5-2013

Dynamic Bioactive Stimuli-Responsive Polymeric Surfaces

Heather Marie Pearson

University of Southern Mississippi

Follow this and additional works at: https://aquila.usm.edu/dissertations

Part of the Polymer Chemistry Commons

Recommended Citation

https://aquila.usm.edu/dissertations/525

This Dissertation is brought to you for free and open access by The Aquila Digital Community. It has been accepted for inclusion in Dissertations by an authorized administrator of The Aquila Digital Community. For more information, please contact Joshua.Cromwell@usm.edu.
The University of Southern Mississippi

DYNAMIC BIOACTIVE STIMULI-RESPONSIVE POLYMERIC SURFACES

by

Heather Marie Pearson

A Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

May 2013
ABSTRACT

DYNAMIC BIOACTIVE STIMULI-RESPONSIVE POLYMERIC SURFACES

by Heather Marie Pearson

May 2013

This dissertation focuses on the design, synthesis, and development of antimicrobial and anticoagulant surfaces of polyethylene (PE), polypropylene (PP), and poly(tetrafluoroethylene) (PTFE) polymers. Aliphatic polymeric surfaces of PE and PP polymers functionalized using click chemistry reactions by the attachment of –COOH groups via microwave plasma reactions followed by functionalization with alkyne moieties. Azide containing ampicillin (AMP) was synthesized and subsequently clicked into the alkyne prepared PE and PP surfaces. Compared to non-functionalized PP and PE surfaces, the AMP clicked surfaces exhibited substantially enhanced antimicrobial activity against Staphylococcus aureus bacteria. To expand the biocompatibility of polymeric surface anticoagulant attributes, PE and PTFE surfaces were functionalized with pH-responsive poly(2-vinyl pyridine) (P2VP) and poly(acrylic acid) (PAA) polyelectrolyte tethers terminated with NH$_2$ and COOH groups. The goal of these studies was to develop switchable stimuli-responsive polymeric surfaces that interact with biological environments and display simultaneous antimicrobial and anticoagulant properties. Antimicrobial AMP was covalently attached to –COOH terminal ends of protected PAA, while anticoagulant heparin (HEP) was attached to terminal –NH$_2$ groups of P2VP. When pH < 2.3, the P2VP segments are protonated and extend, but for pH > 5.5, they collapse while the PAA segments extend. Such surfaces, when exposed to
*Staphylococcus aureus*, inhibit bacterial growth due to the presence of AMP, as well as are effective anticoagulants due to the presence of covalently attached HEP. Comparison of these “dynamic” pH responsive surfaces with “static” surfaces terminated with AMP entities show significant enhancement of longevity and surface activity against microbial film formation. The last portion of this dissertation focuses on the covalent attachment of living T1 and Φ11 bacteriophages (phages) on PE and PTFE surface. This was accomplished by carbodiimide coupling between –COOH groups on PE and PTFE surfaces and –NH$_2$ moieties present on T1 and Φ11 phages. These studies show that covalently attached T1 and Φ11 phages retain their antimicrobial activity manifested by the effective destruction of both Gram negative *Escherichia coli* (Φ11) phages and Gram positive *Staphylococcus aureus* bacteria (T1).
DYNAMIC BIOACTIVE STIMULI-RESPONSIVE POLYMERIC SURFACES

by

Heather Marie Pearson

A Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

Approved:

____________________________________
Director

____________________________________

____________________________________

____________________________________

____________________________________
Dean of the Graduate School

May 2013
ACKNOWLEDGMENTS

I would like to thank my research advisor, Dr. Marek W. Urban, for his encouragement and unwavering support throughout my graduate experience. His guidance and enthusiasm for research has been motivational and has allowed for me to expand my creative capacity, making my Ph.D. experience stimulating and productive. I appreciate his time, ideas, and insightful comments which have not only benefited my graduate research but will help me throughout my professional career. I would also like to thank him for acquiring the funding necessary to pursue my graduate degree in Polymer Science and Engineering.

Additionally, I would like to thank my graduate committee members Dr. William L. Jarrett, Dr. Sarah E. Morgan, Dr. Sergei I. Nazarenko, and Dr. Mohamed O. Elasri for their support and advice during these studies.

Special appreciation goes to the past and present members of the Urban Research Group for their friendship and helpful discussions. Financially, this work was primarily funded by the National Science Foundation and the National Institutes of Health.

Lastly, I would like to thank my parents, Danny and Judy Anderson, as well as my children, Jade and Lance Pearson. Without their continuous love and support, I would have never made it through graduate school.
# TABLE OF CONTENTS

ABSTRACT................................................................................................................................. ii

ACKNOWLEDGMENTS.............................................................................................................. iv

LIST OF TABLES....................................................................................................................... vii

LIST OF ILLUSTRATIONS....................................................................................................... viii

CHAPTER

I. INTRODUCTION................................................................................................................. 1

II. RECENT ADVANCES IN SURFACE MODIFICATIONS OF POLYMERS UTILIZED IN BIOLOGICAL ENVIRONMENTS........ 4

   Introduction
   Selected Polymer Surface Reactions
   Bioactivity of Modified Polymer Surfaces
   References

III. SIMPLE TWO-STEP CLICK REACTIONS ON ALIPHATIC POLYMER SURFACES LEADING TO ANTIMICROBIAL BEHAVIOR...................................................................................... 32

   Introduction
   Experimental
   Results and Discussion
   Conclusion
   References

