionalized Cholic acid and PEG-based Diblock copolymer

The above post-modified CA-PEG diblock copolymer (300 mg) was placed in a 25 mL round bottom flask and sealed with a rubber septum and dissolved in DMF (4 mL). Then, Excess 1,1,3,3-tetramethyl guanidine (1 mL) was added to the reaction mixture and stirred for 24 h at 55 °C. After cooling and concentrating the reaction mixture, the resultant solution was precipitated in THF and centrifuged to collect the product. The product was washed with THF and dried under high vacuum. Finally, the product was dissolved in deionized water, and further purified by dialysis against deionized (DI) water (1 L × 3) for 24 hrs. The solution in the dialysis bag was collected and freeze-dried to obtain a white product. Final product was characterized by $^1$H NMR (Figure 2.17).
**Figure 2.16** Guanidine functionalization of CA-PEG diblock copolymer.

**Figure 2.17** $^1$H NMR of Guanidine functionalized CA-PEG Diblock copolymer.
Synthesis of Cholic acid and PEG-based Random copolymer

CA-PEG Methacrylate monomers (MAECA and PEGMA) were copolymerized using a typical RAFT polymerization process. For example, R1 (CA-PEG diblock copolymer) was synthesized using the predetermined ratios (e.g. [Monomer] : [AIBN] : [CTP] = 30: 0.2: 1). MAECA (0.40 g, 0.769 mmol), PEGMA (0.384 g, 0.769 mmol), CTP (7.16 mg, 0.0256 mmol), and AIBN (0.84 mg, 5.12 µmol) were placed in a 10 mL Schlenk flask and dissolved in DMF (1 mL). The mixture was performed with three freeze-pump-thaw cycles under nitrogen and immersed into a preheated oil bath set at 70 °C. After a certain period of time, the polymerization was quenched by exposure to air and cooled under an ice water bath. The reaction mixture was precipitated twice into a mixture of hexane and DCM (80: 20) and finally dissolved in THF and precipitated into hexane.

Post polymerization modification of Cholic acid and PEG-based Random copolymer

Post-polymerization modification was carried out by following a previously reported method. For example, bromine functionalized R1 (CA-PEG random copolymer) (400 mg) was placed in a 25 mL round bottom flask and dissolved in anhydrous DMF (2 mL). An excess amount of 6-bromohexanoyl chloride (3 mL) was added to the polymer solution dropwise at room temperature. The reaction mixture was allowed to stir at 55 0 C for 48 hrs. After the completion of reaction, the reaction mixture was precipitated into methanol. The product was redissolved in DCM (2 mL), precipitated in methanol twice, and dried under high vacuum. The reaction was confirmed by 1H NMR (Figure 2.19). Similarly, all copolymers were modified.
Figure 2.18 Synthetic route of Brominated CA-PEG Random copolymers.

Figure 2.19 $^1$H NMR of brominated CA-PEG Random copolymers.
Synthesis of Guanidine functionalization of Cholic acid and PEG-based Random copolymer

The above-brominated R1 (CA-PEG random copolymer) (300 mg) was placed in a 25 mL round bottom flask and sealed with a rubber septum and dissolved in DMF (4 mL). Then, Excess 1,1,3,3-tetramethyl guanidine (1 mL) was added to the reaction mixture and stirred for 24 h at 55 °C. After cooling and concentrating the reaction mixture, the resultant solution was precipitated in THF and centrifuged to collect the product. The product was washed with THF and dried under high vacuum. The reaction was confirmed by $^1$H NMR (Figure 2.21).

![Synthesis reaction diagram](image)

**Figure 2.20** Synthesis of Guanidine functionalized CA-PEG Random copolymer.
Quaternization of Guanidine functionalized R_CA_PEG copolymer

Quaternization of all copolymers was carried out by following a previously reported method.\textsuperscript{8} As an example: the above guanidine functionalized R1 (CA-PEG random copolymer) (300 mg) was placed in a 25 mL round bottom flask and sealed with a rubber septum and dissolved in DMF (4 mL). Then, Excess 1-bromo ethane (3 mL) was added to the reaction mixture and stirred for 24 h at 55 °C. After cooling and concentrating the reaction mixture, the resulting solution was precipitated in THF and centrifuged to collect the product. The product was washed with THF and dried under vacuum. Finally, the product was further purified by dialysis against DI water (1 L × 3) for 24 h. The solution in the dialysis bag was collected and freeze-dried to obtain a white product. The reaction was confirmed by \textsuperscript{1}H NMR (Figure 2.23).

