Discovery and Development of Synthetic and Natural Biomaterials for Protein Therapeutics
and Medical Device Applications

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Controlling nonspecific protein interactions is important for applications from medical devices to protein therapeutics. The presented work is a compilation of efforts aimed at using zwitterionic (ionic yet charge neutral) polymers to modify and stabilize the surface of sensitive biomedical and biological materials. Traditionally, when modifying the surface of a material, the stability of the underlying substrate. The materials modified in this dissertation are unique due to their unconventional amorphous characteristics which provide additional challenges. These are poly(dimethyl siloxane) (PDMS) rubber, and proteins. These materials may seem dissimilar, but both have amorphous surfaces, that do not respond well to chemical modification.

PDMS is a biomaterial extensively used in medical device manufacturing, but experiences unacceptably high levels of non-specific protein fouling when used with biological samples. To
reduce protein fouling, surface modification is often needed. Unfortunately conventional surface modification methods, such as Poly(ethylene glycol) (PEG) coatings, do not work for PDMS due to its amorphous state. Herein, we demonstrate how a superhydrophilic zwitterionic material, poly(carboxybetaine methacrylate) (pCBMA), can provide a highly stable nonfouling coating with long term stability due to the sharp the contrast in hydrophobicity between pCBMA and PDMS.

Biological materials, such as proteins, also require stabilization to improve shelf life, circulation time, and bioactivity. Conjugation of proteins with PEG is often used to increase protein stability, but has a detrimental effect on bioactivity. Here we have shown that pCBMA conjugation improves stability in a similar fashion to PEG, but also retains, or even improves, binding affinity due to enhanced protein-substrate hydrophobic interactions. Recognizing that pCBMA chemically resembles the combination of lysine (K) and glutamic acid (E) amino acids, we have shown how zwitterionic nonfouling peptides can be genetically engineered onto a protein to form recombinant protein-polymer conjugates. This technique avoids the need to post-modify proteins, that is often expensive and time consuming in protein manufacturing.

Finally, we have developed two new peptide screening methods that were able to select for nonfouling peptide sequences. The selection for nonfouling sequences is not possible using traditional methods (phage display, yeast display, bacterial display and resin display) due to the presence of background interference. In our first nonfouling peptide screening method, we measured the fouling properties of peptides that were immobilized on the surface of solid glass beads. Peptides first needed to be rationally designed, and then subsequently evaluated for protein binding. Using this method, we were able to screen of 10’s of sequences. Our second nonfouling peptide screening method is able to screen thousands of peptide sequences using a
combinatorially generated peptide library. This was accomplished using controlled pore glass (CPG) beads as substrates to develop one-bead-one-compound (OBOC) peptide libraries. The choice of a porous substrate made it possible to synthesize enough peptide material to allow for peptide sequencing from a single bead using mass spectrometry techniques.
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Chapter 1

Introduction

Controlling nonspecific protein interactions is important for several applications, from medical devices to protein therapeutics. Nonspecific interactions, when interfacing with the human body, can lead to loss of device function and rejection from the body. The best way to avoid these problems has been to modify the surface of the material to prevent nonspecific protein adsorption. Materials attached to surfaces to prevent nonspecific protein adsorption are known as nonfouling materials. For the last several decades, the most commonly used nonfouling material to attach to desired surfaces has been poly(ethylene glycol), commonly known as PEG.1 PEG is a simple polymer, consisting of alternating ether and ethylene groups (PEG, Scheme 1.1) that binds strongly many water molecules through hydrogen bonding. Zwitterions are molecules that possess an equal number of positively and negatively charged groups. Due to their ionic nature, zwitterions also interact strongly with water. Our work focuses on the use of zwitterionic polymers as nonfouling materials, where PEG was often the only prior option. We have found that polymeric zwitterions, such as poly(sulfo betaine methacrylate) (pSBMA) and poly(carboxy betaine methacrylate) (pCBMA, Scheme 1.1), are able to resist nonspecific protein adsorption even better than PEG.2 We have also applied our materials towards applications involving nanoparticles,3,4 hydrogels,5 wound dressings,6 marine coatings,7 and several others.8-10

The presented work is a compilation of efforts aimed at using zwitterionic polymers to modify and stabilize the surface of highly sensitive biomedical and biological materials, where
PEG does not work, due to its amphiphilic properties (solubility in both hydrophilic and hydrophobic phases). Traditionally, when modifying the surface of a material, the stability of the underlying substrate. The materials modified in this dissertation are unique due to their unconventional amorphous characteristics which provide additional challenges. These are poly(dimethyl siloxane) (PDMS) rubber, and proteins. These materials may seem dissimilar, but both have amorphous surfaces and hydrophobic cores that do not respond well to chemical modification. Poly(ethylene glycol) (PEG) due to its organic soluble properties, does not form stable surface modifications with PDMS or proteins.

It is often thought that PEG’s water hoarding characteristics come from it being a hydrophilic material, but this is misleading. PEG (depending on molecular weight) only possesses modest dielectric constants. It’s hydrophilic nature, in fact, is due to the precise positioning of ether groups along its backbone, which act as ideal hydrogen bond acceptors for water. It is interesting to observe that polymers with similar structures are found to be insoluble in water, such as poly(methylene oxide) and poly(propylene oxide). While PEG is unique compared to poly(methylene oxide) and poly(propylene oxide) for water solubility, all three share solubility in solvents such as toluene, dichloromethane and acetone. PEG’s solubility in these nonpolar solvents is more representative of what would be expected from chemical makeup, with solubility in water being the exception rather than the rule. While PEG’s solubility in nonpolar solvents is useful during organic synthesis, this characteristic can have negative consequences by undesirably interfering with hydrophobic regions in many applications. This fundamental concept is the foundation for this dissertation.

While both PEG and zwitterionic polymers, like pCBMA, are able to provide resistance to protein adsorption to surfaces, they use different mechanisms. PEG will specifically bind to large
amounts of water, which sterically prevents proteins from interacting with the surface. Displacing bound water from PEG is an energetically unfavorable event. pCBMA’s interactions with proteins can be described by a theory referred to as the Hofmeister Series. The Hofmeister Series is an arrangement of ions based on their ability to stabilize or destabilize proteins (Figure 4.8). The pattern shows that ion pairs consisting of high charge density anions with a low charge density cations (i.e. F\(^-\) and Cs\(^+\)) should stabilize proteins, while low charge density anions with high charge density cations (i.e. I\(^-\) and Li\(^+\)) should destabilize proteins.\(^\text{16}\) This phenomenon is most commonly observed in ammonium sulfate precipitation used for protein purification. pCBMA consists of an acetate and ammonium group, which is one of the most stabilizing pairs of monovalent ions. With this understanding, it is not that pCBMA sterically forces away proteins from surface, as with PEG. Instead, pCBMA stabilizes proteins and prevents them from unfolding.\(^\text{17}\) If proteins are kept from unfolding, they are unable to adhere to the surface, and thereby remain in solution. Nature has already made use of zwitterions for stabilizing cytosolic machinery. Organisms that exist in extreme environments are often found with high levels of zwitterions, which are a type of “osmolyte”.\(^\text{18}\)

Zwitterionic materials differentiate themselves from PEG in two fundamental ways. Firstly, zwitterions are truly superhydrophilic due to their ionic nature, and thereby have little to no solubility in non-aqueous environments. Secondly, they act as nonfouling materials by chemically resisting protein unfolding as described by the Hofmeister Series, rather than through steric hindrance. Herein, we show how pCBMA provides stability to unconventional surfaces, such as poly(dimethyl siloxane) (PDMS) and proteins. Previously, PEG has been used to stabilize these types of materials, but has failed due to its miscibility with the PDMS substrate and a proteins core. Finally, we introduce new zwitterionic materials that are comprised of
natural occurring peptides. These provide the same nonfouling properties and stabilizing effects as synthetic zwitterionic polymers, but have the additional benefit of being natural materials.
1.1 Schemes

Scheme 1.1: Chemical structures of poly(ethylene glycol) (PEG) and poly(carboxybetaine methacrylate) (pCBMA)
Chapter 2

Suppressing Surface Reconstruction of Superhydrophobic PDMS using a Superhydrophilic Zwitterionic Polymer

Poly(dimethyl siloxane) (PDMS) is extensively used for biomedical applications due to its low cost, ease of fabrication, high durability and flexibility, oxygen permeability, and self-healing properties. PDMS, however, has some significant drawbacks. PDMS experiences unacceptably high levels of non-specific protein fouling when used with biological samples due to its super-hydrophobic characteristics. Unfortunately, conventional surface modification methods such as do not work for PDMS due to its low glass transition temperature. This phenomenon has been a well-known problem for years as “hydrophobic regeneration”. For the same reason, it is also very difficult to bring functionalities onto PDMS surfaces. Herein, we demonstrate how a superhydrophilic zwitterionic material, poly(carboxybetaine methacrylate) (pCBMA), can provide a highly stable coating with long term stability due to the sharp contrast in hydrophobicity between pCBMA and PDMS. This material is able to suppress nonspecific protein adsorption in complex media and functionalize desired biomolecules needed in applications, such as diagnostics, without sacrificing its nonfouling characteristics.

2.1 Introduction

Due to its advantageous material properties, PDMS has been used to manufacture catheters, contact lenses, prosthetics, and many other devices. In recent years, PDMS has also found an exciting role in the manufacturing of microfluidic devices. Unfortunately, like many other hydrophobic materials, PDMS is susceptible to high levels of protein adsorption. For implants,
this can lead to rejection from the body by the immune system, and when in contact with blood, protein fouling can result in thrombosis and clotting.23,24

Currently, the easiest and most widely used method to reduce protein fouling of PDMS is to make the surface more hydrophilic by oxidizing the surface using UV-ozone,25 oxygen plasma,26 or an oxidative wet chemical method.27 These strategies introduce hydroxyl groups directly onto the PDMS surface to increase surface hydrophilicity, reduce non-specific protein fouling, and provide functional groups for functionality. Due to their superhydrophobicity and low glass transition temperature, PDMS polymer chains will rearrange and undo most surface coatings.15,28-30 This is commonly referred to as hydrophobic regeneration (Figure 2.1a).26 Thus, these modifications often last only a few hours or sometimes a matter of minutes (Figure 2.1b).31 More effective methods introduce material coatings onto the surface, such as with sol-gel, layer-by-layer, surfactant coatings, silane coatings, polymer attachment, and surface polymerizations.22,32,33 In recent years, poly(ethylene glycol) (PEG) has been the polymer most widely used to modify the surface of PDMS for interacting with biological samples. Despite its ubiquitous use, PEG coatings are unstable, which has been a well-known problem for years due to reconstruction that occurs at the interface between the PDMS and PEG polymers (Figure 2.1c).15,30,34 While PEG is often considered a hydrophilic polymer, this material is in fact amphiphilic, being soluble in such non-polar solvents as dichloromethane and toluene, in addition to water.

Herein, we modify the surface of PDMS with the zwitterionic polymer pCBMA (Figure 2.1d and 2.1e). When compared to PEG, zwitterionic polymers are chemically very different. Zwitterionic polymers are strongly hydrated via electrostatically-induced hydration and have extremely poor solubility in non-aqueous solvents, making surface rearrangement with PDMS
thermodynamically unfavorable. Furthermore, zwitterionic polymers have strong chain-chain interactions formed by ionic bridging between the zwitterionic polymers,\textsuperscript{35} enhancing the coating’s stability (Figure 2.1d). These properties make zwitterionic polymers ideal for the interface between biological media and PDMS.

2.2 Results and Discussion

2.2.1 Surface polymerization and characterization

CBMA was introduced to the PDMS surface via surface initiated atom transfer radical polymerization (SI-ATRP). This was done by first treating the surface with an ATRP trichlorosilane initiator using chemical vapor deposition. Polymerization of CBMA occurred in 100% water. This is worth noting because many organic solvents absorb into PDMS, deforming the material, which is important to avoid when dealing with medical devices. Polymer film thickness was measured as a function of reaction time using multiple-angle ellipsometry (Figure 2.2a), with a final film thickness around 30 nm. It is worth noting that this method for introducing pCBMA to the PDMS surface was done without needing to be exposed to organic solvents and only water.

The surface of the pCBMA modified PDMS was imaged using atomic force microscopy (AFM) to evaluate the structure of the modified surface in both wet and dry conditions. When compared to unmodified PDMS (Figure 2.2b), pCBMA polymer domain formations were obvious to see (Figure 2.2c). Under wet conditions (Figure 2.2d), the pCBMA film became hydrated and the dried polymer domains were no longer visible, leaving a smooth surface. The smooth hydrated film demonstrates the uniformity of the polymer film that is typical of SI-ATRP.

2.2.2 Long-term resistance to protein and serum fouling
Protein fouling was measured as a function of polymerization time using a fibrinogen adsorption enzyme-linked immunosorbent assay (ELISA) assay, as shown in Figure 2.3a, with a minimum at reaction times from 8 to 24 hours. By controlling reaction time, protein adhesion can be kept at ~2% compared to unmodified PDMS. Next, long-term stability was evaluated with samples that had undergone 18 hours of polymerization time. These were incubated in either wet conditions in phosphate buffered saline (PBS) or dry conditions in a desiccator. The results for fibrinogen adsorption relative to incubation time can be seen in Figure 2.3b for both conditions. Almost no protein adhesion was seen for up to 31 days for samples incubated under dry conditions. Eventually, the dry surface became unstable and fouling increased at 74 days. When incubated in wet conditions, the nonfouling properties of pCBMA-coated PDMS remained ultralow, with <2% protein adsorption after 74 days. These results are significant, as most PDMS-based biomedical devices operate under aqueous conditions. To fully stress the performance of the coating, the pCBMA-coated PDMS surface was exposed to undiluted human serum. After washing, Sulfo-NHS activated biotin was used to non-specifically tag any adsorbed proteins that fouled. Next, fluorescein isothiocyanate (FITC) labeled NeutrAvidin was used to visualize any adsorbed protein. This was a particularly useful method we developed for detecting adsorption from protein mixtures on PDMS, as most traditional labeling methods using direct tagging with activated fluorescent tags will physically adsorb into PDMS. Our results showed that uncoated PDMS showed significant serum protein binding (Figure 2.3c), while little to no serum binding occurred on the pCBMA-coated PDMS sample (Figure 2.3d). With complete resistance to undiluted human serum, it is promising that pCBMA coatings can be used on PDMS devices interacting with whole blood.

2.2.3 Functionalization
While the nonfouling capacity and long term stability of pCBMA-modified PDMS are considerable, the unique structure of pCBMA allows for covalent immobilization of biomolecules such as RGD-containing peptides for cell adhesion or antibodies for biomarker detection\textsuperscript{36, 37} without sacrificing the nonfouling characteristics of the surface. We chose FITC labeled bovine serum albumin (FITC-BSA) as a model biomolecule to demonstrate functionality. This was done first by activating the exposed carboxylic acid groups with reactive N-hydroxysuccinimide (NHS) esters using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). The activated NHS groups are able to react with biomolecules containing accessible primary amines. Once the proteins are covalently bound to the surface, the surface can be deactivated under slightly basic conditions (pH ~ 10) to hydrolyze unreacted NHS groups back to carboxylic acids, thereby restoring the zwitterionic nonfouling background. In Figure 2.4a, a pCBMA-PDMS surface is shown that was exposed to FITC-BSA without any activation, showing no visible protein adhesion. Figure 2.4b shows the pCBMA-PDMS surface activated with EDC/NHS, which was then exposed to FITC-BSA. Figure 2.4c shows a pCBMA-PDMS surface that was activated with EDC/NHS, but then deactivated with 10 mM sodium carbonate, pH 10.0, before being exposed to FITC-BSA. This confirms that the pCBMA surface can controllably immobilize desired biomolecules without affecting its nonfouling properties.