IV. COVALENT ATTACHMENT OF MULTILAYERS (CAM) AS A PLATFORM FOR PH SWITCHABLE ANTIMICROBIAL AND ANTICOAGULANT POLYMERIC SURFACES........................................ 47

   Introduction
   Experimental
   Results and Discussion
   Conclusion
   References
V. PHAGE-BACTERIUM WAR ON POLYMERIC SURFACES; CAN SURFACE-ANCHORED BACTERIOPHAGES ELIMINATE MICROBIAL GROWTH

Introduction
Experimental
Results and Discussion
Conclusion
References

APPENDIXES
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Chemical species for preventing blood coagulation, fouling, and microbial</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>processes</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Schematic illustration of physical adsorption (non-covalent) and covalent attachment.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>(A) Schematic diagram of lbl structure on PVC surface. (B) Schematic diagram of biotinylated green fluorescent proteins deposited on SAMs through biotin-avidin recognition and fluorescent nanopatterns generated.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PDMS surface patterning by microwave plasma reactions using a masking technique to create distinct areas of functional surface groups to which the antibiotic amoxicillin is attached. Surfaces were analyzed by AFM and IR spectroscopy.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Schematic diagram of lbl PET film formation consisting of alternating layers of PAA and antimicrobial CTAB. Antimicrobial activity is shown by the zone of inhibition of bacterial growth.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Schematic of the attachment of antimicrobial species to a polymer surface via microwave plasma reactions.</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Inactivation of clotting enzymes by heparin; A: no binding of clotting enzyme to AT-III; B: binding of clotting enzymes to AT-III due to the presence of heparin; C: dissociation of heparin from the AT-III-clotting enzyme complex.</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Two modes of adsorption of a particle on a polymeric layer grafted to a substrate. A: primary adsorption in which proteins penetrate the polymer brush, and B: secondary adsorption at the brush-solvent interface.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Schematic diagram of ‘clickable’ polymeric surfaces exhibiting alkyne functionalities for ‘clicking’ any azide containing molecule.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Surface reactions leading to the formation of alkyne surface groups; (A) Microwave plasma reactions in the presence of MA; (B) and alkyne functionalization using propargylamine.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>(a) Reaction sequences on PE and PP substrates leading to AMP clicked surfaces; (b) ATR-FTIR spectra of A: PE, B: PE-MA, C: PE-MA-PPA, D: PE-MA-PPA-AMP; (c) ATR-FTIR spectra of A: PP, B: PP-MA, C: PP-MA-PPA, D: PE-MA-PPA-AMP.</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Raman spectra of A: PP; B: PP-MA; C: PP-MA-epoxide-PPA; D: PP-MA-DCC-PPA; and E: PP-MA-OC-PPA.</td>
<td></td>
</tr>
</tbody>
</table>
Antimicrobial activity against *S. aureus* of (a) PE (blue) and PP (red) surfaces; (b) PE-MA and PP-MA surfaces; (c) PE-MA-PPA and PP-MA-PPA surfaces; (d) PE-MA-PPA-AMP and PP-MA-PPA-AMP surfaces..................42

(A) Schematic diagram of dual stimuli-responsive polyelectrolyte surfaces terminated with –NH₂ and –COOH moieties; (B) Structures of (a) HEP and (b) AMP, with the moieties available for attachment circled.........................60

Reaction sequences on polymeric substrates that form stimuli-responsive polyelectrolyte surfaces terminated with –COOH and –NH₂ functionalities and exhibiting bioactive molecules............................................61

(a) Reaction sequences (circles represent reaction sites) on Si, PE, and PTFE surfaces leading to terminal –COOH groups; (b) ATR-FTIR spectra of A: Si, B: Si-MA, C: Si-MA-EDN, D: Si-MA-EDN-PrBA; (c) ATR-FTIR spectra of A: PE, B: PE-MA, C: PE-MA-EDN, D: PE-MA-EDN-PrBA; (d) ATR-FTIR spectra of A: PTFE, B: PTFE-MA, C: PTFE-MA-EDN, D: PTFE-MA-EDN-PrBA.................................................................62

(a) Reaction sequences (circles represent reaction sites) on Si, PE, and PTFE surfaces leading to terminal –NH₂ groups (circled); (b) ATR-FTIR spectra of A: Si, B: Si-MA, C: Si-MA-EDN, D: Si-MA-EDN-P2VP; (c) ATR-FTIR spectra of A: PE, B: PE-MA, C: PE-MA-EDN, D: PE-MA-EDN-P2VP; (d) ATR-FTIR spectra of A: PTFE, B: PTFE-MA, C: PTFE-MA-EDN, D: PTFE-MA-EDN-P2VP...........................................................................63

ATR-FTIR spectra of PTFE-MA-EDN-PPA/P2VP switching behavior observed at (A) pH 10, (B) pH 2, and (C) pH 4.9 (isoelectric point)..........................64


(a and a’) Chemical structures from reactions with AMP to PE-MA-EDN-PPA and PTFE-MA-EDN-PPA and HEP to PE-MA-EDN-P2VP and PTFE-MA-EDN-P2VP (circles represent reaction sites); (b, b’, c, and c’) ATR-FTIR spectra of PE-MA-EDN-PPA-AMP, PTFE-MA-EDN-PPA-AMP, PE-MA-EDN-P2VP-HEP, and PTFE-MA-EDN-P2VP-HEP........................................................................66