Figure 2.21 \textsuperscript{1}H NMR of Guanidine functionalized CA-PEG Random copolymer.
Figure 2.22 Quaternization of Guanidine based CA-PEG Random copolymer.

Figure 2.23 $^1$H NMR of Quaternized Guanidine based CA-PEG Random copolymer.
2.4 Results and Discussion

Polymer design and synthesis

Guanidine functionalized cholic acid-based cationic homopolymers were prepared. First, (2-methacryloyloxy)ethyl cholate (MAEC) monomer was polymerized via reversible addition fragmentation transfer (RAFT) polymerization utilizing 4-cyano-4-(thiobenzylthio)pentanoic acid as a chain transfer agent and followed by postmodification to yield guanidine-containing polymers. Hydroxyl groups of these homopolymers were modified through an esterification reaction with bromohexanoyl chloride. After post modification, the peaks next to the alcohol group in $^1$H NMR at ~ 3.2 to 3.8 ppm shifted to 4.7 to 5.2 ppm (Figure 2.5). The methylene group next to the bromine group appears at ~ 3.4 to 3.6 ppm, indicating the formation of an ester linkage.

Then the bromine groups were substituted by 1,1,3,3-tetramethyl guanidine to offer guanidine-containing polymers. The successful synthesis was confirmed by the $^1$H NMR peaks of protons on -N(CH$_3$)$_2$ (δ 2.8 ppm, s) and -N(CH$_3$)$_2$ (δ 2.9 ppm, s) (Figure 2.7). Finally, quaternization was done by adding 1-bromo ethane. The appearance of an intense peak at ~ 3.0 ppm for all the methyl groups of guanidine and bromoethane and a peak at ~ 3.2 ppm for the methylene group of bromo ethane in $^1$H NMR (Figure 2.9) spectra confirmed the formation of quaternary guanidine-containing polymers. A series of different molecular weight polymers H1, H2, H3 and H4 (Mn = 7,000-12,000 g mol$^{-1}$) were further prepared (Table 2.1). The molecular weight of all four cholic-acid polymers was controlled with low dispersity as determined by Gel Permeation Chromatography (GPC).

To improve the solubility in water, a series of amphiphilic copolymers bearing hydrophilic PEG and facial amphiphilic bile acid derivatives were synthesized via
reversible addition−fragmentation chain-transfer (RAFT) polymerization by following a recently reported method. The random copolymer was made where linear poly (ethylene glycol) methyl ether (PEGMA, Mn = 500 Da) was chosen as a neutral hydrophilic block. Hydroxyl groups on the cholic acid (CA) moiety of the copolymers were functionalized to introduce Quaternized Guanidine groups in the copolymers via esterification with bromohexanoyl chloride, then substitution with 1,1,3,3-tetramethyl guanidine and quaternization with 1-bromo ethane (Figure 2.22). according to a reported procedure. To study the influence of hydrophilicity on the copolymer self-assembly, we prepared three cholic acid and PEG-based random copolymers R1, R2 and R3 (Table 2.1) with different fractions of PEG (20–40% molar ratio). The polymerization was well controlled with a molecular weight in the range of Mn = 9,000–18,000 Da and low dispersity (D = 1.19–1.6), as determined by gel permeation chromatography (GPC). The post-polymerization modification was confirmed by $^1$HNMR (Figure 2.19) the peaks at $\sim$3.6–3.8 ppm for $-\text{CH}$ on the tetracyclic rings, adjacent to the alcohol group, shifted to 4.7–5.2 ppm). The appearance of a peak at $\sim$3.5 ppm for the methylene group confirms the presence of PEG. The incorporation of guanidine was confirmed by the presence of a peak at $\sim$2.5 ppm for the protons on $-\text{N(CH}_3}_2$ (Figure 2.21).