2.3 Conclusions

By employing a superhydrophilic zwitterionic polymer, we are able to modify zwitterionic PDMS and to suppress surface reconstruction for long-term stability. We have been able to introduce excellent nonfouling properties in undiluted blood serum and provide the ability to specifically functionalize desired biomolecules. Results show that a thin and uniform pCBMA layer can be introduced onto PDMS to create a nonfouling surface that will last up to 31 days in
dry air, and >74 days in aqueous conditions (saline). It is worth noting that, due to the swelling properties of PDMS in non-aqueous solvents, surface modification was accomplished without exposure to any solvents but water. This greatly increases amenability to biomedical applications because it avoids any surface deformation.\textsuperscript{38} Due to long-term stability, nonfouling properties, and functionality, this surface modification method based on pCBMA will enable a wider range of biomedical applications that require specific binding or targeting.

2.4 Experimental Section

2.4.1 Materials

Copper(I) bromide (99.999\%), Copper(II) bromide (>99.0\%), 1,1,4,7,10,10-hexamethyltriethylene tetramine (\textit{HMTETA}), d-Biotin, bovine plasma fibrinogen, and phosphate-buffered saline (PBS, 0.01 M phosphate, 0.138 M sodium chloride, 0.0027 M potassium chloride, pH 7.4) were purchased from Sigma Chemical Co. N-(3-Dimethylaminopropyl)-N'\-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS), Sulfo-N-Hydroxysuccinimide (Sulfo-NHS), and fluorescein isothiocyanate were purchased from Acros Organics. NeutrAvidin was purchased from Pierce Biotechnology, Inc. Sylgard \textregistered 184 Silicone Elastomer Kit was used from Dow Corning. 2-Carboxy-\textit{N,N,}-dimethyl-\textit{N}-(2\'-\textit{methacryloyloxy})ethyl) ethanaminium inner salt (carboxybetaline methacrylate, CBMA) was synthesized as previously reported.\textsuperscript{39} Synthesis of the ATRP initiator trichlorosilane:10-undecen-1-yl 2-bromo-2-methylpropionate was synthesized as previously reported.\textsuperscript{40}

2.4.2 PDMS preparation initiator immobilization

Sylgard 184 Silicone Elastomer (Dow Corning) was prepared by thoroughly mixing 10 parts base to 1 part curing agent. The mixture was poured into a 20 cm petri dish and placed under vacuum to remove bubbles. The PDMS was cured at 60 degrees overnight. After curing, samples
were cut and soxhlet extracted against acetone for one day, then dried under vacuum and mild heating. Clean samples of PDMS were treated with 3% ozone at 2 L/min for 1 hour. After oxidation, samples were exposed briefly to humid air and dried with nitrogen. For vapor deposition, the samples were placed on a clean glass cover slip in a vacuum desiccator with a small amount of ATRP trichlorosilane initiator. A strong vacuum was pulled on the desiccator, which was then sealed and placed at 80°C for 4 hours. After the chemical vapor deposition of the trichlorosilane, the PDMS samples were briefly dipped in a 1mM solution of NaOH (pH = 11) to crosslink the surface silanes and neutralize the HCl byproducts.

2.4.3 Atom transfer radical polymerization (ATRP)

Cu(I)Br (7.17 mg, 0.0500 mmol) and Cu(II)Br₂ (2.79 mg, 0.0125 mmol) were placed in a small test tube under nitrogen protection and sealed with a rubber septum. 1.375 g (6 mmol) CBMA monomer and a PDMS substrate with immobilized initiator were placed in a large test tube, also under nitrogen protection and sealed with a rubber septum. Both test tubes were deoxygenated by five repetitions of a strong vacuum followed by nitrogen backfill. Deoxygenated water (deoxygenated by bubbling with N₂ gas) was then added to both test tubes (2 mL to the small tube, 30 mL to the large tube). While stirring, 17 uL of HMTETA was added to the copper solution and stirred for 30 min for ligand complexation. To initiate polymerization, 1.2 mL of the copper catalyst solution was added to the CBMA monomer and PDMS substrate. Reaction time was controlled to adjust film thickness.

2.4.4 Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to measure fibrinogen adsorption on the modified and unmodified PDMS surfaces. Substrates were soaked in PBS for at least 30 minutes before the experiment. Next, the substrates were incubated in 1 mg/mL fibrinogen in PBS for 60 min. The substrates were then
rinsed several times with fresh PBS. The samples were next incubated in PBS solution containing 5.5 μg/mL horseradish peroxidase (HRP)-conjugated anti-fibrinogen (USbiological) for 30 min. The substrates were again rinsed several times with PBS. Finally, the substrates were incubated in 1 mL of 0.1 M citrate–phosphate buffer (pH 5.0) containing 1 mg/mL of o-phenylenediamine (OPD) and 0.03% hydrogen peroxide for 60 minutes. Absorbance at 490 nm was measured using a spectrophotometer. Values of fibrinogen adsorption are represented as percentages of the adsorption levels from unmodified PDMS.

2.4.5 Bovine serum albumin and NeutrAvidin labeling with fluorescein isothiocyanate (FITC)

FITC-BSA and FITC-NeutrAvidin were prepared by protein conjugation with fluorescein isothiocyanate (FITC). Briefly, 30 mg of protein was dissolved in 3 mL of 100 mM NaHCO₃, pH 9. From a solution of 15 mg/mL FITC in DMSO, 300 μL was added to the protein solution to initiate labeling. After 2 hours, the FITC labeled conjugates were purified twice using 10 mL Bio-Gel P-6DG Bio-Rad disposable size exclusion columns.

2.4.6 Serum binding

9.77 mg (40 mmol) d-Biotin was dissolved in 200 μL of DMSO. 7.67 mg EDC (40 mmol) and 8.69 mg Sulfo-NHS (40 mmol) were dissolved in 200 μL of DI water. The EDC/Sulfo-NHS solution was slowly added to the biotin while frequently vortexing. The coupling reaction was allowed to react for 20 minutes. Next, 5 μL of 2-mercaptoethanol was added to quench unreacted EDC and allowed to stand for 10 minutes. This solution was diluted 25x into 0.1 M phosphate, pH 7.4, containing the serum exposed sample for 60 minutes. Samples were then washed several times with PBS to remove unbound biotin. Next, they incubated in a solution of 1 mg/mL BSA
and 0.01 mg/mL FITC-NeutrAvidin in PBS for 30 min. Samples were again washed with PBS and imaged using a fluorescence microscope.

### 2.4.7 Functionalization

57.4 mg EDC and 7.67 mg NHS were dissolved in 1.5 mL ultrapure (18.2 MΩ) water. pCBMA-coated PDMS was incubated in the activation solution for 10 minutes. The sample was removed, rinsed with water and dried with air. Next, 2 μL of FITC-BSA at 1 mg/mL in 10 mM phosphate, pH 7.4, was spotted on the activated pCBMA-PDMS surface and allowed to react for 30 min. The surface was then rinsed with PBS and incubated in 300 mM NaCl and 10 mM Na₂CO₃, pH 10.0, for 1 hour to deactivate any unreacted NHS ester groups still remaining on the surface. A similar procedure was followed, but without NHS in the activation solution, as a control. Another control was performed where FITC-BSA was not spotted on the activated surface, but instead the surface was deactivated first with basic solution. FITC-BSA was then spotted to test the effectiveness of deactivation. Samples were washed with PBS and imaged using a fluorescence microscope.
2.5 Figures

Figure 2.1: (a) Over time PDMS chains will reconstruct, resulting in a surface that is constantly being renewed, known as hydrophobic regeneration; (b) PDMS surface is made more hydrophilic by oxidation, but this surface will degrade over time; (c) The same occurs with polymers, particularly like PEG of amphiphilic nature; (d) pCBMA’s unique characteristics form a super stable nonfouling surface due to its zwitterionic nature. The structure of pCBMA is shown in (e).
Figure 2.2: (a) pCBMA film thickness as a function of reaction time, as measured by multiple-angle ellipsometry. Error bars represent standard deviation. AFM images of an (b) unmodified PDMS surface, (c) pCBMA-modified PDMS surface in dry state, and (d) pCBMA-modified PDMS surface in phosphate buffered saline solution.
Figure 2.3: (a) Fibrinogen adsorption as a function of reaction time during polymerization. (b) Shows fibrinogen adsorption on pCBMA-modified PDMS after incubation under wet and dry conditions up to 74 days after polymerization for 18 hours. (c) Bare PDMS and (d) pCBMA-modified PDMS (18 hour polymerization) were exposed to 100% human serum. The surface was labeled by biotinylation and FITC-NeutrAvidin to visualize total surface binding.
Figure 2.4: (a) Inactivated pCBMA-PDMS surface exposed to FITC-BSA showed no protein binding. (b) NHS/EDC activated pCBMA-PDMS surface exhibited high levels of controlled protein attachment. (c) NHS/EDC activated pCBMA-PDMS surface followed by deactivation demonstrated no protein binding. This showed that the surface does not lose its nonfouling characteristics during activation.
Chapter 3

A Simple and Robust Approach for Passivating and Functionalizing Surfaces for use in Complex Media

Pluronic is a popular triblock copolymer used as a surfactant to introduce hydrophilic coatings onto many different types of material surfaces, from engineering to biomedical applications. Unfortunately, this leaves the surface inert to further modification and is limited in its ability to resist fouling from complex media such as blood. Herein, we report a simple and robust approach for passivating and functionalizing surfaces based on zwitterionic poly(carboxybetaine) (pCB) based triblock copolymer, which can be directly applied to surfaces to prevent nonspecific protein adsorption from undiluted blood plasma, and to provide additional functionalities needed for the attachment of biomolecules. Various hydrophobic surfaces including poly(dimethyl siloxane) (PDMS), silanized silica and self-assembled monolayers are tested to demonstrate its applicability to a wide range of systems. This approach provides a robust, convenient, and effective surface modification method for real-world applications from simple surface passivation to specific targeting in complex media.

3.1 Introduction

Surfactants take advantage of differences in hydrophobicity to mediate the interface between materials. One of the most widely used is the nonionic polymer, Pluronic. Pluronic has found broad use in chemical, environmental, and pharmaceutical industries as an antifoaming agent,\textsuperscript{41} emulsifier,\textsuperscript{42} and colloidal dispersion stabilizer.\textsuperscript{43} More recently, Pluronic has found several applications in the biomedical field: coating contact lenses to resist irritation and protein
buildup,\textsuperscript{44} lining silicone based diagnostic devices to reduce fouling,\textsuperscript{45, 46} entering and repairing cell membranes,\textsuperscript{47, 48} and many others.\textsuperscript{49-53} Pluronic micelles are also a popular method to increase drug solubility and stability, while improving drug pharmacokinetics and biodistribution in the body.\textsuperscript{54, 55}

Pluronic is a triblock copolymer made up of two terminal hydrophilic polyethylene glycol (PEG) segments and one center hydrophobic polypropylene oxide (PPO).\textsuperscript{56} Pluronic functions by assembling onto hydrophobic materials and making the surface more hydrophilic in order to suppress protein adsorption from biological media.\textsuperscript{46, 56-62} Despite its popularity, Pluronic’s abilities to resist nonspecific protein adsorption from complex media and to introduce surface functionalities have not been achieved for many medical applications. In addition, it has been reported that Pluronic with a shorter PEG block is toxic to certain cells.\textsuperscript{4}

Zwitterionic polymers, such as poly(carboxybetaine) (pCB), have been recently recognized as excellent non-fouling materials\textsuperscript{10, 63, 64} and applied to various surfaces to suppress non-specific protein adsorption. Ultra-low fouling surface properties (<5 ng/cm\textsuperscript{2}) can be achieved during exposure to undiluted human blood plasma and serum. PCB is particularly unique in that it can be also easily functionalized with biomolecules after attachment of an active N-Hydroxysuccinimide (NHS) group.\textsuperscript{4} Although pCB has excellent non-fouling properties and an ability to be specifically functionalized, current methods to attach it to surfaces often involving \textit{ex situ} surface polymerization on a limited number of specific materials.

Here we report a simple and robust approach for passivating and functionalizing surfaces for use in complex media based on a zwitterionic triblock copolymer, poly(carboxybetaine methacrylate)-poly(propylene oxide)-poly(carboxybetaine methacrylate) (PCB-PPO-PCB) (\textbf{Figure 3.1a}). This copolymer contains PCB moieties which are more compatible with proteins
and lipid membranes due to their zwitterionic nature, along with additional functionalities unique to pCB for direct biomolecular immobilization. We demonstrate that pCB-PPO-pCB can be directly applied onto a surface via a simple “graft to” method based on hydrophobic-hydrophobic interactions to prevent nonspecific protein adsorption from undiluted blood plasma and to provide additional functionalities needed for the attachment of biomolecules for specific targeting. Various hydrophobic surfaces including PDMS, silanized silica, and self-assembled monolayer (SAM) surfaces have been tested to demonstrate the facilities of this simple method. A dihydroxy-terminated PPO48 was esterified with bromoisobutyryl bromide to afford the PPO based difunctional atom transfer radical polymerization (ATRP) initiator. It was then used to initiate the ATRP polymerization of CB. A series of pCB-PPO48-pCB triblock copolymers with different PCB block lengths were obtained by varying the feed amount of CB monomer. 1H NMR analysis shows that the conversion rate of polymerization was over 99%. It is well known that there is a high level of protein adsorption on any hydrophobic surfaces, particularly super hydrophobic PDMS (Figure 3.1b). The pCB-PPO-pCB triblock copolymer was coated onto hydrophobic surfaces (Figure 3.1c) by simply exposing it to the polymer solution. A polymer coating quickly forms with the PPO block sticking to the surface and the CB block facing the aqueous solution. Unlike all other low fouling materials, CB groups can be activated to react with biomolecules. Molecules containing primary amines, such as antibodies and other proteins, can be covalently attached to activated CB groups by commonly used EDC/NHS chemistry (Figure 3.1d). Activated CB groups that do not form a covalent attachment can be converted back to zwitterionic groups to maintain their nonfouling properties on post-functionalized surfaces.
3.2 Results and Discussion

3.2.1 Surface modification and functionalization of PDMS

PDMS is commonly used for the fabrication of medical devices, artificial implants and microfluidic devices. Although excellent mechanical properties make PDMS an attractive material for device manufacturing, its superhydrophobic surface causes a high level of protein fouling, leading to rejection from the body or device failure. Various PDMS surface modification methods have been reported in the literature to suppress nonspecific protein adsorption. However, most of these methods are time-consuming. Furthermore, unlike other surfaces, it is particularly difficult to modify PDMS using PEG-based polymers due to its continual surface reconstruction. Resulting surface modification can last only for a relatively short period of time. Compared to these methods, the triblock copolymer reported in this work is simple, robust, and effective at preventing non-specific protein adsorption and providing surface functionalities. PDMS surfaces previously exposed to 1 mg/mL of three different triblock copolymers showed significant reduction in protein adsorption (Figure 3.2a). Fibrinogen adsorption levels were measured by enzyme-linked immunosorbent assay (ELISA). PCB40-PPO48-PCB40 shows the lowest fibrinogen adsorption, 3% compared to that on the uncoated PDMS surface. The triblock copolymer composition has influence on the self-assembly of the copolymer on the surface. If the pCB block is too short relative to the PPO block, there will not be enough pCB on the surface to prevent protein adsorption. If the pCB block is too long, there will not be enough interactions between the PPO block and the surface. In addition, in order to form a dense polymer layer on a hydrophobic surface, the PPO block also needs to pass through the superhydrophilic pCB layer before it can reach the hydrophobic substrate surface when a loosely packed polymer layer has been formed. Figure 3.2b demonstrates the ability to functionalize a PDMS surface coated with
the PCB$_{40}$-PPO$_{48}$-PCB$_{40}$ triblock copolymer: after activation with EDC/NHS chemistry, the surfaces can be easily functionalized with a protein such as fibrinogen. After functionalization, the EDC/NHS activated surface can be deactivated with pH 10 buffer solution, restoring a nonfouling surface again with fibrinogen adsorption as low as 5% compared to uncoated PDMS surface.