Antimicrobial activity against *S. aureus* of (a) PE (blue) and PTFE (red) surfaces; (b) PE-MA and PTFE-MA surfaces; (c) PE-MA-EDN-PPA and PTFE-MA-EDN-PPA surfaces; (d) PE-MA-EDN-PPA-AMP and PTFE-MA-EDN-PPA-AMP surfaces........................................................................67
Antimicrobial activity against *S. aureus* of PE-MA-EDN-PAA-AMP (blue) and PTFE-MA-EDN-PAA-AMP (red) surfaces (a, c, and e); PE-MA-PEG-AMP and PTFE-MA-PEG-AMP surfaces (c, d, and f) after first (1), second (2), and third (3) 4 hour exposure to *S. aureus*........................68

Raman images of the 1620 cm$^{-1}$ band before/after exposure to whole blood to (a and a’) PTFE; (b and b’) PTFE-MA; (c and c’) PTFE-P2VP; (d and d’) PTFE-P2VP-HEP; (e) blood reference. Each image represents a 10 x 10 µm area.....................................................69

Raman images of the 1620 cm$^{-1}$ band before/after exposure to whole blood to (a and a’) PE; (b and b’) PE-MA; (c and c’) PE-P2VP; (d and d’) PE-P2VP-HEP; (e) blood reference. Each image represents a 10 x 10 µm area........70

A: Covalent attachment of acid groups to polymeric surfaces; B: Reactions of NH$_2$ groups of T1 with polymer surface acid groups; C: Attachment of phages to bacteria and injection of DNA; D: Replication of DNA and destruction of bacteria.................................................................82

A: ATR-FTIR spectra of PE (a) and PTFE (b) surfaces; B: after plasma reactions on PE and PTFE surfaces in the presence of maleic anhydride; C: after T1 phage covalent attachment to MA modified surfaces; D: Reference spectrum of T1 phage (at 20% scale)..................................................83

A: ATR-FTIR spectra of PE (a) and PTFE (b) surfaces; B: after plasma reactions on PE and PTFE surfaces in the presence of maleic anhydride; C: after Φ11 phage covalent attachment to MA modified surface; D: Reference spectrum of Φ11 phage (at 20% scale)........................................84

(A): AFM height image of Si wafer surface; (B): Height image of Si-MA surface; (C): AFM height image of T1 bacteriophages attached to Si-MA surfaces; (D): Height image of Si-MA surfaces with covalently attached Φ11 phages..................................................85

Plaque formation assays for covalently attached T1 phages on A: PE and B: PTFE surfaces against *E. coli*; C: PE and D: PTFE with covalently attached 1:1 mixture of T1 and Φ11 phages against *E. coli*. Plaque formation assays for covalently attached Φ11 phages on E: PE and F: PTFE surfaces against *S. aureus*; G: PE and H: PTFE with covalently attached 1:1 mixture of T1 and Φ11 phages against *S. aureus*.........................86
CHAPTER I

INTRODUCTION

This dissertation focuses on the design, synthesis, and development of biocompatible polyethylene (PE), polypropylene (PP), and poly(tetrafluoroethylene) (PTFE) surfaces that dynamically respond to environmental changes. The goal was to create stimuli-responsive polymeric surfaces that interact with biological environments and display simultaneous antimicrobial and anticoagulant properties.

Chapter II reviews the recent advances of polymeric materials used for biomedical applications. Here the emphasis is on the surface reactions, especially those involving covalent attachment to inert polymeric surfaces. Advantages and disadvantages of numerous surface modifications are discussed as well as various methods for imparting antimicrobial, anticoagulant, and antifouling properties into polymeric surfaces.

Chapter III reports the development of stimuli-responsive model polymeric surfaces utilizing click chemistry. These studies show for the first time that inert PP and PE surfaces can be effectively functionalized with alkyne functional groups that participate in Copper(I)-catalyzed Azide-Alkyne Cycloaddition reactions to achieve surface modification with any desired molecule. Azide functional ampicillin was synthesized and subsequently “clicked” onto alkyne-containing PP and PE surfaces; these materials exhibited remarkable antimicrobial activity against S. aureus bacteria.

Chapter IV focuses on responsive and functional polyethylene (PE), poly(tetrafluoroethylene) (PTFE), and silicon (Si) surfaces. These were functionalized using the covalent attachment of multilayers (CAM) approach by tethering pH-responsive
“switching” polyelectrolytes consisting of poly(2-vinyl pyridine) (P2VP) and poly(acrylic acid) (PAA) chains terminated with NH$_2$ and COOH groups, respectively. These polymers have either collapsed or extended localized macromolecular domains depending on pH. At pH values < 2.3, the P2VP segments become protonated and will have an extended conformation. However, at pH values > 5.5, the PAA segments adopt an extended conformation, whereas the P2VP segments form a collapsed structure. In addition, ampicillin and heparin were attached to PE and PTFE surfaces using the NH$_2$ or COOH end groups of the polyelectrolyte surface tethers. These studies showed that surfaces containing terminal ampicillin groups exhibited antimicrobial properties as manifested by bacterial growth inhibition, whereas those modified with heparin showed anticoagulant behavior. The simultaneous presence of antibiotic and anticoagulant species facilitates both functions.

