DEVELOPMENT OF MULTIFUNCTIONAL AND ELECTRICAL CONDUCTING CARBOXYBETAINE BASED POLYMERS

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DEVELOPMENT OF MULTIFUNCTIONAL AND ELECTRICAL CONDUCTING CARBOXYBETAINE BASED POLYMERS

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ABSTRACT

This dissertation describes the design and development of three novel multifunctional carboxybetaine (CB) based zwitterionic polymer platforms, which integrate superior antifouling property, enhanced mechanical property, switchable antimicrobial property and electrical conductivity.

In the first zwitterionic biomaterial platform, the switchable multifunctional polymers with integrated antimicrobial and antifouling properties were developed. The side chains of hydroxyethyl functionalized CB derivatives could reversely switch between a cationic ring form and a zwitterionic form by changing acidic and basic conditions. In the ring form, cationic polymeric surfaces could catch and kill the attached bacteria; in the zwitterionic form, the surfaces released killed bacterial cells, and resisted protein adsorption and bacterial attachment. The hydrogels of hydroxyethyl functionalized CB derivatives also showed dramatically improved mechanical properties, which are highly desired for biomedical applications. In a follow-up work, a systematic study was carried out to understand how the structure of zwitterionic materials affected their elasticity, switchability, stability and antifouling properties. This study provides a general design guideline for the development of new zwitterionic materials in many biomedical applications.
In the second zwitterionic biomaterial platform, a facile one-pot zwitteration method was developed to synthesize biodegradable zwitterionic polysaccharides. Dextran was selected as a model polymer. Cell attachment and protein adsorption studies were carried out on hydrogels made of dextran with various degrees of the CB substitution. It was demonstrated that the zwitteration with CB groups endows dextran superior antifouling property, switchability and enhanced optical transparency. This work has shed light on ingenious designing of zwitterionic material and provided a new avenue of generating high performance multifunctional polysaccharides.

In the last zwitterionic biomaterial platform, zwitterionic conjugated polymers, which contain conducting polythiophene backbones and multifunctional side chains, were developed. Cell attachment study showed that a controllable antifouling property could be simply achieved through grafting specific peptides onto the readily available reactive sites. Zwitterionic materials gained electrical conductivity and optical properties through the conjugated polymer backbone, and the non-biocompatible conjugated polymer obtained excellent antifouling properties, enhanced electrical conductivity, functional groups of bioconjugation and response to environmental stimuli via multifunctional zwitterionic side chains. This platform can potentially be adapted to a wide range of applications, which require high performance conducting materials with excellent antifouling/biocompatibility at biointerfaces.
DEDICATION

To my parents: Cao, Shaoqin and Ren, Xijiao

To my wife: Li, Linlin
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CHAPTER I
INTRODUCTION

Zwitterionic polymers represent a unique subcategory of polyampholytes, which bearing equivalent cationic and anionic charges on the same repeating unit. Over the past decade, zwitterionic polymers have attracted considerable attention in the field of biomaterials due to the outstanding antifouling properties, ease of functionalization and design flexibility.[1] The superior antifouling properties of zwitterionic polymers in resisting undiluted human plasma and serum[2-6] were attributed to their strong interaction with water via ionic solvation, in contrast to that of polyethylene glycol (PEG), which relies on hydrogen bond to bind water.[7] Within the class of zwitterionic materials, polycarboxybetaine (pCB),[8-10] polysulfobetaine (pSB)[11, 12] and poly(methacryloyloxyethyl phosphorylcholine) (pMPC)[13-15] are three types of the most widely investigated zwitterionic polymers. pCBs have drawn my special interests for their several advantages over other zwitterionic materials, including versatile structural options,[8, 9] simple synthetic approaches,[16] capability of further functionalization,[8, 17-19] ultra-low protein fouling and biofouling levels[20, 21] and good biocompatibility.[22-25] This dissertation will mainly focus on the design and development of novel pCB based material platforms, aiming to solve several challenges and improve some intrinsic weakness that associated with zwitterionic pCB materials.
Currently, one of the major challenges for many biomaterial researchers is to maintain a controllable bio-interface, promote specific binding and resist nonspecific binding of protein and cells, thus minimize biofouling and potential infections. Many successful examples of PEGs, zwitterionic polymers and their derivatives have been developed to achieve this goal, but the development of mono-functional antifouling material has reached a bottle-neck. Now new material platforms are urgently needed to address the intrinsic drawbacks and improve the needed properties of the biomaterials (such as mechanical property, degradability, biocompatibility, etc.), while realizing many other functions beyond antifouling property, including antimicrobial properties, ionic conductivity, electrical conductivity, etc. This dissertation is focused on the design and development of three novel pCB based zwitterionic biomaterial platforms: switchable antifouling and antimicrobial pCB with enhanced mechanical property, biodegradable polysaccharide based pCB with improved optical transparency, electrically and ionically conducting pCB with controllable antifouling property.

1.1 Development of switchable antifouling and antimicrobial hydrogel

Zwitterionic material-based hydrogels have attracted notable attention due to their ultra-low-fouling property, good biocompatibility and high water content;[25, 26] however, the potential biomedical applications of zwitterionic hydrogels are limited by their low mechanical strength.[27, 28] Several approaches have been proposed to improve mechanical properties of zwitterionic materials. One commonly used method is to add a second component via either copolymerizing zwitterionic monomer(s) with non-ionic monomer(s), such as hydroxyethyl methacrylate (HEMA)[29, 30] or blending
zwitterionic polymers with other (nano)composites, such as clay;[31] however, resulting products will no longer be pure zwitterionic materials and their antifouling properties might be compromised as the increase of introduced non-zwitterionic moieties with lower antifouling properties. Another strategy to enhance mechanical properties of zwitterionic hydrogel is to control the ratio of zwitterionic crosslinker content[32-34] or to create a physical-chemical double network[27] by pre-mixing linear zwitterionic polymer solution with a certain concentration of zwitterionic monomers and then polymerizing monomers with the existence of polymers. The hydrogel networks are consolidated with both high chemical crosslinking density and interpenetrating liner polymer physical crosslinking networks. As a result, the compression modulus of hydrogels was significantly improved.[34, 35] However, the water content of hydrogels was relatively low and their elasticity was not improved with this method. It should be noted that the composition of materials made of fewer ingredients can be more accurately controlled during the processing and can provide more constant results for each application.

It is known that the positively charged surface can disrupt the integrity of negatively charged cell membranes in microorganisms, which will lead to cell death.[36-38] However, cationic materials show poor biocompatibility due to the permanent charge and high protein adsorption.[39] Cationic surfaces can kill attached bacterial cells, but killed cells and their debris remaining on the surface can trigger the inflammation.[10] To address these challenges, several switchable antimicrobial and antifouling materials have been developed, including hydrolysable[40, 41] [42] and UV-cleavable cationic CB-ester.[43] They were able to catch and kill bacterial cells in their cationic CB-ester form and then switch to a zwitterionic antifouling surface to release killed bacterial cells upon
hydrolysis or UV irradiation. The resulting zwitterionic surface can further prevent the adsorption of proteins, the attachment of cells and the formation of biofilm on the surface. However, all these example are all one-way process and not reversible.

So it is highly desired to have a hydrogel material bearing the excellent antifouling property/biocompatibility to prolong the lifetime of implanted materials, reversely switchable antimicrobial property to eliminate infection and inflammation and good mechanical property to avoid the failure of the implanted material. My approach relies on the re-design of material structure at the molecular level: I hypothesize to enhance the overall mechanical properties of zwitterionic hydrogels by introducing additional functional groups thus improving the hydrogen bonding interactions within the polymer networks.

1.2 Understanding of structure-function-property relationships of novel zwitterionic CB

Zwitterionic type polymers have been extensively explored in recent years as a new generation of biomaterials.[44-47] However, most of the studies are application oriented. In order to solve the intrinsic drawbacks associated with conventional zwitterionic polymers and advance the material development to a new stage, a systematic structure-function-property relationships study of this type of materials is urgently needed. For example, several pCB derivatives (Scheme 1) have been developed with the variations in, 1) polymer backbones of (meth)acrylate[8, 10, 23] or (meth)acrylamide,[9, 21, 48] 2) different number (n) of carbon spacers between carboxylate and quaternary ammonium groups.[9, 49-53] The molecular structure not only determines the polymerization condition but also affect other properties of the materials. It was found
that although the increase of the chain length between positive and negative charges resulted in an increase of the hydrophilicity of the material,[9] only pCBs with one and two carbon spacers can effectively resist non-specific protein adsorption from undiluted blood serum.[50] pCBs with three or more carbon spacers do not perform well in resisting protein adsorption. In another study from Carr and co-workers, hydrogels made from monomers with a vinylimidazole backbone showed significantly improved mechanical properties than the one made from monomers with a methacrylate backbone.[54]

Scheme 1. Structure of zwitterionic CB derivatives

I propose to investigate the impact of structures on pCB materials’ mechanical property, functionality and stability, since the stability of zwitterionic materials is equally important as other desired properties and is rarely investigated. A better understanding of structure-function-property relationships is critical to design more advanced materials with all desired properties.
1.3 Development of facile zwitteration of natural polysaccharide with CB.

In order to effectively prevent the nonspecific protein adsorption and minimize biofoulings at the interfaces, development of novel zwitterionic polymers with both good biocompatibility and degradability is one of the major challenges. Most conventional zwitterionic polymers, synthesized with a methacrylate or methacrylamide backbone, are not degradable so that their uses are limited especially for \textit{in vivo} applications. Biodegradable materials, that can be broken down and excreted out after certain function has been served, are highly desired for application such as surgical sutures and implants.

Several approaches have been proposed to address this issue. Dr. Jiang and co-workers developed an antifouling and biodegradable zwitterionic polypeptide,[55] but the solid-phase synthesis technique that requires repeated protection and de-protection steps would be very challenging. In another work, a degradable reduction-sensitive disulfide crosslinker was introduced in zwitterionic polymer based nanogel and hydrogels, for controllable drug delivery[56] and cell encapsulation,[57] respectively. A few more degradable zwitterionic derivatives were developed attempting to reach the same goal, including the pH-responsive polypeptide synthesized from amidation of poly (α,β-l-aspartic acid),[58] and zwitterionic chitosan derivatives from amidation of chitosan with succinic anhydride.[59] However, polypeptide synthesis is not quite convenient for further functionalization, while chitosan has limited solubility at neutral conditions.

In this study, dextran is of special interest because it is a type of polysaccharides and one of the most abundant and commonly used natural polymers in many biotech and biomedical applications, including coatings,[60, 61] biosensing,[62, 63] tissue engineering,[64-66] drug delivery,[67-69] bioseparation/purification.[70, 71] However,
there are several challenges to be addressed to let the potential of polysaccharide materials be fully realized in biotech and biomedical applications. Firstly, their antifouling properties are unsatisfactory in applications that deal with the complex medium. For example, dextran-derivative antifouling surface[72] used in biosensing is not effective in resisting protein fouling from blood sample.[73] Secondly, natural polysaccharides do not carry both antifouling property and functionality to conjugate other moieties (such as capture ligand and cell adhesion molecule), which are needed in affinity bioseparation, biosensing, tissue engineering and drug delivery. Functional groups such as tetrazole [74] and carboxylate [75] groups have to be incorporated into polysaccharides. Excessive unreacted functional groups cause non-specific protein adsorption, thus either reducing the sensitivity of the biosensor or leading to low purity in bioseparation. Thirdly, natural polysaccharides can resist bacterial attachment and but cannot kill a small amount of attached microbes.[76, 77] Microorganisms can be introduced into patients during surgical procedures, and colonized microorganisms on the surface of the implanted material/device will trigger inflammation and immune response.[78] Therefore, I hypothesize to develop a zwitterionic dextran integrating all desired properties including excellent antifouling property to prolong the lifetime of implanted materials, antimicrobial property to eliminate surgical infection and chronic inflammation, and good mechanical properties/stability to avoid the structure failure of the implanted material.
1.4 Development of novel antifouling and electrical conducting pCB

Conjugated polymers have been widely used in organic electronic and bioelectronics as the key component for their many advantages over their inorganic counterparts, including structure flexibility, tunable functionality and good biocompatibility and stability. It was found that conjugated polymers could improve communications between electrochemical devices and biological systems;[79-81] however, conjugated polymers, such as polyacetylene (PA),[82] polyaniline (PANI),[83] polypyrrole (PPy),[84] polythiophene (PTH) and poly(3,4-thylenedioxythiophene) (PEDOT),[85] are originally designed for complex biological applications. These conjugated polymers consist of hydrophobic or charged side chains. Biomacromolecules, proteins and lipids, tend to adsorb on charged or hydrophobic surfaces. The adsorption of proteins will reduce the sensitivity or lead to the failure of the embedded device.[86, 87] To increase their biocompatibility, PTH,[88] PANi[89] and PPy[90] hydrogels have been developed to combine the electrical properties from conjugated polymers with the properties of hydrogels.[91] Conducting hydrogels are typically generated through blending or physically crosslinking CPs with non-conducting polymers. For example, metal ions (Fe$^{3+}$ or Mg$^{2+}$) were used to crosslink PEDOT-polystyrene sulfonate (PSS) with non-conducting polyvinylpyrrolidone.[92] Polyethylene glycol (PEG) was used to crosslink PANi for glucose sensing.[93] However, non-conducting components compromise electrochemical properties of conducting hydrogels.[94] Physically crosslinked hydrogels are less stable[95] and excessive metal ions reduce their biocompatibility.[94] Furthermore, non-conducting components of current conducting hydrogels are not effective enough to prevent biofouling and foreign body response.
Embedded conducting hydrogels require the integration of bioactive molecules (such as growth factor,[96] cell adhesion protein[97, 98] and anti-inflammatory drug[99, 100]) with the hydrogel to promote the formation of a stable interface prior to the growth of scar tissue. Hydrogels should provide functional groups for the conjugation of biomolecules under mild conditions. In existing CPs, excessive unreacted functional groups impair the biocompatibility and sensing sensitivity due to the nonspecific adsorption of biomacromolecules. Hydrophobic or charged components in current conducting hydrogels may cause the denaturation of biomolecules and reduce the service life of the device. Moreover, the long spatial distance between sensing biomolecules and CPs also significantly limit electron transfer. Hence, a big challenge remains to synthesize conjugated polymers and hydrogels with good biocompatibility, functionality, facile processability and tunable electrochemical activity.[94]

In this chapter, a novel biomaterial platform consisting of the conjugated polymer backbone and multifunctional zwitterionic side chains is proposed. It is hypothesized that zwitterionic materials will gain electrical conductivity and interesting optical properties through conjugated polymer backbones, and non-biocompatible conjugated polymers will obtain excellent antifouling properties, enhanced electrical conductivity, functional groups of bioconjugation and response to environmental stimuli via multifunctional zwitterionic side chains. The integrated zwitterionic conducting polymers with good biocompatibility and controlled antifouling properties will be promising candidates for the next generation bioactive materials.
1.5 Overview of the dissertation

In this dissertation, I am proposing to solve several challenges and improve some intrinsic weaknesses that are associated with zwitterionic pCB materials. The first challenge has been their unsatisfactory mechanical properties. Instead of following the commonly used method of adding a second component or blending with other (nano)composites, I propose to enhance the mechanical property with a single component material by re-designing its structure at the molecular level. In Chapter II, to address the unsatisfactory mechanical properties of pCB materials, switchable hydrogels were developed by introducing hydroxyethyl groups into CB monomers. I studied the mechanical properties, switchable ring formation properties, antifouling and antimicrobial properties. The new pCB hydrogels showed a dramatically improved mechanical properties. Under acidic conditions, pCB hydrogels undergo self-cyclization and can catch and kill bacteria. Under neutral/basic conditions, hydrogels undergo ring-opening process and can release killed bacterial cells, and resist protein adsorption and bacterial attachment. In Chapter III, a more advanced all-in-one pCB material was developed by replacing methyl group with hydroxyethyl on the quaternary ammonium and substituting methacrylate backbone with methacrylamide backbone. The effect of carbon spacer length on elasticity, switchability and stability were systematically studied. The material with one carbon spacer shows higher elasticity, switchability and stability. In Chapter IV, a facile method for zwitteration of dextran with CB was developed. The zwitterionic CB-dextran integrated superior antifouling properties, switchability between zwitterionic and cationic forms, as well as enhanced optical transparency into one polysaccharide material. Their properties can also be fine-tuned by adjusting the CB
substitution ratio. This study provides a new avenue for generating multifunctional zwitterionic CB materials in situ. In Chapter V, to improve the antifouling property and biocompatibility of conventional conjugated polymers, I have proposed a new platform that consists of a conjugated polymer backbone and zwitterionic side chains. A series of poly(carboxybetaine thiophene) (pCBTh) based zwitterionic conjugated polymers, with different type of functional side chains, were developed. Zwitterionic materials gain electrical conductivity and interesting optical properties through conjugated polymer backbones, and non-biocompatible conjugated polymers obtain excellent antifouling properties, enhanced electrical conductivity, functional groups of bioconjugation and response to environmental stimuli via multifunctional side chains. I believe my studies that stated above will dramatically broaden the application spectrum of zwitterionic pCB materials.
CHAPTER II
SWITCHABLE ANTIMICROBIAL AND ANTIFOULING HYDROGELS WITH ENHANCED MECHANICAL PROPERTIES

2.1 Introduction

Fouling is an undesired process in which molecules or living organisms from environment attach and accumulate onto a surface.[101] This phenomenon has significant impact on a variety of fields, including implantable sensors, biomedical devices, food processing and marine industries.[102-104] For example, undesired surface adsorption of biomacromolecules can cause the failure of biomedical devices.[105] Thus, materials with superior anti-fouling properties are urgently needed. In recent years, there is an extensive study of zwitterionic materials, such as pCB, pSB and pMPC, being used as anti-fouling platforms.[106-109] Compared with poly(ethylene glycol) (PEG), the ease of further functionalization, as well as superior anti-fouling properties[110] makes them more promising candidates for many biomedical applications. These materials have been proven to effectively reduce bacterial attachment, biofilm formation and highly resist nonspecific protein adsorption even from undiluted blood plasma.[106] Zwitterionic coatings can reduce initial attachment and delay biofilm formation on surfaces, but they are not able to kill attached microorganisms. It is possible to introduce pathogenic microbes into the patient during implantation operations and catheter insertions which
cause the failure of implanted devices; however, it will be necessary to use antimicrobial agents to eliminate these microbes. Surface-responsive materials with antimicrobial properties have been developed well for a broad spectrum of applications,[111] but it is still a great challenge to develop materials bearing antimicrobial, biocompatibility and anti-fouling capabilities. To address this challenge, previously I synthesized a cationic derivative of pCBMA. The surface coated with the cationic derivative of pCBMA is able to catch and kill bacterial cells, switch to a zwitterionic non-fouling surface, and release killed bacterial cells upon its hydrolysis.[112] However, this material can only switch once from an antimicrobial state to an anti-fouling state and the process is not reversible. The alcohol leaving groups also may not be suitable for applications, which require no small molecule to be leaked out. Therefore, a material that can reversibly switch between an antifouling surface and an antimicrobial surface is highly desired.