### 3.2.2 In situ passivation of microfluidic device

PDMS based microfluidic devices have taken on an expanding role in the field of bioengineering. These devices can be fabricated to make very complex fluidic systems for manipulating fluid flow of very small samples. These systems require easy in situ surface modification to prevent fouling from biological samples and Pluronic has previously been the only option. It is often desired to immobilize desired biomolecules to the walls of microfluidic channels, but Pluronic is not easily functionalized. Here, we have demonstrated that the PCB-PPO-PCB triblock copolymer is ideal for the surface modification of microfluidic devices.

**Figure 3.3** shows how PCB$_{40}$-PPO$_{48}$-pCB$_{40}$ prevents nonspecific protein adsorption from a mixed solution of fibrinogen and BSA. **Figure 3.3a-d** shows an unmodified 50um PDMS microfluidic channel exposed to a mixture of 50 ug/mL Alexa Fluor 555 tagged BSA and 50 ug/mL Alexa Fluor 488 tagged Fibrinogen. **Figure 3.3e-h** shows the same experiment performed but with the channel previously exposed to 1 mg/mL PCB$_{40}$-PPO$_{48}$-PCB$_{40}$. These results demonstrate the ability of the triblock copolymer to prevent nonspecific protein adsorption.

### 3.2.3 Controllable immobilization of biomolecules in situ

A controlled functionalization of a biomolecule onto a PCB$_{40}$-PPO$_{48}$-pCB$_{40}$ modified PDMS based microfluidic channel was demonstrated in **Figure 3.4**. Here, the triblock coated channel (**Figure 3.4a**) was first activated by 0.2M EDC and 0.05M NHS for 10 minutes. After washing,
100 ug/mL Alexa Fluor 555 tagged BSA was injected to react with the surface (Figure 3.4b). CB monomer units that were activated with an NHS ester can react with exposed primary amines on BSA. When washed with high pH buffer (pH 10), the unreacted NHS esters are hydrolyzed and washed away with the free BSA leaving only the restored zwitterionic CB surface and the now covalently immobilized BSA (Figure 3.4c). Upon addition of other proteins, such as 100 ug/mL Alexa Fluor 488 tagged fibrinogen (Figure 3.4d), the surface prevents any further surface binding, leaving only the immobilized BSA (Figure 3.4e). The ability to perform a surface modification in situ that can both specifically immobilize a desired molecule and resist all nonspecific protein adsorption is something that has not been achieved before.

### 3.2.4 Surface modification and functionalization of silanized silica beads

The pCB based triblock copolymer can also be coated onto micro or nano structured hydrophobic surfaces easily, such as silanized silica beads (Figure 3.5). Shown in Figure 3.5a are beads that have not been treated with the triblock copolymer and fluorescently tagged proteins can adsorb to the hydrophobic surfaces easily, as evidenced by the strong fluorescence in the image. After coating with the triblock copolymer, no protein adsorption can be detected (Figure 3.5b). Triblock copolymer-coated surfaces can also be easily functionalized with fluorescently tagged proteins after EDC/NHS activation (Figure 3.5c). Activation does not destroy the surface as shown with triblock copolymer-coated beads that were activated then deactivated before being exposed to fluorescent protein (Figure 3.5d).

### 3.2.5 Biomolecular detection with surface plasmon resonance biosensors

A surface plasmon resonance (SPR) sensor was used to quantify nonspecific protein adsorption, functionalization level, and antigen detection on the polymer coated surfaces. A hydrophobic surface was initially created on an SPR chip using a 1-undecanethiol self assembled
monolayer (SAM). The surface was then coated by flowing the triblock copolymer solution (1mg/mL in 10mM NaCl) over the hydrophobic surface for 60 min in situ. **Figure 3.6a** shows a typical SPR curve for protein adsorption to a coated surface, in this case, undiluted blood plasma. Single solutions of fibrinogen and lysozyme adsorption were measured to be less than 2±1.0ng/cm² while protein adsorption from undiluted plasma was 5.2±0.20ng/cm². Here, we were able to quantifiably show that pCB40-PPO48-pCB40 was able to resist fouling in several biological solutions. Recently, a PEG-PPO-PEG triblock copolymer (PluronicTM F108) was also used to coat hydrophobic SAMs for SPR.⁵⁷ Results showed that the PEG-PPO-PEG polymer modified surface was able to achieve low fouling surface only from single proteins or 20% diluted plasma. There is a sharp chemical difference between amphiphilic PPO and superhydrophilic pCB. Zwitterionic pCB thereby is able to bind strongly to surrounding water molecules by electrostatically induced hydration, while PEG interacts with water via hydrogen bonding.⁶⁶, ⁶⁷ This makes pCB based zwitterionic polymers well suited to suppress non-specific protein adsorption from complex media. Previous studies have shown that although PEG-coated surfaces and gold nanoparticles were stable in 10% blood serum, only PCB-coated surfaces ² and gold nanoparticles⁶² were stable in 100% blood serum. SPR was also used to demonstrate the possibility of introducing functionalities onto hydrophobic surfaces (**Figure 3.6b**). After activation with EDC/NHS, we attached a model antibody (antiALCAM) onto the surface at a level of 70.6±5.0ng/cm². The functionalized surface was shown to detect ALCAM at the level of 58.2±2.0ng/cm². This ability is unique to pCB, which shows great promise for its use in real-world application involving specific target in complex media such as diagnostics and drug delivery.
3.3 Conclusions

In this work, a zwitterionic based triblock copolymer with a PPO middle segment has been synthesized and shown to self-assemble onto various surfaces from aqueous media to generate dense polymer brush films. Unlike Pluronic triblock copolymers, the pCB based triblock copolymer has been demonstrated to effectively resist nonspecific protein adsorption from undiluted human blood plasma and to provide unique functionalities onto hydrophobic surfaces for attaching biomolecules to PDMS surfaces, PDMS-based microfluidic channels, silanized silica particles, and SPR sensor surfaces. Since pCB shares similarities in chemistry to polymers found on protein surfaces and cell membranes, it is expected that this zwitterionic based triblock copolymer will have better biocompatibility with proteins and cells. Due to its effectiveness in complex media, unique functionalization capability, better biocompatibility, ease in use, and suitability for many surfaces, this pCB based triblock copolymer will exceed the existing PEG based triblock polymer for a wide range of applications in sensing and detection, cosmetics, pharmaceuticals, drug delivery, biological response modifiers, and nonspecific protein adsorption suppressers.

3.4 Experimental Section

3.4.1 Materials

CB monomer was synthesized according to previously reported protocol. Poly(propylene glycol) (PPO; Mn=2700), copper(I) bromide (99.999%), 2,2'-bipyridine (BPY, 99%), bromoisobutyryl bromide (98%), N-hydroxysuccinimide (NHS), and N-ethyl-N'-(3-diethylaminopropyl) carbodiimide hydrochloride (EDC), 1-undecanethiol (98%), bovine serum albumin, Alexa Fluor 555 tagged bovine serum albumin, Alexa Fluor 488, fibrinogen from bovine, and methanol were purchased from Aldrich and were used as received. Phosphate-
buffered saline (PBS, 0.01 M phosphate, 0.138 M sodium chloride, 0.0027 M potassium chloride, pH 7.4) was purchased from Sigma Chemical Co. Ethanol (absolute 200 proof) was purchased from AAPER Alcohol and Chemical Co. The water used in these experiments was purified using a Millipore water purification system with a minimum resistivity of 18.0 MΩ cm. Sylgard ® 184 Silicone Elastomer Kit was used from Dow Corning.

3.4.2 Synthesis of PPO macroinitiator

In a typical example, OH-PPO$_{48}$-OH (27.0 g, 0.01mol) was dissolved in 100 mL of chloroform in a 250 mL three-neck flask. Triethylamine (6.95 mL, 0.05 mol) was added and the solution mixture was cooled to 0 °C. Then, 2-bromoisobutyryl bromide (3.7 mL, 0.03 mol) was added dropwise via syringe over 1 h, and the reaction mixture was stirred overnight at room temperature. The solution was then washed with NaHCO$_3$ solution for 5 times and DI water for 2 times. Solvent was removed under vacuum to afford a yellowish liquid.

3.4.3 Synthesis of pCB-PPO-pCB triblock copolymer

A typical protocol for the synthesis of the pCB-PPO-pCB triblock copolymer via ATRP using the Br-PPO-Br bifunctional macroinitiator was as follows: Br-PPO-Br (0.5 g, 0.179 mmol, 1 equiv.) and CB (3.27 g, 14.2 mmol, 40 equiv.) were dissolved in methanol (5.0 ml). After purging with nitrogen for 30 min., the Cu(I)Br catalyst (0.0511 g, 0.357 mol, 2 equiv.) and bpy ligand (0.1114 g, 0.714 mmol, 4 equiv.) were added to this stirred solution under nitrogen. The reaction mixture immediately became dark brown and progressively more viscous, indicating the onset of polymerization. After 18 h, $^1$H NMR analysis indicated that more than 99 % of the CB had been polymerized (disappearance of vinyl signals between δ 5.5 and 6.0). The reaction solution turned blue on exposure to air, indicating aerial oxidation of the Cu(I) catalyst. The resulting triblock copolymer was diluted with methanol and passed through a silica column to
remove the spent ATRP catalyst. The polymer solution was dried under vacuum to remove the solvent.

### 3.4.4 Fabrication of microfluidic devices

A 50um thick negative SU8-2050 photoresist was spin coated on an HF dipped silicon wafer. A Heidelberg uPG 101 laser pattern generator was used to generate 20,000 um X 50 um microstructures. Patterned wafer was then developed and silanated to generate a stable hydrophobic master. Sylgard 184 Silicone Elastomer (Dow Corning) was prepared by thoroughly mixing 10 parts base to 1 part curing agent, and poured over the master in a Petri dish. Silicone tubing was placed vertically on the master where ports were needed before the PDMS solution was poured. Sylgard 184 was also spin coated on a large glass microscope slide. The microscope slide was previously silanated with allyltrimethoxysilane to anchor the PDMS. Both PDMS materials were allowed to cure at 60°C overnight. After curing, the PDMS mold was peeled off the silicon master, and both the patterned mold and the PDMS coated glass were exposed to oxygen plasma. After treatment, both materials were pressed together to form a covalent seal for the microfluidic channels.

### 3.4.5 Surface plasmon resonance (SPR)

Fibrinogen, lysozyme and plasma adsorption were measured with a custom-built surface plasmon resonance (SPR) sensor, which is based on wavelength interrogation. The chip was first cleaned with UV ozone, and then immersed in 1-undecanethiol solution overnight to form a self-assembled monolayer (SAM) on a gold SPR chip. The chip was then attached to the base of the prism. The optical contact was established by using a refractive index matching fluid (Cargille). A baseline signal was established by flowing PBS buffer at a rate of 50 μL/min through the sensor for 10 min. Triblock copolymer solution (1mg/mL) was then flowed through the SPR
channels for 60 min., followed by PBS buffer solution (30 min.) to remove loosely bounded polymers, and to re-establish the baseline. Freshly prepared 1 mg/mL protein solutions of fibrinogen, lysozyme and undiluted blood plasma were flowed through independent channels for 10 min, followed by PBS buffer solution to remove unbound protein molecules and to re-establish the baseline. Adsorption was quantified by measuring the change in wavelength before and after protein adsorption. The wavelength change was converted to an amount of adsorbed protein. For the SPR sensor used in this work, a 1 nm SPR wavelength shift at 750 nm represents a surface coverage of ~17 ng/cm² adsorbed proteins. A temperature controller was employed to maintain the temperature during experiments.

3.4.6 Antibody immobilization and antigen detection in SPR

The antibody functionalization and antigen detection procedure were monitored in real time by the SPR sensor. The carboxylate groups of triblock copolymer coated surface were activated by the injection of a freshly prepared solution of 0.1 M NHS and 0.4 M EDC in water for 10 min at 25 °C. Sodium acetate buffer (10 mM), pH 5.0 at 25 °C (SA), was briefly injected to obtain a stable baseline. A solution of antibodies (anti-ALCAM) with an antibody concentration of 50 μg mL⁻¹ in 10 mM HEPES buffer pH 7.5 was flowed over spots of the activated triblock coated surface for 14 min. The functionalized surface was then washed for a short time with 10 mM sodium carbonate buffer containing 0.3 M NaCl at pH 10 to remove noncovalently bound ligands. Along with protein immobilization, the residual activated groups of triblock polymer were also deactivated. To monitor the amount of immobilized antibodies, the SA buffer was injected again. After antibody immobilization, the functionalized surface was washed with PBS buffer. ALCAM solution in PBS (1 ug mL⁻¹) was injected for 10 minutes followed by washing with buffer.
3.4.7 Modification of PDMS samples

PCB-PPO-PCB triblock copolymer was first dissolved in water at 1 mg/mL. PDMS samples (5 mm diameter punch-outs from 2 mm thick PDMS) were incubated in the polymer solutions for 60 minutes. After polymer deposition, PDMS punch-outs were washed with PBS. For functionalization, samples were first activated using a solution of 0.20 M EDC and 0.05 M NHS for 10 minutes. Next, the samples were washed briefly with 10 uM HCl-Water, and then exposed to the desired protein to immobilize (in the current case, fibrinogen) at 1 mg/mL in 10 mM sodium phosphate, pH 7.4, for 30 min. The surface was then washed with deactivation buffer (300 mM NaCl, 10 mM sodium carbonate, pH 10.0) for 10 minutes, followed by washing with PBS for 60 min. To evaluate nonspecific protein adsorption, samples were incubated in 1 mg/mL fibrinogen in PBS. Fouling was measured using enzyme-linked immunosorbent assay (ELISA).

3.4.8 Modification of microfluidic channels

pCB-PPO-pCB triblock copolymer was first dissolved in water at 1 mg/mL. Microfluidic channels were injected with 100 uL of polymer solutions for 60 minutes. After polymer deposition, the channels were washed with 100 uL of PBS. For functionalization, samples were first activated using a solution of 0.20 M EDC and 0.05 M NHS for 10 minutes. Next, the samples were washed briefly with 10 uM HCl-Water, and then exposed to the desired protein to immobilize (in the current case, Alexa Fluor 555 tagged BSA) at 0.1 mg/mL in 10 mM sodium phosphate, pH 7.4, for 30 min. The surface was then washed with deactivation buffer (300 mM NaCl, 10 mM sodium carbonate, pH 10.0) for 10 minutes, followed by washing with PBS for 60 min. To evaluate nonspecific protein adsorption, 100 uL injections were made of either 0.1 mg/mL of Alexa Fluor 555 tagged BSA, 0.1 mg/mL of Alexa Fluor 488 tagged fibrinogen, or a
mixture of 0.05 mg/mL of Alexa Fluor 555 tagged BSA and 0.05 mg/mL of Alexa Fluor 488 tagged fibrinogen. Protein was detected using a fluorescent microscope.