Chapter V describes the synthetic methods used to covalently attach T1 and Φ11 bacteriophages (phages) to inert polymeric surfaces without affecting their ability to kill bacteria. The first step involved formation of acid (COOH) groups on polyethylene (PE) and poly(tetrafluoroethylene) (PTFE) surfaces using microwave plasma reactions in the presence of maleic anhydride. This is followed by covalent attachment of T1 and Φ11 species via primary amine groups. Retention of the phages biological activity was confirmed by the modified surfaces ability to kill *Escherichia coli* and *Staphylococcus aureus* human pathogens. These investigations showed that simultaneous covalent attachment of two biologically active phages effectively destroyed both *E. coli* and *S. aureus* bacterial colonies and eliminated biofilm formation, thus offering an opportunity
for an effective combat against multi-bacterial colonies as well as surface detection of other pathogens.
CHAPTER II
RECENT ADVANCES IN SURFACE MODIFICATIONS OF POLYMERS UTILIZED IN BIOLOGICAL ENVIRONMENTS

Introduction

Polymers having tunable physical and chemical properties offer a number of unique applications when utilized in biological systems. Notable uses range from medical implants,\textsuperscript{1-3} microarrays,\textsuperscript{4} biosensors and bio-actuators\textsuperscript{5} to tissue engineering,\textsuperscript{6-8} and gene and drug delivery systems.\textsuperscript{7,9-11} Inertness, mechanical strength, and biocompatibility are attributes that allow polymeric materials to be successfully integrated into biological systems without adverse effects. Polymers often used in biomedical applications include poly(tetrafluoroethylene) (PTFE), poly(ethylene terephthalate) (PET), ultra-high molecular weight poly(ethylene) (UHMWPE), poly(polypropylene) (PP), polyether ether ketone (PEEK), poly(methylmethacrylate) (PMMA), poly(pyrrrole) (PPy), polythiophene (PT), poly(lactide-co-glycolide) (PLGA), poly(N-isopropylacrylamide) (PNIPAAm), poly-L-lactic acid (PLLA), poly(N,N'-(dimethylamino)ethyl methacrylate) (pDMAEMA), poly(ethylene glycol) (PEG), poly(ε-caprolactone) (PCL), poly(acrylic acid) (PAA), poly(allylamine) (PAH), and polyaniline (PANI). There are others, each offering specific and unique attributes pertinent to their functions.

Although these materials provide specific functionalities, their mechanical strength, low toxicity, and inertness are also essential attributes in biological environments. For example, chemically and thermally resistant non-toxic polymers, such as PTFE and PET, are utilized in implant vascular grafts.\textsuperscript{1,4} UHMWPE, a self-
lubricating polymer with low moisture adsorption, is used in hip and knee replacements as well as artificial joint implants,\(^1,3\) which require high impact strength, low frictional coefficient, and resistance to abrasion. Due to its good physical strength and excellent impact resistance, PP\(^4\) is often used for heart valves,\(^1\) hemodialysis membranes and mesh for hernia repairs. PEEK’s mechanical strength and biocompatibility makes it useful in long-term medical implant devices.\(^1\) In contrast, PMMA’s transparency and UV resistance, allow its use in intraocular implants, bone cement, and dentures,\(^1,4\) as well as oligonucleotide microarrays,\(^4\) which facilitate the immobilization of DNA. Biosensors capable of sensing biomolecules such as DNA or glucose, require PPy and PT integrated with electrical transducers.\(^5\) Other types of bio-applicable polymers are represented by biodegradable polymers such as PLGA, PLLA, and PCL.\(^2,6,7,9\) These materials are used in sutures, stents, and scaffolding materials for tissue engineering; here biodegradation kinetics, good mechanical properties, low immunogenicity, and non-toxicity are essential properties.\(^12,13\) Stimuli-responsive polymers, such as PNIPAAm, PAA, and PAH play a crucial role in drug delivery systems.\(^7,10\) Their ability to shrink or expand at specific pH values or temperatures, allow them to controllably release their drug cargo. Other stimuli-responsive polymers, such as cationic pDMAEMA, is reportedly an effective transfection agent for gene delivery,\(^9\) whereas PEG is widely utilized to enhance biocompatibility due to its antifouling properties and ability prolong resident time within the body.\(^7\) The growing field of artificial muscle actuators\(^5\) requires conductive polymers such as PPy and PANI; here mechanical force is generated by the application of electrical current.
This comprehensive summary clearly shows that polymeric materials are highly attractive to biomedical researchers. However, many of these materials, when in contact with biological systems, can become susceptible to microbial growth and undesirable protein adsorption. Although the primary driving force for these phenomena is the low surface energy of polymer surfaces, morphological properties also play a role. This dissertation will focus on the control of morphology by chemical modification which in turn affects interactions with biological systems. In order to circumvent these biological interactions while maintaining useful bulk characteristics, polymer surfaces can be physically or chemically altered to exhibit antimicrobial, anticoagulant, as well as pH sensitive properties.

Modification of polymeric surfaces to create biocompatible as well as bioactive materials has been explored utilizing an array of approaches ranging from physisorption to covalent attachment. Numerous studies involving the attachment of biologically active species having antimicrobial\textsuperscript{14-16} and anticoagulant\textsuperscript{14} properties have been investigated for application in various bio-medical areas.\textsuperscript{17-20} Reactions for modifying polymer surfaces range from simply adding bioactive molecules to a polymer matrix during processing\textsuperscript{18, 19} or non-covalent physisorption using layer-by-layer deposition,\textsuperscript{11, 21, 22} and self-assembled monolayers (SAMs),\textsuperscript{20, 23, 24} to covalent bonding using grafting-to\textsuperscript{18, 25} and grafting-from\textsuperscript{18, 19, 26} methods. The latter offers greater control over surface chemistry and morphologies;\textsuperscript{14} for example, chemical spacers can be used to impart mobility and accessibility to the bioactive species of interest. Their surface modifications are critical, because polymers in contact with biological systems potentially become susceptible to microbial growth and undesirable protein adsorption.\textsuperscript{14} The adhesion strength (A) of
biologically active species to polymer surfaces is driven by the surface energy (γ) and polymer matrix modulus (E), with this relationship expressed as $A = (\gamma E)^{1/2}$. In order to circumvent these interactions with bioorganisms, while maintaining useful bulk characteristics, the surface energy of the polymer surface must be decreased.