In addition to the random copolymers, a diblock copolymer was also synthesized in order to improve the solubility of the polymers in water and the effect of polymer architecture on the antimicrobial activity. Linear PEG (M$n = 2000$ Da) was modified to PEG-based RAFT agent (PEG-CTP) and used to polymerize cholic acid-based methacrylate monomer (2-methacryloyloxy)ethyl cholate (MAECA), via reversible addition−fragmentation chain transfer (RAFT) polymerization and followed by
postmodification to yield guanidine functionalized copolymer D (Figure 2.16), according to a method recently reported.\textsuperscript{20}

**Table 2.1 Guanidine functionalized cholic acid-derived polymers characterized by GPC.**

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Cholic acid content (%)</th>
<th>PEG content</th>
<th>$M_n$ (g mol(^{-1}))</th>
<th>$D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>100</td>
<td></td>
<td>7,000</td>
<td>1.09</td>
</tr>
<tr>
<td>H2</td>
<td>100</td>
<td></td>
<td>7,200</td>
<td>1.10</td>
</tr>
<tr>
<td>H3</td>
<td>100</td>
<td></td>
<td>9,000</td>
<td>1.16</td>
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<tr>
<td>H4</td>
<td>100</td>
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<td>1.19</td>
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<tr>
<td>D</td>
<td>80</td>
<td>20</td>
<td>12,000</td>
<td>1.12</td>
</tr>
<tr>
<td>R1</td>
<td>80</td>
<td>20</td>
<td>12,500</td>
<td>1.19</td>
</tr>
<tr>
<td>R2</td>
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<td>30</td>
<td>9,000</td>
<td>1.21</td>
</tr>
<tr>
<td>R3</td>
<td>60</td>
<td>40</td>
<td>18,000</td>
<td>1.6</td>
</tr>
</tbody>
</table>

**Self-Assembly Behavior**

Recently, we showed that facial amphiphilicity of cholic acid moiety can induce the self-assembly of the amphiphilic polymers.\textsuperscript{19, 27} To investigate the self-assembly behavior of homopolymer, dynamic light scattering (DLS) experiment was performed in an aqueous solution of guanidine factionalized homopolymer. The hydrodynamic diameter ($D_h$) of H3 and H4 polymers are around 70 nm and 120 nm respectively, confirming that these copolymers formed aggregates in aqueous solution. The size of the aggregation increases with the molecular weight.
Then the morphology formed by different homopolymers in water was visualized by atomic force microscopy (AFM). The aqueous solution of polymers was incubated at 37 °C temperature for one day; then the solution was drop cast onto a silicon wafer and dried at room temperature for AFM imaging. A spherical morphology was observed from the H3 homopolymer solution and, rod-like nanostructures were formed from the H4 homopolymer (Figure 2.25). The average size of the spherical aggregates is around 200 nm and rods are around 220 nm length with 90 nm diameter. In the sphere, the hydrophobic moiety of cholic acid forms the core by hydrophobic integration and the quaternized guanidine stays on the corona.²⁸
AFM images were taken from an aqueous solution at a concentration of 5 mg/mL.

**Hemolysis Activities**

The hemolysis activity of cholic based guanidine functionalized cationic homopolymers was evaluated by measuring hemoglobin release from mouse red blood cells (RBCs) at various concentrations. The HC50, the concentration that causes 50% hemolysis of RBCs, is measured for H3 and H4 homopolymers. The results showed that the guanidine functionalized cholic acid homopolymers exhibit some toxicity. This is maybe due to the presence of a large four fused-ring structure and the methyl/ethyl groups in the guanidine derivative. To overcome these issues, neutral PEG was incorporated into the guanidine-based polymers to increase the hydrophilicity of bile acid-based copolymers. Interestingly, we observed that diblock copolymer (D) showed very low hemolysis activity (Figure 2.26). The HC50 value for diblock copolymer is >1000 µg/mL, demonstrating that the PEG can increase the biocompatibility by increasing the hydrophilicity of the guanidine-based diblock polymer.
**2.5 Conclusions**

In summary, we synthesized guanidine functionalized cholic acid based amphiphilic homopolymers, diblock, and random copolymers. These facial amphiphilic polymers can be a potential antimicrobial agent against both Gram-positive and Gram-negative bacteria. Guanidine functionalized homopolymers form spheres and rod-shaped aggregates. Incorporation of PEG improved the hemolysis activity of guanidine functionalized cholic acid polymers. Guanidine functionalized diblock copolymers showed better biocompatibility than homopolymers.