3.4.9 Modification of hydrophobic beads

pCB-PPO-pCB triblock copolymer was first dissolved in water at 1 mg/mL. Silane treated hydrophobic glass beads (300 um) were incubated in the polymer solution for 60 minutes. After polymer deposition, the beads were washed with PBS. For functionalization, samples were first activated using a solution of 0.20 M EDC and 0.05 M NHS for 10 minutes. Next, the samples were washed briefly with 10 uM HCl-Water, and then exposed to the desired protein to immobilize (in the current case, Alexa Fluor 488 tagged BSA) at 0.1 mg/mL in 10 mM sodium phosphate, pH 7.4, for 30 min. The surface was then washed with deactivation buffer (300 mM NaCl, 10 mM sodium carbonate, pH 10.0) for 10 minutes, followed by washing with PBS for 60 min. To evaluate nonspecific protein adsorption, samples were incubated in 0.1 mg/mL of Alexa Fluor 488 tagged BSA in PBS.
3.5 Figures

**Figure 3.1:** a) pCB-PPO-pCB triblock copolymer with hydrophilic pCB ends for protein fouling resistance and functionalization and a hydrophobic PPO center for binding. b) A typical hydrophobic materials, like PDMS, when exposed to biological solutions, result in proteins adsorbing on the surface. c) pCB-PPO-pCB will assemble on hydrophobic surfaces in solution, and remain after washing as a thin layer to prevent nonspecific protein adsorption. d) pCB-PPO-pCB has the unique ability to be activated with NHS groups, which react with a desired biomolecules, useful for a wide range of biomedical applications.
Figure 3.2: Amounts of adsorbed fibrinogen (Fg) measured by ELISA. a) PDMS surfaces coated with different pCB-PPO-pCB triblock copolymers. b) PDMS surface coated with pCB40-PPO48-pCB40 compared to unmodified PDMS. Once the surface is activated by EDC/NHS chemistry, the surface is able to be functionalized with fibrinogen by covalent immobilization. By treating an activated surface with pH 10 buffer without it having previously been exposed to fibrinogen, the surface is able to maintain its nonfouling properties.
Figure 3.3: a) A mixture of fluorescently tagged BSA, red, and fibrinogen, green, were flown through an unmodified PDMS microfluidic channel. b) Light field, c) red fluorescence, and d) green fluorescence images. e) The same fluorescent image was flown through a PDMS microfluidic channel that was previously exposed to a solution of pCB40-PPO48-pCB40. f) Light field, g) red fluorescence, and h) green fluorescence images. The solution was comprised of 50 ug/mL Alexa Fluor 555 tagged BSA and 50 ug/mL Alexa Fluor 488 tagged fibrinogen.
Figure 3.4: a) A PDMS microfluidic channel previously exposed to 1 mg/mL pCB40-PPO48-pCB40. b) The channel was activated by EDC/NHS chemistry, followed by 100 ug/mL Alexa Fluor 555 tagged BSA. The tagged BSA reacted with NHS activated CB groups, covalently immobilizing the protein on the surface. c) After being washed with high pH buffer, unreacted NHS groups hydrolyzed and wash away with the free protein. d) Addition of 100 ug/mL Alexa Fluor 488 tagged fibrinogen was added to the channel but unable to bind, e) and was easily washed away leaving only the covalently immobilized BSA.
Figure 3.5: a) Bare hydrophobic beads exposed to Alexa Fluor 488-BSA. b) Hydrophobic beads pretreated with 1 mg/mL pCB40-PPO48-pCB40 exposed to Alexa Fluor 488 tagged BSA. c) Triblock copolymer coated hydrophobic beads activated by EDC/NHS, followed by Alexa Fluor 488 tagged BSA for covalent functionalization. d) Triblock copolymer coated hydrophobic beads activated by EDC/NHS, then deactivated in pH 10.0 buffer, followed by Alexa Fluor 488-BSA.
Figure 3.6: SPR sensorgrams of (a) undiluted human plasma fouling over a pCB40-PPO48-pCB40 coated gold surface; (b) Surface functionalization with anti-ALCAM and corresponding ALCMA detection. The functionalization level was 70.6±5.0ng/cm² with an antigen response of 58.2±2.0/cm². I: Anti-ALCAM solution; II: Deactivation solution; III: Running buffer IV: PBS; V: Antigen Solution; VI: PBS
Chapter 4

Poly(Zwitterionic) Protein Conjugates offer Increased Stability without Sacrificing Binding Affinity or Bioactivity

Treatment with therapeutic proteins is an attractive approach to targeting a number of challenging diseases. Unfortunately, the native proteins themselves are often unstable in physiological conditions, reducing bioavailability and therefore increasing the dose that is required. Conjugation with poly(ethylene glycol) (PEG) is often used to increase stability, but this has a detrimental effect on bioactivity. Here, we introduce conjugation with zwitterionic polymers such as poly(carboxybetaine). We show that poly(carboxybetaine) conjugation improves stability in a manner similar to PEGylation, but that the new conjugates retain or even improve the binding affinity as a result of enhanced protein–substrate hydrophobic interactions. This chemistry opens a new avenue for the development of protein therapeutics by avoiding the need to compromise between stability and affinity.

4.1 Introduction

The attachment of PEG, known as PEGylation, has been the benchmark for protein stabilization and increased body circulation time for decades.\textsuperscript{68, 69} Although greatly beneficial as a protein stabilizer, PEGylation is also known to cause loss in binding affinity, thereby reducing overall bioactivity.\textsuperscript{70, 71} Efforts have been made to control the location of attachment of PEG in order to minimize this effect,\textsuperscript{72} but the material problem has remained unresolved for the last 30 years. For marketed PEGylated interferon-α2a (Pegasys®), activity has been seen to drop to 7%
when compared to its native form. When PEG is attached to monoclonal antibodies, large reductions in activity are found. The effect is also true for PEGylated enzymes. For this reason, the current pool of available therapeutic proteins has been limited to either humanized proteins or proteins with small molecule substrates that are able to resist inhibition by PEGylation, such as Pegasparagase (Oncaspar®) and Pegademase Bovine (Adagen®). These are just some of many examples.

Over the last several years, we have demonstrated how poly(zwitterions) act as super non-fouling surfaces that prevent adsorption from complex biological media. We have also shown how poly(zwitterions), such as pCB, outperform PEG in terms of resistance to non-specific protein adsorption from blood serum and plasma. This is noteworthy given that PEG has been universally used as a highly resistant non-fouling surface in many applications. PEG receives its non-fouling characteristics from its ability to bind large amounts of water through hydrogen bonding among its many ether groups. PEG therefore takes on a ballooning-type conformation that increases solution viscosity and presents a water barrier that prevents proteins from approaching the surface. PEG, although able to form many bonds with water, is also known to have amphiphilic character, being soluble in both polar and non-polar organic solvents. Zwitterions, alternatively, are polyelectrolytes that possess both positively and negatively charged groups, but have an overall neutral charge. Poly(zwitterions) such as pCB receive their non-fouling characteristics through strong ionic structuring of water, which creates a super hydrophilic surface. The properties of how ions, such as those found in pCB, affect water structure can be found described in what is referred to as the Hofmeister series. It is through understanding how ions affect protein stability/activity that we propose the development of a pCB protein conjugate. Although pCB is comparable to PEG as a nonfouling material, the
underlying mechanisms by which it receives its biocompatible properties are so different that there is potential for a nonfouling polymer-protein conjugate that does not have the paralyzing effect on activity that is seen with PEGylation.

4.2 Results and Discussion

In this work, pCB was conjugated to a model protein to demonstrate its stabilizing properties, while using PEG for comparison. Ideally, PEG and pCB polymers of similar size would have been chosen. However, poly(zwitterions) are known to have strong intramolecular interactions through ionic binding which give pCB a higher density in solution than predicted. This is referred to as the anti-polyelectrolyte effect.\textsuperscript{80} In contrast, PEG, which binds to a large number of water molecules, has an artificially larger size than would be expected in solution. For this reason two different sizes of pCB polymers were synthesized to compare to PEG: pCB $M_n$ (equivalent molecular weight to 5 kDa PEG determined by NMR, Figure 4.1) and pCB $R_h$ (equivalent hydrodynamic size to 5 kDa PEG determined by size exclusion chromatography (SEC), Table 4.1). The synthesis and conjugation can be seen in Figure 4.2. The well-studied and characterized enzyme $\alpha$-chymotrypsin (CT) was chosen as the model protein. The second parameter adjusted, after polymer size, was the number of polymers per protein. This parameter was varied by adjusting the feed ratio to give three degrees of conjugation for each polymer-conjugate. The degree of modification (# polymers/CT protein) was determined by an amine-sensitive assay. Conjugation was also confirmed from the SEC chromatograms (Figure 4.3).

To stress the stability of the prepared conjugates, activities were tested in a 5M urea (Figure 4.4). Values are represented as percentages relative to their activities before urea was added. As anticipated, PEG was seen to provide a stabilizing effect which correlated with an increasing degree of conjugation, with the least conjugated PEG having no improvement relative
to the bare enzyme. Increased stability was also seen for the pCB conjugates, but with little dependence on polymer size or the number of polymers. All of the pCB conjugates had comparable stability relative to the most stable PEG conjugate. A thermal stability test was also performed, measuring the effect of temperature on enzyme activity. It was observed that both pCB \( M_n \) and PEG conjugates had stabilizing effects at elevated temperatures, resisting loss in activity compared to the conjugated enzyme (Figure 4.4b). Even though PEG is a known stabilizing agent against thermal denaturation,\(^8\) pCB \( M_n \) was seen to provide increased stability when compared to PEG conjugates with comparable numbers of polymers/protein. In Figure 4.4c, it was seen that pCB \( R_h \) conjugates provided a drastic improvement over the PEG samples. These results show that pCB has a strong stabilizing effect against chemical and thermal denaturation. It should be pointed out that, although both polymers have stabilizing properties, the proposed mechanisms by which they function are very different. PEG, as previously mentioned, acts as a protective barrier around the protein. This phenomenon results in an increased local viscosity, thereby reducing the structural dynamics of the protein, making it more difficult to unfold.\(^8\) pCB, on the other hand, stabilizes globular proteins by strengthening the hydrophobic interactions holding the protein together by reducing water interactions with the surface.\(^8\) By reducing the degree water can interfere with the structure of the protein, the conjugates become less likely to unfold. The different mechanisms become apparent when evaluating their effect on binding affinity.

Affinities of the prepared conjugates were determined by measuring their Michaelis constants (\( K_m \)). N-Succinyl-Ala-Ala-Pro-Phe \( \beta \)-nitroanilide was used as a substrate. This is a peptide-based substrate with an explicit binding region for cleavage by \( \alpha \)-chymotrypsin. This system was chosen since substrates of this size are affected by polymer inhibition.\(^7\) Small
molecule substrates are known to already have high affinities (<$K_m$) due to their high diffusivities. As was expected, PEG conjugates were seen to have decreasing affinity for the peptide substrate as the number of polymers per protein increased (Figure 4.5a). Lower affinity was observed as an increase in $K_m$, with $K_m$ being the concentration of substrate required to achieve 50% full activity ($k_{cat}$). For the pCB conjugates of both polymer sizes, substrate inhibition was seen to be absent. Most notably, for the larger pCB $R_h$ conjugates, there was observed a negative correlation between amount of conjugated pCB and $K_m$. This meant the addition of the pCB polymer assisted in binding.

A general mechanism for molecular binding is to have two species possess higher solubility for each other than their environment (Figure 4.6a). With proteins, hydrophobic-hydrophobic interactions play a major role. Attaching polymers, such as PEG, to the protein surface imposes steric hindrances as well as competitive interactions with the substrate and binding site, thereby reducing binding affinity (Figure 4.6b). For PEG, amphiphilicity reduces the hydrophobic-hydrophobic driving force of the substrate-binding site interaction while also imposing a steric hindrance. Contrarily, as seen with the pCB conjugates, affinity was either unaltered or improved. Due to the super hydrophilicity of the polymer, water is drawn away from hydrophobic regions of the protein, shifting the equilibrium to allow the substrate and binding site to complex (Figure 4.6c). To support this model, the solubility of the peptide substrate was measured in solutions of PEG and pCB polymers. This was performed by measuring the partitioning of the substrate molecule into free polymer solutions of PEG and pCB through a semi permeable membrane (Figure 4.7a). These results showed that the substrate had higher solubility in PEG and lower solubility in pCB compared to the buffer control (Figure 4.7b). These results support how the amphiphilic PEG polymer can interfere with the substrate-binding,
and also how the super-hydrophilicity of the pCB polymer has low affinity for hydrophobic materials.

To confirm that the changes in affinity were originating from the differences in local hydrophilicity, $K_m$ values of the bare enzyme were measured in solutions of ammonium acetate ($\text{NH}_4\text{OAc}$) salt and 650 M$_n$ PEG. It was observed that the same improvement in binding affinity occurred with ammonium acetate as was seen for the pCB conjugates (Figure 4.5b). The effect of adding 650 M$_n$ PEG to solution also was seen to correlate with what was observed of the conjugated PEG form; contributing significant losses in affinity. This provides evidence that the promising results obtained from the pCB conjugates originated from the ionic characteristics of the polymer. Ions, and their effect on protein stability and activity, follow a principle referred to as the Hofmeister series (Figure 4.8). The trend seen in the series correlates greater enzyme performance (stability, activity and affinity) with high surface charge density (kosmotropic) anions and low surface charge density (chaotropic) cations. These are referred to as hard anions and soft cations, respectively. This phenomenon can also be seen with zwitterionic molecules in solution.\textsuperscript{85} The effect has been shown with several enzyme and substrate pairs, possessing trends of increasing values of $k_{\text{cat}}$ and decreasing values of $K_m$, resulting in overall greater activities.\textsuperscript{86} This has also been seen with non-enzyme-based binding.\textsuperscript{87} Unfortunately, this type of stabilization and increased activity through altering the environment with salts is lost when considering application \textit{in vivo}. However, this phenomenon can be retained by covalently attaching a zwitterionic polymer, like pCB, to the protein surface. The monomer unit itself is a derivative of glycine betaine, which is a natural molecule found in many living systems as a stabilizing agent. We can see that when compared to pCB’s molecular structure (Figure 4.6b), the betaine monomer corresponds to the most stabilizing and active monovalent ions in the
series, ammonium and acetate. This is the mechanism by which our polymer conjugation technology with pCB enhances binding affinity (Figure 4.6c).

To ensure the observed effects of the polymer conjugates were originating from the attached polymer and to show the sensitivity substrate size has on polymer inhibition, a small-molecule control substrate was also evaluated (resorufin bromoacetate, Figure 4.5c). It can be seen that compared to the larger peptide based substrate, the presence of conjugated polymer showed no observable effect on affinity. The accessibility of the active site by the control substrate can be seen by noting the overall lower values in $K_m$ relative to the larger peptide based substrate. Values of $k_{cat}$ were also calculated for all kinetic experiments and can be seen in Figure 4.9. For most conjugates, there was no significant effect, except for pCB $R_h$ conjugates which had an improved activity ($k_{cat}$) at higher degrees of conjugation.

4.3 Conclusion

It has been demonstrated in this work that zwitterionic pCB polymers are able to maintain stability of proteins without sacrificing their binding affinity, which has never been achieved before. This finding is a major step forward over the current PEGylation technique, which maintains stability of proteins at the expense of binding affinity. Zwitterionic pCB polymers derive their unique properties by being comprised of the most stabilizing and bioactive of monovalent ions as defined in the Hofmeister series, resulting in pCB both strengthening the stability of the protein folded structure while also encouraging specific binding at the hydrophobic binding site. The mechanism of action is contributed to mimicking that of protein stabilizing ions (kosmotropic anions and chaotropic cations). This work opens a new direction for protein therapeutics, by allowing accessibility to a pool of potential protein therapeutics that was originally thought not possible. Other limitations of PEG have been addressed, including
oxidative degradation, non-biodegradability, and toxicity of side-products.\textsuperscript{88} These limitations extend beyond PEG protein conjugation, giving potential for zwitterionic pCB polymers to also be used with therapeutic nanoparticles.\textsuperscript{89} In addition, the acting monomer unit of pCB is comprised of the naturally occurring glycine betaine. Thus, it is expected that zwitterionic polymers can substitute the current benchmark PEG and take a key role in the future of protein therapeutics.