Selected Polymer Surface Reactions

Approaches to immobilize bioactive molecules on polymeric surfaces by either non-covalent or covalent bonding are of significant interest. Non-covalent bonding methods include physisorption, electrostatic interactions, and ligand-receptor recognition, whereas covalent bonding is achieved by chemical reactions of functional groups for form stable surface entities. Figure 1 illustrates physical adsorption and covalent attachment methods to immobilize molecules onto surfaces.

Physisorption of bioactive molecules onto polymer surfaces is the simplest method for temporarily altering biopolymer surface properties. For example, gentamicin is used to soak PP mesh implants before surgery in order to prevent infections. Similarly, by combining mammalian cells with polymers such as PLA, it is possible to create new skin for burn patients.

The layer-by-layer (lbl) approach takes advantage of electrostatic interactions by dipping of a charged substrate into dilute aqueous solutions of polyelectrolytes with opposite charges, thus allowing adsorption of alternating charges to the substrate. Multilayer films are obtained by sequential adsorptions of anionic and cationic polyelectrolytes, resulting in heterogeneous surfaces with limited stability. An example of a lbl surface modification is multilayer thromboresistant thin films containing
poly(ethylenimine) (PEI), dextran sulfate (DS), and heparin (HP) deposited on poly (vinyl chloride) (PVC) surfaces, depicted in Figure 2-A.\textsuperscript{22}

In addition to electrostatic interactions, ligand-receptor recognitions such as biotin-streptavidin, the strongest reported non-covalent bond (disassociation constant $K_d$ of $10^{-15}$ M$^{31}$), offers an alternative for specific attachment. Streptavidin exhibits a specific affinity for biotin molecules, with its four receptor sites creating a specific orientation of the immobilized species onto surfaces.\textsuperscript{31} Figure 2-B illustrates the deposition process of biotinylated green fluorescent proteins on SAMs via streptavidin-biotinylated ligands to generate surface nanopatterns.\textsuperscript{24} Although non-covalent bonding can effectively bind bio-molecules to polymer surfaces, covalent attachment is utilized to achieve significantly more stable surfaces.

The first step to covalently modify polymer surfaces is the generation of a surface reactive species whereby bio-relevant molecules can be anchored. For example, the reaction of maleic anhydride forms carboxylic acid groups, which followed by reactions with a spacer molecule\textsuperscript{16} provides mobility to the attached species of interest. Well-studied chemical reactions for surface attachment via linkages between functional groups include esterification,\textsuperscript{16} amidation,\textsuperscript{32} and etherification.\textsuperscript{33}

UV photografting using photoinitiators and photosensitizers can also affix bioactive molecules to polymer surfaces.\textsuperscript{34} This approach has been used to fabricate DNA oligonucleotide arrays on PMMA via UV treatment to generate carboxylic acid functionality.\textsuperscript{4, 35} In addition to photografting, surface initiated polymerization methods involving reversible-addition fragmentation chain transfer (RAFT) and atom transfer radical polymerization (ATRP) have gained popularity due to their ability to provide
controllable chain lengths as well as higher grafting density.\textsuperscript{25, 36, 37} Examples include RAFT surface grafting polymerization of pDMAEMA onto cellulose fiber surfaces to incorporate antimicrobial quaternary ammonium groups.\textsuperscript{25} RAFT polymerization was also utilized to synthesize cell mimicking polymer brushes on the surface of polysulfone.\textsuperscript{37} Additionally, ATRP based surface initiated polymerization to graft poly(poly(ethylene glycol) methyl ether monomethacrylate) (PPEGMA) to poly(dimethyl siloxane) (PDMS) gave the modified surface nonfouling properties.\textsuperscript{36}

Solvent based chemical functionalization can modify polymer surfaces using liquid reagents to create reactive groups on polymer surfaces via aminolysis, alkaline and acidic hydrolysis, and hydrogen peroxide reactions.\textsuperscript{4, 34} Examples of this approach include submerging PE into an aqueous solution of chromium trioxide and sulfuric acid to generate COOH groups,\textsuperscript{38} and introducing oxygen-containing moieties on PE and PP using a mixture of chromic acid, potassium permanganate, and sulfuric acid.\textsuperscript{4} These methods offer an easy approach to modify polymer surfaces; however they produce toxic chemical waste, are non-specific in introducing functionalization, and may cause etching of the surface.\textsuperscript{34}