Hydrogels, which can trap water molecules inside their three-dimensional network, have been widely used as wound dressings,[113] drug delivery carriers,[114] tissue engineering scaffolds[115, 116] and coatings for implantable biosensors.[117] Zwitterionic material-based hydrogels have attracted notable attention due to their ultralow fouling property, good biocompatibility and high water content;[118, 119] however, the potential biomedical applications of zwitterionic hydrogels are limited by their low mechanical strength.[120, 121] Although the mechanical property can be improved through the blending[121] or co-polymerization[119] of the zwitterionic monomers with other materials, such as 2-hydroxyethyl methacrylate (HEMA) and N-isopropylacrylamide (NIPAm), anti-fouling properties are compromised in many cases. It should be noted that the composition of materials made of fewer ingredients can be more
accurately controlled during processing and can provide more consistent results for each application.

It is highly desired to have a hydrogel material bearing excellent anti-fouling property and biocompatibility to prolong the lifetime of implanted materials, switchable antimicrobial property to eliminate infection and inflammation and good mechanical property to avoid the failure of the implanted material. I hypothesize derivatives of zwitteronic CB with hydroxyl group(s) can switch between the lactone form (antimicrobial) and the zwitterionic form (anti-fouling) and that the intramolecular hydrogen bonds will enhance the mechanical property of the zwitterionic hydrogel. To test our hypotheses, I developed two novel zwitterionic materials, poly(2-((2-hydroxyethyl)(2-(methacryloyloxy)ethyl)(methyl) ammonio)acetate) (pCBOH1) and poly(2-(bis(2-hydroxyethyl)(2-(methacryloyloxy)ethyl) ammonio)acetate) (pCBOH2). Under neutral or basic condition, these materials are in zwitterionic forms with ultralow-fouling property; under acidic conditions, they will automatically convert into cationic charged forms, which have antimicrobial ability. Bacteria can be trapped and killed through contact, then released under neutral, or basic environment. This process is reversible by simply changing the acidic/basic environment of the medium. Their ultra-antifouling property was tested by a surface plasmon resonance (SPR) sensor and the switchable ability was tested on the hydrogel surface. To the best of our knowledge, such switchable antimicrobial, anti-fouling and mechanically enhanced hydrogel has never been achieved.
2.2 Experimental methods

All reagents and chemicals were purchased from Alfa and Sigma-Aldrich and used without further purification. All solvents used for reaction here were purchased from Acros. Column chromatography was carried out on flash silica gel obtained from Sigma. Carboxybetaine dimethacrylate (crosslinker) was synthesized following a published procedure.[122] Mercaptoundecyl bromoisobutyrate was synthesized by the reaction of bromoisobutyryl bromide and 11-mercapto-1-undecanol using a published method.[123]

2.2.1 Synthetic procedures and instrumentation

All NMR experiments were performed at 303.2 K unless stated otherwise and on Varian 300-, 500-, and 750-MHz spectrometers. The $^1$H and $^{13}$C NMR experiments were performed on Varian Mercury 300 MHz spectrometer. The $^1$H{$^{13}$C} gHMBC NMR spectra of CBOH1 and CBOH2 were collected on a Varian VNMRS 500 MHz spectrometer. Each NMR sample was prepared by dissolving 30 mg of product in about 700 μL of the chloroform-d or deuterium oxide solvent contained in 5 mm NMR sample tubes. Quantitative $^1$H NMR spectra were collected with 3 s acquisition time, 5 to 10 s relaxation delay, 32 transients and 90° pulse width of 22.9 μs. The data was zero-filled 131 k points, exponentially weighted with a line broadening of 0.5 Hz and Fourier transformed. Qualitative $^{13}$C NMR spectra were collected with $^1$H decoupling, 1.3 s acquisition time, 1 s relaxation delay, 512 to 1024 transients and 90° pulse width of 10.2 μs. The data was zero-filled 131 k points, exponentially weighted with a line broadening of 1.5 Hz and Fourier transformed. The $^1$H{$^{13}$C} gHMBC spectra were obtained with 90° pulse widths for $^1$H and $^{13}$C of 9.0 μs and 8.1 μs, respectively, a 1.0 s relaxation delay, Δ
= 1.8 ms (based on \(^1J_{CH} = 140\ Hz\) and \(^8J_{CH} = 8\ Hz\)) and a 0.128 s acquisition time. 8 transients were averaged for each of 512 increments during t1. The spectral width in the f1 and f2 dimensions are 23.8k Hz and 4k Hz, respectively. The experiment time was ca. 50 min. Data were zero filled to a 2 k x 4 k matrix, and weighted with a Gaussian function before Fourier transformation. Quantitative \(^1\)H NMR spectra were collected with 3 s acquisition time, 5 s relaxation delay, 32 transients and 90° pulse width of 22.9 \(\mu\)s. The data was zero-filled 256 k points, exponentially weighted with a line broadening of 0.5 Hz and Fourier transformed.

2.2.1.1 Synthesis of 2-((2-hydroxyethyl)(methyl)amino)ethyl methacrylate (compound 2a).

20 mL (174 mmole) of N-methyl diethanol amine, 120 mL of anhydrous tetrahydrofuran (THF) and 60 g (566 mmole) of anhydrous sodium carbonate powder were added to a 500 mL three-neck round bottom flask. The mixture was cooled down to 0 °C with an ice-bath. 17 mL (174 mmole) of methacryloyl chloride (diluted with 30 mL of anhydrous THF) was added dropwise. Then the ice-bath was removed, and the reaction was stirred at room temperature overnight. After the reaction was completed, the reaction solution was filtered. The solvent in the filtrate was removed with a rotary evaporator, and the residue was dried with a vacuum pump fitted with a liquid nitrogen cold trap. The product was further purified by silica gel column chromatography (ethyl acetate/hexane, 1/1 (v/v)). Pure product was obtained as a colorless liquid. (Yield: 53%).\(^1\)H NMR (300 MHz, CDCl3) δ6.12 (s, 1H), 5.58 (s, 1H), 4.26 (t, \(J = 5.6\ Hz\), 2H), 3.58 (t, \(J = 5.3\ Hz\) 2H), 2.77 (t, \(J = 5.7\ Hz\), 2H), 2.62 (t, \(J = 5.4\ Hz\), 2H), 2.35 (s, 3H),
1.95 (s, 3H) 1 hydroxy peak not seen due to overlapping signals. $^{13}$C NMR (75 MHz, CDCl$_3$) δ167.61, 136.38, 125.96, 62.50, 59.06, 58.50, 55.94, 42.16, 18.53.

2.2.1.2 Synthesis of 2-(tert-butoxy)-N-(2-hydroxyethyl)-N-(2-(methacryloyloxy)ethyl)-N-methyl-2-oxoethanaminium bromide (compound 3a).

17.1 g (79 mmole) of compound 2a was dissolved in 150 mL of acetonitrile in a nitrogen filled flask, followed by adding 14 mL (95 mmol) of t-buty1 bromoacetate. The mixture was stirred at 60 °C for 2 days. After solvent removal with a rotary evaporator, the residue was precipitated in diethyl ether and dried under vacuum to obtain a white solid with quantitative yield. $^1$H NMR (300 MHz, CDCl$_3$) δ6.06 (s, 1H), 5.58 (s, 1H), 4.92 (s, 1H), 4.63 (m, 2H), 4.56 (s, 2H), 4.34 (m, 2H), 4.06 (m, 2H), 4.00 (m, 2H), 3.58 (s, 3H), 1.87 (s, 3H), 1.41 (s, 9H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ166.30, 163.62, 135.19, 127.32, 85.45, 64.87, 62.21, 61.19, 58.18, 55.61, 50.84, 28.02, 18.31.

2.2.1.3 Synthesis of 2-((2-hydroxyethyl)(2-(methacryloyloxy)ethyl)(methyl)ammonio) acetate (compound 4a: CBOH1).

3 g of compound 3a was dissolved in 6 mL of trifluoroacetic acid (TFA) and 6 mL of dichloromethane for 1.5 hours at room temperature to remove the tert-butyl group. The mixture was precipitated in diethyl ether and dried under vacuum. The product was redissolved in acetonitrile, neutralized over an ion exchange resin (IRA-400 OH form), and further purified by silica gel column chromatography (Ethyl Acetate/Methanol 1/1 v/v). (Yield: 86%). $^1$H NMR (300 MHz, D$_2$O) δ6.21 (s, 1H), 5.83 (s, 1H), 4.70 (m, 2H), 4.31 (m, 2H), 3.91 (m, 2H), 4.11 (m, 4H), 3.40 (s, 3H), 1.99 (s, 3H). $^{13}$C NMR (75 MHz,
D$_2$O) δ168.77, 168.58, 135.11, 127.68, 64.35, 62.32, 61.00, 58.53, 55.31, 50.10, 17.24 (see Figure 1). $^{13}$C NMR spectrum of CBOH1 is shown in Figure 2.

2.2.1.4 Synthesis of 2-(bis(2-hydroxyethyl)amino)ethyl methacrylate (compound 2b).

Compound 2b was synthesized following a similar procedure to that for compound 2a. $^1$H NMR (300 MHz, CDCl$_3$) δ6.13 (s, 1H), 5.59 (s, 1H), 4.27 (t, J = 5.6 Hz, 2H), 3.62 (t, J = 5.3 Hz, 4H), 2.89 (t, J = 5.6 Hz, 2H), 2.75 (t, J = 5.3 Hz, 4H), 1.95 (s, 3H) 1 hydroxy peak not seen due to overlapping signals. $^{13}$C NMR (75 MHz, CDCl$_3$) δ167.82, 136.32, 126.14, 62.83, 59.93, 56.89, 53.72, 18.48.

2.2.1.5 Synthesis of 2-(bis(2-hydroxyethyl)(2-(methacryloyloxy)ethyl)ammonio)acetate (compound 4b: CBOH2).

Compound 3b and 4b were synthesized following similar procedures to that for compound 3a and 4a. The crude product from the second step was not purified and directly used for the next step. $^1$H NMR (300 MHz, D$_2$O) δ6.21 (s, 1H), 5.83 (s, 1H), 4.70 (m, 2H), 4.31 (m, 2H), 4.16 (s, 2H), 4.10 (m, 4H), 4.01 (m, 4H), 1.99 (s, 3H). $^{13}$C NMR (75 MHz, D$_2$O) δ168.74, 168.58, 135.10, 127.68, 61.37, 60.49, 58.82, 58.50, 55.11, 17.25 (see Figure 3). $^{13}$C NMR spectrum of CBOH2 is shown in Figure 4.
Figure 1. $^1$H NMR of CBOH1 spectrum at 300 MHz, D$_2$O.

Figure 2. $^{13}$C NMR of CBOH1 spectrum at 75 MHz, D$_2$O.
Figure 3. $^1$H NMR spectrum of CBOH2 at 300 MHz, D$_2$O

Figure 4. $^{13}$C NMR of CBOH1 spectrum at 75 MHz, D$_2$O.
si-ATRP: The polymer brushes were synthesized via surface initiated ATRP. Gold-coated sensor chips were treated followed the previous reported procedures.[112] The initiator SAMs were formed by soaking gold-coated substrates in a pure ethanol solution containing 0.1mM ω-mercaptoundecyl bromoisobutyrate[109] at room temperature for 24 h. A monomer solution (0.12 g/mL) in DMF/water (3/1) was degas under a positive nitrogen flow, two treated chips with initiator SAM, 71.3 mg of copper(I) bromide and 154.3 mg of 2,2’-bipyridine (BPY) were placed in a reaction tube under nitrogen. The degassed monomer solution was then transferred into the reactor and left shaking on an orbital shaker for 16 h. After the reaction, the chips were rinsed with ethanol and water, and stored in PBS.

2.2.2 Protein adsorption test

The polymer brushes were grafted from the gold surface covered with initiators by surface-initiated ATRP. A custom-built four-channel SPR sensor was used to measure protein adsorption on pCBOH1 and pCBOH2 polymer brushes. First, a PBS buffer at 50 μL/min flow rate was used to obtain a baseline signal. 1 mg/mL bovine fibrinogen solution or 100% human blood plasma was then injected for 10 minutes followed by a PBS wash to remove any loosely bound proteins. The amount of adsorbed proteins was calculated as the change in wavelength before and after protein injection.

2.2.3 Hydrogel preparation for antimicrobial test and compression test

For antimicrobial test, both CBOH1 and CBOH2 monomers were kept in their ring form and directly photopolymerized in DMSO. The reaction solution contains 3 M
mononor, 0.06 M carboxybetaine dimethacrylate and 0.5 wt % 2-hydroxy-2-methylpropiophenone. Carboxybetaine dimethacrylate was synthesized following a published procedure. The solution was transferred into a mold made of two quartz slides separated by a 2 mm thick PTFE spacer and polymerized under UV (362 nm) for 1 hour. The gels were immersed in acetonitrile for 3 days. Before the antimicrobial study, the gels were equilibrated in water for 2 hours to obtain hydrogels. Because of the poor solubility in organic solvents, pCBMA hydrogel were prepared at the same concentration with the same method in H₂O. Hydrogels for compression test were prepared using CBOH1 and CBOH2 monomers under the zwitterionic form in water following a similar procedure.

2.2.4 Water content measurement

The water content of hydrogels is a basic property of the hydrogel materials for biomedical applications. Wet weight of the hydrogel samples were measured after removal of excess water from the samples. Dry weight was recorded after the samples had been dried at 65 °C under vacuum for 72 hours. The water contents of hydrogels (Table 1) are calculated by (Wet weight – Dry weight)/Wet weight x 100%.

<table>
<thead>
<tr>
<th></th>
<th>pCBMA</th>
<th>pCBOH1</th>
<th>pCBOH2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water content of hydrogel (1.5 M)</td>
<td>90.6 %</td>
<td>84.2%</td>
<td>87.2%</td>
</tr>
<tr>
<td>Water content of hydrogel (3 M)</td>
<td>85.3 %</td>
<td>71.7%</td>
<td>73.7%</td>
</tr>
</tbody>
</table>
2.2.5 Compression test

At least five disks of each hydrogel (2 mm thickness when cast) were compressed to failure at a rate of 1 mm/min using an Instron 5543 mechanical tester with a 100 N load cell. The Young’s modulus was calculated from the linear portion between 1-2% and 11-12% strain.

2.2.6 Bactericidal activity

The method for evaluating the antibacterial efficiency of polymer surfaces was modified from a previously published method.[124] *E. coli* K12 was cultured at 37 °C in Luria-Bertani (LB) medium (20 g/L) to reached an optical density of 0.8 at 600 nm. After wash with PBS, cells were suspended in PBS to get a final concentration of 5 x 10⁷ cells/mL. 20 μL of *E. coli* suspension was pipetted onto a hydrogel disc (8 mm in diameter) and incubated at room temperature for 1 hour. Then each sample was placed into one well of a sterile 24-well plate with 1 mL of LB medium and cultured at 37 °C for 18 hours. Bacterial culture in each well was diluted serially in water and spread on LB agar plates. After 18 hours at 37 °C, the number of the colony on agar plates was recorded to calculate the concentration of live bacterial cells.

2.2.7 Bacteria attachment, viability and releasing test

50 μL of fresh *E. coli* suspension in PBS was pipetted onto a hydrogel disc (8 mm in diameter) and incubated at room temperature for 1 hour. To analyze the density of bacteria accumulated on hydrogel surfaces, samples were gently rinsed with water, and stained with LIVE/DEAD *BacLight* Bacterial Viability assay kit. After the staining, the
number of live and dead cells was determined with an Olympus IX81 fluorescent microscopy with 60x oil lens through FITC and Cys3 filters, and the results are shown in Table 2. Following imaging, the sample was placed in PBS buffer for 16 hours. The number of remaining *E. coli* was again determined by fluorescent microscopy. Four separate samples were analyzed for each hydrogel sample.
2.3 Results and discussion

CBOH1 and CBOH2 monomers were synthesized by a three-step reaction as shown in Scheme 2.

2.3.1 Synthesis and structural switch characterized by NMR

$^1$H, $^{13}$C NMR data and heteronuclear multiple-bond correlation (gHMBC) two-dimensional (2D) NMR spectrum, which provides two- and three-bond correlations between $^1$H and $^{13}$C, were used to confirm the ring structure formation of both CBOH1 and CBOH2 in deuterated trifluoroacetic acid (TFA-d).