**Figure 2.26** Hemolysis activity of polymers measured by hemoglobin release from mouse RBCs at various concentrations.
2.6 References


CHAPTER 3

SYNTHESIS OF COBALTOCENIUM CONTAINING POLYMERS FOR GENE DELIVERY
3.1 Abstract

Gene therapy is an attractive therapeutic option in order to correct disease at the genetic level by replacing abnormal genes using exogenous DNA. Polymers are synthetic gene delivery vectors that possess the ability to deliver genetic material to target cells. Metal-containing polymers are excellent candidates for drug delivery due to their unique combination of organic and inorganic components in one macromolecular system. Cationic polymers neutralize the negative charge on plasmid DNA resulting in the formation of nanoscale polymer-DNA complexes (polyplexes). However, the use of cobaltocenium for gene delivery vehicles is still unexplored. We have synthesized cationic cobaltocenium based polymers with PEG which can be a potential candidate for gene delivery because they can actively bind with DNA by electrostatic interactions and are non-toxic to mammalian cells.

3.2 Introduction

Nucleic acid-based therapeutics has gained extensive attention over the past few decades due to their promising approach for the treatment of many untreatable, life-threatening and chronic diseases, ranging from genetic disorders to cancer.\(^1\) However, there are no potentially active nucleic acid loaded drugs realized clinically due to their delivery challenges.\(^2\) Viral and non-viral vectors are used to transport the nucleic acid-based therapeutics including plasmid DNA and small interfering RNA.\(^3\) Though viral gene delivery vectors are more operative for higher transfection efficiency, their medical applications are limited by immunogenicity, toxicity, and difficulties of large-scale production as well as high manufacturing costs.\(^4\) Therefore, our primary focus was to design the effective non-viral vectors that can
circumvent these problems for efficient gene transfection. These non-viral vectors need to prevent potential degradation of the polyplexes by serum endonucleases in the physiological fluids and the extracellular space needs to extravasate from the bloodstream to reach target tissues and facilitate cell entry via endocytosis.\textsuperscript{5} In endocytosis, vectors have to reach into the endocytic vesicles. Another critical challenge of the non-viral vectors is the endosomal escape for delivering nucleic acid therapeutics.\textsuperscript{6}

One of the promising non-viral vectors cationic polymers attracted much popularity due to their limited immunogenicity and straightforward synthesis, as well as their amenability to structural modification.\textsuperscript{7} Cationic polymer forming a complex with DNA through electrostatic interactions are called polyplex.\textsuperscript{8} Numerous cationic polymers have been explored so far, Poly(ethyleneimine) (PEI) is one of the efficient gene carriers due to their binding ability with DNA using their primary amine.\textsuperscript{9} Despite the tremendous success of PEI under \textit{in vitro} conditions, their \textit{in vivo} application was limited due to their cytotoxicity and instability of polyplex under the physiological environment.\textsuperscript{10} Most of the existing cationic polymers are cytotoxic and unstable under physiological conditions and cannot overcome the other barriers due to their weak interactions with DNA. Therefore, it is an urgent need to develop nontoxic cationic polymer vectors with a high binding affinity towards DNA for overcoming undesirable effects.

Metal-containing polymer has gained significant attraction due to its emerging biomedical applications.\textsuperscript{11} Metallopolymers especially cobaltocenium based polymers can be potential candidates for nucleic acid (DNA/RNA) delivery due to their less cytotoxicity, redox properties and charged state as well as their unique interaction with DNA/RNA.\textsuperscript{11}
Cobaltocenium cation can bind strongly to negatively charged DNA/RNA through electrostatic interactions.\(^{12}\)

Coating the surface of nanoparticles with polyethylene glycol (PEG), or “PEGylation”, is a commonly used method for improving the efficiency of drug and gene delivery to the target cells.\(^{13}\) Polyethylene glycol (PEG) is the most widely used “stealth” polymer in the drug delivery field, due to its long history of safety in humans.\(^{14}\) Due to its hydrophilic nature, PEG coat the surface of the therapeutic with an inert polymer which changes the positive surface to neutral surface and resist the interaction of polycation with the components of the bloodstream.\(^{15}\) Therefore, it increases colloidal stability and reduces the toxicity of cationic polymer.\(^{15}\) Herein, we have synthesized the cationic cobaltocenium based polymer with a certain amount of PEG to deliver the nucleic acid-based therapeutics.