4.4 Experimental Section

4.4.1 Materials

α-chymotrypsin from bovine pancreas type II, lyophilized powder, ≥40 units/mg protein (sigma), copper(I) bromide (CuBr, 99.999%, Aldrich), copper(II) bromide (CuBr\textsubscript{2}, 99.999%, aldrich), 1,1,4,7,10,10-Hexamethyltriethylene tetramine (HMTETA 97%, aldrich), N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (Sigma), and methoxy PEG succinimidyl carboxymethyl ester, MW 5,000 (Jenkem Technology, China).

4.4.2 N-Hydroxysuccinimide terminated poly(carboxybetaine methacrylate) polymer synthesis

Synthesis of the N-Hydroxysuccinimide (NHS) ATRP initiator\textsuperscript{90} and poly(carboxybetaine) methacrylate with t-butyl protected ester (CBMA-1-tBut)\textsuperscript{3} were performed as published. Atom transfer radical polymerization was carried out in anhydrous dimethylformamide (DMF) using a Cu(I)Br/HMTETA catalyst (Supplementary Figure S1a online). In a typical polymerization, DMF and HMTETA were separately purged of oxygen by bubbling with nitrogen. 1g (3.67 mmol) of CBMA-1-tBut monomer and 125 mg (0.5 mmol) of NHS-initiator were added to a Schlenk tube. To a second Schlenk tube was added 71.7 mg (0.5 mmol) of Cu(1)Br. Both tubes were deoxygenated by cycling between nitrogen and vacuum three times. 8 and 2 mL of
deoxygenated DMF were added to the monomer/initiator and Cu(I)Br tubes respectively. 136 uL (0.5 mmol) of deoxygenated HMTETA was added to the Cu(I)Br containing solution and was stirred for 30 min under nitrogen protection. The catalyst solution (Cu(I)/HMTETA) was then all added to the monomer/initiator solution to start the reaction. The reaction was run to completion at room temperature with monitoring by NMR. After polymerization, the reaction was fully precipitated in ethyl ether. The precipitate was then dried under vacuum and redissolved in minimal DMF (3-5 mL). The solution was reprecipitated in anhydrous acetone to remove the soluble catalyst and trace monomer. This was repeated for a total of 3 times to fully remove the catalyst. The remaining ester polymer was dried overnight under vacuum and analyzed by NMR. To hydrolyze the tert-butal group, 500 mg NHS-pCBMA-1-tBut was dissolved in 5 mL trifluoroacetic acid (Supplementary Figure S1b online). This was allowed to sit for 2 hours. The solution was then precipitated in ethyl ether, dried overnight under vacuum and subsequently analyzed by NMR (MW = 4.2 kDa) and GPC (MW = 2.5 kDa, PDI = 1.05).

4.4.3 α-chymotrypsin polymer conjugation and purification (Supplementary Figure S1c online)

α-chymotrypsin conjugates were prepared in 200 mM HEPES buffer, pH 8.5. The α-chymotrypsin concentration was fixed at 4 mg/mL, while the polymer solutions varied at 4 mg/mL, 8 mg/mL and 16 mg/mL. This was done for all three polymers: PEG, pCB Mn and pCB Rh. Solutions were mixed with a stir bar for 30 min, then incubated at 4°C for 3 hours. The conjugates were purified and concentrated by ultrafiltration using a 30kDa MWCO membrane. The pCB Rh conjugates were further purified using size exclusion column with a Superdex 200 10/300 column. These samples were then again concentrated using ultrafiltration. The number of polymers per protein for each sample was determined using a trinitrobenzene sulfonate (TNBS)
assay to determine the number of unreacted surface amines, along with AUC of SEC chromatograms from size exclusion chromatography.

### 4.4.4 Urea stability

Conjugates were incubated in 0.1 M Tris at pH 8.0 for 8 hours at 0.5 µg/mL with and without 5M Urea. Measurements were performed in triplicate. Solutions were also incubated with 0.1 mg/mL BSA as a blocking agent to prevent depletion of enzyme from surface adsorption. After 8.0 hours, activities were measured of all samples by adding N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide at 667 µg/mL. Activities were measured as the increase in absorbance at 412nm with time. Values were recorded as percentage of activity with urea, relative to without urea.

### 4.4.5 Thermal stability

Conjugates were incubated in 0.1 M Tris, pH 8.0, at temperatures of 40, 45, 50 and 55°C at a concentration of 5.0 µg/mL. After 10 min at each temperature, each conjugate was diluted into refrigerated buffer to a concentration of 0.5 µg/mL. Solutions were also incubated with 0.1 mg/mL BSA as a blocking agent. Activities were measured of all samples by adding by adding N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide to a final concentration of 667 µg/mL. Activities were measured as the increase in absorbance at 412nm with time. Values were recorded as percentage of each conjugates activity at 40°C.

### 4.4.6 Conjugate kinetics with N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide

Conjugates were incubated at 20 pM in 0.1 M Tris, pH 8.0 with 0.1 mg/mL BSA as a blocking agent. Low enzyme concentrations were required to achieve accurate activity measurements at low substrate concentrations. N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide was added at varying concentrations from 0 to 667 µg/mL (0-1,070 µM). Concentrations of each
substrate were prepared in triplicate and activity measured as an increase in absorbance with time at 412nm.

4.4.7 Conjugate kinetics with resorufin bromoacetate

Conjugates were incubated at 20 pM in 0.1 M MES, pH 6.0 with 0.1 mg/mL BSA as a blocking agent. A lower pH was required to retain the stability of the base liable substrate. Resorufin bromoacetate was added at varying concentrations from 0 to 50 ug/mL (0-150 µM). Concentration of each substrate were prepared in triplicate and activity measured as an increase in absorbance with time at 571nm.

4.4.8 Evaluation of polymer-substrate solubility

350 uL, 5 mg/mL solutions of 6.45 kDa PEG and the larger pCB polymer (pCB R_h) were added to separate 0.5 mL dialysis containers. A third dialysis container was used as a control containing only buffer, and no polymer. The dialysis containers were 3,500 Da molecular weight cut-off (MWCO) so polymer could not pass through the membrane, but substrate could. Both the PEG and pCB polymers were purified by dialysis and lyophilized before the experiment to ensure the polymers would not escape from the containers. The three containers were exposed to a large bath (35 mL) containing 2.5 ug/mL of peptide substrate (N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide). After 3 days, the sample solutions were measured by HPLC for peptide concentration.
Figure 4.1: NMR spectrum of the NHS functionalized pCB M<sub>a</sub> polymer in D<sub>2</sub>O. The ratio of the monomer and the terminal NHS peak were used to determine the degree of polymerization. The arrow in the inset plot is the location of where hydrolyzed NHS would appear. Due to the susceptibility of NHS to hydrolysis, this figure shows that full functionality it retained. However, functionality has been seen to decrease over long periods of time. This can be seen as a drop at 2.95 (s, 4H), and an increase at 2.67 (s, 4H). There is also the presence of contaminant ether that was unable to be removed by vacuum.
Table 4.1: Molecular weights determined by NMR and GPC including PDI’s for all polymers.

<table>
<thead>
<tr>
<th>Name</th>
<th>NMR</th>
<th>GPC</th>
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<tbody>
<tr>
<td></td>
<td>M_n (kDa)</td>
<td>M_n (kDa)</td>
</tr>
<tr>
<td>PEG</td>
<td>5.0</td>
<td>4.9</td>
</tr>
<tr>
<td>pCB M_n Eq (PEG molecular weight equivalent)</td>
<td>4.2</td>
<td>2.5</td>
</tr>
<tr>
<td>pCB R_h Eq (PEG hydrodynamic size equivalent)</td>
<td>12.0</td>
<td>6.7</td>
</tr>
</tbody>
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Figure 4.2: Synthesis and formation of carboxybetaine-protein conjugates.
Figure 4.3: Size exclusion chromatographs of a, PEG, b, pCB $M_n$, and c, pCB $R_h$ chymotrypsin conjugates respectively. Similar traces for the pCB conjugates, relative to the PEG conjugates, are contributed to the large differences in polymer solution conformation. While PEG polymers are heavily hydrate and greatly increase the conjugates hydrodynamic radius with each additional polymer, pCB polymers will have strong inter- and intra-molecular interactions that do not provide large differences hydrodynamic size. This is what we attribute to the similarities in chromatograms by the pCB conjugates. To accurately determine the degree of conjugation, a TNBS assay we performed.
Figure 4.4: Stability of PEG- and pCB-enzyme conjugates. a, Activity of conjugates after 8.0-hour incubation in 5M Urea. Values calculated as percentages of each conjugates activity in buffer without urea. Error bars represent standard deviation. b, Relative activity of PEG and pCB Mₙ conjugates (similar molecular weight) and c, PEG and pCB Rₜ conjugates (similar hydrodynamic size) after 10-minute incubation at different temperatures. 40°C is known as the enzyme’s optimum operating temperature.
Figure 4.5: Affinities of prepared conjugates measured as values of Michaelis constants ($K_m$). Error bar are values of standard error (s.e.m.). a, $K_m$ of prepared conjugates to the large peptide substrate. b, $K_m$ values of native enzymes to the large peptide substrate in the presence of 650 M$_n$ PEG and ammonium acetate solutions. c, $K_m$ values of prepared conjugates to the small molecule substrate. In kinetics, $K_m$ represents the concentration of substrate required to achieve 50% maximum activity. A decrease in $K_m$ represents an increase in binding affinity.
Figure 4.6: Mechanism of how PEG and pCB polymers influence binding affinity. 

**a**, Relationship between the enzyme and the substrate without polymer. **b**, PEG impedes affinity by reducing enzyme-substrate hydrophobic-hydrophobic interactions due to its amphiphilic characteristics. **c**, Super hydrophilic pCB has a strong effect on the structure of water, creating a local environment that increases enzyme-substrate hydrophobic-hydrophobic interactions, thereby increasing the substrate’s affinity for the binding pocket. **d**, The structural relationship between poly(carboxybetaine) (pCB) and ammonium acetate, both of which contain protein-stabilizing ions found in the Hofmeister series. The R group represents a methacrylate backbone.
Figure 4.7: Evaluation of polymer-substrate solubility. a, Dialysis setup with buffer (control), PEG or pCB, exposed to a large bath in excess volume containing peptide substrate. b, Substrate concentration in each container after 3 days.
Figure 4.8: The Hofmeister Series for monovalent ions.
Figure 4.9: Values of $k_{\text{cat}}$ for a, conjugates with the N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide, b, bare α-chymotrypsin with the peptide molecule substrate in solutions of ammonium acetate (NH$_4$OAc) and 650 M$_n$ PEG, and c, the conjugates with resorufin bromoacetate.
Chapter 5

Genetically Engineered Poly(zwitterionic)-Protein Conjugates

Conjugation of synthetic polymers and proteins has been widely used as a method to increase stability and prolong circulation of therapeutic proteins in the body. Often, polyethylene glycol (PEG) is used as the polymer for conjugation due to its nonfouling characteristics that resist bodily clearance. Zwitterionic polymers have recently been shown to outperform PEG in respect to nonfouling characteristics. Here, we have investigated a recombinant method for producing zwitterionic polymer protein conjugates. This is possible due to the similarities between nonfouling zwitterionic polymers and naturally occurring amino acids. We chose β-lactamase (28.9 kDa) as a model protein, which was easily measured for bioactivity. Three different length zwitterionic polymers (30.9 kDa, 15.4 kDa, and 10.6 kDa) consisting of perfectly alternating lysine (K) and glutamic acid (E) residues were recombinantly introduced to the C-terminus of the beta-lactamase gene. β-lactamase polymer conjugates were seen to have increased stability compared to the unconjugated control when challenged with increasing temperature.

5.1 Introduction

Protein based drugs introduced into the circulatory system often face challenges from the body’s immune system that lead to short circulation times, resulting in reduced therapeutic effect. A drug’s time in the body is greatly affected by its size, shape, charge and surface chemistry. Bodily clearance can occur by renal filtration, innate opsonization through the liver and spleen, and adaptive opsonization by the development of antibodies against the drug.
The primary technique for extending the circulation time of a protein therapeutic has been to conjugate synthetic polymers to the protein. PEGylation, the attachment of PEG to a drug, has been shown to improve a protein’s pharmacokinetics by increasing circulation half-life due to reduced kidney clearance, by protecting against degrading enzymes, and by reducing uptake by the reticulo-endothelial system (RES). However, PEG has been found to greatly reduce a protein’s bioactivity as well as be subject to autooxidation. Discovery of a PEG-specific immune response has triggered further investigation into its use to avoid antibody production. Recently, we have shown how zwitterionic polymers are able to provide the same stability to proteins that is seen through PEGylation, but without the loss in bioactivity. While changing the type of synthetic polymer used in protein-polymer conjugation is valuable, the use of synthetic methods have fundamental limitations.

The use of synthetic polymers to modify proteins often leads to a large diversity of polymer-protein variants. Expensive and difficult methods are required for purification and analysis of the desired product (Figure 5.1a-b). FDA approval also becomes difficult when dealing with heterogeneous drug types. Due to these limitations, amino acid based polymers could be a promising solution because they can be introduced onto proteins genetically. Recombinant protein engineering allows precise control over the number, position, and size of the attached amino acid polymers. Unfortunately, since PEG is not one of the naturally occurring amino acids, it cannot be used with this technique. Polymers only consisting of the twenty natural occurring amino acids can be used. Previously, researches have tried designing amino acid type polymers by selecting hydrophilic or hydrophilic and anionic amino acids. These amino acid based polymers, however, are not designed as ultralow fouling materials. Recently, we have shown how surfaces made of mixed charge amino acids, glutamic acid (negative, E) and
lysine (positive, K), are able to resist ultralow levels of protein adsorption. This was developed with the understanding that glutamic acid and lysine together are chemically very similar to synthetic ultralow fouling carboxybetaine zwitterionic polymers. Recognizing this, we have engineered a gene to encode for an amino acid based polymer consisting of perfectly alternating glutamic acid and lysine residues. β-lactamase, a well-studied model enzyme, was chosen to demonstrate the construction of a protein-polymer conjugate with alternating glutamic acid and lysine residues (Figure 5.1c).

5.2 Results and Discussion

β-lactamase was extracted from the plasmid pJG108 which included the OmpA (outer membrane protein A) signal sequence. The signal sequence was required to direct the protein into the periplasmic space of the E. coli. The OmpA signal sequence has been shown previously as an efficient signal sequence yielding high levels of β-lactamase expression in the periplasm. Three different length alternating glutamic acid (E) and lysine (K) genes were purchased: EK1 = 240 residues (30.9 kDa), EK2 = 120 residues (15.4 kDa), and EK3 = 82 residues (10.6 kDa). These synthetic genes were engineered into the 3’ end of the OmpA-β-lactamase gene (C-terminus of protein). This would result in the EK polymers being translated after the β-lactamase enzyme. By being translated last, it was less likely to interfere with protein folding and chaperoning to the periplasmic space. The β-lactamase C-terminus was also ideal because it was away from the enzyme’s active site. This gave a total of four genes including OmpA-β-lactamase (Bla, control), OmpA-β-lactamase-EK1 (EK1), OmpA-β-lactamase-EK2 (EK2) and OmpA-β-lactamase-EK3 (EK3). All the genes were constructed in pBLN200 expression vectors and transformed into BL21(DE3) cells for protein expression.
LB media was used during expression, except with the Bla control. Bla required growth in LB media supplemented with 15% sucrose. This was needed to prevent aggregation of the protein in the periplasmic space which interfered with osmotic shock. This was also observed in previous works. In the whole cell fractions (WC), as evaluated by SDS-PAGE (Figure 5.2), greater total protein expression was seen with lesser amounts of EK polymer (Bla>EK3>EK2>EK1), with expression of EK1 not easily distinguishable. However, after osmotic shock (Peri fraction), a phenylboronic acid-agarose affinity column, specific for β-lactamase, greatly enhanced the protein purity (BA fraction). A final size exclusion separation resulted in highly pure products (SEC fraction).