![Scheme 2. Synthetic route of CBOH1 and CBOH2. Reaction conditions: (i) Na$_2$CO$_3$, THF; (ii) tert-butyl bromoacetate, acetonitrile, 60 °C; (iii) TFA, CH$_2$Cl$_2$, ion-exchange resin.](image)

The crosspeak in red circle shown in the 2D NMR spectrum (Figure 5a) is the three-bond correlation between the resonances of the methylene protons adjacent to
hydroxyl group of CBOH1 and the resonances of the carbon on negatively charged carboxylate. This is the solid evidence of ring formation. It should be noted that, after cyclization, the protons from ethylene group in blue dotted circle change from a single peak into two doublets. A similar correlation of CBOH2 is shown in Figure 5b.

Figure 5. 500 MHz $^1$H($^{13}$C) gHMBC NMR spectra of CBOH1 (a) and CBOH2 (b) which switch between zwitterionic form and cationic ring form.

The kinetics of lactone ring formation was monitored by $^1$H NMR at different time points. The switching concept was first demonstrated by Cao and co-workers[125]
that 2-morpholinone ring can be obtained in both strong and weak acids, and will hydrolyze into zwitterionic form in basic and physiological condition (pH 7.3). To study the ring formation, CBOH1, CBOH2 monomers were dissolved in deuterated trifluoroacetic acid (TFA-d) and deuterated acetic acid (HAc-d) at a concentration of 0.2 M, respectively. Both vinylic protons showed downfield shift after ring formation. Conversion was calculated based on the ratio of vinylic protons from each form. As shown in Figure 6a, 96% of CBOH1 was converted in to the ring form in TFA within an hour, and for CBOH2 the conversion was 99% within 15 minutes. In acetic acid Figure 6b, CBOH1 and CBOH2 were able to reach 55% and 84% conversion within 20 hours, respectively.
Figure 6. The conversion kinetics of zwitterionic CBOH1 (black square) and CBOH2 (red dot) to their cationic ring form in TFA-d (a) and HAc-d (b).
In both cases, the sterically favored ring structure gives them better sensitivity to the stimuli from acidic environment compared with previously reported CBOH (TFA: 90 % conversion in 2 hours, HAc: 50 % conversion in 20 hours).[125] The results indicated that CBOH2 structure is more favorable for lactone ring formation than CBOH1 under the same condition. The symmetrically substituted CBOH2 is cyclizable on either side, which gives it a better chance to react with the carbonyl group and result in a much faster response for ring formation. The ring open kinetics (Figure 7) was studied by dissolving CB-Rings in 0.2 M Na2CO3 buffer solution in D2O at pH 7.3. Calculations were performed with the same method as ring-formation; the final conversion within 5 hours

Figure 7. The conversion kinetics of cationic CBOH1 (black square) and CBOH2 (red dot) to zwitterionic CBOH1 and CBOH2 monomers in pH 7.3 solution.
was 89% for CBOH1 and 65% for CBOH2. From the aspect of thermal dynamics, CBOH2 is expected to have a lower dissociation constant compared to CBOH1 under the same condition. From the aspect of kinetics, CBOH2 is expected to have a higher reaction coefficient for ring formation process and has a lower reaction coefficient for ring open compared to CBOH1. Our results reflected both expected trends.

Besides the pH of a bulk solution, the kinetics of ring formation and ring open in the polymerized hydrogel is also affected by the local pH and the steric hindrance of polymer backbone and side chains. The formation of one lactone ring consumes one proton. The consumption of protons subsequently leads to the increase of the local pH and slow down the ring formation, but the effect will not be dramatic. Since zwitterionic/cationic hydrogels have very high water contents and high porous structures, ions in the hydrogel can reach the equilibrium with bulk solution within a few minutes or less.[124] Further investigation is needed to have a better understanding on the steric hindrance and local pH effect on the kinetics of ring open and formation.

2.3.2 Antifouling study by SPR

In many biofouling processes, protein adsorption on a surface is the initial but critical step. This is particularly true in the field of biomedicine, since materials often contact blood or body fluid. Adsorbed proteins can facilitate the attachment and accumulation of bacteria or cells from the immune system and cause infection or inflammation, which subsequently leads to the foreign body response and cause the failure of implanted devices or materials. Beside the surface chemistry, protein adsorption on surfaces can be affected by many factors, including surface packing density,
surface roughness, and surface thickness. To minimize the effect of surface packing
density and surface roughness and evaluate the intrinsic anti-fouling properties of the
material, the anti-fouling properties of pCBOH1 and pCBOH2 were evaluated on the
polymer brush surfaces via surface initiated atom transfer radical polymerization (si-
ATRP) method. si-ATRP method has been widely used to prepare high packing, well-
defined and uniform surfaces.[126, 127]
Figure 8. SPR sensorgrams showing ultra-low fouling properties of zwitterionic pCBOH1 (a) and pCBOH2 (b) polymer brushes against 1 mg mL⁻¹ fibrinogen (black solid line) and undiluted human plasma (red dash line).
The protein-resistant properties of pCBOH1 and pCBOH2 polymer brushes were characterized by SPR on gold-coated sensor chips using a single protein solution (1 mg mL\(^{-1}\) bovine fibrinogen) and a complex solution (100% human blood plasma). As shown in Figure 8, both materials were highly resistant to the adsorption of both fibrinogen (0.8 ng cm\(^{-2}\) for pCBOH2 and < 0.3 ng cm\(^{-2}\) (detection limit of the sensor) for pCBOH1) and 100% human blood plasma (0.2 ng cm\(^{-2}\) for pCBOH1 and 2.4 ng cm\(^{-2}\) for pCBOH2). Horbett et al. found that the materials contacting blood with less than 5 ng cm\(^{-2}\) fibrinogen adsorption can delay the blood coagulation caused by platelet activation.\[128\] Protein adsorption values on both pCBOH1 and pCBOH2 surfaces are below the criteria for ultralow fouling materials.

Results from both experiments and molecular dynamic simulation suggest that the hydration of a surface plays a key role to resist protein adsorption.\[3, 129, 130\] Zwitterionic materials bind water through their carboxylate anions and quaternary ammonium cations via ionic solvation. Previous study indicated that the zwitterionic poly(carboxybetaine acrylamide) polymer brush surface achieved its optimal anti-fouling performance at a film thickness of ~20 nm.\[110\] For zwitterionic materials, the increase of the film thickness will lead to an increased intramolecular charge-charge interaction which will reduced the hydration of the surface and compromise its anti-fouling properties. In this work, the film thickness of the polymer brushes can be controlled through adjusting reaction time and monomer concentration. The film thickness was measured by SPR as ~13 nm for pCBOH1 and ~26 nm for pCBOH2. In CBOH1 and CBOH2, hydroxyl groups are linked to quaternary ammonium cation. The substitution of methyl group of quaternary ammonium cation with a larger group will change the
hydration of quaternary ammonium cation and affect the anti-fouling properties of the zwitterionic materials. I hypothesized that the loss of the hydration of quaternary ammonium in CBOH1 and CBOH2 can be countered by the hydrophilic hydroxyl group(s) in hydroxyethyl substitution group. SPR results showed that the anti-fouling properties of pCBOH1 and pCBOH2 surfaces are very close to that of pCBMA, which supported our hypothesis.

2.3.3 Antimicrobial study on hydrogel surfaces

The antimicrobial efficiency of cationic materials depends on their charge density. To keep a high ratio of cationic form units in the hydrogel and reduce the possibility of ring-opening to a minimum during the experimental process, both CBOH1 and CBOH2 monomers were kept in their ring form and directly photo-polymerized in dimethyl sulfoxide (DMSO) with 2 % carboxybetaine dimethacrylate as a crosslinker. pCBMA hydrogel were prepared at the same concentration with the same method in H2O, as both a positive anti-fouling control and a negative antimicrobial control. It should be noted that hydroxyl group(s) in CBOH1 and CBOH2 dramatically improves their solubility in organic solvents, such as DMSO, Dimethylformamide (DMF), acetonitrile and chloroform, compared with CBMA.
Table 2. Antimicrobial efficiency of hydrogel surfaces measured by the colony counting method (n = 6).

<table>
<thead>
<tr>
<th></th>
<th>pCBMA</th>
<th>pCBOH1</th>
<th>pCBOH2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live bacterial concentration (CFU/mL)</td>
<td>5.36E+08</td>
<td>&lt; 200</td>
<td>&lt; 200</td>
</tr>
<tr>
<td>Antimicrobial efficiency (%)</td>
<td>-</td>
<td>&gt;99.99996</td>
<td>&gt;99.99996</td>
</tr>
</tbody>
</table>

To evaluate antimicrobial properties, a strain of gram negative E. coli K12 was used as a model species. Before the test, hydrogel samples were punched into 8 mm diameter discs and equilibrated in PBS buffer solution. 20 µL of E. coli K12 suspension at a concentration 5 x 10⁷ cells mL⁻¹ were added onto one hydrogel disk. The hydrogel disk was kept at room temperature for 60 minutes. Colony-forming unit (CFU) of E coli K12 on each hydrogel was measured by a conventional colony counting assay to determine viable bacterial numbers, and the results are shown in Table 2. pCBMA hydrogel is used as the negative antimicrobial control. The antimicrobial efficiency was calculated from the amount of live cells on the tested surfaces relative to those on pCBMA hydrogel surfaces. Both hydrogels made from CBOH1 and CBOH2 in cationic ring form inhibited bacterial growth and killed more than 99.99996 % E. coli K12 within 1 hour relative to pCBMA-2 surfaces. Since cationic compounds, unlike antibiotics, kill bacterial cells non-specifically, they have less chance to generate antibiotic-resistant strains. This property is particularly important in long-term applications such as chronic infection treatment and wound healing.
Figure 9. Representative fluorescence microscopy images of bacterial attachment on pCBOH1 in cationic form (a), pCBOH2 in cationic form (b), and pCBMA (c) hydrogels before hydrolysis and on pCBOH1 in zwitterionic form (d), pCBOH2 in zwitterionic form (e), and pCBMA (f) hydrogels after 16 hours hydrolysis in PBS. Bacterial cells were stained with LIVE/DEAD BacLight Bacterial Viability assay kit. Cells with damaged cytoplasm membrane are in red and cells with intact cytoplasm membrane are in green.
Figure 10. Attachment of *E. coli* K12 from a suspension with 5 x10^7 cells mL^-1 for 1 hour exposure to cationic pCBOH1, cationic pCBOH2 and zwitterionic pCBMA hydrogels before and after hydrolysis (n = 6).

It is known that positively charged polymers or small molecule compounds can interrupt the membrane integrity of negatively charged microorganisms and lead to the death of the cells. Permanent cationic materials can kill attached bacterial cells, but killed cells and their debris remain on the surface that can trigger the inflammation.[112] Permanent cationic materials also have poor biocompatibility due to the high protein adsorption. The switchable antimicrobial and anti-fouling surface can be used to address
these challenges. The ability of catch, kill and release of E. coli K12 were tested on pCBOH1 and pCBOH2 hydrogel surfaces before and after hydrolysis. 50 µL of E. coli K12 suspension at a concentration 5 x 10^7 cells mL\(^{-1}\) were added onto one hydrogel disk. The hydrogel disk was kept in a sterile humidity chamber and incubated at room temperature for 60 minutes. The number and viability of attached cells were characterized by LIVE/DEAD BacLight Bacterial Viability assay (Invitrogen, Carlsbad, CA). As shown in Figure 9, a large amount of bacteria were caught and trapped on the cationic pCBOH1 (ring form) and pCBOH2 (ring form) hydrogel surfaces before hydrolysis, whereas only few bacterial cells were found on the zwitterionic pCBMA surface. The quantitative data for the amount of bacterial cells remaining on hydrogel surface before and after hydrolysis is shown in Figure 10. The surface density of attached E. coli on cationic pCBOH1 and pCBOH2 (ring form) hydrogels are 3.8 x 10^6 and 2.8 x 10^6 cells cm\(^{-2}\) respectively, and after overnight hydrolysis, the surfaces released 99.6% and 99.4% of attached cells for pCBOH1 and pCBOH2 hydrogels at zwitterionic form respectively.

Table 3. Cell viability of *E. coli* K12 on hydrogels in the ring form before hydrolysis. The results are averaged from six replicates.

<table>
<thead>
<tr>
<th></th>
<th>pCBMA</th>
<th>pCBOH1</th>
<th>pCBOH2</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of cells with</td>
<td>38.89 ± 37.51</td>
<td>99.13 ± 0.45</td>
<td>99.42 ± 0.34</td>
</tr>
<tr>
<td>membrane damage (in red)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The cell viability assay (Table 3) showed that pCBOH1 and pCBOH2 in their cationic ring form can cause cell membrane damage of over 99% attached cells. Upon overnight hydrolysis in PBS buffer solution, both pCBOH1 and pCBOH2 hydrogels became anti-fouling and released most of the killed bacterial cells, while CBMA hydrogel retained very low bacterial adhesion, but it cannot kill the small amount of attached bacterial cells. The large standard deviation on pCBMA surface is due to the low attachment number. Through this design, I demonstrate that pCBOH1 and pCBOH2 hydrogels in the ring form can kill bacteria and then release the attached bacterial cells after hydrogels are converted to zwitterionic form via hydrolysis.

2.3.4 Mechanical property study

The utility of current zwitterionic hydrogel is limited due to its unsatisfactory mechanical property. I hypothesized that the mechanical property of zwitterionic materials can be improved by incorporating hydrogen bond forming group into the molecular structure. To test our hypothesis, the compression moduli of pCBOH1 and pCBOH2 hydrogels at two concentrations were tested. Each hydrogel (2 mm thickness when cast) was compressed to failure at a rate of 1 mm min\(^{-1}\) using an Instron 5543 mechanical tester with a 100 N load cell. The Young’s modulus was calculated from the linear portion between 1-2% and 11-12% strain. Compared with pCBMA hydrogels, pCBOH1 hydrogels show significantly increased modulus, about 73 % higher at 1.5 M, and 43 % higher at 3 M. pCBOH1 hydrogels also show about two times higher break stress and about 30 % higher break strain than pCBMA hydrogels, at both low and high concentration. The higher compression modulus, break stress, and strain at break were
attributed to asymmetrically fused hydroxyethyl groups with neighboring carbonyl groups. As shown in Figure 11, hydrogen bonding is more predominant at the lower concentration than the higher concentration. More recently, the dynamic simulation studies by He et al.[131] proved that incorporation of hydroxyl groups can effectively enhance the mechanical properties. The detailed elaboration of molecular interaction within hydrogels was also in good agreement with our hypothesis. However, it is possible that more hydroxyl groups will make the material softer and more elastic, so that pCBOH2 hydrogel only show a relatively higher break strain compared with CBMA, but no big difference regarding to modulus and break stress.

It also should be noted that the ring formation of both pCBOH1 and pCBOH1 hydrogels will affect their mechanical properties. Two hydrophilic groups, hydroxyl and carboxylate, are consumed after one ring is formed. The loss of hydrophilic groups reduces the water content of the hydrogel, and is expected to increase the compression modulus; however, the net charge of polymer side chains changes from neutral to positive after ring formation. Therefore, the repulsion force among positively charged side chains leads to the conformation change of polymers from random coil at their zwitterionic state to more stretched form at their cationic state. This conformation change subsequently increases the mesh size and water content of hydrogels, and reduces their compression modulus.

I found the size changes of hydrogels are very small (<2%) after hydrogels switch to the cationic state from the zwitterionic state. Therefore, I expected that compression modulus of pCBOH1 and pCBOH2 hydrogels in their cationic form is lower than that in
their zwitterionic form because of the loss of hydroxyl groups. Further tensile test and systematic characterization of hydrogen bond formation are in progress.
Figure 11. Comparison of compression stress vs. strain curve for pCBOH1 (red dash line), pCBOH2 (blue dot line), and pCBMA (black solid line) hydrogels prepared at 1.5 M (a) and 3M (b) respectively.
2.3.5 Internal morphology study by SEM

To study the internal morphology of the polymeric networks, the cross-section morphology of the freeze-dried hydrogel was studied by using a scanning electron microscope (Hitachi TM-3000 Tabletop SEM). A dramatic morphology difference was observed (Figure 12).

pCBOH1 hydrogel shows the best homogeneity among all three samples. Additional intermolecular hydrogen bond may play an important role during photopolymerization process. Compared with the randomly distributed big holes appeared on pCBMA hydrogel, pCBOH1 hydrogel shows much stronger internal network across the boundary. In contrast, pCBOH2 hydrogel shows the finest structure and very thin fibrillar-like network, resulting in the lowest resistance to compression but higher break strain compared with pCBMA hydrogel.
Figure 12. SEM images of the cross-section from 3M hydrogels after freeze-drying: pCBOH1 (a) and (d), pCBOH2 (b) and (e), and pCBMA (c) and (f) at different resolutions. (scale bars: a-c 300 μm, d-f 30 μm.)
2.4 Conclusion

In conclusion, two switchable CB derivatives, CBOH1 and CBOH2, were synthesized and systematically investigated. In the cationic ring form, pCBOH1 and pCBOH2 hydrogels are able to kill over 99.99996 % of attached E. coli K12 on the surface. After hydrolysis under neutral or basic condition, they changed into zwitterionic form, which will release dead bacterial cells and resist further bacterial adhesion. These processes are reversible by changing the pH of the environment between acidic or basic/neutral. The hydrogen bond-forming hydroxyl group greatly improves the mechanical property of hydrogels, which significantly broadens their applications. Additionally, SPR and bacterial attachment experiments show that the introduction of the hydroxyl groups did not compromise their anti-fouling properties of the zwitterionic polymers.
3.1 Introduction

Over past two decades, there have been booming research interests in antifouling materials, for their great significance in biomedicine.[44-47] Zwitterionic materials, especially CB-based materials, have attracted great attention due to their outstanding antifouling properties of resisting proteins, mammalian cells and microbes, as well as the capability of further functionalization. pCB and their derivatives have been used as the hydrogel to prolong the half-life of implantable biosensors,[26] the surface coating to resist biofilm,[132, 133] the stealth coating to increase stability of the protein[134] and the blood half-life of nanomedicine,[135, 136] the vector to delivery nucleic acids for gene therapy,[137] etc.