3.3 Experimental Section

Materials

Cyclopentadiene (95%, Acros) dimer was distilled via a 30cm column to obtain cyclopentadiene unimer, Sodium (Sigma-aldrich), n-BuLi solution in hexane, 4-(dimethylamino)pyridine (99%, DMAP), 2-aminoethyl methacrylate hydrochloride (90%), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC-HCl, 98%), Tetrabutylammonium chloride (TBACl) salt was purchased from Sigma Aldrich and used directly. 2, 2-Azobisisobutyronitrile (AIBN), Cobalt (II) bromide (anhydrous, Alfa Aesar), Sodium hexafluorophosphate (98%, Oakwood Products) were used as received. Poly(ethylene glycol) methyl ether (Mn = 2000 Da), poly(ethylene glycol) methyl ether methacrylate (PEGMA, Mn = 500 Da) and 4-Cyano-4-(thiobenzylthio)pentanoic acid (CTP, 97%) was purchased from Strem Chemicals.
Characterization

Monomer and compound purity, and polymer conversion were monitored by 300 MHz proton nuclear magnetic resonance (1H NMR) spectroscopy using Bruker Avance III HD 300 spectrometer. Spectra were recorded in deuterated chloroform, deuterium oxide or dimethylsulfoxide solvent in ppm (\(\delta\)) with tetramethylsilane as an internal standard.

Synthesis of trimethylsilylethynylcobaltocenium hexafluorophosphate (TMSCoPF\(_6\))

Trimethylsilylethynylcobaltocenium hexafluorophosphate was prepared by following a recently reported method by our group.\(^{31}\) A 500 mL round-bottom flask was charged with a magnetic stirring bar and 200 ml of dry THF, 2.40 mL of (trimethylsilyl) acetylene (17 mmol) under protection from the air by a nitrogen atmosphere. The mixture was cooled using dry ice/acetone and 6.28 mL (15.6 mmol) of n-BuLi solution was added. It was let to stir for 50 minutes followed by the addition of 5 g of cobaltocenium hexafluorophosphate (14.9 mmol). The cobaltocenium hexafluorophosphate was synthesized according to the literature.\(^{16,17}\) The reaction mixture was slowly cooled to room temperature to let it react further. There was a color change from yellow to rusty red which is because of the nucleophilic addition. The solvent was removed and passed through an alumina column using hexane. After removing hexane in vacuo 3.5 g of solid cobaltocenium was obtained which was then further dissolved in 150 mL of dry DCM. 6.15 g of triphenylcarbenium hexafluorophosphate was added to the stirring solution and let it react in dark condition for 15 minutes. Then, the reaction mixture was filtered and washed with ether and water until filtrate came off clear. The obtained solid was dried in vacuum to give 60 % (5.08 g) overall yield.
Synthesis of carboxycobaltocenium hexafluorophosphate

Carboxycobaltocenium hexafluorophosphate was prepared by a recently reported method. For example, 2 g of TMSCoPF$_6$ (4.65 mmol) was dissolved in 90 mL of acetonitrile and sodium fluoride (4.65 mmol) was added. Then, an aqueous potassium permanganate solution (2.7 equivalent, 12.56 mmol in 150 mL water) was added into it. The reaction mixture was refluxed for 4 hrs at 80 °C. The resulting mixture was then filtered and washed with hot water. The filtrate was concentrated in a vacuum at 100 °C and let it cool down. Hexafluorophosphoric acid (1.3 equivalent, 6.05 mmol) was added and the product was allowed to crystallize overnight. Next, the obtained yellow powder was filtrated, washed with ice-cooled water and dried in vacuum to give 70 % yield (1.30 g).