Other solvent based functionalization methods include the click chemistries developed by Sharpless \textit{et al.},\textsuperscript{39} which are relatively fast, high yielding reactions performed under mild conditions.\textsuperscript{40} Examples of click methods include Diels-Alder reactions, nucleophilic substitution, thiol-yne reactions, and Cu(I)-catalyzed azide-alkyne cycloaddition.\textsuperscript{41} These reactions have been utilized to functionalize polymeric micelles and vesicles.\textsuperscript{42} However, their use on polymeric substrates has not been explored, because the surfaces must contain moieties necessary for click reactions to occur.
High energy radiation is an effective means to modify biopolymer surfaces.\textsuperscript{34} These include electron beam radiation\textsuperscript{43} and radio or microwave plasma reactions.\textsuperscript{44, 45} Microwave plasma offers a clean, fast, and solventless route to surface functionalization without affecting bulk properties. Plasma reactions require vacuum conditions with low concentrations of gases such as N\textsubscript{2}, O\textsubscript{2}, CO\textsubscript{2}, He, or Ar present. These are ionized by the microwave radiation, allowing them to create reactive groups in the presence of a desirable monomer. This provides controllable chemical conditions for covalently attaching desirable chemical entities within 0.5-10 seconds.\textsuperscript{44, 46} Glow discharge plasma is created in which the gases are ionized to generate highly reactive species within the reactor.\textsuperscript{34} These reactive species chemically and physically alter the polymer surface at depths ranging from several angstroms to microns,\textsuperscript{34} depending on the energy input and exposure time. The first step is to attach an anchor molecule, such as maleic anhydride, followed by addition of a spacer to provide mobility to the desired species attached to the other end of the spacer. Previous studies employing plasma reactions to modify biopolymer surfaces include patterning of PDMS followed by ammonolysis with amoxicillin to form antimicrobial surfaces.\textsuperscript{15, 47-49} Patterning is achieved by masking PDMS substrates during microwave plasma reaction; Figure 3\textsuperscript{15} clearly shows the masked areas of the substrate are not chemically altered.

Many biopolymer materials require surface modifications for enhanced antifouling, anticoagulant and antimicrobial properties. Prevention of undesirable protein adsorption, blood coagulation, or bacterial biofilm formation is critical in many applications. Thus, these attributes are necessary for polymer substrates with biological environments.
Bioactivity of Modified Polymer Surfaces

Polymers, when in contact with biological systems, are potentially susceptible to microbial growth and undesirable protein adsorption due to their low surface energies. Microbial growth, thrombosis, and protein absorption are biological events that must be minimized or eliminated for a polymer surface to function in biological environments. Several specific strategies have been implemented to prevent these problems, with the modification of polymer surfaces playing an essential role in enhancing their antimicrobial, anticoagulant and antifouling properties. This is especially significant considering that each year the number of deaths resulting from infection continues to increase, with approximately 64% of infections acquired at hospitals being attributed to the attachment of bacteria to medical devices and implants. Many of these infections could be avoided by introducing antimicrobial agents onto the surfaces of medical devices and implants to prevent bacterial attachment and biofilm formation. In addition, surface reactions leading to anticoagulant properties will minimize or eliminate thrombosis. The summary of chemicals exhibiting anticoagulant, antifouling, and antimicrobial properties is shown in Table 1. The majority of these chemicals are utilized in a liquid phase. Therefore, the challenge is to anchor these materials onto polymeric surfaces in such a manner that retains their anticoagulant, antifouling, and antimicrobial properties.

Antimicrobial Surfaces

Several methods have been employed in an attempt to introduce antimicrobial agents into biomaterials to prevent biofilm formation. One approach is adding antimicrobial agents into the polymer during processing, with their release controlled by
diffusion from a polymer matrix. Antimicrobial agents used in this manner include quaternary ammonium (or phosphonium) salts (QAS), chitosan, antimicrobial peptides (AMPs), silver ions (Ag\(^+\)), bacteriophages and antibiotics. Unfortunately these materials exhibit different and for the most part unknown mechanisms of bacterial growth inhibition. Whereas metals displace essential Ca\(^{2+}\) and Zn\(^+\) ions in bacteria, quaternary ammonium salts and chitosan bind to negatively charged bacterial surfaces, causing leakage of intracellular components. The attachment of poly(quaternary ammonium) to PP surfaces using photochemical synthesis and ATRP successfully inhibited *S. aureus* and *E. coli* bacterial growth. Here the molecular weight and the density of the QA was controlled. An example of adding antimicrobial QAS on surfaces is illustrated in Figure 4. This technique involves the lbl assembly of alternating anionic poly(acrylic acid) (PAA) and cationic cetyltrimethylammonium bromide (CTAB) layers. The antimicrobial activity is due to the diffusion of the CTAB moiety to the surface of the assembly. The covalent attachment of poly(vinyl-N-hexylpyridinium) onto HDPE and PET surfaces also show bactericidal activity against *S. aureus* and *E. coli*. In contrast, Ag\(^+\) ions bind to electron donor groups containing sulfur, oxygen, or nitrogen, present in biological molecules as thio, amino, imidazole, carboxylate and phosphate groups. By displacing other ions, such as Ca\(^{2+}\) and Zn\(^+\), Ag\(^+\) effectively interrupts a number of cellular transport and oxidation processes. For that reason, Ag\(^+\) ions have been incorporated onto PET films by lbl surface modification using PEI and PAA. These PET films exhibit biocidal activity against *S. aureus* and *E. coli*. 
Aminoglycoside and beta-lactam-based antibiotics have different modes of action for antimicrobial activity. Aminoglycosides, such as gentamicin, kanamycin, and streptomycin, prevent bacterial protein synthesis by entering the bacteria and binding to ribosomes within the cell; beta-lactams, such as penicillin (PEN), amoxicillin, and ampicillin (AMP), inhibit bacterial cell wall formation. PEN\textsuperscript{16,57} and AMP\textsuperscript{58} were successfully attached to ePTFE surfaces via microwave plasma reactions and grafting to carboxylic acid groups. These materials showed efficient inhibition of both Gram positive and Gram negative bacteria. Microwave plasma reactions and subsequent attachment of antibiotics are illustrated in Figure 5.