Despite intense interests in zwitterionic materials for many biomedical applications, there are several challenges to be solved to let the potential of zwitterionic materials fully realized. Firstly, existing zwitterionic materials are fragile and not stretchable,[138, 139] which significantly limits their utility for flexible medical devices (such as heart valve, implantable biosensors, and tissue scaffolds). These applications require implanted materials to be elastic and biocompatible;[87] however most of
biomedical elastic materials are made of hydrophobic materials (such as silicone and polyurethane),[140] which lack biocompatibility due to high protein adsorption.[141] Secondly, hydrophobic elastic materials cannot resist bacterial attachment and zwitterionic materials cannot kill a small amount of attached microbes.[76, 77] Microorganisms can be introduced into patients during invasive surgical procedures, and colonized microorganism on the surface of the implanted material/device will trigger inflammation and immune response. Therefore, it is highly desired to have a material integrating all desired properties including excellent antifouling property/biocompatibility to prolong the lifetime of implanted materials, antimicrobial property to eliminate surgical infection and chronic inflammation, and good mechanical property to avoid the failure of the implanted material.

Although compression properties of zwitterionic materials can be improved by copolymerization[138] or increasing crosslinking density,[139] a good elasticity cannot be easily obtained due to their high water solubility and high water content. Previously I developed two switchable materials which can switch between their zwitterionic forms (antifouling) and cationic forms (antimicrobial). In these materials, incorporated hydroxyl group(s) improves the compression property through intramolecular hydrogen bonding. However, these materials are still rigid and barely stretchable. To overcome the infection challenge, cationic CB derivatives were synthesized to catch and kill bacterial cells, and then release killed bacterial cells upon its hydrolysis into the zwitterionic antifouling state; however the conversion is nonreversible.[76] Furthermore, the stability of zwitterionic materials, which is equally important as other desired properties, is rarely investigated. A
good understanding on structure-function-stability relationships is necessary to rationally design a single material with all desired properties.

The objectives of this work are to design and synthesize elastic zwitterionic materials with uncompromised antifouling and switchable properties, and to investigate the effect of molecular structure on their switchability, stability and elasticity. I hypothesized that the elasticity and stability of zwitterionic materials can be improved by replacing methacrylate backbone with methacrylamide backbone. Amide group can function as both hydrogen bond donor and acceptor, and therefore the mechanical property can be enhanced through inter/intra molecular hydrogen bond formation.[142] Based on our rational molecular design, two novel CB-based monomers bearing hydroxyl group and acrylamide backbone, 2-((2-hydroxyethyl)(2-methacrylamidoethyl)(methyl)ammonio) acetate (CBMAA-1) and 3-((2-hydroxyethyl)(2-methacrylamidoethyl)(methyl)ammonio) propanoate (CBMAA-2), were devised in this study. Hydrogels prepared by both monomers show high elasticity. Especially CBMAA-1, it also shows high stability in both acidic and basic condition, as well as reversible switchability between zwitterionic carboxylate form (antifouling) and cationic six-membered ring form (antimicrobial). It is reported for the first time that a single material combines the advantages (elasticity, switchability, stability, antifouling and antimicrobial properties) while overcomes the disadvantages of both antifouling materials and elastic materials.
3.2 Experimental methods

All reagents and chemicals were purchased from Alfa and Sigma-Aldrich and used without further purification. All solvents were purchased from Acros.

3.2.1 Synthetic procedures and instrumentation

Column chromatography was carried out on flash silica gel obtained from Sigma. Carboxybetaine dimethacrylate (crosslinker) was synthesized following a published procedure.[1] All NMR experiments were performed at 303.2 K on Varian 300-, 500- and 750-MHz spectrometers under the same condition as described in previous section unless stated otherwise.

Scheme 3. Synthetic routes to CBMAA-1 and CBMAA-2. Reaction conditions: (i) ethanol, water, NaOH; (ii) ethanol, tert-butyl bromoacetate, NaOH; (iii) tert-butyl acrylate; (iv) CH₃I, acetonitrile, TFA, CH₂Cl₂, ion-exchange resin.
3.2.1.1 Synthesis of N-(2-((2-hydroxyethyl)amino)ethyl)methacrylamide (compound 2)

4.8 g (0.12 mole) of NaOH was dissolved in a mixture of 30 mL water and 70 mL ethanol in a 250 mL three-neck round bottom flask, followed by 10.1 mL (0.1 mole) of 2-((2-aminoethyl)amino)ethanol. The mixture was cooled down to 0 °C with an ice bath. 17.4 mL (0.11 mole) of methacrylic anhydride was added drop-wise under a positive nitrogen flow. After stirring at 0 °C for 2 hours, the ice-bath was removed, and the reaction was stirred at room temperature for another 3 hours. Without purification of this intermediate, the crude product was directly submitted to the next reaction. For verification purposes, a small portion of the crude product was purified by silica gel column chromatography (ethyl acetate/methanol, 2/1 (v/v)). Pure product was obtained as a colorless liquid. \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 5.79 (s, 1H), 5.27 (s, 1H), 3.77 (t, \(J = 5.7\) Hz, 2H), 3.50 (t, \(J = 6.3\) Hz 2H), 2.94-2.86 (m, 4H), 1.99 (s, 3H) 1 hydroxy peak not seen due to overlapping signals (Figure 13). \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 171.90, 138.92, 121.19, 59.65, 49.62, 47.11, 38.47, 17.66 (Figure 14).

3.2.1.2 Synthesis of tert-butyl N-(2-hydroxyethyl)-N-(2-methacrylamidoethyl)glycinate (compound 3a).

17.7 mL (0.12 mol) of t-butyl bromoacetate was added into the mixture of the crude intermediate compound 2 and heated at 60 °C under a positive nitrogen flow for overnight with stirring. Ethanol was removed with a rotary evaporator, and the residue pH was adjusted to ~10 with NaOH. Then it was extracted with ethyl acetate (2 times) and the organic layer was washed by de-ionized water, dried with anhydrous magnesium sulfate. After filtration, the liquid was concentrated and purified by silica gel column
3.2.1.3 Synthesis of tert-butyl 3-((2-hydroxyethyl)(2-methacrylamidoethyl)amino) propanoate (compound 3b).

The synthesis procedures of compound 3b were similar to that of compound 3a, tert-butyl acrylate was used instead of tert-butyl bromoacetate. Product was purified by silica gel column chromatography (ethyl acetate/hexane, 4/1 (v/v)). (Two-step yield: 33 %). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 6.78 (s, 1H), 5.67 (s, 1H), 5.24 (s, 1H), 3.52 (t, $J = 5.0$ Hz, 2H), 3.34 (m, 2H), 3.11 (s, 1H), 2.71 (t, $J = 6.3$ Hz, 2H), 2.56 (m, 4H), 2.32 (t, $J = 6.3$ Hz, 2H), 1.90 (s, 3H), 1.38 (s, 9H) (Figure 17). $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 172.61, 168.62, 140.06, 119.50, 81.03, 59.34, 55.78, 53.30, 49.45, 37.50, 33.82, 28.14, 18.72 (Figure 18).

3.2.1.4 Synthesis of 2-((2-hydroxyethyl)(2-methacrylamidoethyl)(methyl)ammonio) acetate (compound 4a: CBMAA-1).

10.3 g (36 mmol) of compound 3a was dissolved in 75 mL of acetonitrile in a nitrogen filled flask, followed by adding 4 mL (50 mmol) of CH$_3$I. The mixture was stirred at 60 °C under a positive nitrogen flow for 24 hours. After solvent removal with a rotary evaporator, the residue was precipitated in anhydrous diethyl ether and dried under
vacuum. The obtained white solid was sequentially treated with a mixed solvent of 15 mL trifluoroacetic acid (TFA) and 15 mL of dichloromethane for 1.5 hours at room temperature to remove the tert-butyl group, concentrated with a rotary evaporator, precipitated in ether, dried under vacuum, redisolved in methanol, neutralized over an ion exchange resin (Amberlyst® A26, OH-form), and further purified by silica gel column chromatography (Ethyl Acetate/Methanol 1/2 v/v). (Yield: 73 %). $^1$H NMR (300 MHz, D$_2$O) δ 5.81 (s, 1H), 5.56 (s, 1H), 4.11 (m, 2H), 4.05 (s, 2H), 4.01-3.80 (m, 6H), 3.38 (s, 3H), 1.99 (s, 3H) (Figure 19). $^{13}$C NMR (75 MHz, D$_2$O) δ 171.77, 168.69, 138.50, 121.81, 63.90, 62.13, 60.70, 55.30, 49.93, 33.42, 17.49 (Figure 20).

3.2.1.5 Synthesis of 3-((2-hydroxyethyl)(2-methacrylamidoethyl)(methyl)ammonio) propanoate (compound 4b: CBMAA-2)

The synthesis of compound 4b was followed the same procedures as that of compound 4a. Pure product was obtained by silica gel column chromatography (Ethyl Acetate/Methanol 1/2 v/v). (Yield: 70 %). $^1$H NMR (300 MHz, D$_2$O) δ 5.81 (s, 1H), 5.57 (s, 1H), 4.13 (m, 2H), 3.82-3.72 (m, 4H), 3.65-3.63 (m, 4H), 3.25 (s, 3H), 1.99 (s, 3H) (Figure 21). $^{13}$C NMR (75 MHz, D$_2$O) δ 175.98, 171.92, 138.45, 121.88, 63.37, 60.03, 59.87, 55.06, 49.08, 33.23, 30.19, 17.47 (Figure 22).
Figure 13. $^1$H NMR Spectra of compound 2 at 300 MHz, D$_2$O.

Figure 14. $^{13}$C NMR Spectra of compound 2 at 75 MHz, D$_2$O.
Figure 15. $^1$H NMR Spectra of compound 3a at 300 MHz, CDCl$_3$.

Figure 16. $^{13}$C NMR Spectra of compound 3a at 75 MHz, CDCl$_3$. 
Figure 17. $^1$H NMR Spectra of compound 3b at 300 MHz, CDCl$_3$.

Figure 18. $^{13}$C NMR Spectra of compound 3b at 75 MHz, CDCl$_3$. 
Figure 19. $^1$H NMR Spectra of compound 4a at 300 MHz, D$_2$O.

Figure 20. $^{13}$C NMR Spectra of compound 4a at 75 MHz, D$_2$O.
Figure 21. $^1$H NMR Spectra of compound 4b at 300 MHz, D$_2$O.

Figure 22. $^{13}$C NMR Spectra of compound 4b at 75 MHz, D$_2$O.
The cross-peaks in $^1$H$^{13}$C gHMBC 2D-NMR spectrum, showing the correlations between methylene protons and carbonyl carbon, indicated the seven membered ring structure formation of for CBMAA-2 (Figure 23) in deuterated trifluoroacetic acid (TFA-d).

Figure 23. 750 MHz $^1$H$^{13}$C gHMBC NMR spectrum of CBMAA-2 as the evidence of seven membered ring formation in TFA.

3.2.2 Surface-initiated photoiniferter-mediated polymerization (si-PIMP)

The polymer brushes were synthesized via si-PIMP. The photoiniferter, 11-mercaptoprundecane-1-[4-(phenyl)carbamate] (DTCA), was synthesized following the procedure reported previously.[143] Gold-coated sensor chips were treated with previous reported procedures.[144] The photoiniferter self-assembled monolayer (SAM) was formed by soaking cleaned chips in 1 mM photoiniferter in tetrahydrofuron (THF)
overnight at room temperature. Chips were then rinsed with THF and dried with a stream of filtered air. One of treated chips with initiator SAM was placed in a quartz reaction tube under nitrogen. 10 mL of a monomer solution (50 mg/mL) in PBS was deoxygenated under a positive nitrogen flow. The deoxygenated monomer solution was transferred into a reactor with a syringe and then irradiated with a 302 nm UV lamp (UVP, model UVM-57) coupled with a 280 nm cutoff filter for 2 hours. The cutoff filter was used to avoid the cleavage of the thiol-gold bond of the photoiniferter SAM. After the reaction, the chip was rinsed with water and ethanol, and then stored in PBS before use.

3.2.3 Protein adsorption study

A custom-built four-channel SPR sensor was used to measure protein adsorption on pCBMAA-1 and pCBMAA-2 polymer brushes. Firstly, PBS solution at 50 µL min⁻¹ flow rate was used to obtain a baseline signal. 1 mg mL⁻¹ bovine fibrinogen solution, 100% human blood plasma or 100% human serum was then injected for 10 minutes followed by a PBS wash to remove any loosely bound proteins. The amount of adsorbed proteins was calculated as the change in wavelength before and after protein injection.[76]

3.2.4 Hydrogel preparation for antimicrobial test and mechanical tests

To prepare hydrogels for antimicrobial test, CBMAA-1 monomer was equilibrated in pure acetic acid to obtain above 70% conversion of its cationic ring form. After checked the conversion ratio with NMR, the monomer was precipitated in ether, vacuum dried, then photopolymerized in DMSO. The reaction solution contains 1.5 M
monomer, 0.045 M carboxybetaine dimethacrylate and 0.5 wt % 2-hydroxy-2-methylpropiophenone. The solution was transferred into a mold made of two quartz slides separated by an 2 mm thick PTFE spacer and polymerized under UV (362 nm) for 1 hour. The gel was immersed in acetonitrile for 2 days. Before the antimicrobial study, the gel was equilibrated in water for 2 hours to obtain hydrogels. Because of the poor solubility of CBMA in organic solvents and instability of CBMAA-2 in its ring form, pCBMA and pCBMAA-2 hydrogels were prepared in their zwitterionic form at the same concentration with the same method in H₂O. Hydrogels for compression and tensile tests were prepared with all four monomers (zwitterionic form) at the same concentration in water following a similar procedure.

3.2.5 Water content measurement

The water content is a basic property of hydrogel materials for biomedical applications. The wet weight of the hydrogel sample was measured after the removal of excess water. Dry weight was recorded after the samples had been freeze-dried for 48 hours. The water contents of hydrogels (Table 4) are calculated by (Wet weight – Dry weight)/Wet weight x 100%.

Table 4. Equilibrium water content (average of 3 samples)

<table>
<thead>
<tr>
<th></th>
<th>pCBMAA-1</th>
<th>pCBMAA-2</th>
<th>pCBOH-1</th>
<th>pCBMA-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water content of hydrogel (1.5 M)</td>
<td>94.3%</td>
<td>97.8%</td>
<td>84.2%</td>
<td>90.6%</td>
</tr>
</tbody>
</table>
3.2.6 Compression and tensile tests

At least five disks of each hydrogel (about 2 mm thickness when swell to equilibrium in water) were compressed to failure at a rate of 0.5 mm min\(^{-1}\) using an Instron 5543 mechanical tester with a 100 N load cell. For tensile test, hydrogel samples were cut into rectangular shape with about 15 mm width, 40 mm length, and 2-3 mm thickness. All samples were pulled to failure at a rate of 0.1 mm/s using a TA.XT Plus Texture Analyzer (Texture Technologies) with a 500 g load cell.

3.2.7 Bacterial attachment, viability and releasing study

_E. coli_ K12 was cultured at 37 °C in Luria-Bertani (LB) medium (20 g L\(^{-1}\)) to reached an optical density of 0.8 at 600 nm. After three wash with PBS, cells were suspended in PBS to get a final concentration of 5 x 10\(^7\) cells mL\(^{-1}\). 50 μL of fresh _E. coli_ suspension in PBS was pipetted onto hydrogel discs (8 mm in diameter) and incubated at room temperature for 1 hour. To analyze the density of bacteria accumulated on hydrogel surfaces, samples were gently rinsed with water, and stained with LIVE/DEAD BacLight Bacterial Viability assay kit. After the staining, the number of live and dead cells was determined with an Olympus IX81 fluorescent microscopy with 60x oil lens through FITC and Cys3 filters, and results are shown in Table 5. Following imaging, the sample was placed in PBS solution for 16 hours. The number of remaining _E. coli_ was again determined by fluorescent microscopy. Three separate samples were analyzed for each hydrogel sample.
Table 5. Cell viability of *E. coli* K12 on hydrogels in the ring form before hydrolysis. The results are averaged from three replicates.

<table>
<thead>
<tr>
<th></th>
<th>pCBMAA-1</th>
<th>pCBMAA-2</th>
<th>pCBMA-2</th>
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</thead>
<tbody>
<tr>
<td>% of cells with</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>membrane damage</td>
<td>99.5 ± 0.5</td>
<td>10.8 ± 6.6</td>
<td>19.2 ± 8.3</td>
</tr>
</tbody>
</table>

Table 6. Bacteria attachment, viability and releasing test of hydrogel surfaces (n = 3).

<table>
<thead>
<tr>
<th></th>
<th>pCBMAA-1</th>
<th>pCBMAA-2</th>
<th>pCBMA-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell density before hydrolysis (cells cm$^{-2}$)</td>
<td>1.0 ± 0.1 E6</td>
<td>8.3 ± 2.3 E4</td>
<td>4.0 ± 0.9 E4</td>
</tr>
<tr>
<td>Cell density after hydrolysis (cells cm$^{-2}$)</td>
<td>4.4 ± 2.1 E4</td>
<td>3.5 ± 1.1 E4</td>
<td>4.9 ± 1.2 E4</td>
</tr>
<tr>
<td>Antimicrobial efficiency (%)</td>
<td>&gt;99.5</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
3.3 Results and discussion

Two new monomers, CBMAA-1 and CBMAA-2, were synthesized via three-step reactions as shown in Scheme 3 (see supporting information).