Thermal stability was measured at different temperatures to evaluate the effects of the EK polymers. Enzymes were incubated at the listed temperatures for 10 minutes, then removed and measured for activity using the substrate, Cefazolin. From Figure 5.3, it can be observed EK1, with the longest EK polymer, possessed the greatest resistance to deactivation from thermal denaturation. EK2 and EK3 showed similar denaturation patterns, with β-lactamase (Bla), the unmodified control, showing the greatest denaturation. These results show that the alternating glutamic acid and lysine (EK) polymers provide resistance to enzyme unfolding from thermal denaturation.

Bioactive proteins, such as enzymes, often experience losses in activity when chemically modified. Protein modification can lead to unpredictable alterations to tertiary structure which in turn affects how the protein is meant to interact with a substrate or receptor. Polymer modification, such as PEGylation (discussed previously), can competitively interact with the active site, thereby reducing binding affinity. Additionally, recombinant modifications can interfere with protein folding during translation as well as interfering with protein transport.
through the cell. For these reasons, it is essential to evaluate the enzyme kinetics (Michaelis-Menten) of the modified β-lactamase. Figure 5.4a has the values of $K_m$ (Michaelis constant) for β-lactamase (Bla) and the three β-lactamase-EK conjugates (EK1, EK2 and EK3). $K_m$ represents the binding affinity of the substrate for the enzymes, with lower $K_m$ values equating to greater binding affinities. The β-lactamase control showed the greatest binding affinity, with the lowest value of $K_m$. The EK conjugates, EK1, EK2 and EK3, had higher $K_m$ values, but had stronger binding affinities with longer EK polymers (EK1>EK2>EK3). This corresponds with the effect of zwitterionic polymers on protein binding studied previously. $k_{cat}$, (maximum turnover rate, Figure 5.4b), showed β-lactamase with the greatest performance, but little activity was lost with even the largest EK polymer, EK1.

5.3 Conclusions

We have developed a method for constructing zwitterionic polymer-protein conjugates using recombinant engineering techniques. By mimicking synthetic zwitterionic polymers using alternating sequences of glutamic acid and lysine amino acids, we were able to construct a polymer-protein conjugate during protein expression in one step. This eliminates the need for constructing polymer-protein conjugates in two steps: protein expression and polymer conjugation. The presented technique also provides precise control over polymer location on the protein, polymer size, and eliminates the possibility of producing heterogeneous constructs.

5.4 Experimental Section

5.4.1 Plasmid construction

The β-lactamase gene was amplified out of the pJG108 vector with the OmpA signal sequence using (OmpA-bla) PCR with primers: 5’-TAATAACATATGAAAAGACAGCTATCGCGATTG-3’ and 5’-
TAATAAAAGCTTACCAATGCTTAATCAGTGAGGC-3’. The PCR fragment was introduced with NdeI and HindIII sites at the 5’ and 3’ ends, respectively. The pBLN200 expression vector, along with the OmpA-bla insert, was digested using NdeI and HindIII, and the insert was dropped into pBLN200. In order to express the EK genes at the C-terminus of protein, the stop codon needed to be removed. This was done using QuikChange mutagenesis with primers 5’-GATTAAGCATTGGAAGCTTGCGGCC-3’ and 5’-GGCCGCAAGCTTCCAATGCTTATCC-3’. This brought in frame the HindIII cut site, adding a lysine (K) and leucine (L) linker after the β-lactamase gene and before the EK gene.

Three different genes encoding for different length polymers of alternating glutamic acid (E) and lysine (K) were purchased from GenScript USA Inc. in pUC57 vectors: EK1 (735 base pairs), EK2 (375 base pair gene) and EK3 (261 base pair gene). These genes were ordered with HindIII and XhoI cut sites at the 5’ and 3’ ends, respectively. The pBLN200-OmpA-β-lactamase (pBLN200-bla), pUC57-EK1, pUC57-EK2 and pUC57-EK3 vectors were digested using HindIII and XhoI. The cut EK1, EK2 and EK3 inserts were directly dropped into the cut pBLN200-bla vector without PCR amplification. The final plasmids for this work were: pBLN200-bla, pBLN200-bla-EK1, pBLN200-bla-EK2 and pBLN200-bla-EK3. The constructed vectors were stored in TOP10 cell lines for long term storage, and BL21(DE3) cell lines for protein expression.

5.4.2 Protein expression and purification

500 mL of LB media containing 50 µg/mL kanamycin was inoculated with 25 mL of overnight culture, grown with the same media. The culture for expressing the β-lactamase wild type was grown with the addition of 15% sucrose. This was to prevent what was believed to be protein aggregation in the periplasmic space that prevented the cells from undergoing osmotic
Cultures were grown, shaking at 37°C, to mid-exponential phase. At OD$_{600}$ = 0.5, cultures were induced with 0.2% arabinose to begin protein expression. After 3 hours, 1 mL of each cell culture was centrifuged at 14000g for 10 minutes to separate the cells from the media. The cell pellet was resuspended in 1x SDS-PAGE running buffer for the whole cell fraction. The remaining cells were centrifuged at 2000g and resuspended in 30 mM Tris-HCl, pH 7.4. This step was then repeated. The cells were then incubated in 20% sucrose, 30 mM Tris-HCl, 2mM EDTA, pH 7.4 for 10 minutes. The cells were then centrifuged at 2000g and resuspended in ice cold water. This released the periplasmic fraction of the cells, where the recombinant products were expressed. The cells were then centrifuged at 2000g, and the soluble periplasmic fraction was separated from the spheroplasts. The periplasmic fractions were dialyzed in 3.5 kDa molecular weight cut-off (MWCO) regenerated cellulose under refrigerated conditions for 24 hours against 20mM triethanolamine, 100mM NaCl, pH 7.0 buffer. The dialyzed protein solutions were then purified over a phenylboronic acid-agarose column. Loading was done using 20mM triethanolamine, 500mM NaCl, pH 7.0 buffer and elution was done using 500mM borate, 500mM NaCl, pH 7.0 buffer. A final purification was performed using size exclusion chromatography with a superdex 75 10/300 column. This gave a purity of >95% visible from SDS-PAGE.

5.4.3 Thermal stability

Conjugates were incubated in 20mM phosphate, pH 7.0, at temperatures of 22, 30, 40, 50, 60, 70, 80 and 90°C at a concentration of 24 nM. 10 ug/mL of bovine serum albumin (BSA) was added to the buffer to prevent enzyme loss due to nonspecific surface adsorption. After 10 min at each temperature, each conjugate was diluted into cold buffer to a concentration of 6 nM. Enzyme activity was measured using cefazolin at 80uM. Activity was measured as the loss in
substrate absorbance at 270nm with time. Values were recorded as percentage of each conjugate’s activity at 22°C.

5.4.4 Conjugate kinetics with Cefazolin

Conjugates were incubated in 20mM phosphate, pH 7.0 with 10 μg/mL BSA as a blocking agent. Cefazolin was added at varying concentrations from 0-4000 μM. Activity was measured as the loss in substrate absorbance at 270nm with time. Enzyme activity vs. substrate concentration was modeled using standard Michaelis-Menten kinetics. Experiments were performed in triplicate.
5.5 Figures

**Figure 5.1**: a) The gene for the enzyme β-lactamase (Bla) with the OmpA signal sequence (SS) used for expression of the β-lactamase protein. b) Costly secondary steps are required if synthetic polymer conjugation is needed. c) Genes for three different length alternating glutamic acid (E) and lysine (K) polymers were added to the c-terminus of the Bla gene. Using this type of genetic modification, synthetic modification and post processing is avoided.
**Figure 5.2:** SDS-PAGE gel (12.5%) of all expressed proteins at sequential purification steps. From left to right: whole cell fraction (WC), cytosolic fraction (Cyto), periplasmic fraction with product (Peri), purification from the periplasmic fraction using phenylboronic acid-agarose column (BA), and purified product after final size exclusion column purification (SEC).
Figure 5.3: Temperature stability of β-lactamase and three β-lactamase-EK conjugates (EK1, EK2 and EK3). EK1, with the longest EK polymer, possessed the greatest resistance to deactivation from thermal denaturation. EK2 and EK3 showed similar denaturation patterns, with β-lactamase (Bla), the unmodified control, showing the greatest denaturation.
Figure 5.4: Kinetics parameters of β-lactamase (Bla) and three β-lactamase-EK conjugates (EK1, EK2 and EK3). a) Michaelis constant ($K_m$) represent the binding affinity of the substrate for the enzyme. Lower values represent high affinities. The β-lactamase control showed the greatest binding affinity, but of the polymer conjugates, stronger binding affinities were seen with larger EK polymers. b) $k_{cat}$, (maximum turnover rate), showed β-lactamase with the greatest performance, but little value was lost with even the largest EK polymer, EK1.
Chapter 6

Screening Nonspecific Interactions of Peptides without Background Interference

The need to discover new peptide sequences to perform particular tasks has lead to a variety of peptide screening methods: phage display, yeast display, bacterial display and resin display. These are effective screening methods because the role of background binding is often insignificant. In the field of nonfouling materials, however, a premium is placed on chemistries that have extremely low levels of nonspecific binding. Due to the presence of background binding, it is not possible to use traditional peptide screening methods to select for nonfouling chemistries. Here, we developed a peptide screening method, as compared to traditional methods, that can successfully evaluate the effectiveness of nonfouling peptide sequences. We have tested the effect of different peptide lengths and chemistries on the adsorption of protein. The order of residues within a single sequence was also adjusted to determine the effect of charge segregation on protein adsorption.

6.1 Introduction

It has been established that surfaces modified with hydrophilic synthetic polymers, such as poly(ethylene glycol) (PEG), are able to resist types of surface fouling, such as nonspecific protein adsorption and bacterial adhesion/biofilm formation in complex media. More recently, synthetic zwitterionic poly(carboxybetaine) and poly(sulfo betaine) as well as synthetic mixed positively and negatively charged polymers have been developed and shown to achieve ultralow levels of fouling that have not been achieved before. Despite some
progress in the fundamental understanding of molecular-level nonfouling mechanisms and the
development of new materials, only a handful of nonfouling materials are currently available.
Mixed charge nonfouling polymers are particularly attractive for practical applications because
of their simplicity, broad variations, and low-cost. Among these, peptides are of particular
interest due to their being natural materials. Mimicking the chemistry of zwitterionic polymers,
we have also been able to achieve ultralow levels of fouling with peptide based surfaces. For
evaluating nonfouling materials, peptide-based nonfouling polymers offer an indispensable
advantage; potentially infinite variability in structure and property, with precise control over
length and sequence. In addition to the already rich diversity of naturally occurring amino acids,
inhibiting synthetically derived unnatural amino acids allows for an even greater number of
potential materials.

Peptide libraries are tools often used to search for new functional chemistry, such as
identifying enzyme binding motifs, protease cleavage sites, mineral binding peptides, and antibiotics. To accomplish this, peptide libraries require a display mechanism; commonly phage display, yeast display, bacterial display, or resin display. These methods work by
evaluating the binding performance of individual peptide sequences to specific materials. The
peptide of interest usually makes up only a small portion of the presenting scaffold (i.e. cell,
virus, etc.). The presence of background binding from the display mechanism does not interfere
because the affinity of the peptide is far greater than the affinity of the background for the
material being tested.

6.2 Results and Discussion

In this work, we screened a large library of peptide sequences to determine nonfouling
properties. Since we were looking to determine what peptide sequences have the lowest amount
of nonspecific binding, traditional peptide display mechanisms would not work, due to the presence of background binding.\textsuperscript{122} Background binding would eliminate the ability to differentiate between nonfouling sequences. For this reason, a new method for screening nonfouling peptides was developed using amino functionalized glass beads to introduce peptides to a surface through solid phase Fmoc peptide synthesis. The full surface of the glass beads were coated with synthesized peptide, thereby presenting only the peptide of interest to the protein adsorbent. We also demonstrate how this new method compared to traditional peptide screening when evaluating known nonfouling peptide sequences.

In order to grow peptides off glass beads, the glass surface was needed to be modified with accessible primary amines (Figure 6.1a). To accomplish this, an epoxide functionalized silane, GPTMS, was first coated on the surface. The presence of the epoxide then allowed coupling of a tetraethylene glycol diamine linker. Tetraethylene glycol linker was used as an alternative to shorter diamines in order to improve amino acid coupling. To ensure efficient surface modification, the amino functionalized glass was evaluated by XPS. Data were collected for the unmodified bare glass beads, the silane functionalized glass beads with the epoxides deactivated, as well as the amino functionalized glass beads. As seen in Table 6.1, the unmodified glass consisted or primarily of silicon and oxygen with some carbon contamination and no detectable nitrogen. Using such surface sensitive analysis techniques, contaminant carbon if often unavoidable. After silination, a significant increase in carbon was observed representing what is likely a monolayer, given the deposition conditions. After reacting the epoxide surface with the diamine linker, 2.0% nitrogen surface content was measured. This gave a 9.1/1 carbon to nitrogen ratio compared to a theoretical ratio of 7.0/1. This meant most of the deposited silane was reacted with diamine linkers. Accounting for the presence of contaminant carbon would
likely bring the measured values closer to the theoretical. Overall these data show that there is a high surface density of available amine for peptide synthesis.

To evaluate the performance of this new method of peptide screening, five amino acids were chosen to rationally design peptide sequences of interest. Glutamic acid (E) and lysine (K) were chosen due to their negative and positive charges, respectively. Glycine (G) was included because it constitutes the bare amide backbone found in all peptides, since it has no side-chain. Leucine (L) was included due to its hydrophobic characteristics, and glutamine (Q) for its uncharged, hydrophilic amide side-chain. The structures of these amino acids, with their chemical characteristics, can be seen in Figure 6.1b. The beads were synthesized using standard Fmoc peptide synthesis. Since the peptide surfaces were synthesized as large groups of beads, samples were able to be taken during the syntheses to provide surfaces that had shorter length peptides, thereby increasing the number of peptide surfaces capable of being analyzed.

Fouling was evaluated by performing protein adsorption experiments using 0.5 mg/mL fluorescently labeled (Alexa Fluor 488) bovine fibrinogen in phosphate buffered saline (PBS). Fibrinogen is a large protein found in the blood that is highly susceptible to surface adsorption and plays a vital role initiating the foreign body response in the body. Fibrinogen is therefore useful to determine the nonfouling characteristics of surfaces. Beads were incubated in the protein solution, and then washed with PBS to remove unbound protein. The beads were analyzed using confocal microscopy in order to accurately measure the fluorescent intensity of bound protein at a bead’s surface.

Figure 6.2 demonstrates the effectiveness of the new screening method as compared to a traditional screening method that uses TentaGel resin. The fluorescent images are cross sections of the beads or resin taken using a confocal microscope. Like most resins, TentaGel resin
consists of a polymer scaffold that has peptides synthesized throughout the scaffold. As seen in **Figure 6.2**, both the “non peptide” and poly(glycine, G) samples had moderate to low levels of fibrinogen adsorption, however, the mixed charge peptide (EKEKEKEK) had extremely high levels of adsorption for the TentaGel resin and very low levels of adsorption for the glass beads. As published previously, this mixed charge sequence was shown to have ultralow levels of adsorption.\textsuperscript{105, 106} The unintended result observed from the TentaGel resin was likely due to the protein entering the resin and salting out within the surface of the polymer scaffold. The salting out was likely caused by the presence of the ionic species of the peptide (E, negative; K, positive). These complex adsorption events can be avoided if the peptides are presented on a rigid surface, removing background effects. The lower row in **Figure 6.2** shows the new method with expected adsorption levels, in contrast to what was observed with TentaGel.