Figure 3.2 Synthetic route of carboxycobaltocenium hexafluorophosphate.
Synthesis of 2-Cobaltoceniumamidoethyl methacrylate hexafluorophosphate (CoAEMAPF$_6$)

An amidation reaction was performed$^{31}$ to synthesize CoAEMAPF$_6$. 4g of cobaltoceniumcarboxylic acid hexafluorophosphate (1.0 equiv, 10.58 mmol), 2.28g of 2-aminoethyl methacrylate hydrochloride (1.3 equiv, 13.76 mmol), and 0.38 g of 4-(Dimethylamino) pyridine (0.3 equiv, 3.18 mmol) were dissolved in 40 mL dry DCM and the solution was cooled to 0°C. A solution of EDC-HCl (2.64 g, 1.3 equiv 13.76 mmol) was then slowly added into the previously cooled solution. Then, dry triethylamine (3.2, 2.5 equiv, 26.44 mmol) was immediately added into the reaction mixture and stirred for 4 hours at room temperature. The solution was then extracted by saturated sodium hexafluorophosphate aqueous solution three times to remove unreacted starting materials. The organic phase was condensed and precipitated into diethyl ether. 3 g of yellow solids were collected and dried under vacuum overnight.

![Chemical structure](image)

**Figure 3.3** Synthesis of CoAEMAPF$_6$ monomer.
Figure 3.4 $^1$H NMR of CoAEMAPF$_6$.

Synthesis of PEGylated Raft agent

4-Cyano-4-(thiobenzylthio)pentanoic acid (CTP) (100 mg, 0.358 mmol), Poly(ethylene glycol) methyl ether ( 644mg, 0.322 mmol) and DMAP ( 8.73mg, 0.07 mmol) were dissolved in dry DCM (4 mL) under nitrogen atmosphere. To this solution was added DCC (81.29 mg, 0.39 mmol) in 3 mL dry CH$_2$Cl$_2$ drop-wise under nitrogen. Then, the reaction mixture was stirred for 3 days at room temperature. The resulting solution was then filtered and precipitated in Diethyl Ether. The product was washed with Diethyl Ether and dried under vacuum. Final product was characterized by $^1$H NMR (Figure 3.6).
Synthesis of Cobaltocenium-PEGMA Diblock copolymer

Cobaltocenium monomer CoAEMAPF₆ and PEGMA were copolymerized using a typical RAFT polymerization process by using the predetermined ratios (e.g. [Monomer] : [AIBN] : [CTP] = 60: 0.3: 1). CoAEMAPF₆ (0.30 g, 0.613 mmol), PEGMA (0.205 g, 0.411 mmol), CTP (3.78 mg, 0.0171 mmol), and AIBN (0.56 mg, 3.43 µmol) were placed in a 10 mL Schlenk flask and dissolved in DMF (1 mL). The polymerization was started by heating the mixture at 90°C. Samples were taken out periodically to check the monomer conversion. Once the desired conversion was reached, the polymerization reaction was quenched by exposure to air. The reaction mixture was then precipitated in cold DCM three times and vacuum dry overnight.

Figure 3.6 ¹H NMR of PEGylated RAFT agent.
Figure 3.7 Synthesis of P (CoAEMAPF$_6$ and PEGMA) diblock copolymer.

Figure 3.8 $^1$H NMR of P (CoAEMAPF$_6$ and PEGMA) diblock copolymer.
Synthesis of P(PEG-PCoAEMAPF₆) diblock copolymer

Cobaltocenium monomer CoAEMAPF₆ monomer were polymerized using PEG-based RAFT agent by using the predetermined ratios (e.g. [Monomer] : [AIBN] : [CTP] = 20: 0.3: 1). CoAEMAPF₆ (0.30 g, 0.62 mmol), PEG-CTP (70.46 mg, 0.03 mmol), and azobisisobutyronitrile (AIBN) (1.52 mg, 0.0094 mmol) were placed in a 10 mL Schlenk flask and dissolved in N,N-dimethylformamide (DMF) (4 mL). The polymerization was started by heating the mixture at 90°C. Samples were taken out periodically to check the monomer conversion. Once the desired conversion was reached, the polymerization reaction was quenched by exposure to air. The reaction mixture was then precipitated in cold DCM three times and vacuum dry overnight. The final product was characterized by ¹H NMR (Figure 3.10).