3.3.1 Synthesis and structural switch characterized by NMR

Carboxybetaine methacrylate (CBMA-2) and 2-((2-hydroxyethyl)(2-(methacryloyloxy)ethyl)(methyl) ammonio)acetate (CBOH-1) were synthesized using previously published methods [76, 145] and were used as control materials. Heteronuclear multiple-bond correlation (gHMBC) 2D-NMR spectrum, which provides two- and three-bond correlations between $^1$H and $^{13}$C, were used to verify the ring structure formation of CBMAA-1 (Figure 24) and to monitor whether there is the lactone ring formation for CBMAA-2 (Figure 23) in deuterated trifluoroacetic acid (TFA-d). As expected, CBMAA-1 formed six-membered lactone ring and showed well resolved correlations in the NMR spectrum, which is similar to the 2D-NMR spectrum of CBOH1 reported in previous study.[145] The $^1$H NMR data shown in Figure 25A were recorded at different time points to observe the dynamic ring formation process of CBMAA-1. The conversion ratio was calculated based on the integral value of vinylic protons in each form. As shown in Figure 25B, 98% of CBMAA-1 converted into the six-membered ring form in TFA within an hour. In acetic acid (Figure 25C), a 70% conversion was achieved for CBMAA-1 within 24 hours. The ring opening kinetics (Figure 25D) was studied by dissolving monomers in their cationic forms in pure D$_2$O. Calculations were performed with the same method as ring formation, and the final conversion was 86% for CBMAA-1 in 10 hours. In the case of CBMAA-2, seven-membered ring structure formation was
also observed in TFA (see supporting information), but was at a much slower rate (after 52 hours) due to a much higher ring strain upon closure. Surprisingly, it did not undergo cyclization in acetic acid, while elimination of the carboxylate to acrylic acid occurred. Hofmann type elimination of quaternary ammoniums was commonly seen under basic conditions;[146, 147] however elimination under acidic (acetic acid) conditions has been rarely reported.

Scheme 4. Chemical structure of CB derivatives. All listed CB derivatives showed excellent antifouling properties.
Figure 24. 750 MHz $^1$H($^{13}$C) gHMBC NMR spectrum of CBMAA-1 in its pure cationic ring form. 2D-NMR can provide superior resolution and unique atomic connectivity information, so that unambiguous resonance assignment can be made for molecules.
Figure 25. The change of NMR spectra of CBMAA-1 from zwitterionic form to its cationic ring form in TFA-d (A). The conversion kinetics of zwitterionic CBMAA-1 from zwitterionic form to its cationic ring form in TFA-d (B) and HAc-d (C). The conversion kinetics of cationic CBMAA-1 from ring form to its zwitterionic form in D$_2$O (D).
3.3.2 Chemical stability study by NMR

Zwitterionic monomers may undergo two types of decomposition: Hofmann elimination (side group elimination of quaternary ammonium) and side chain-backbone linkage hydrolysis; however, there is little attention on these possible and undesired transformations, which have a significant influence on the life and performance of the material. In this work a systematic study of four monomers, as shown in Scheme 4, was carried out to investigate the effect of structures on their stability in acidic (pure acetic acid (HAc)) and basic (0.2 M Na$_2$CO$_3$ solution, pH 12.1) conditions. In terms of elimination, monomers with one carbon spacer between carboxylate and quaternary ammonium, CBMAA-1 and CBOH-1, were very stable under both acidic (Figure 26A) and basic (Figure 26B) conditions, while CBMA-2 and CBMAA-2 monomers bearing a two-carbon spacer underwent elimination in both conditions. The elimination reaction in HAc was relatively faster, compared to that in 0.2 M Na$_2$CO$_3$ solution. The elimination reaction of CBMA-2 and CBMAA-2 completed in ten days in HAc, while it took over twenty five days for the elimination reaction to finish in 0.2 M Na$_2$CO$_3$ solution. Although the carboxylate group in monomers with a two-carbon spacer are slightly easier to be further functionalized for conjugating other moieties, one carbon spacer dramatically increases the stability of materials. Once eliminations occur, materials will lose their zwitterionic characteristics.

As a result, desired antifouling properties will disappear. In our design, I replaced methacrylate backbone with methacrylamide backbone, and it is expected that amide bond is much more stable that ester bone under both acidic and basic conditions. As expected, results (Figure 26C) show that methacrylamide group is highly resistant to
hydrolysis under basic condition. Methacrylate group was completely hydrolyzed within 7 hours in 0.2 M Na₂CO₃ solution, while methacrylamide group was still stable after 30 days under the same condition. It should be noted that the hydrolysis and elimination reactions may occur simultaneously, but the rate of ester hydrolysis is much faster than that of Hofmann elimination. Hofmann elimination of CBMA-2 was observed after the completion of its hydrolysis. Through this study, CBMAA-1 demonstrated the highest stability under both harsh acidic and basic conditions.

Figure 26. Stability of quaternary ammonium in pure HAc (A) and in 0.2 M NaCO₃ (B), and the stability of methacrylate and methacrylamide backbone in 0.2 M NaCO₃ (C) of CBMAA-1 (blue), CBMAA-2 (green), CBOH-1 (red) and CBMA-2 (black) monomers. Note that straight lines were slightly shifted for clarity. Data were calculated from integral values of the corresponding peaks in NMR spectra.
3.3.3 Elimination mechanism study

CBMAA-2 was chosen as a model molecule to further study elimination mechanisms. Under acidic conditions, nucleophilicity of the negatively charged acetate anion could be the reason of elimination reaction. CBMAA-2 may undergo elimination via the mechanism described in Figure 27A. The superior electronegative nature of -CF₃ group makes TFA a 100,000-fold stronger acid than HAc; however the nucleophilicity of trifluoroacetate anion is much lower.\[148\] This mechanism can explain why the slow seven-membered ring formation of CBMAA-2 was observed but there was no elimination detected in TFA. In addition, NMR detected the presence of some complexes of CBMAA-2 in TFA, however their structures can not be determined now. Under basic conditions, the hydrogen's acidity of the beta carbon is important for possible elimination reactions, since protons adjacent to carbonyl groups are more acidic than other protons.\[148\] The proposed mechanism is illustrated in Figure 27B.

Theoretically, any side group of the quaternary ammonium may undergo elimination reactions; however, the resonance stabilized carboxylate make it a preferential thermodynamically favored leaving group in this case (this may apply to both acidic and basic conditions). Thus only one tertiary amine product, \(\text{(N-(2-((2-hydroxyethyl)(methyl)amino)ethyl)methacrylamide)}\) was observed and isolated (Figure 27). It is possible that more complicated eliminations may occur at higher temperatures. Further investigation is needed to verify proposed elimination mechanism under both acidic and basic conditions.
Figure 27. Proposed mechanism for eliminations of CBMAA-2 in acetic acid (A) and Na$_2$CO$_3$ (B) solutions.
3.3.4 Mechanical compression and tensile test of hydrogel

Our previous studies demonstrated that hydroxyl group can enhance the compression modulus of CB-based hydrogel.[145] It is known that amide groups can function as both hydrogen bond donor and acceptor.

![Tensile (A) and compression (B) study for pCBMAA-1 (blue), pCBMAA-2 (green), pCBOH-1 (red), and pCBMA-2 (black) hydrogels prepared at 1.5 M.]

Therefore, I hypothesized that the replacement of the methacrylate backbone with methacrylamide backbone, a more hydrophilic and stronger hydrogen bond forming group, would make the materials softer, more elastic, and more stable. As shown in Figure 28A and 28B, pCBMAA-1 hydrogel shows typical elastomeric stress-strain curves with low Young's modulus and high yield strain, in both tensile and compression test. About 65% tensile strain and 85% compressive strain are achieved for this hydrogel. In the tensile study, the breaking strain of pCBMAA-1 hydrogel is increased 11-fold and 6-
fold compared to pCBMA-2 and pCBOH-1 hydrogels respectively. The compressive breaking strain of pCBMAA-1 hydrogel has 1.8-fold and 1.5-fold increases compared to pCBMA-2 and pCBOH-1 hydrogels respectively and its compressive breaking stress is comparable to that of pCBOH-1 hydrogel. Our results indicate that pCBMAA-1 hydrogel is stronger in compression and much more elastic in tensile than existing zwitterionic hydrogels. pCBMAA-2 hydrogel also shows an improved elasticity, but the change is not as dramatic as pCBMAA-1 hydrogel. Higher equilibrium water content (EWC) and swelling ratio of pCBMAA-2 may be the cause of the lower elasticity than that of pCBMAA-1 hydrogels. Crosslinking density can be adjusted to obtain a moderate EWC and optimum elasticity. To the best of our knowledge, no zwitterionic material with such a good elastic property has ever been made. This huge improvement may dramatically broaden their applications where high mechanical strength is required.

3.3.5 Antifouling test of polymer brush

In the field of biomedicine, materials often contact with blood and/or body fluid and fouling process usually start with protein adsorption on a surface. Adsorbed proteins can aid bacterial attachment and colonization to cause infection and inflammation, and it can also trigger the foreign body response and cause the insulation of implanted devices or materials. Biomaterials with superior antifouling properties are highly desired. Since the protein adsorption on hydrogel surface is not be easily quantified, the antifouling properties of pCBMAA-1 and pCBMAA-2 were evaluated on high packing and well-defined polymer brush surfaces via a surface-initiated photoiniferter-mediated polymerization method.\textsuperscript{[143]} The film thickness was measured by Ellipsometry as
~18.07 nm for pCBMAA-1 and ~9.26 nm for pCBMAA-2. The protein-resistant properties of pCBMAA-1 and pCBMAA-2 polymer brushes were characterized by SPR on gold-coated sensor chips using bovine fibrinogen (1 mg mL⁻¹), 100% human blood plasma and 100% human blood serum. As shown in Figure 29, both pCBMAA-1 and pCBMAA-2 surfaces highly resist the protein adsorption from the single protein (fibrinogen) and complex solutions (blood plasma and serum). The amount of adsorbed protein is below the detection limit (0.3 ng cm⁻²) of our SPR sensor. SPR results show that both pCBMAA-1 and pCBMAA-2 can achieve excellent antifouling results with very thinner film. The results support our hypothesis that the hydration of a surface is the key to resist protein adsorption. Materials contacting blood with less than 5 ng cm⁻² adsorbed fibrinogen is called ultralow fouling materials and it is found that ultralow fouling materials is less likely trigger blood coagulation through platelet activation passway.[149] Ultralow fouling materials are highly desired to be used as the top layer to contact blood. Both pCBMAA-1 and pCBMAA-2 surfaces are qualified as ultralow fouling materials.
Figure 29. SPR sensorgrams showing ultra-low fouling properties of zwitterionic pCBMAA-1 (A) and pCBMAA-2 (B) polymer brushes against the adsorption of 1 mg mL\(^{-1}\) fibrinogen (black solid line), undiluted human plasma (red dash line) and undiluted human blood serum (blue dotted line).

3.3.6 Antimicrobial test on hydrogel surface

Cationic compounds, unlike antibiotics, kill bacterial cells via a non-specific mechanism, so they are less likely to generate antibiotic-resistance and are particular useful for long-term antimicrobial applications such as chronic infection treatment and wound healing. However conventional cationic materials lack biocompatibility due to permanent positive charge. These challenges can be solved using the switchable antimicrobial and antifouling materials. Since only CBMAA-1 shows good switchability and ring stability. The ability of catch, kill and release of *Escherichia coli* K12 was tested on pCBMAA-1 hydrogel surfaces before and after hydrolysis with zwitterionc
pCBMAA-2 and pCBMA-2 hydrogels as both negative antimicrobial control and positive antifouling control.

As shown in Figure 30, a large amount of bacteria was caught on cationic pCBMAA-1 (ring form) hydrogel surfaces before hydrolysis, whereas only few bacterial cells were found on the zwitterionic pCBMAA-2 and pCBMA-2 surfaces. pCBMAA-1 hydrogel in the cationic ring form could effectively catch and cause the membrane damage of *E. coli* K12 within 1 hour compared to zwitterionic pCBMAA-2 and pCBMA-2 hydrogel surfaces, since positively charged compounds can interrupt the membrane integrity of microorganisms and cause the death of cells. Zwitterionic pCBMAA-2 and pCBMA-2 surfaces had fewer attached cells and they were not able to kill attached cells. The quantitative results are shown in Table 6. The surface density of attached *E. coli* on cationic pCBMAA-1, zwitterionic pCBMAA-2 and pCBMA-2 hydrogels were 1.0 x 10^6, 8.3 x 10^4 and 4.0 x 10^4 cells cm^{-2} respectively. After overnight hydrolysis, pCBMAA-1 surfaces released 95% of attached cells. The results demonstrate that pCBMAA-1 hydrogels in its ring form can kill bacteria and then release killed bacteria via hydrolysis and that zwitterionic pCBMAA-2 and pCBMA-2 hydrogels can effectively resist bacterial adhesion.
Figure 30. Representative fluorescence microscopy images of bacterial attachment on pCBMAA-1 in cationic form (A), pCBMAA-2 (B), and pCBMA-2 (C) hydrogels before hydrolysis and on pCBMAA-1 in zwitterionic form (D), pCBMAA-2 (E), and pCBMA-2 (F) hydrogels after 16 hours hydrolysis in PBS. Bacterial cells were stained with LIVE/DEAD BacLight Bacterial Viability assay kit. Cells with damaged cytoplasm membrane are in red/yellow and cells with intact cytoplasm membrane are in green.
3.4 Conclusions

In conclusion, a new elastic zwitterionic material, pCBMAA-1, was synthesized to carry all desired properties (elasticity, stability, antimicrobial and antifouling properties) for biomedical applications. This study provided a better understanding on relationships among structure, function and stability of zwitterionic materials. It is found that one-carbon spacer is necessary to obtain good switchability and stability in CB-based materials. To the best of our knowledge, such an all-in-one material has never been reported. The material will help to address several key challenges of current materials in many biomedical applications, and I believe that this all-in-one material will broaden the application spectrum of zwitterionic materials to a great extent.
CHAPTER IV
ZWITTERATION OF DEXTRAN: A FACILE ROUTE TO INTEGRATE
ANTIFOULING, SWITCHABILITY AND OPTICAL TRANSPARENCY INTO
NATURAL POLYMERS

4.1 Introduction

Polysaccharides are most abundant and most commonly used natural polymers, which have been used in many biotech and biomedical applications, including coatings,[61] biosensing,[62, 63] tissue engineering,[64, 66] drug delivery,[67-69] and bioseparation/purification.[70, 71] Polysaccharide-based materials have attracted a great attention due to their ability of resisting proteins,[134, 150] mammalian cells and microbes,[60] biocompatibility,[65] biodegradability,[151] capability of further functionalization for biosensing and drug delivery,[152] as well as design flexibility for a broad range of applications. Despite intense interests in polysaccharide materials, there are several challenges to be addressed to let the potential of polysaccharide materials fully realized in biotech and biomedical applications. Firstly, antifouling properties of natural polysaccharides are unsatisfactory in applications dealing with the complex medium.[72] For example, antifouling surface from dextran-derivatives in biosensing is not effective in resisting protein fouling from blood sample.[73] Agarose-based affinity protein purification system is troubled by non-specific protein adsorption.[153, 154]
Secondly, natural polysaccharides do not carry both antifouling property and functionality to conjugate other moieties (such as capture ligand and cell adhesion molecule), which are needed in affinity bioseparation, biosensing, tissue engineering and drug delivery. In most cases, functional groups such as tetrazole[74] and carboxylate[75] groups have to be incorporated into polysaccharides. Excessive unreacted functional groups cause non-specific protein adsorption, thus either reducing the sensitivity of the biosensor or leading to low purity in bioseparation. Thirdly, natural polysaccharides can resist bacterial attachment but cannot kill a small amount of attached microbes.[76, 77] Microorganisms can be introduced into patients during surgical procedures, and colonized microorganisms on the surface of the implanted material/device will trigger inflammation and immune response.[78] Therefore, it is highly desired to have a material integrating all desired properties including excellent antifouling property to prolong the lifetime of implanted materials, antimicrobial property to eliminate surgical infection and chronic inflammation, and functionality for conjugating bioactive moieties to promote tissue integration.

The objective of this work is to develop a versatile and high performance zwitterionic polysaccharide platform and understand the structure-property-function relationships of zwitterionic polysaccharides, so that this platform can be readily adapted to address these key challenges for biomedical and biotech applications. Herein I report a facile method of zwitteration of dextran with CB. The integrated zwitterionic polysaccharide consists of a degradable polysaccharide backbone and multifunctional zwitterionic side chains. Polysaccharides can obtain excellent antifouling property, sensitivity to environmental stimuli, functional groups for bioconjugation and
antimicrobial property via zwitterionic side chains, while zwitterionic materials can obtain biodegradability from the polysaccharide backbone. To test our hypotheses, the impact of the CB substitution on the switchability, hydrophilicity, antifouling and optical transparency of dextran was studied in this work.

4.2 Experimental methods

All NMR experiments were performed at 303.2 K on Varian 300-MHz spectrometers under the same condition as described in previous section unless stated otherwise.

4.2.1 Materials and general instrumentation

Dextran (70k), N,N-Dimethylglycine ethyl ester, epichlorohydrin, sodium hydroxide, glycidyl methacrylate, trifluoroacetic acid-d (TFA-d), 2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropionone, cellulose dialysis membrane (14k cut-off), phosphate-buffered saline (PBS), fluorescein isothiocyanate (FITC) and human fibrinogen (Fg), fluorescein diacetate used as cell viability stain were purchased from Sigma-Aldrich (St. Louis, MO). Bovine aorta endothelial cells (BAECs) was purchased from American Type Culture Collection (Rockville, MD). Dulbecco’s Modified Eagle’s Medium (DMEM) was purchased from Invitrogen.