An almost entirely unexplored area of inhibition of bacterial growth is the use of bacteriophages. These living bacterial viruses are capable of selectively binding to specific receptors of the target bacteria. Upon binding, they inject their DNA, which reproduces inside the bacteria, thereby killing the host, and releasing the phage progeny.\textsuperscript{60} The use of these species has been only sporadically explored.\textsuperscript{54,61,62} Although control of their progeny is critical, under controllable conditions bacteriophages potentially offer an alternative and powerful approach for inhibiting bacterial infections.

**Anticoagulant Surfaces**

Due to their low surface energies, most polymeric materials exhibit highly thrombogenic surfaces and consequently can adsorb fibrin, thrombus, or other proteins, resulting in clot formation.\textsuperscript{19} Thrombosis, or blood clotting within minor wounds, is necessary for hemostasis to allow healing. After serving its purpose, the clot is dissolved through a process called fibrinolysis.\textsuperscript{63,64} Naturally occurring hirudin and synthetically produced bivalirudin peptides prevent thrombosis.\textsuperscript{19} Covalent immobilization of peptides
onto polymer surfaces such as PET\textsuperscript{65} and PLGA\textsuperscript{66} have been widely studied due to their ability to inhibit thrombus formation. Another anticoagulant species, heparin (HEP), a linear polysaccharide consisting of uronic acid-(1,4)-D-glucosamine repeating disaccharide subunits,\textsuperscript{67} binds to antithrombin III (AT-III), a thrombin inhibitor.\textsuperscript{67,68} As illustrated in Figure 6, the HEP molecule dissociates from the complex and can be reutilized.\textsuperscript{68} Several efforts to develop hemocompatible devices such as dialysis membranes, catheters, coronary stents, and vascular grafts by immobilizing heparin onto polymer surfaces such as PET,\textsuperscript{69} PTFE,\textsuperscript{69,70} and PU\textsuperscript{71} using lbl surface modification have been made in order to prevent blood coagulation in variety of biomedical applications.

Acetyl salicylic acid (aspirin) and dipyridamole are also important antithrombosis agents. Their anticoagulant activities involve inactivation of clotting enzymes which facilitate thrombus formation. Aspirin has been incorporated into polymer matrices such as PLCA\textsuperscript{72} and PVA\textsuperscript{73} to enhance blood compatibility. Similarly, dipyridamole has been covalently attached onto PUR surfaces via photomodification.\textsuperscript{74} Minimization of biofouling and blood coagulation at the surface by surface modification greatly enhances the biocompatibility of polymeric materials. Selected chemicals having anticoagulant properties are listed in Table 1.

\textit{Antifouling Surfaces}

The adsorption of proteins onto a polymer substrate in contact with biological environments is detrimental to biocompatibility, because the adsorbed proteins may initiate platelet adhesion and activation.\textsuperscript{75,76} Protein adsorption is a complex process involving electrostatic interactions between the surface and protein, protein concentration levels, and surface energy.\textsuperscript{34} There are two recognized modes of interaction involved in
protein adsorption onto the surface, depicted in Figure 7. Primary adsorption (A) occurs at the polymer surface-brush interface and occurs when small proteins are able to penetrate through the polymer brush and adsorb to the substrate surface. Secondary adsorption (B) is due to larger proteins being attracted by van der Waals interactions at the solvent-brush interface.

The adhesion strength of bio-molecules onto polymer surfaces is a function of surface energy and polymer modulus; thus attaching hydrophilic PEG to a low surface energy polymer substrate will increase resistance to biofouling by providing a high activation barrier for protein adsorption as well as steric repulsion, also important for protein resistance. PEG-functionalized polymer brushes synthesized by surface-initiated ATRP have been extensively investigated; these surfaces exhibited significant resistance to protein adsorption as well as cell adhesion. In contrast, elastomeric polymers such as PDMS have low modulii, but high surface energies resulting in increased protein adhesion strength. To circumvent these problems, several polymer architectures and surface modification techniques involving PDMS have been explored. For example, zwitterionic phosphorylcholine polymers that resemble the structure of natural membrane lipids have been grafted onto PDMS via photo-induced polymerization, greatly improving surface hydrophilicity and antifouling properties. Several zwitterionic polymers including phosphorylcholine, polybetaine, carboxybetaine, and sulfobetaine, serve as antifouling polymers due to their hydrophilicity as well as electroneutrality. These are shown in Table 1.

In summary, significant advances have been made in the surface modification of commodity polymers to improve their antimicrobial, anticoagulant, and antifouling
properties. Nevertheless, there are major unresolved scientific and technological problems associated with the safe use of polymers in biological environments. Understanding surface modification of polymers and developing reproducible technologies are timely due to devastating effects of microbial infections and thrombosis. These undesirable events can be minimized or eliminated if covalent attachment of desirable bioactive species to polymeric surfaces is successful. This dissertation presents a platform for surface reactions that successfully eliminate bacterial film growth and thrombosis.
Figure 1. Schematic illustration of physical adsorption (non-covalent) and covalent attachment.