Figure 3.9 Synthesis of P(PEG-PCoAEMAPF₆) diblock copolymer.
Ion-exchange of P(PEG-PCoAEMAPF$_6$) diblock copolymer

1 mL of PEGylated PCoAEMAPF$_6$ polymer solution (30 mg/mL in acetonitrile) was slowly added into 5 mL tetrabutylammonium chloride salt solution (40 mg/mL in acetonitrile) under vigorous stirring. After stirring for 3~5 minutes, the precipitated polymers were collected and washed by acetonitrile three times to remove PF$_6^-$ anions and excess tetrabutylammonium salts. The solid polymers were then vacuum-dried and collected.
3.4 Results and Discussion

Synthesis and Characterization

We have synthesized pure monosubstituted cobaltocenium monomer to prepare side-chain cobaltocenium containing polymers. The statistical reaction was done by using unsubstituted cobaltocenium and organolithium reagents were followed as shown in Figure 3.13. This method consists of facile nucleophilic addition and selective hydride abstraction of endo-proton followed by the oxidative cleavage by potassium permanganate with an overall yield of around 81%.
The monosubstituted cobaltocenium acid was reacted with 2-aminoethyl methacrylate hydrochloride in acetonitrile with EDC as a coupling agent, as shown in Figure 3.3. The protons of –CH2 groups at 3.5 ppm and 4.36 ppm and amide proton at 8.3 ppm showed the successful synthesis of monomer, as shown in Figure 3.4. The peaks at 5.67 ppm and 6.12 ppm corresponded to the vinyl protons of methacrylate monomer.

Methacrylate monomer of cobaltocenium (CoAEMAPF6) and Poly(ethylene glycol) methyl ether methacrylate was used to make block copolymer via reversible addition-fragmentation chain transfer (RAFT) polymerization (Figure 3.7). The ratio of [Monomer] / [RAFT] was adjusted to achieve the target molecular weight (15,000 g/mol). The polymerization was done by targeting 50% monomer conversion and the conversion was checked periodically by comparing the vinyl proton peaks with cobaltocenium peak. Compared to the 1H NMR spectrum of monomer, the vinyl protons disappeared, and a new broad peak showed up at 1.5 ppm in the 1H NMR (Figure 3.8). As cobaltocenium-containing polymers cannot be characterized by GPC due to the interaction between the cationic polymers and stationary phase of columns, end group analysis was used to confirm the final degree of polymerization.20. According to recent work by our group, cobaltocenium-containing polymers depend on the counterions.31 Polymers with different anions show different hydrophobicity such as polymer with PF6− and BPh4− are
hydrophobic and polymers with inorganic counterions like chloride, bromide are extremely hydrophilic. Thus, we attempted to synthesize water-soluble polymers through the ion exchange phenomenon so that we can use these polymers for biological applications. Tetrabutylammonium chloride (TBACl) was used to carry out the anion exchange. However, no precipitation was found when PCoAEMAPF$_6$-PEGMA solution in acetonitril was added into the salt solution. Therefore, we have tried another effective synthetic strategy to prepare linear PEG-based cobaltocenium block copolymer.

**Figure 3.14** Synthetic route of P(PEG-PCoAEMAPF$_6$) diblock copolymer.

PEG-based macro chain transfer agent was prepared by the esterification between 4-Cyano-4-(thiobenzylthio)pentanoic acid (CTP) and Poly(ethylene glycol) methyl ether (Mn= 2000 Da). This PEG-RAFT agent was used to polymerize methacrylate monomer of cobaltocenium (CoAEMAPF$_6$). The ratio of [Monomer]:[RAFT] was 20:1 to achieve
target molecular weight 10,000 g/mol. The polymerization was done by targeting 85% monomer conversion and the degree of polymerization was 20. Final molecular weight was 6,000 g/mol. The reaction was confirmed by the $^1$HNMR (Figure 3.10). In the $^1$H NMR the appearance of a new peak at around 3.5 ppm for the methylene group and a peak at 3.3 ppm for the methyl group of PEG-RAFT agent confirmed that the reaction was successful.