4.2.2 Synthesis and characterization of CB-functionalized dextran (CB-Dex)

4.9 mL (33.7 mmole) of N,N-Dimethylglycine ethyl ester was dissolved and hydrolyzed in 15 mL of NaOH solution containing 1.35 g NaOH (33.7 mmole) at 50 °C
for overnight. After the removal of byproduct (ethanol) with rotavap, the solution was mixed with 1 g of dextran (70k) (6.13 mmole of glucose unit) in water, followed by the addition of 2.5 mL of epichlorohydrin (30.6 mmole) as grafting agent. The mixture was heated at 55 °C with stirring for 2 days. After the reaction, the product was purified by cellulose dialysis membrane (14k cut off) and lyophilized to have CB-functionalized dextran (CB-L-Dex). Higher degrees of CB substitution were achieved by repeating the addition reaction with 10 equivalent of reactant to have CB-functionalized dextran (CB-H-Dex) with a higher degree of substitution. The lyophilized CB-Dex from the first step was then grafted with methacrylate (MA) as a crosslinking functional groups for hydrogel preparation following one of the published method.[155] Three methacrylated dextran derivatives, with different ratio of CB substituent, from 0% (Dex-MA), 35% (CB-L-Dex-MA), to 100% (CB-H-Dex-MA) were prepared in this study. All samples was kept at a similar MA ratio around 25 % (one MA unit per four glucose units). These CB-based materials were expected to have superior nonfouling property, two-state switchability compared to Dex-MA. The degree of CB substitution was assessed by quantitative ¹H NMR by integrating the peaks at 5.1 ppm (anomeric proton of dextran) against 4.8 ppm (CH₃ of CB) (Figure 32).

The molecular weights of dextran derivatives (dissolved in running buffer, concentration: 5 mg/mL) were determined by GPC (Waters 1515 isocratic hplc pump, 7725i injector, 2414 refractive index detector, USA) coupled with two analytical Agilent PL aquagel-OH MIXED-M 8um 300 mm x 7.8 mm columns connected in series, using 0.01 M NaH₂PO₄ and 0.3 M NaNO₃ as a mobile phase at a flow rate of 1 mL/min at room
temperature. Dextran standards (Fluka, Switzerland) in the molecular range of 1–356 kDa were used as calibration standards.

4.2.3 Hydrogel preparation

Dextran hydrogels were prepared via photopolymerization as follows. All samples were dissolved at the same concentration (2 M regarding to glucose unit) with 0.5 wt % 2-Hydroxy-4’-(2-hydroxyethoxy)-2-methylpropiophenone in water. Then the solution was transferred into a mold made of two quartz slides separated by an 1 mm thick PTFE spacer and polymerized under UV (362 nm) for 1 hour. The gel was hydrated and equilibrated in water for 3 days. The wet weight of the hydrogel sample was measured after the removal of excess water. Dry weight of each hydrogel was recorded after the sample was freeze-dried for 48 hours. The water contents of hydrogels are calculated by \((\text{Wet weight} - \text{Dry weight}) / \text{Wet weight} \times 100\%\).

4.2.4 Protein adsorption study

After reaching equilibrium, Dex-MA, CB-L-Dex-MA and CB-H-Dex-MA hydrogels were cut into discs with a biophysical punch (8 mm in diameter and 1 mm thick), washed thoroughly with DI water and transferred into a sterile 24-well plate. 1 mL of FITC-labeled fibrinogen (FITC-Fg) solution (0.1 mg / mL) was added into each well. All samples were immersed in the solution for 30 minutes to allow protein adsorption on hydrogel surfaces. To remove loosely absorbed proteins on sample surfaces, hydrogel samples were rinsed with PBS buffer three times. Protein adsorption on hydrogel surface was visualized with an Olympus IX81 fluorescent microscopy (Olympus, Japan) with
40x lens through FITC filter at a fixed exposure time for all samples, so that different protein surface adsorption amount will result in different fluorescent intensity on images. To make sure that they all focused on the same plan, all pictures were taken on the edge of hydrogel samples. ImageJ software was used to quantify the fluorescent intensity of each sample.

4.2.5 Cell adhesion study

BAECs were chosen to study cell adhesion on all hydrogel surfaces, since their attachment on a surface depend on the protein adsorption on the surface. After hydrogels were equilibrated in water, BAECs were seeded on different hydrogel substrates at 8 x 10^4 cells / well with serum medium consisting of DMEM, 10% fetal bovine serum (FBS), and 1% penicillin–streptomycin and kept in an incubator with 5% CO2 at 37 °C for 24 hours. Tissue culture polystyrene (TCPS) plate was used as positive fouling control surface. Fluorescein diacetate was used to stain the cells. Surface cell coverage and cell morphology was visualized with fluorescence microscope with a FITC filter.

4.3 Results and discussion

As shown in Scheme 5, CB-Dex was synthesized via one pot reaction. The molecular weight and the degree of substitution were characterized by GPC (Figure 31) and 1H NMR spectroscopy (Figure 32), respectively.
4.3.1 Dextran synthesis and characterization

Zwitterionic CB side chains were introduced onto dextran backbone using our rational molecular design. GPC results show a decrease in retention time as the substitution ratio increases from unmodified dextran, CB-L-Dex, to CB-H-Dex. Especially for CB-H-Dex polymer, as expected, after repeated the zwitteration step with higher ratio of reactant, the molecular weight increased significantly. Same trend was found in $^1$H NMR spectra (Figure 32). As the ratio of CB substitution increases, the integrated signal attributed to CH$_3$ group around 3.4 ppm increases significantly. Compared to unmodified dextran (Figure 32A), signal from vinylic protons was seen around 6 ppm after grafting methacrylate units.

![Scheme 5. Synthetic route of CB-Dex-MA.](image)

I expected that hydroxyl and carboxylate groups generated from the reaction can undergo cyclization to form cationic lactone ring structure under acidic condition, while ring opens under neutral or basic conditions. Thus the material can switch between two
different states (zwitterionic and cationic ring form) while achieve two different functions (antifouling and antimicrobial). This structure is similar as previous reported CBOH by Dr. Jiang and coworkers.[125]

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Figure 31. GPC trace of Dextran-70k, CB-L-Dex, CB-H-Dex.
Figure 32. $^1$H NMR spectra of A) Dextran, B) Dex-MA, C) CB-L-Dex, D) CB-H-Dex at 300 MHz, D2O
Figure 33. GPC trace of enzyme degradation products of A) Dextran after 0 minute (black), 5 minutes (green), 60 minutes (blue); B) CB-L-Dex after 0 minute (black), 5 minutes (blue), 60 minutes (green) in 0.1 U/mL of dextranase.
Figure 34. 750 MHz $^1$H($^{13}$C) gHMBC NMR spectrum of CB-H-Dex in its cationic ring form. The cross-peak in dotted circle indicated the ring structure formation of CB side chain.

To confirm our hypothesis, heteronuclear multiple-bond correlation (gHMBC) 2D-NMR spectrum, which provides two- and three-bond correlations between $^1$H and $^{13}$C, was used to verify the ring structure formation of CB-Dex (Figure 34) in TFA-d. CB groups on dextran formed six-membered lactone ring structure and showed well-resolved correlations in the 2D NMR spectrum. The crosspeak in dotted circle shows the two-bond correlation between the resonances of methylene proton adjacent to carboxylate and the resonances of carbonyl carbon. It changes from a single peak into a doublet of doublet as the evidence of ring structure formation. $^1$H NMR data was collected at different time
points to record the dynamic ring formation process (Figure 35B). The conversion ratio was calculated based on the integral ratio from the methyl (-CH3) protons in each form.

As shown in Figure 35C, about 90% of CB-Dex converted into the six-membered ring form in TFA within 2 hours. The ring open kinetics (Figure 35D) was studied by dissolving polymer in their cationic forms in pure D2O. Calculations were performed with the same method as ring formation, and the final conversion was 86% for CB-Dex in 10 hours. Dex-MA was synthesized, according to one of the published method, and used as a control material. No ring formation was observed with Dex-MA in acidic condition.

Zwitterionic coatings can reduce bacterial attachment and delay biofilm formation on surfaces, but they cannot kill attached microorganisms. It is know that biofilm will cause the drop of local pH in vitro and in vivo. It will be ideal if a surface can switch from an antifouling surface to an antimicrobial surface in response to environmental pH drop. In our previous studies,[156, 157] two switchable antimicrobial/antifouling materials were developed and they can switch to an antimicrobial material from a zwitterionic material under acidic conditions. It can kill 99.5% of attached bacteria in its cationic antimicrobial form and then release 95% of killed cells at their zwitterionic antifouling state. It is expected that switchable CB-Dex would have same antimicrobial/antifouling functions.
Figure 35. A) Diagram of CB-L-Dex structural switch between zwitterionic form and cationic form. B) The change of NMR spectra of CB-L-Dex from zwitterionic form to its cationic ring form in TFA-d. C) The conversion kinetics of CB-L-Dex from zwitterionic form to its cationic ring form in TFA-d. D) The conversion kinetics of CB-L-Dex from ring form to its zwitterionic form in D$_2$O.
4.3.2 Protein adsorption test on hydrogel

Protein fouling on the surfaces of devices in biotech and biomedical applications can cause the failure of the device or affect the service life or sensitivity of the devices. One of main reasons for the underperformance of polysaccharides is their unsatisfactory capability to resist protein adsorption from the complex medium. I hypothesize that grafted zwitterionic CB groups can dramatically reduce non-specific protein adsorption on polysaccharide materials. Protein adsorption studies were carried out on the hydrogel surfaces and visualized with fluorescence microscope. Three types of samples were compared. Hydrogels of Dex-MA without CB unit were used as controls in the study. After hydration and equilibrium in PBS, hydrogel samples were briefly rinsed with DI-water, and immersed in 0.1 mg / mL FITC-Fg solution for 30 minutes. Hydrogel with no protein solution contact was used as the positive nonfouling control.

Firstly, the exposure time of the microscope was adjusted with the sample with no contract of FITC-Fg, until a completely dark background was obtained. All four image were obtained from fluorescence microscope at the same excitation light intensity and exposure time thereafter. As shown in Figure 36, among all four samples tested, Dex-MA, as the negative antifouling control, shows the highest fluorescence intensity. The one with highest CB ratio (CB-H-Dex) shows the lowest amount of protein fouling, while lower ratio of CB substituted dextran (CB-L-Dex) shows medium fluorescence intensity. Image-J software was utilized to quantify the fluorescence intensity values of each image by dividing the fluorescence with area. Compared with the negative control, Dex-MA hydrogel, CB-L-Dex and CB-H-Dex hydrogels showed 26.6 % and 4.6 % of fluorescent signal intensities respectively. Hydrogel samples with different CB ratio show similar
equilibrium water content (Table 7), which mean water amount kept in hydrogel has no direct relationship with the ratio of CB substitution. So that the difference in protein adsorption amount on hydrogel surface was not because of water content but rather the hydrophilicity of the materials.

Figure 36. Protein (FITC-Fg) fouling test on hydrogels visualized under fluorescence microscope at the same excitation light intensity and exposure time. Pictures were focused on the edge of the upper surface of each hydrogel sample. A) Dex-MA, B) CB-L-Dex, C) CB-H-Dex, and D) Control hydrogel surface with no protein contact.
Table 7. Protein adsorption (quantified by ImageJ) and equilibrium water content of hydrogels (average of 3 samples).

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4.3.3 Cell adhesion test on hydrogel

For implantable materials, protein adsorption can trigger the cell attachment, which can further trigger foreign body reaction and lead to chronic inflammation or isolation of implanted materials. [158] To further test antifouling properties of CB-Dex hydrogels, cell adhesion studies were performed with BAECs. After incubated at 37 °C for 24 hours, the control TCPS surface turned out full coverage of BAECs. However, there was almost no cell adhesion on CB-H-Dex surface (Figure 37). These results demonstrated that I have successfully create a zwitterionic CB-Dex hydrogel that highly resist protein adsorption and cell adhesion. It is expected that the CB-H-Dex coating can prolong the service life of implanted materials by minimizing protein adsorption and cell attachment.
Figure 37. BAECs attachment test on hydrogel surfaces. A) TCPS, B) Dex-MA, C) CB-H-Dex, D) CB-H-Dex.
4.3.4 Optical transparency

The optical transparency is also important especially for the applications, such as contact lens, optical sensors, etc, which requires the optical clarity of a material. The optical clarity of dextran hydrogels with different degrees of CB substitution (Figure 38) is dramatically different. Dex-MA hydrogel without any CB unit shows white color, and mostly opaque. When the ratio of CB units reach 35%, it became translucent, while the higher ratio substituted CB-Dex-H was completely transparent.

It is important to have a optically transparent clear material to meet the needs of optical sensor or devices, which work in the complex fouling environments. It is possible that ionic interaction between the zwitterionic domains with water trapped inside of hydrogel network increase the solubility of the complex so that the matrix became more transparent with increased CB ratio.

Figure 38. Digital image of dextran hydrogels. Left to right: Dex-MA, CB-L-Dex, CB-H-Dex.
4.4 Conclusions

In this work, it is demonstrated that zwitteration of dextran can be simply achieved in one pot. Zwitterionic CB-Dex show superior nonfouling property, enhanced optical transparency, as well as two state (cationic and zwitterionic) switchability. This work has shed light on ingenious designing of zwitterionic material, and provided a new avenue/direction of generating nonfouling zwitterionic CB units in situ of polysaccharide backbone, who property can be readily tuned through controlling the ratio of substitution. Unique properties from two distinct materials (polysaccharides and zwitterionic materials) were integrated into one material without sacrificing any properties. This strategy can be easily adopted into other systems. To the best of our knowledge, such facile zwitteration method has not been reported. All the advantages, including: simple one pot synthetic pathway in aqueous solution, switchability of two distinct functions, low cost and natural abundance of raw materials, relatively easy purification steps, together with quantitative high yield make this a very promising zwitteration pathway of nature products. Through this study, I also developed a fundamental understanding of basic properties of zwitterionic polysaccharides, and this platform can be adapted to a range of applications (e.g. biosensing, drug delivery, tissue engineering, implantable medical devices, and bioseparation).
CHAPTER V
INTEGRATED ZWITTERIONIC CONJUGATED POLY(CARBOXYBETAINE THIOPHENE) AS A NEW BIOMATERIAL PLATFORM

5.1 Introduction

Conjugated polymers (CPs) have attracted significant interests for numerous biomedical and biotech purposes, including bioelectronics and biosensing,[97, 99, 159] tissue engineering,[160, 161] wound healing,[162] robotic prostheses,[80] biofuel cell,[163] etc., due to their great design flexibility, tuneable conductivity, compatible mechanical properties with soft tissues and ease of fabrication over inorganic conducting or semiconducting materials.[164] As core components in these devices, CPs improve communications between electrochemical devices and biological systems by allowing the delivery of smaller charges or the detection of very low electrical signals, so devices can perform more efficiently.[79-81] However, biomacromolecules, such as proteins and lipids, tend to adsorb to hydrophobic CPs surfaces that are originally designed for non-biological and non-aqueous systems. The nonspecific adsorption of biomacromolecules on electrochemical device surfaces reduces the sensitivity and performance of the device and triggers foreign body response that eventually leads to the failure of implanted devices.[165] \textit{In vivo} studies have shown that the improved electrochemical performance of devices by CP coatings could not be sustained after implantation due to the formation
of non-conductive scar tissues around devices.[166-169] Therefore, materials with tunable electronic and ionic conductivity, good biocompatibility and multi-functionality for allowing specific cell adhesion and proliferation are highly desired and urgently needed.

Herein, I present a zwitterionic conjugated poly(carboxy-betaine thiophene) (PCBTh) based biomaterial platform (Scheme 6) to address key challenges associated with existing CPs for biomedical applications.[170] Conjugated polythiophene (PTh) was selected as the backbone due to its good electronic conductivity, chemical stability, low redox potential, moderate band gap and optical properties in its conducting state.[171, 172] CB was introduced as the side chains because of its excellent antifouling property,[133] high water solubility, ionic conductivity, electrical conductivity,[173] functionality and biocompatibility.[174] Therefore, zwitterionic conjugated PCBTh was expected to carry highly desired properties of both zwitterionic and conjugated polymers, including superior antifouling properties, enhanced electrical conductivity and improved biocompatibility. The objective of this work is to prove the first concept of the integrated zwitterionic conjugated polymer based biomaterial platform and to lay the foundations for its further development.
Scheme 6. Schematic illustration of PCBTh-co-ThMAA hydrogel consists of the conducting backbone and multifunctional zwitterionic side chains.

To test our hypothesis, I designed and synthesized a PCBTh homopolymer and a poly(carboxybetaine thiophene-co-thiophene-3-acetic acid) (PCBTh-co-ThAA) random copolymer with PCBTh as the key functional component. PCBTh-co-ThAA was further functionalized with methacrylamide, thiol and cell adhesion molecules and studied in the form of polymer film and hydrogel for their electrical conductivity, antifouling property to resist protein adsorption/cell attachment and functionality to incorporate cell adhesion molecules to allow the attachment of specific cells. Thus, this new platform can be readily adapted into different forms for further evaluations and different applications.
5.2 Experimental methods

All NMR experiments were performed at 303.2 K on Varian 300-MHz spectrometers under the same condition as described in previous section unless stated otherwise.