**Figure 6.3** shows fluorescent cross sections in the XY plane of beads with different peptide surfaces after exposure to fluorescent protein. A glass bead was also silinated with an alkyl silane (octadecyltriethoxysilane) as a positive control for protein binding due to its hydrophobicity, labeled as “alkyl”. It is apparent that there are significant differences based on peptide sequence. It was observed that hydrophobic and positively charged surfaces showed increased levels of protein adsorption while the negative and hydrophilic beads had low levels of protein adsorption. Unfortunately, quantifying protein adsorption using XY cross sectional images was inaccurate, varying greatly with the selected Z plane. This made it difficult to quantify the difference between sequences. In order to achieve reproducible fluorescent surface intensities, XZ fluorescent cross sections were measured. Data achieved in this manner gave consistent measurements of protein adsorption. All collected fibrinogen fouling data for synthesized peptide surfaces can be seen in **Figure 6.4a**. Values were calculated as percentages relative to the
positive control: octadecyltriethoxysilane modified beads. An example of a XZ fluorescent cross sectional image can be seen in Figure 6.4b. Integrating across the surface of the bead was used to quantify the amount of protein fouling (Figure 6.4c).

The collected fouling data was organized to elucidate trends and patterns based on a peptide’s sequence and chemistry. Figure 6.5 shows the trend in fouling of homologous sequences for the five amino acids used. Since fibrinogen is a negatively charged protein, there is a distinct effect of surface charge on fouling. Glutamic acid surfaces showed low levels of fouling, with lower fouling correlated to the number of amino acids in length. The opposite was apparent with lysine a positively charged residue, with longer chains having corresponding to higher levels of fouling. Hydrophobic surfaces were also susceptible to higher levels of protein fouling, due to hydrophobic interactions with the protein core, resulting in irreversible unfolding and adsorption. Glycine with no side-chain and glutamine with a non-charged amide side chain display low fouling properties. The lower fouling levels seen for glutamine compared to glycine show that its amide side-chain plays a significant role for reducing protein fouling. At longer peptide lengths, glutamine was seen to be one of the lowest fouling peptides.

Charge position was also investigated to determine how the arrangements of positive and negative charges (lysine and glutamic acid) affected fouling. It is expected that surfaces with positive and negative charges, that are uniformly mixed, give the lowest levels of fouling by minimizing charge localization and dipole formation. In contrast, with enough charge localization, with separated areas of negative and positive charges, on an overall net neutral surface, there will be higher degrees of fouling. Figure 6.6a shows levels of fouling on several mixed charged surfaces, which have a net surface charge of zero. Little distinction was seen between peptides that had alternating charges or charges fully segregated. These results indicate
two possibilities. First is the presence of salt bridging between positive and negative regions of peptides, which reduces the degree of charge separation experienced on the surface. Second, the maximum peptide length tested was only 8 residues. It is expected that longer peptides with larger regions of segregated charges will experience higher degrees of fouling. It was apparent that sequences tested in this study where not long enough to experience this effect. Here, the primary variable affecting protein fouling was peptide length. This was due to higher degrees of surface hydration, with an overall increase in hydrated peptide chains. However, for glutamic acid and lysine mixtures that were not charge neutral, there were observable differences in fouling as expected (Figure 6.6b). Electrostatics between a charged surface and charged protein can play a dominant role in attracting or repelling a protein onto a surface, depending on different charges on a protein and a surface. A charged surface which repels the adsorption of similarly charged proteins cannot be considered a nonfouling surface since proteins of the opposite charge would certainly lead to fouling.

6.3 Conclusions

Here, we developed a new platform of peptide screening that eliminates the effect of background binding. This was done by using a bead display that allowed for the synthesis of peptides off the full surface of the bead. This is a valuable technique for measuring the performance of nonfouling peptide sequences since it is essential to eliminate the role of background binding. We have shown how this technique can be used to differentiate between beads consisting of different peptide coatings. Confocal microscopy was an ideal method for observing bound fluorescent protein, which was able to accurately measure the adsorbed protein film across the peptide surface. From our results, it was observed that glutamine and mixtures of charged residues of overall neutral charge had the lowest degrees of fouling. Using this new
method, the problem of background binding found in traditional peptide screening methods can be avoided, allowing for the screening of nonfouling chemistries.

6.4 Experimental Section

6.4.1 Materials

Triisopropylsilane (TIPS), Trifluoroacetic acid (TFA), γ-glycidoxypropyltrimethoxysilane (GPTMS), N,N-Diisopropylethylamine (DIPEA), glass beads (212 - 300 µm), Fibrinogen from Bovine, and octadecyltrithoxysilane were purchased from Sigma-Aldrich. Tetraethyleneglycol diamine (PEO4-Bis Amine) was purchased from Molecular Biosciences. TentaGel MB NH2 resin (capacity: 0.51 mmol/g) was purchased from RAAP Polymere. Alexa Fluor® 488 Carboxylic Acid, 2,3,5,6-Tetrafluorophenyl Ester (Alexa Fluor® 488 5-TFP) was purchased from Fisher Scientific. Hydroxybenzotriazole (HOBt), O-Benzotriazole-N,N,N’,N’-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and Fmoc protected amino acids were purchased from AAPPTec.

6.4.2 Glass bead surface modification for peptide synthesis

100 mL of 37% hydrochloric acid was added to 20 grams of glass beads and refluxed overnight. The beads were washed several times with water and methanol, then dried under reduced pressure and heating. The glass beads were next treated with a 3:1 volumetric ratio of concentrated sulfuric acid and 30% hydrogen peroxide, and allowed to sit for 30 minutes. The beads were then rinsed several times with water, then treated with a 5:1:1 volumetric ratio of water to 29% ammonium hydroxide to 30% hydrogen peroxide, and allowed to sit for 30 minutes. The beads were washed with water several times and then washed several times with methanol. The glass was then dried under reduced pressure and heating. The beads were placed in an oven at 130°C for 2 hours to remove and trace water then allowed to cool to room
temperature. For silination, the beads were added to a solution of anhydrous toluene, GPTMS, and DIPEA (94/5/1). The silination occur at 80°C for 18 hours. After silination, the beads were washed with anhydrous toluene, dichloromethane (DCM), and then dried under reduced pressure and heated at 100°C for 2 hours. The silanized beads were next added to a solution of 5% PEO4-Bis Amine in anhydrous acetonitrile and heated to 80°C for 18 hours. The beads were then washed with acetonitrile and dried. Unreacted surface epoxide groups were deactivated by incubating in 2% sulfuric acid in water overnight. Then beads were then finally washed with water and methanol, then dried under reduced pressure and heating.

6.4.3 Peptide synthesis

Solid phase peptide synthesis was performed using a Titan 357 peptide synthesizer (AAPPTec inc., Louisville, KY). Coupling of Fmoc protected amino acids occurred in 80 mM amino acid, 80 mM HBTU, 80 mM HOBt, 160 mM DIPEA in dimethylformamide (DMF) for 60 minutes. Piperidine/DMF (20/80) was used to deprotect the Fmoc protected amine from the newly bound amino acid residue. A solution of DMF/DCM (50/50) was used for washing between coupling and deprotection steps. After synthesis was complete, all terminal amine group were acetylated using acetic anhydride/pyridine/DMF (5/5/90). Deprotection of the acid cleavable side-chains occurred as the final step using a solution of TFA/phenol/water/TIPS (88/4/4/4) for 3 hours. The beads were then washed with DCM and dried under reduced pressure.

6.4.4 Protein adsorption and screening

Alexa Fluor 488 labeled fibrinogen was prepared by conjugation with Alexa Fluor® 488 5-TFP. Briefly, 30 mg of fibrinogen was dissolved in 3 mL of 100 mM NaHCO₃, pH 9. This solution was added to a vial containing ~1 mg of Alexa Fluor® 488 5-TFP. After 2 hours, the
labeled conjugates were twice purified using 10 mL Bio-Gel P-6DG Bio-Rad disposable size exclusion columns.

Before adding labeled fibrinogen, peptide coated beads were washed several time with phosphate buffered saline (PBS). The beads were then incubated in a 0.5 mg/mL solution of Alexa Fluor 488 labeled fibrinogen for 60 minutes. After adsorption, the beads were washing several times with PBS to remove unbound protein and quickly analyzed using a Zeiss LSM 510 confocal microscope. Cross-sectional fluorescent images of the beads were taken (X-Z plane) using 488 nm argon laser for excitation, and detecting emission signal between 500-550 nm.

6.4.5 X-ray photonelectron spectroscopy (XPS) of glass beads

XPS experiments were performed on an S-Probe spectrometer using a monochromatic Al Ka X-ray source \( (h\nu = 1486.6 \text{ eV}) \) operated at 10 mA and 15 kV. Survey spectra were acquired with an analyzer pass energy of 80 eV. High-resolution O 1s, N 1s, C 1s and Si 2p spectra were acquired with an analyzer pass energy of 20 eV. All of the XPS data were acquired at a nominal photoelectron takeoff angle of 0°, where the takeoff angle is defined as the angle between the surface normal and the axis of the analyzer lens. Three spots on each sample were examined. The compositional data are averages of the values determined at each analysis spot.
6.5 Figures

Figure 6.1: Steps for surface modification of glass beads. a) GPTMS was first used to introduce epoxide groups to the glass surface. The epoxidized surface was exposed to PEO4-Bis Amine in order to attach reactive amine groups needed for peptide synthesis. b) Finally, amino acids, with diverse chemical properties, were selected to grow peptides from the glass beads using standard Fmoc peptide synthesis.
Table 6.1: Summary of XPS-Determined Elemental Composition of bare glass, deactivated epoxy and amine surfaces (atom %). *Theoretical C/N ratio for 100% coupling efficiency of the diamine linker onto the surface.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Atom %</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>O</td>
</tr>
<tr>
<td>Bare Glass</td>
<td>67.5 ± 1.0</td>
</tr>
<tr>
<td>Deactivated Epoxy</td>
<td>57.6 ± 2.3</td>
</tr>
<tr>
<td>Amine Surface</td>
<td>57.0 ± 2.1</td>
</tr>
</tbody>
</table>
Figure 6.2: Comparison between the traditionally used TentaGel resin and our glass beads for evaluating nonspecific protein adsorption. It can be observed for peptides like EKEKEKEK that TentaGel resins are susceptible to swelling and protein entrapment, where the glass beads provide a more rigid surface that represents the chemistry of the surface more accurately.
Figure 6.3: Fluorescent images in the XY plane of peptide coated glass beads after exposure to Alexa Fluor 488 labeled fibrinogen. The first tile is a light field image without fluorescence of the alkyl surface.
Figure 6.4: a) Nonspecific fibrinogen adsorption of all synthesized peptide surfaces. Values listed of percents fouling compared to an alkyl silane surface. b) An example of a protein bound bead scanned in the X-Z plane. The top of the bead is integrated c) to measure the level of adsorbed protein.
Figure 6.5: The effect of peptide length on protein adsorption for homologous peptide sequences. The addition of positively charged lysine and hydrophobic leucine resulted in increased protein adsorption. Increased length on glycine, glutamic acid and glutamine showed reduced protein adsorption, with glutamic acid and glutamine showing the lowest adsorption levels (5%).
Figure 6.6: a) The effect of charge position on protein adsorption for charge neutral peptide sequences. No effect was observed as a result of charge segregation except for peptides of only two residues. The primary factor in reducing protein adsorption for charge neutral ionic peptides was length. b) Net charged peptides showed differences in protein adsorption only as a result of total charge.
Chapter 7
Peptide Screening Using One-Bead-One-Compound Combinatorial Approach for Discovery of Nonfouling Peptide Chemistries

Peptide libraries have been used for discovering many types of new enzymatic substrates, surface adherents, antimicrobial agents, and several other unique chemistries. However, these traditional methods (phage display, yeast display, bacterial display and resin display) are only useful for finding peptides that are able to be isolated due to strong binding to a particular ligand or substrate. In this work, we describe a new peptide library technology this is able to elucidate ultralow binding, as well as high binding peptides. A diverse one-bead-one-compound (OBOC) peptide library was created using mix-and-split combinatorial peptide synthesis on small control pore glass (CPG) substrates (d = 150 um). We show how peptide sequences can be screened for protein fouling using fluorescent confocal microscopy and sequenced using partial Edman degradation matrix assisted laser desorption/ionization time of flight mass spectroscopy (PED MALDI-TOF MS).

7.1 Introduction

It has been established that surfaces modified with zwitterionic and mixed charge polymers such as poly(carboxybetaine) and poly(sulfobetaine) can effectively resist nonspecific protein adsorption and bacterial adhesion/biofilm formation in complex media. Mixed charge nonfouling polymers are also attractive for practical applications because of their simplicity, broad variations, and low-cost. Among mixed charge polymers, alternating charge peptides are of particular interest. In conjunction with material development, molecular-level nonfouling mechanisms are studied, enabling the rational design of some new nonfouling
materials. Despite some progress in the fundamental understanding of molecular-level nonfouling mechanisms and the development of several new nonfouling materials, only a handful of nonfouling materials are available. Biological environments are complex, containing thousands of potential adsorbents. Thus, it is necessary to provide a deeper understanding of nonfouling mechanisms and to search for a series of chemistries suitable as nonfouling materials.

Peptide-based nonfouling polymers do offer one indispensable advantage over other commonly used materials: potentially infinite variability in structures and properties. In searching for new peptides that serves a unique application (enzymatic substrate, surface adherent, antimicrobial agent, etc.), phage, yeast, bacterial and resin display technologies are typically used. It should be pointed out that for the combinatorial identification of specific-binding peptides as studied by others, phage display is most popular. There are two major advantages of phage display: (a) synthesis and sequencing can be done using developed DNA technologies, since the variable peptide sequence is found in the phage DNA sequence; (b) multiple rounds of scanning can be done to enhance resolution since biological amplification is easy. However, the phage display method used for the identification of specific-binding peptides is not suitable for the screening of non-specific binding peptides proposed in this work, since envelope proteins will exhibit nonspecific binding therefore preventing analysis of the variable region. This will also hold true for any of the other common methods for peptide screening.

Previously, we developed a new type of peptide screening technology that selected for both ultralow binding, as well as high binding peptides. This was accomplished by measuring the fouling properties of peptides that were immobilized on the surface of solid glass beads. Peptides first needed to be rationally designed, and then subsequently evaluated for protein binding. Using this method, we were able to screen of 10’s of sequences. In this work we demonstrate a new
type of peptide screening technology that is able to screen thousands of peptide sequences using a combinatorially generated peptide library. To accomplish this, controlled pore glass (CPG) beads were used as substrates to develop one-bead-one-compound (OBOC) peptide libraries. The choice of a porous substrate made it possible to synthesize enough peptide material to allow for peptide sequencing from a single bead using mass spectrometry techniques. Each bead possessed total homologous peptide coverage of one unique peptide sequence. This prevented the chosen adsorbent form interacting with anything but the bound peptide. Binding was measured using fluorescent confocal microscopy with a fluorescent adsorbent (Alexa Fluor® 488-fibrinogen). Single bead peptide sequencing was possible using partial Edman degradation matrix assisted laser desorption/ionization time of flight mass spectroscopy (PED MALDI-TOF MS).