To make the polymer water-soluble we performed the ion exchange of P(PEG-PCoAEMAPF$_6$) diblock copolymer. Tetrabutylammonium chloride (TBACl) was used to carry out the anion exchange. P(PEG-PCoAEMAPF$_6$) diblock copolymer solution in acetonitrile was precipitated in the salt solution (10 times molar ratio of cobaltocenium moiety) under vigorous stirring. The precipitated polymer was washed three times with a salt solution and finally by acetonitrile to remove any left-over polymer. Fluorine NMR ($^{19}$F) (Figure 3.12) confirmed the complete ion-exchange. After the ion-exchange, the resulting polymer was not soluble in acetonitrile but soluble in water.

3.5 Conclusions

In summary, we have synthesized linear PEG-based cobaltocenium block copolymer after preparing the PEG-based macro chain transfer agent which can be a stable nanoparticle complex with DNA/RNA. We have also synthesized cobaltocenium monomer and Poly (ethylene glycol) methyl ether methacrylate-based cationic polymer through ion exchange procedure that needs to be improved for this synthetic strategy.
3.6 References


CHAPTER 4

SUMMARY AND OUTLOOK
4.1 Dissertation Summary

There were two major goals of this dissertation. First, to develop a novel antimicrobial polymer with repeat units possessing local facial amphiphilicity from a multicyclic natural product such as cholic acid. Guanidine functional group was incorporated into facial amphiphilic cholic acid-based macromolecular architectures to synthesize different polymer architectures such as diblock and random copolymers, which can be a potent anti-microbial material of their selective interactions with negatively charged bacterial cell membranes. In this approach, a neutral polyethylene glycol (PEG) component was chosen to prepare polymer nanoparticle with cholic acid to study the effect of cationic and hydrophobic functionalities on antimicrobial polymers over their selectivity between bacteria and a mammalian cell. The hemolysis data showed that PEG can increase the biocompatibility by increasing the hydrophilicity of the guanidine-based diblock polymer.

The second goal was to prepare non-toxic gene delivery vectors by copolymerizing the cobaltocenium and PEG that can form a safe and stable polyplex with DNA or RNA. Linear PEG was incorporated into the cationic cobaltocenium by preparing a PEG-based macro-RAFT agent which can condense DNA through electrostatic interaction to build the complex (called polyplex) core. Cationic cobaltocenium was polymerized with a certain amount of PEG so that the PEG segment can surround the polyplex like a corona and prevent the nanoparticles from precipitation by increasing the colloidal stability and reduce the adsorption of serum.
4.2 Future Work

Antimicrobial biomaterial development using hydrocarbon-rich biomass such as rosin and bile acids has more avenues for future developments to make them more effective antimicrobial agents with low toxicity. Guanidine-based polymers, as one of the antimicrobial polycations, show excellent antimicrobial properties, which stem from the special structure of guanidine. Guanidine is protonated and positively charged under physiological conditions. The guanidine derivative with the multicyclic natural product (cholic acid) derived facial amphiphilicity by post polymerization modification has been developed as a potent antimicrobial agent. New molecular biomass with true facial amphiphilicity can be explored in the future. Only guanidine-based polymers can be synthesized by the conversion of guanidine in the side chain with bile acid polymers or by the direct polymerization of guanidine-functionalized monomers. The antimicrobial activity of all newly synthesized polymer will also be evaluated.

To improve transfection efficacy by controlling polymer topology and polycation contents bioreducible disulfide bonds will be inserted from cystamine into the cobaltocenium based polymer. A couple of simple amidification reactions will yield cystamine containing a macro raft agent. The pEG-based macro chain transfer agent will also be prepared by coupling reaction from methoxy polyethylene glycol amine and 4-cyanopentanoic acid dithiobenzoate (CTA). PEG-CTA will be used to polymerize 2-cobaltocenium amidoethyl methacrylate hexafluorophosphate (CoAEMAPF$_6$) in the presence of AIBN. Cobaltocenium based homopolymers are almost non-toxic. Hemolysis study will be conducted to evaluate the cytotoxicity of the cobaltocenium containing copolymers. The in vitro gene delivery efficiency of cobaltocenium based polyplexes will
also be tested in different cancer cell lines (HeLa and KB cervical carcinomas, A549 and HCC1299 lung carcinoma, and Z310 choroidal epithelial cells) using a luciferase reporter plasmid.