5.2.1. Materials and general instrumentation

Thiophene-3-acetic acid (ThAA) was purchased from Matrix Scientific (Columbia, SC, USA). 1,1’-Carbonyldiimidazole (CDI), 1-ethyl-3-(3-dimethylaminopropyl) carbo-diimide (EDC), N-hydroxysuccinimide (NHS) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Chem-Impex International (Wood Dale, IL, USA). Thermo initiator 2,2'-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044) was purchased from Wako Chemicals USA, Inc. (Richmond, VA, USA). Anhydrous tetrahydrofuran (THF), anhydrous chloroform, methanol, dichloromethane, ethyl acetate, acetonitrile, cystamine dihydrochloride, N,N’-Dimethylethlyenediamine, ethyl bromoacetate, anhydrous FeCl₃, sodium hydroxide, phosphate-buffered saline (PBS), human fibrinogen (Fg), bovine serum albumin (BSA), fetal bovine serum (FBS), 100X penicillin–streptomycin solution and fluorescein diacetate used as a cell viability stain were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were used as received without further purification. Bovine aorta endothelial cell (BAEC) was purchased from American Type Culture Collection (Manassas, MD, USA). Dulbecco’s Modified Eagle’s Medium (DMEM) was purchased from Life Technologies (Carlsbad, CA, USA). Water used in all experiments was purified using a Millipore Milli-Q Direct 8 Ultrapure Water system (Billerica, MA, USA).
The AC impedance spectrum was measured by a Solartron Model 1260 Impedance/Gain-phase Analyzer with a Model 1287 potentiostat/galvanostat (UK). The UV-vis absorption spectra of polymers were collected on a Hewlett Packard 8453 UV-vis spectrophotometer (Palo Alto, CA, USA). The fluorescence emission spectra of polymers were collected on a PerkinElmer LS 55 fluorescence spectrometer (Waltham, MA, USA). SEM images were obtained on a Hitachi TM-3000 Tabletop scanning electron microscope.

5.2.2 Synthesis of monomers and polymers

M2 (N-(2-(dimethylamino) ethyl)-2-(thiophen-3-yl)acetamide). 3-Thiophene-acetic acid (4.26 g, 30 mmol) was dissolved in 100 mL of anhydrous THF in a three-necked round bottom flask, followed by the addition of 5.88 g (36 mmol) of 1, 1'-Carbonyldiimidazole (CDI). The mixture was cooled in an ice-bath (0 °C) and kept stirring for 20 minutes under a positive nitrogen flow. 3.28 mL of N, N'-dimethylethylenediamine (30mmol) diluted in 10 mL of anhydrous THF was added dropwise with a dropping funnel. After the complete of addition, the mixture was warmed up to room temperature and kept stirring overnight. THF was removed with a rotary evaporator, and the product was purified with silica gel column chromatography (MeOH/CH₂Cl₂/ethyl acetate, 1/10/10 (v/v/v)). Pure product was obtained as a light yellowish liquid at 67 % yield. ¹H NMR (300 MHz, CDCl₃) δ 7.31 (m, 1H), 7.15 (s, 1H), 7.02 (d, 1H, J= 4.8 Hz), 6.14 (s, 1H), 3.58 (s, 2H), 3.30 (m, 2H), 2.37 (t, 2H, J= 6.0 Hz), 2.18 (s, 6H) (Figure 39). ¹³C NMR (75 MHz, CDCl₃) δ 170.72, 135.26, 128.62, 126.38, 123.15, 57.89, 45.23, 38.29, 37.18 (Figure 40).
Figure 39. $^1$H NMR spectrum of monomer M2 at 300 MHz, CDCl$_3$

Figure 40. $^{13}$C NMR spectrum of monomer M2 at 75 MHz, CDCl$_3$

M3 (methyl thiophene-3-acetate) was synthesized following a previously reported method.[175] Briefly, 3-thiopheneacetic acid (8.52 g, 60 mmol) was dissolved in 50 mL
of methanol with 2 drops of concentrated H₂SO₄. The mixture was heated in an oil bath and refluxed for 24 hours. After the removal of methanol, the crude product was re-dissolved in diethyl ether, washed with DI water and dried with anhydrous magnesium sulfate. Pure product was obtained after filtration and evaporation of solvent. Structure was confirmed with ¹H NMR and the data was in agreement with the previous report.

Homo-polymer P1. 6.11 g (37.7 mmole) of anhydrous FeCl₃ was suspended in 60 mL of anhydrous chloroform under a positive nitrogen flow. The mixture was cooled in an ice-bath (0 °C) and kept agitated for 30 minutes. 2.0 g (9.42 mmol) of compound 2 dissolved in 30 mL of dry chloroform was slowly added into the mixture during a period of one hour. Then the reaction was stirred for 24 hours at room temperature under nitrogen. After the reaction, the product was washed with chloroform and dried with rotary evaporator. Then it was re-dissolved in DI-water and purified through dialysis with cellulose dialysis membrane (1 k cut off). Water was changed daily for a week, and the solution was lyophilized to obtain P1 at 20 % yield. ¹H NMR (300 MHz, D₂O) δ 6.6-7.6 (m, thiophene ring proton, 1H), 3.0-4.3 (m, thiophene ring -CH₂- and -NH-CH₂-, 4H), 2.7-3.0 (m, -CH₂-N(CH₃)₂, 2H), 2.0-2.7 (s, -CH₃, 6H) (Fig. S3).
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Figure 41. $^1$H NMR spectrum of homo-polymer P1 at 300 MHz, D$_2$O

Homo-polymer P2. 130 mg (0.6 mmole) of P1 was dissolved in 15 mL of methanol, followed by the addition of 0.2 mL (1.8 mmole) of ethyl bromoacetate. The mixture was heated at 60 °C for 2 days under nitrogen. After concentrated with rotary evaporator, the product was precipitated in diethylether and dried under vacuum to obtain P2 with quantitative yield. $^1$H NMR (300 MHz, D$_2$O) δ 6.9-7.7 (m, thiophene ring proton, 1H), 4.2-4.6 (m, N(CH$_3$)$_2$-CH$_2$-C=O and -CH$_2$-CH$_3$, 4H), 3.6-4.1 (m, thiophene ring -CH$_2$- and -NH-CH$_2$-, 4H), 3.2-3.6 (s, -N(CH$_3$)$_2$, 6H), 2.9-3.1 (m, -CH$_2$-CH$_2$-N(CH$_3$)$_2$, 2H), 1.1-1.5 (s, -CH$_2$-CH$_3$, 3H) (Fig. S4).
Homo-polymer P3 (PCBTh). P2 was dissolved in DI water and passed through an ion exchange resin (Amberlite IRA-400 OH form) filled column to hydrolyze ethyl ester into the final zwitterionic form. Pure PCBTh was obtained as a red powder after freeze-drying with 95% yield. $^1$H NMR (300 MHz, D$_2$O) $\delta$ 6.5-8.0 (m, thiophene ring proton, 1H), 3.5-4.5 (m, N(CH$_3$)$_2$-CH$_2$-C=O, thiophene ring -CH$_2$- and -NH-CH$_2$-, 6H), 3.0-3.5 (s, -N(CH$_3$)$_2$, 6H), 2.2-2.6 (s, -CH$_2$-CH$_2$-N(CH$_3$)$_2$, 2H) (Fig. S5.).
Figure 43. $^1$H NMR spectrum of homo-polymer P3 at 300 MHz, D$_2$O

Copolymer P7 (PCBTh-co-ThMAA) and P8 (PCBTh-co-ThSH) (Scheme 7). Monomers M2 and M3 were pre-mixed at a feeding ratio of 80:20 for the oxidative polymerization with anhydrous FeCl$_3$. The synthesis from copolymers P4 to P6 were following similar procedures to that of homopolymer P3. After purification from dialysis with cellulose dialysis membrane (1 k cut off), P6 (PCBTh-co-ThAA) was separated into two portion sand submitted to two separate reactions to synthesize P7 (PCBTh-co-ThMAA) and P8 (PCBTh-co-ThSH).

In the first reaction, P6 was reacted with 2-aminoethyl methacrylamide hydrochloride[176] in the presence of EDC to obtain self-crosslinkable copolymer P7 (PCBTh-co-ThMAA). After dialysis, the substitution ratio of methacrylamide double bond to thiophene unit was about 10 % based on $^1$H NMR integral values. $^1$H NMR (300 MHz, D$_2$O) $\delta$ 6.6-8.0 (m, 1H), 5.5-5.8 (m, 1H), 5.2-5.5 (m, 1H), 3.5-4.5 (m, 8H), 3.0-3.5 (s, -N(CH$_3$)$_2$, 6H), 2.5-3.0 (m, 4H), 1.7-2.0 (s, 3H) (Fig. S6).
Scheme 7. The synthetic route of copolymers P7 and P8.
In the second reaction, P6 was reacted with cystamine dihydrochloride using EDC/NHS chemistry,[176] followed by the reduction of disulfide with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) to obtain copolymer P8 (PCBTh-co-ThSH). The incorporation of free thiol groups were designed for the immobilization of copolymers P8 (PCBTh-co-ThSH) on gold-coated SPR sensor chips. Since the resonance from thiol (SH) containing side chain was not resolved from the overlapping signals, the actual substitution ratio of thiol groups cannot be calculated from $^1$H NMR. $^1$H NMR (300 MHz, D$_2$O) $\delta$ 6.6-8.0 (m, 1H), 3.5-4.5 (m, 8H), 2.8-3.5 (s, -N(CH$_3$)$_2$, 6H), 2.6-2.8 (m, 2H), 2.2-2.4 (m, 2H) (Fig. S7).
Figure 45. $^1$H NMR spectrum of co-polymer P8 at 300 MHz, D$_2$O

Copolymers P9 (PCBTh-co-ThRGD). A cysteine containing cell adhesion peptide, CRGDS, was conjugated to the double bond on P7 (PCBTh-co-ThMAA) via the thiol-methacrylamide Michael type reaction in D$_2$O solution. $^1$H NMR was used to monitor the reaction in real time. The ratio of double bonds to thiophene units changed from 10-12% (before conjugation) to 9% (after conjugation). So the RGD substitution ratio is estimated to be about 1-2%, equals to the final consumption of methacrylamide double bond that reflected from the NMR integral values. $^1$H NMR (300 MHz, D$_2$O) $\delta$ 6.6-8.0 (m, 1H), 5.4-5.6 (m, 1H), 5.1-5.4 (m, 1H), 3.4-4.2 (m, 8H), 2.9-3.3 (s, -N(CH$_3$)$_2$, 6H), 2.5-3.0 (m, 4H), 1.6-2.2 (s, 7H) (Fig. S8).
5.2.3 Hydrogel preparation

Both PCBTh-co-ThMAA and PCBTh-co-ThRGD hydrogels were prepared via similar thermo-initiated polymerizations as follows. 100 mg of copolymers was dissolved in 400 μL aqueous solution with 0.5 wt % of thermo-initiator (VA-044). Then the solution was transferred into a mold made of two quartz slides separated by a 1 mm thick PTFE spacer and polymerized at 50 °C for overnight. The gel was equilibrated in DI water and water was changed daily for 7 days. The wet weight of the hydrogel samples was measured after the removal of excess water. PThAA hydrogel was prepared according to a reported method and used as a control in this study.[177]
5.2.4 Polymer film preparation

Polymer thin films were prepared with a graft-to method. Copolymer P8 (PCBTh-co-ThSH) with free thiol end group was prepared at the concentration of 10 mg/mL in a mixed solvent of 90% DI-water and 10% methanol by volume. 400 µL of polymer solution was drop-casted on a gold-coated SPR chip. It was put in a petri-dish and left undisturbed until solvent evaporated at room temperature. Sample was washed with PBS and dried with filtered air before the SPR measurement.

5.2.5 Electrochemical study

The AC impedance spectrum was measured by a Solartron Model 1260 Impedance/Gain-phase Analyzer with a Model 1287 potentiostat/galvanostat in the frequency range from 0.1 mHz to 100 kHz at low amplitude voltage (~10 mV) [22]. The hydrogel sample were cut into a disc with a diameter of 6.8 mm and put between to stainless steel electrodes. The ionic and electronic conductivities of hydrogels were calculated with a previously reported method.[178] The ionic resistance, Ri, can be determined from the relationship 1/R1 = 1/Ri +1/Re, where R1 is the high-frequency semi-circle resistance from impedance data and Re is the electrical resistance measured under small applied DC potentials (-30 mV - +30 mV) using the potentiostat. Cyclic voltammetry (CV) can provide potentiodynamic electrochemical measurements and stability measurement.[83] Fig.S9 shows CV curves and the impedance curve and of PCBTh-co-ThMAA hydrogel based electrodes using a two electrode system. Fig.S9A shows the rate-dependent CVs with the potential window of 0 to 1 V at scan rates of 5, 10, 20, 30 and 50 mV/s. CV were recorded in the potential range of 0–1 V using the
potentiostat. The complex diagram shows a linear behavior at low frequencies, which indicates that the mass transport is the dominant mechanism. The capacitive response at medium frequencies denotes the current carries within the material.

Figure 47. (A) Cyclic voltammogram curves of the PCBTh-co-ThMAA hydrogel electrode at different scan rates (5 – 50 mV·s⁻¹) and (B) Impedance curve of a PCBTh-co-ThMAA hydrogel electrode.

5.2.6 Protein adsorption study

Protein adsorption study: A custom-built four-channel SPR sensor was used to measure protein adsorption on pCBTh-co-ThSH coated surface. Firstly, PBS solution at 50 μL min⁻¹ flow rate was used to obtain a baseline signal. 1 mg mL⁻¹ of Fg solution and 1 mg mL⁻¹ of BSA were then injected into different channels for 10 minutes followed by a PBS wash to remove any loosely bound proteins. The amount of adsorbed proteins was calculated as the change in wavelength before and after protein injection.
5.2.7 Cell adhesion study

BAECs were chosen to study cell adhesion on hydrogel surfaces, following a similar procedure as in a previous work.[179] Hydrogel samples were equilibrated in DI-water and then transferred to sterilized PBS, exposed under UV for half an hour before the experiment. BAECs were seeded on different hydrogel and control surfaces at a concentration of $10^5$ cells mL$^{-1}$ in DMEM containing 10% FBS and 1% penicillin-streptomycin, and kept in an incubator with 5% CO$_2$ at 37 °C for 24 hours. After the incubation, medium was removed from the wells and changed to the staining solution that prepared in sterilized PBS as follows. Fluorescein diacetate was dissolved at a concentration of 10 mg mL$^{-1}$ in acetone, then 50 µL of the solution was diluted in 10 mL sterilized PBS and used for staining the cells. After incubated for 5 min with the staining solution, surface cell coverage and cell morphology was visualized and imaged with an Olympus IX70 fluorescence microscope equipped with a FITC filter at ×10 magnification.

5.2.8 Water content measurement

The water content is a basic property of hydrogel materials for biomedical applications. The wet weight of the hydrogel sample was measured after the removal of excess water. Dry weight was recorded after the samples had been freeze-dried for 48 hours. The water contents of hydrogels (Table 7) are calculated by (Wet weight – Dry weight)/Wet weight x 100%.
5.2.9 Cytotoxicity study

The cytotoxicity of the zwitterionic polymer was studied with various concentrations of pCBTh. 100 μL of BAEC cells solution, at a concentration of $10^5$ cells/mL, were incubated in a 96 well plate for 24 hours with different concentrations (0.5, 5x$10^{-2}$, 5x$10^{-3}$, 5x$10^{-4}$ and 5x$10^{-5}$ mg/mL) of pCBTh. 6 replicates were used for each concentration. As a control, the same cells were also incubated at the same conditions without adding pCBTh. After 24 hours incubation, cells were stained with the same method as discussed in cell adhesion study. Representative fluorescence images of surviving cells were taken for each condition (Figure 48), with an Olympus IX70 fluorescence microscope equipped with a FITC filter at ×10 magnification. The number of cells was counted by three replicates and relative viability was calculated and summarized in Figure 49.
Figure 48. Representative fluorescence images of BAECs treated with a series of dilutions of pCBTh polymer A) 0.5 mgmL$^{-1}$, B) 5x10$^{-2}$mgmL$^{-1}$, C) 5x10$^{-3}$mgmL$^{-1}$, D) 5x10$^{-4}$mgmL$^{-1}$, E) 5x10$^{-5}$ mgmL$^{-1}$ and E) untreated cells, after 24 hours incubation in DMEM medium.
Figure 49. Representative cytotoxicity assay of BAECs treated with a series of dilutions of pCBTh polymer in culture media, expressed as a percentage of control untreated cells.

5.2.10 Optical properties study

The UV-vis absorption spectra of pCBTh were collected on a Hewlett Packard 8453 UV-vis spectrophotometer. Samples were prepared in 20 mM PBS buffer solution at different pH, from pH 2 to pH 12. Fluorescence emission spectra were collected on a PerkinElmer LS 55 fluorescence spectrometer, excited at 411 nm.
5.3 Results and discussion

Electrical conducting hydrogels, which can transport both electrons and ions, are of great interest for biomedical and biotech applications, since they provide not only a favourable electrical conducting environment but also a highest level of hydration and similarity to tissues.[91]

5.3.1 Hydrogel synthesis and morphological characterizations

Since macromonomers are less toxic to cells compared to highly reactive small molecular monomers and crosslinkers for hydrogel synthesis,[180] in this work, PCBTh homopolymer and PCBTh-co-ThAA random copolymer with 20 mol% ThAA repeat unit
were first synthesized using oxidative polymerization with iron (III) chloride in anhydrous chloroform. PCBTh-co-ThAA was further modified with 2-aminoethyl methacrylamide to generate crosslinkable PCBTh-co-ThMAA copolymer (ratio of MAA to thiophene: 10%), where pendant methacrylamide (MAA) function as crosslinking groups (Scheme 8).

Scheme 8. Synthetic routes to PCBTh homopolymer and its random copolymers: PCBTh-co-ThAA, PCBTh-co-ThMAA and PCBTh-co-ThSH.