### 7.2 Results and Discussion

Peptides were synthesized on the surface of amino functionalized control pore glass (CPG). CPG beads were used with a diameter of ~150 um and pores of 300nm (± 10%) because A) this allowed sufficient surface area to synthesize enough peptide on a single bead to detect by MALDI and B) had a large enough pore size to prevent protein entrapment. The CPG was modified with amines by subsequent surface modification with epoxy silane and a tetraethylene glycol diamine linker. This flexible linker allowed for high coupling efficiency of amino acid residues. To ensure that peptide coated CPG could be used to discriminate between peptides with different surface fouling properties, 3 control peptide sequences were individually synthesized: LLLLLLLL = (LL)$_4$, GGGGGGGG = (GG)$_4$, and EKEKEKEK = (EK)$_4$. “L” refers to leucine, “G” glycine, “E” glutamic acid, and “K” lysine. Refer to Figure 7.2 for amino acid abbreviations, structures and chemical properties. Peptide synthesis was performed using standard Fmoc peptide synthesis. From previous work, we know that surfaces of repeating
hydrophobic amino acid residues, such as (LL)$_4$, experience high levels of nonspecific protein adsorption. Hydrophilic amino acid surfaces, such as (GG)$_4$, are able to resist protein adsorption, while super hydrophilic zwitterionic surfaces, such as (EK)$_4$, have been found to perform the best. From Figure 7.1, CPG as a substrate for peptide surfaces was able to greatly discriminate between the control peptide sequences. Bright field (Figure 7.1a,c,e) and their corresponding fluorescent images (Figure 7.1b,d,f) are presented.

Next, we constructed a random, combinatorial peptide library, for the goal of finding new peptide sequences that had ultralow fouling properties. Since the method of sequencing involved Edman degradation and mass spectrometry (MS) techniques, some of the 20 naturally occurring amino acids had to be left out. Proline was avoided due to its incompatibility with Edman degradation. Cysteine was not used because of the propensity of disulfide formation. Amino acids of the exact same molecular weight could also not be used because MS sequencing is unable to discriminate: glutamine and lysine both 146 g/mol, and leucine and isoleucine both 131g/mol. Glutamine was not used, due to similar chemistry available in asparagine, and isoleucine was not used. Since tryptophan and tyrosine are both difficult to synthesize and have protecting groups that have likelihoods of causing side reactions, these were also excluded. Methionine was also left out because the thioether side group interferes with the final peptide cleavage. Finally, 13 amino acids remained that were used to create random peptide libraries (Figure 7.2).

One-bead-one-compound (OBOC) peptide libraries were constructed using a mix and split synthesis technique (Figure 7.3a). This involved having one reaction vessel for each desired amino acid. For our system we needed 13. The beads were equally distributed into the 13 reaction vessels, where the coupling of 1 amino acid to the bead took place. The beads were then
removed from the 13 vessels, recombined, mixed, and evenly redistributed. This process was repeated to create libraries that were 2, 4, 6 and 8 amino acids in length. Figure 7.3b shows the peptide library with length 8 amino acids after being exposed to fluorescently labeled fibrinogen using confocal microscopy imaging. Integrating across the surface of the bead, numerical values can be assigned to individual beads. Figure 7.3c shows the fouling data from 100 randomly selected beads ranked in order of fouling included with the 3 control peptide surfaces from Figure 7.1. When compared to the control surfaces, 37% of the library was found to have lower fouling than ultralow fouling (EK)_4.

Performing PED-MALDI-TOF MS allows for the sequencing the peptide surface chemistry of individual beads (Figure 7.4). Here is an example of a sequenced peptide from a single bead from a library with residues of length 4. The weight up to 911.7 m/z, represented by the dashed line, comes from the c-terminus linker and the n-terminus Fmoc. The extra atomic weight was required for detection by MALDI-TOF MS. See experimental section for details.

7.3 Conclusions

Herein, we described a new peptide screening technology that was capable of screening for ultralow binding, as well as high binding peptide sequences. Predetermined peptide sequences were also synthesized with known levels of protein binding to demonstrate the robustness of the technique. A diverse one-bead-one-compound (OBOC) peptide libraries were created using mix-and-split combinatorial peptide synthesis on CPG substrates consisting of 13 natural occurring amino acids and 2, 4, 6 and 8 amino acids in length. Additionally, PED MALDI-TOF MS was shown to determine the peptide sequence from a single library bead. This peptide display technology can be used to discover new types of nonfouling peptide sequences that traditional peptide screening methods cannot.
7.4 Experimental Section

7.4.1 Materials

Triisopropylsilane (TIPS), 9-Fluorenylmethyl N-succinimidyl carbonate (Fmoc-OSU), protein sequencing grade phenyl isothiocyanate (PITC), trifluoroacetic acid (TFA), γ-glycidoxypropyltrimethoxysilane (GPTMS), N,N-diisopropylethylamine (DIPEA), bromotrimethylsilane, thioanisol, phenol, and bovine fibrinogen were purchased from Sigma-Aldrich. Controlled pore glass beads were purchased from Millipore Corp. Tetraethyleneglycol diamine (PEO4-Bis Amine) was purchased from Molecular Biosciences. Alexa Fluor® 488 Carboxylic Acid, 2,3,5,6-Tetrafluorophenyl Ester (Alexa Fluor® 488 5-TFP) was purchased from Fisher Scientific. Hydroxybenzotriazole (HOBt), O-Benzotriazole-N,N,N’,N’-tetramethylumonium-hexafluoro-phosphate (HBTU) and Fmoc protected amino acids were purchased from AAPPTec.

7.4.2 Glass bead surface modification for peptide synthesis

The glass beads were treated with a 3:1 volumetric ratio of concentrated sulfuric acid and 30% hydrogen peroxide, and allowed to sit for 30 minutes. The beads were then rinsed several times with water, then treated with a 5:1:1 volumetric ratio of water to 29% ammonium hydroxide to 30% hydrogen peroxide, and allowed to sit for 30 minutes. The beads were washed with water several times and then washed several times with methanol. The glass was then dried under reduced pressure and heating. The beads were placed in an oven at 130°C for 2 hours to remove and trace water then allowed to cool to room temperature. For silination, the beads were added to a solution of anhydrous toluene, GPTMS, and DIPEA (94/5/1). The silination occurred at 80°C for 18 hours. After silination, the beads were washed with anhydrous toluene, dichloromethane (DCM), and then dried under reduced pressure and heated at 100°C for 2 hours.
The silanized beads were next added to a solution of 5% PEO4-Bis Amine in anhydrous acetonitrile and heated to 80°C for 18 hours. The beads were then washed with acetonitrile and dried. Unreacted surface epoxide groups were deactivated by incubating in 2% sulfuric acid in water overnight. Then beads were then finally washed with water and methanol, then dried under reduced pressure and heating.

7.4.3 Peptide synthesis

Solid phase peptide synthesis was performed using a Titan 357 peptide synthesizer (AAPPTec inc., Louisville, KY). Coupling of Fmoc protected amino acids occurred in 80 mM amino acid, 80 mM HBTU, 80 mM HOBt, 160 mM DIPEA in dimethylformamide (DMF) for 60 minutes. The coupling was then repeated a second time to increase overall coupling efficiency. Any remaining unreacted amine groups were acetylated using acetic anhydride/pyridine/DMF (5/5/90). Piperidine/DMF (20/80) was used to deprotect the Fmoc protected amine from the newly bound amino acid residue. DMF was used for washing between coupling and deprotection steps. The beads were then washed with DCM and methanol, then dried under reduced pressure. Acid cleavable side chain protecting groups were cleaved with a solution of TFA, bromotrimethylsilane, thioanisol, and phenol (8:1:1:0.1). Beads we then washed with TFA, DCM, methanol, then dried under reduced pressure.

7.4.4 Peptide library synthesis

For both the libraries and the control peptide sequences, a known peptide linker was first added to all CPG beads. The linker consisted of: surface-methionine(M)-threonine(T)-arginine(R)-threonine(T)-glutamic acid(E)-threonine(T)-NH2. This linker formed two functions: 1) provided addition weight to the peptide required for detection by MALDI and 2) the methionine at the peptide base was used to cleave the peptide from the CPG substrate when
exposed to cyanogens bromide. One-bead-one-compound (OBOC) peptide libraries were constructed using a mix and split synthesis technique (Figure 7.3a). This involved having one reaction vessel for each desired amino acid. For our system we needed 13. The beads were equally distributed into the 13 reaction vessels, where the coupling of 1 amino acid to the bead took place. The beads were then removed from the 13 vessels, recombined, mixed, and evenly redistributed. This process was repeated to create libraries that were 2, 4, 6 and 8 amino acids in length.

7.4.5 Protein adsorption and screening

Alexa Fluor 488 labeled fibrinogen was prepared by conjugation with Alexa Fluor® 488 5-TFP. Briefly, 30 mg of fibrinogen was dissolved in 3 mL of 100 mM NaHCO₃, pH 9. This solution was added to a vial containing ~1 mg of Alexa Fluor® 488 5-TFP. After 2 hours, the labeled conjugates were twice purified using 10 mL Bio-Gel P-6DG Bio-Rad disposable size exclusion columns.

Before adding labeled fibrinogen, peptide coated beads were washed several times with phosphate buffered saline (PBS). The beads were then incubated in a 0.5 mg/mL solution of Alexa Fluor 488 labeled fibrinogen for 60 minutes. After adsorption, the beads were washed several times with PBS to remove unbound protein and quickly analyzed using a Zeiss LSM 510 confocal microscope. Cross-sectional fluorescent images of the beads were taken (X-Z plane) using 488 nm argon laser for excitation, and detecting emission signal between 500-550 nm.

7.4.6 Peptide sequencing using partial Edman degradation matrix assisted laser desorption/ionization time of flight mass spectroscopy (PED MALDI-TOF MS)

CPG bound peptide chains were converted into a series of progressively shorter fragments by multiple cycles of partial Edman degradation and the resulting peptide ladder. To accomplish
this, beads were placed in a small reaction vessel with a porous bottom glass frit to remove solvent. First, several beads were placed into the reaction vessel and washed with water, pyridine and a 2:1 (v/v) pyridine/water solution containing 0.1% triethylamine. Finally, the beads were then suspended in a 160μL 2:1 (v/v) pyridine/water solution containing 0.1% triethylamine.

A stock solution of Fmoc-OSU (17 mM) was prepared in pyridine. To a 160μL aliquot of this solution was added 12 μL PITC (0.10 mmol) to obtain a final degradation reagent mixture containing 313 mM PITC and 16 mM Fmoc-OSU (PITC/Fmoc-OSU ~20:1). This reagent mixture was added to the suspended beads, and the reaction was allowed to proceed at room temperature for ~6 min. The solution was then drained and the beads were washed with pyridine and dichloromethane. The beads were then treated twice with TFA at room temperature for 6 min each to cleave the PITC bound amino acids. The Fmoc bound amino acids remained protected from degradation for the entirety of the procedure. The beads were then washed extensively with dichloromethane and finally pyridine. This PED procedure was repeated for \( n - 1 \) times (\( n \) is the number of residues to be sequenced). At the end, the beads were suspended in 1 mL of TFA containing 20 μL dimethyl sulfide and 25 mg ammonium iodide, and incubated for 20 min to reduce any oxidized methionine. The beads were then washed exhaustively with water and methanol.

Once the beads have been treated to possess surface peptide sequences of various chain lengths the beads were individually isolated manually and treated to detach the peptides from the surface using cyanogen bromide in 70% TFA. After overnight cleavage, the cyanogen bromide and 70% TFA were removed under reduced pressure. The remaining peptide residue was dissolved in MALDI matrix solution, mounted on a MALDI stainless steel plate and analyzed.
The molecular weight differences seen in the MALDI spectrum was used to determine the peptide sequence that was on the chosen CPG bead.
7.5 Figures

Figure 7.1: Fouling of fluorescently labeled fibrinogen on control peptide surfaces on CPG. a) Bright field and b) fluorescent image of (LL)$_4$ coated CPG beads. c) Bright field and d) fluorescent image of (GG)$_4$ coated CPG beads. e) Bright field and f) fluorescent image of (EK)$_4$ coated CPG beads.
**Figure 7.2:** Natural amino acids used for constructing peptide library.
Figure 7.3: a) Here is an example of the one-bead-one-compound (OBOC) synthesis method using just 2 residues with a length of 3. Using this mix and split approach for modifying the CPG beads, a highly diverse library can be constructed, with each bead coated in just one peptide sequence. b) The diversity of surface fouling levels can be seen when peptide coated beads are exposed to fluorescently labeled fibrinogen and visualized using confocal microscopy. This library has a residue length of 8. c) Integrating across the surface of the bead, numerical values can be assigned to individual beads. Here is the fouling data of 100 randomly selected beads ranked in order. When compared to the control surfaces, 37% of the library was found to have lowing fouling levels compared to (EK)₄.
Figure 7.4: Performing PED-MALDI-TOF MS allows for sequencing of the peptide surface chemistry of individual beads. Here is an example of a sequenced peptide from a single bead from a library with residues of length 4. The weight up to 911.7 m/z represented by the dashed line, comes from the c-terminus linker and the n-terminus Fmoc. The extra atomic weight was required for detection by MALDI-TOF MS.
Chapter 8

Conclusions

Herein we have shown that zwitterionic materials are ideally suited for modifying and stabilizing PDMS and proteins. For PDMS, this was due to the sharp solubility differences between the superhydrophilic zwitterionic polymer, and the amorphous hydrophobic substrate. PEG in the past has failed in this application due to its solubility in both organic and aqueous phases, resulting in PEG-PDMS reconstruction at the surface. For proteins, similarly, the superhydrophilic zwitterionic polymer did not interfere with the hydrophobic core or active site due to its ionic nature. This resulted in increased stability and activity of proteins. PEG, we believe due to its solubility in both organic and aqueous phases, resulted in loss in protein activity due to polymer interactions with the protein active site.

Additionally, we showed how glutamic acid (E) and lysine (K) residues can be used as a natural zwitterionic polymer, and could be used to genetically engineer an EK polymer-protein conjugate that mimicked pCBMA-protein conjugates with increased stability and maintained activity. This recombinant method provides a huge advantage over synthetic modification by avoiding costly post processing modification needed for synthetic polymer modifications.

Finally, we developed a new peptide screening method that was able to evaluate the performance of peptide surfaces for resistance to nonfouling. This new method accomplishes evaluating nonfouling peptides, which is not possible with traditional peptide screening methods. An additional method using combinatorial one-bead-one-compound (OBOC) screening and sequencing was developed to increase the number of peptide chemistries that could be tested to
thousands. This was accomplished by being able to sequence a peptide from a single controlled pore glass bead using partial Edman degradation along with mass spectrometry. As discussed in this dissertation, researchers have thus far needed to work around the limitations of PEG as an amphiphilic material. Zwitterionic polymers, both synthetic and natural peptide based, present an intriguing alternative that could potentially replace PEG in several needed areas in the years to come.
References

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**Curriculum Vitae**

Andrew J. Keefe was born in Westwood, MA. He received his Bachelor of Science in Chemical and Biological Engineering at Rensselaer Polytechnic Institute in Troy, NT, in 2007, and his Master’s in Chemical Engineering at University of Washington in Seattle, WA in 2009. In 2013, he plans to receive his Doctor of Philosophy in Chemical Engineering at the University of Washington.

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**Patents:**


**Invited Talks:**

Title: “Poly(zwitterionic)protein conjugates offer increased stability without sacrificing binding affinity or bioactivity” 9th World Biomaterials Conference - June 2012.

Title: “Zwitterionic Poly(carboxybetaine) Hydrogels for Glucose Biosensors in Complex Media” 9th World Biomaterials Conference - June 2012.

Title: “Poly(Zwitterionic) Protein Conjugates: Relieving PEGylation,” Chemical Engineering Graduate Sym.


**Posters:**


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2012 Trending Publication Award, Department of Chemical Engineering, University of Washington. December 2012.

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