PCBTh-co-ThMAA hydrogel was prepared with a thermal free radical initiator, VA-044 (see supporting information). The fouling control hydrogel, poly(thiophene-3-acetic acid) (PThAA),[177] and the antifouling control hydrogel, poly(carboxybetaine methacrylate) (PCBMA),[132] were prepared according to reported methods. The
equilibrium water content of PCBTh-co-ThMAA hydrogel (96.3 wt\%) is close to zwitterionic PCBMA hydrogel (93.7 wt\%), but much higher than the control PThAA hydrogel (80.4 wt\%). The porous structure of conjugated polymer hydrogel is highly favourable for electrochemical processes. Optical and scanning electron microscopy (SEM) images (Figure 51) confirmed the desired porous structure of the PCBTh-co-ThMAA hydrogel.

Figure 51. Morphological characterization of PCBTh-co-ThMAA in the form of A) freeze-dried powder with metallic luster after dialysis (for hydrogel preparation), B) wet hydrogel after equilibrated in water (with PCBMA hydrogel as a reference) (8 mm in diameter), C-E) freeze-dried hydrogel by SEM. (scale bars: (C) 300 μm, (D) 100 μm, (E) 30 μm.)
5.3.2 Hydrogel conductivity measurement

In biological systems, electrochemical processes or devices often require embedded materials to transport both ions and electron. It was expected that the zwitterionic conjugated PCBTh-co-ThMAA hydrogel could conduct electron via the conjugated PTh backbone. I also hypothesized that zwitterionic side chains of PCBTh-co-ThMAA would enhance the overall conductivity of materials, since they can affect the self-ionization of water and subsequently facilitate the ionic conductivity. Electrochemical properties of PCBTh-co-ThMAA hydrogels in water were studied using the alternating current electrochemical impedance spectroscopy and cyclic voltammetry (CV). PCBTh-co-ThMAA hydrogel showed high overall electrical conductivity, which was contributed by both ionic (3.67 mS cm\(^{-1}\)) and electronic (2.73 x 10\(^{-4}\) mS cm\(^{-1}\)) transport. PCBTh-co-ThMAA hydrogel showed a good cyclicability (Fig. 47B) and CV remained the same after 10 cycles.

Traditional biomaterials, such as poly(ethylene glycol) (PEG), zwitterionic polymers and polysaccharides, can only conduct ions instead of electron. Conducting hydrogels, which can facilitate both *electronic* and ionic transport, are typically synthesized through either blending or physical crosslinking CPs with non-conducting polymeric hydrogel networks.[92, 93] however, non-conducting components can compromise electrochemical properties of conducting hydrogels.[94] Our system avoids these problems by integrating all functional groups into one polymer chain. Electrical conductivity of undoped PCBTh-co-ThMAA hydrogel by electron transport was comparable to that of a doped poly(aniline) (PANI)/PEG hydrogel,[181] which was engineered for nerve regeneration; however, the ionic electrical conductivity was
improved significantly due to CB side chains. Pan and co-workers reported that a physically crosslinked PANI hydrogel with phytic acid as the gelator and dopant could reach a much higher electrical conductivity, 0.11 S/cm.[94] Compared to their system, no dopant was added in PCBTh-co-ThMAA hydrogel. Electrons on conjugated polymer backbone can transfer to reactive species, such as ions and oxygen, in biological system, if the redox potential of the conjugated polymer is close to these reactive species. To minimize undesired electron transfer between conjugated backbones and the aqueous environment, the CP with a proper LUMO energy level is required for each application. Higher conductivity of conjugated polymers can reduce the potential gradient of the system and the associated side reactions.

5.3.3 Protein adsorption test with SPR

The body responds to any foreign object by launching a series of physicochemical reactions, which are triggered by nonspecifically adsorbed proteins, and eventually forming thick and non-conductive fibrous tissues.[86, 87] Foreign body response can be attenuated if the surface of an implant can effectively resist protein adsorption and cell attachment. I hypothesized that zwitterionic side chains would endow PTh with superior antifouling properties to resist protein adsorption on their surfaces. A four channel surface plasmon resonance (SPR) sensor was used to evaluate the protein adsorption on polymer coated SPR sensor chips. Cysteamine was conjugated to PCBTh-co-ThAA to obtain PCBTh-co-ThSH. Incorporated thiol groups functioned as anchoring sites. PCBTh-co-ThSH was then immobilized to the surface with a “graft to” approach. Two commonly used proteins, bovine serum albumin (BSA) and human fibrinogen (Fg) that
are most abundant in blood plasma, were used to evaluate the antifouling property of PCBTh-co-ThSH coated surfaces at a concentration of 1 mg mL$^{-1}$. The protein adsorption on PCBTh coated gold chip surface is about 0.3 ng cm$^{-2}$ (the detection limit of the SPR sensor) and 0.45 ng cm$^{-2}$ for BSA and Fg, respectively (Figure 52). In comparison, Fg adsorption on unmodified gold surface is around 300 ng cm$^{-2}$.[182] PCBTh-co-ThSH material is qualified as an ultra-low fouling material, which is defined as a surface with less than 5 ng cm$^{-2}$ adsorbed Fg.
Figure 52. Representative SPR sensorgrams showing the very low protein adsorption of 1 mg mL\(^{-1}\) bovine serum albumin (BSA) (top curve) and fibrinogen (Fg) (bottom curve) in PBS buffer on PCBTh-co-ThSH modified SPR substrates.

Ultra-low fouling materials and surfaces are highly desired for biomedical applications. It was reported in a previous study that blood-contacting materials with the ultra-low fouling property would not trigger the platelet adhesion on the surface and subsequently delay the blood coagulation through contact activation pathway. Beside the surface chemistry, antifouling properties of any solid substrate are also affected by the packing density of the functional polymer coating. To rule out the effect of the surface defect and acquire their intrinsic antifouling fouling properties to resist protein adsorption, antifouling polymers are usually studied in the form of high packing polymer brushes, which can be prepared using surface initiated polymerization techniques on
various solid substrates. Due to the limitation of oxidative polymerization, PCBTh-co-ThSH was immobilized to the surface with the “graft to” approach, which leads to a lower packing density compared to polymer brushes prepared via the “graft from” approach; however, the antifouling property of PCBTh-co-ThSH could still reach the level of the ultra-low fouling material.

5.3.4 Controlled cell adhesion study

To further evaluate antifouling properties of PCBTh at a low packing density, cell attachment studies were performed using bovine aorta endothelial cells (BAECs) on PCBTh-co-ThMAA hydrogel. PThAA hydrogel[177] and tissue culture polystyrene (TCPS) were used as positive fouling control surfaces while PCBMA hydrogel was used as a positive antifouling control surface. After 24 hours’ incubation, PThAA hydrogel and TCPS surfaces were almost fully covered with BAEC cells. However, there was a small amount of cells on antifouling PCBTh-co-ThMAA and PCBMA hydrogel surface (Figure 53). The amount of the attached BAEC on PCBTh-co-ThMAA hydrogel surfaces was 1.5% of that on PThAA hydrogel surfaces (Table 7). To increase the solubility of the hydrophobic PTh in water, PTh needs to be modified with charged or hydrogen bond forming side chains. It is known that both positively and negatively charged surfaces will lead to protein adsorption and promote nonspecific cell attachment.[183, 184] Although hydrogels, which are based on neutral and hydrophilic polymers such as dextran, can reduce cell attachment, our previous study demonstrated that zwitterionic CB modified dextran hydrogel can further reduce the nonspecific protein adsorption and cell attachment compared to unmodified dextran hydrogel.[185] Zhang and co-workers
demonstrated that zwitterionic PCBMA hydrogels with ultra-low fouling properties resisted *in vivo* protein capsule for at least 3 months in mice and they also observed that PCBMA hydrogels promoted angiogenesis in the surrounding tissue.[174] I expect that zwitterionic CB side chains of conjugated polymers will improve their biocompatibility by reducing the foreign body responses.

While implants highly resist the nonspecific attachment of unwanted cells, they may require the attachment and proliferation of specific types of cells (such as endothelial cell, neural cell, etc.) on their surfaces to improve signal transduction and material integration with the biological system.[97, 99, 159] Thus, materials at the biointerfaces are required to provide functional groups to conjugate cell adhesion molecules or other bioactive moieties in a controllable manner. It was demonstrated that a cell adhesion peptide, CRGDS, could be conveniently incorporated into PCBTh-co-ThMAA hydrogel via thiol-acrylamide Michael type reaction and the resulting PCBTh-co-ThRGD subsequently formed a hydrogel via the remaining MAA as crosslinkers by the same method for PCBTh-co-ThMAA hydrogel. BAECs were expected to bind CRGDS-functionalized surfaces via αvβ3 integrin on their surface. As shown in Figure 53 and table 8, the cell density of BAEC cells on the CRGDS-functionalized copolymer (PCBTh-co-ThRGD) hydrogel was 51.7% and 62.3% relative to that on PThAA hydrogel and TCPS respectively.
Figure 53. Representative fluorescence microscopy images of attached bovine aortic endothelial cells (BAEC) on (A) PCBTh-co-ThMAA hydrogel, (B) PCBTh-co-ThRGD hydrogel, (C) PCBMA hydrogel, (D) PThAA hydrogel and (E) TCPS surfaces. (F) Quantitative cell density on these surfaces.
Table 8. Equilibrium water content and BAEC cell density on different surfaces. The percentage of the attached cells relative to PThAA hydrogel surface was calculated and presented. (n = 3)

<table>
<thead>
<tr>
<th></th>
<th>PCBTh-co-ThMAA</th>
<th>PCBTh-co-ThRGD</th>
<th>PCBM A</th>
<th>PThAA</th>
<th>TCPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of water content</td>
<td>96.3</td>
<td>98.8</td>
<td>93.7</td>
<td>80.4</td>
<td>-</td>
</tr>
<tr>
<td>% of cell attachment</td>
<td>1.5±0.5</td>
<td>51.7±5.6</td>
<td>2.2±1.6</td>
<td>100±10.2</td>
<td>82.9±5.0</td>
</tr>
</tbody>
</table>

Specific cell attachment on a substrate depends on the density of the cell adhesion molecule on its surface. According to the nuclear magnetic resonance (NMR) measurement, the degree of substitution (DS) of CRGDS, which is defined as the number of the peptide chain per 100 thiophene repeat unit, is 1-2%. A recent study showed that 1% RGD peptide in an antifouling phosphorylcholine hydrogel could lead to ~70% attachment of C2C12 and SKOV3 cells compared to TCPS.[186] From the antifouling aspect, the low DS of bioactive molecules is desired, since excessive charged or hydrophobic bioactive molecules on a material/surface may compromise its antifouling properties and lead to the nonspecific attachment of unwanted cells. The water content of PCBTh-co-ThRGD hydrogel (98.8 wt%) was comparable to that of PCBTh-co-ThMAA hydrogel. The results indicated that cell attachment was due to the incorporation of cell adhesion molecules. In PCBTh-co-ThRGD hydrogel, DS of incorporated CRGDS or other cell adhesion molecules can be controlled by adjusting the ratio of CRGDS to methacrylamide according to the requirements of different applications.
5.3.5 Optical properties study

CPs are known to have interesting optical properties in response to environmental stimuli such as ionic strength, pH, and temperature. The optical properties of CPs are very attractive for biomedical applications, since they are complimentary to the electrical conducting properties of CPs and can provide the extra freedom for materials/devices to monitor the surrounding environment. Fluorescence spectra of PCBTh were measured in response to pH changes in 20 mM phosphate solution. It was found that the fluorescence intensity of PCBTh was very sensitive to pH changes. As shown in Figure 54, the maximal emission signal occurred at the similar wavelength (around 548 nm) under different pH values, but fluorescence intensity changed dramatically. As the pH value of the solution changed from 12 to 2, the emission intensity of PCBTh at 548 nm gradually decreased and eventually dropped to about 30% of the original intensity. The side chain of CPs determines their overall planarization, solubility in a solvent and assembly behaviour, which subsequently affects their optical properties; however, the detailed mechanism(s) of optical sensitivity of PCBTh in response to pH changes is still unclear.

In CB side chains, the length of the spacer between amine and carboxylate affects the acidity of carboxylate.[187] The pKa of carboxylate of CB with 1-methylene spacer is around 2.[188] The majority of carboxylates remain at the anionic state for pH > 3 conditions.
Figure 54. Fluorescence spectra of pCBTh in 20 mM phosphate solution at pH 2 (purple, the bottom line), 4 (red, second to the bottom line), 6 (green, third to the bottom line), 8 (cyan, third to the top line), 10 (pink, second to the top line) and 12 (black, the top line)

The permanently charged quaternary ammonium groups help PCBTh maintain in the soluble state at all pH conditions. Thus, the pH sensitive phenomenon of PCBTh cannot be explained by the mechanism proposed for anionic CPs in which the self-assembly of polymer chains occurs when the solubility of polymers changes dramatically under different pH conditions. A systematic study is needed to elucidate the mechanism of pH sensitivity.

The performance and lifetime of electrochemical devices are significantly influenced by interfacial mechanisms occurring at the device/biological environment interface, including biofouling,[189, 190] foreign body response,[158] loss of structural
integrity and infection.[191] I believe that zwitterionic CPs can potentially address these challenges in various forms. Linear PCBTh can be used to modify electrode as a thin film. Crosslinked PCBTh hydrogel can be used for application that requires more stable, long term, porous, higher specific surface and/or higher hydration interfaces. Cells, enzymes and/or other macromolecules can be encapsulated into the hydrogel before or after the gel formation. To develop an initial understanding on the toxicity of PCBTh, I studied the acute cytotoxicity of PCBTh polymer at various concentrations. After 24 hr, the results show that PCBTh has little effect on the proliferation of BAEC cell (Figure 48). Systematic chronic toxicity and in vivo biocompatibility tests, which are eventually needed for all implantable materials, will be planned in our future study. It was found that the CB polymer stabilized conjugated proteins and significantly prolonged protein’s activity.[134] This property is particularly useful for biosensing, since the activity of enzymes/biomolecules is another limiting factor for the function of devices. Various CB materials[192] have also demonstrated superior antifouling properties of resisting microbes,[76, 133] excellent biocompatibility,[193] as well as the capability of further functionalization for applications in biosensing[26] and drug delivery[132, 135]. These properties make CB polymers very useful materials to fabricating biomedical devices to prevent protein adsorption, prolong the activity of biomolecules, provide functional groups for conjugation and increase the lifetime of the device.
5.4 Conclusions

I developed a versatile and high performance zwitterionic CP platform, which integrates all desired functions into one material. This zwitterionic CP consists of the conjugated backbone and multifunctional zwitterionic side chains. Non-conducting zwitterionic materials gain electrical conductivity through the conjugated backbone and CPs obtain excellent biocompatibility, sensitivity to environmental stimuli and controllable antifouling properties via multifunctional zwitterionic side chains. Unique properties from two distinct materials (conjugated polymer and zwitterionic polymer) are integrated into one material without sacrificing any properties. Through this study, I also established a better understanding of structure-function-property relationships of zwitterionic CPs. This platform can potentially be adapted for a wide range of applications (e.g. bioelectronics, tissue engineering, wound healing, robotic prostheses, biofuel cell, etc.), which require high performance conducting materials with excellent antifouling/biocompatibility at complex biointerfaces.
CHAPTER VI
CONCLUSIONS AND FUTURE WORK

Integrating zwitterionic CB moieties to the material system to control biofouling and enhance specific bindings has been demonstrated to be an effective and facile strategy for many biomedical and biotech applications. Due to their design flexibility, CB materials can be customized to provide desired properties for each application.

This dissertation details the design and development of three novel zwitterionic CB based polymeric biomaterial platforms, aiming to solve some intrinsic drawbacks, incorporate multifunctionality, improve the performance and broaden the application spectrum of existing zwitterionic materials.

In chapter II and III, a switchable CB biomaterial platform with enhanced mechanical property was developed. By introducing hydroxyethyl groups, the materials can reversely switch between an antimicrobial cationic ring form and an antifouling zwitterionic form. Both hydroxyethyl and amide groups can improve intermolecular hydrogen bonding interaction between polymer chains, thus enhance the overall mechanical strength. A systematic study upon structure-function-stability relationship of this platform shows that CB materials with one carbon spacer between quaternary ammonium cation and negatively charged carboxylate were more stable, while the one with two carbon spacer underwent elimination reaction in both acidic and basic conditions.
conditions, which results in a loss of their zwitterionic functionality. It also provides a general design guideline for new zwitterionic materials targeting biomedical applications.

In chapter IV, a biodegradable CB biomaterial platform was developed. After one step zwitteration, the materials show superior antifouling property, reverse switchability and improved optical transparency. The molecular weight and degradation rate can be customized by controlling the CB substitution ratio. This facile approach can be easily adapted to many material systems where nonfouling property is highly desired.

In chapter V, an electrical conducting CB biomaterial platform was developed. The versatile and high performance zwitterionic CP platform integrates many desired functions into one material. Zwitterionic materials gain electrical conductivity through the conjugated backbone while CPs obtain excellent biocompatibility, sensitivity to environmental stimuli and controllable antifouling properties via multifunctional zwitterionic side chains.

In conclusion, three multifunctional zwitterionic pCB based novel biomaterial platforms have been successfully developed. First switchable antifouling and antimicrobial pCB platform has solved a long-standing issue of poor mechanical strength for most of zwitterionic-based materials. Second dextran based pCB platform provides a facile method to modify biodegradable polysaccharide. Third antifouling and electrical conducting pCB platform prove the concept of integrated zwitterionic conjugated polymer with controlled targeting functionality.

For the future work, the development of more advanced zwitterionic electrical conducting biomaterial platform, a poly(3,4-ethylenedioxythiophene (PEDOT) based system with improved conductivity, stability and optical property, is in progress. It is
expected to find many applications in biosensing, controlled drug delivery, tissue engineering, and protein purification, which will significantly advance the development of conjugated polymers in the field of biomedicine and biotechnology.
REFERENCES


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