Figure 2. (A) Schematic diagram of lbl structure on PVC surface. (B) Schematic diagram of biotinylated green fluorescent proteins deposited on SAMs through biotin-avidin recognition and fluorescent nanopatterns generated.
Attachment of Amoxicillin to Patterned Microwave Plasma PDMS Surfaces

Figure 3. PDMS surface patterning by microwave plasma reactions using a masking technique to create distinct areas of functional surface groups to which the antibiotic amoxicillin is attached. Surfaces were analyzed by AFM and IRIRI spectroscopy.15
Table 1

*Chemical species for preventing blood coagulation, fouling, and microbial processes*¹⁴

<table>
<thead>
<tr>
<th>Anticoagulant</th>
<th>Chemical Structure</th>
<th>Antifouling</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warfarin</td>
<td>![Chemical Structure Image]</td>
<td>Poly(oligo (ethylene glycol) methyl ether methacrylate)⁷⁹</td>
<td>![Chemical Structure Image]</td>
</tr>
<tr>
<td>Heparin</td>
<td>![Chemical Structure Image]</td>
<td>Zwitterionic polymer</td>
<td>![Chemical Structure Image]</td>
</tr>
<tr>
<td>Hirudin</td>
<td>![Chemical Structure Image]</td>
<td>Phosphoryl-choline (PC)</td>
<td>![Chemical Structure Image]</td>
</tr>
<tr>
<td>Argatroban</td>
<td>![Chemical Structure Image]</td>
<td>Sulfobetaine (SB) Carboxybetaine (CB)</td>
<td>![Chemical Structure Image]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antifouling</th>
<th>Chemical Structure</th>
<th>Antimicrobial</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorothalonil</td>
<td>![Chemical Structure Image]</td>
<td>Metals: Silver ion, Tin, Mercury</td>
<td>![Chemical Structure Image]</td>
</tr>
<tr>
<td>Diuron</td>
<td>![Chemical Structure Image]</td>
<td>Quaternary ammonium</td>
<td>![Chemical Structure Image]</td>
</tr>
<tr>
<td>Dichlofluanid</td>
<td>![Chemical Structure Image]</td>
<td>Beta-lactams Penicillin</td>
<td>![Chemical Structure Image]</td>
</tr>
<tr>
<td>Phosphoryl-choline-PDMS³⁴</td>
<td>![Chemical Structure Image]</td>
<td>Ampicillin</td>
<td>![Chemical Structure Image]</td>
</tr>
<tr>
<td></td>
<td>![Chemical Structure Image]</td>
<td>Aminoglycosides Gentamicin</td>
<td>![Chemical Structure Image]</td>
</tr>
<tr>
<td></td>
<td>![Chemical Structure Image]</td>
<td>Streptomycin</td>
<td>![Chemical Structure Image]</td>
</tr>
<tr>
<td></td>
<td>![Chemical Structure Image]</td>
<td>Chitosan</td>
<td>![Chemical Structure Image]</td>
</tr>
</tbody>
</table>
Figure 4. Schematic diagram of lbl PET film formation consisting of alternating layers of PAA and antimicrobial CTAB. Antimicrobial activity is shown by the zone of inhibition of bacterial growth.\(^{28}\)

Figure 5. Schematic of the attachment of antimicrobial species to a polymer surface via microwave plasma reactions.\(^{14}\)
Figure 6. Inactivation of clotting enzymes by heparin;\(^{68}\) A: no binding of clotting enzyme to AT-III; B: binding of clotting enzymes to AT-III due to the presence of heparin; C: dissociation of heparin from the AT-III-clotting enzyme complex.

Figure 7. Two modes of adsorption of a particle on a polymeric layer grafted to a substrate. A: primary adsorption in which proteins penetrate the polymer brush, and B: secondary adsorption at the brush-solvent interface.\(^{77}\)
REFERENCES


21. Bae, W. S.; Convertine, A. J.; McCormick, C. L.; Urban, M. W., Effect of
sequential layer-by-layer surface modifications on the surface energy of plasma-modified

22. Kim, H.; Urban, M. W., Reactions of thrombresistant multilayered thin films on


Ohdomari, I., Hybridization of deoxyribonucleic acid and immobilization of green
fluorescent protein on nanostructured organosilane templates. *Japanese Journal of


26. Lee, S. B.; Koepsel, R. R.; Morley, S. W.; Matyjaszewski, K.; Sun, Y.; Russell,
A. J., Permanent, nonleaching antibacterial surface. 1. synthesis by atom transfer radical

27. Brady, R. F.; Singer, I. L., Mechanical factors favoring release from fouling

28. Dvoracek, C. M.; Sukhonosova, G.; Benedik, M. J.; Grunlan, J. C., Antimicrobial

29. Lichter, J. A.; Vliet, K. J. V.; Rubner, M. F., Design of antibacterial surfaces and
interfaces: polyelectrolyte multilayers as a multifunctional platform. *Macromolecules*
**2009**, *42*, 8573.


37. Ma, Q.; Zhang, H.; Zhao, J.; Gong, Y.-K., Fabrication of cell outer membrane mimetic polymer brush on polysulfone surface via RAFT technique. *Applied Surface


45. Gaboury, S. R.; Urban, M. W., Microwave plasma reactions of solid monomers with silicone elastomer surfaces: a spectroscopic study


453.


56. Huang, J.; Murata, H.; Koepsel, R. R.; Russell, A. J.; Matyjaszewski, K., 

Antibacterial polypropylene via surface-initiated atom transfer radical polymerization. 


57. Aumsuwan, N.; Heinhorst, S.; Urban, M. W., The effectiveness of antibiotic activity of penicillin attached to expanded poly(tetrafluoroethylene) (ePTFE) surfaces: A 


62. Yang, L.-M. C.; Tam, P. Y.; Murray, B. J.; McIntire, T. M.; Overstreet, C. M.; Weiss, G. A.; Penner, R. M., Virus electrodes for universal biodetection
Analytical Chemistry 2006, 78, 3265.


CHAPTER III

SIMPLE TWO-STEP CLICK REACTIONS ON ALIPHATIC POLYMER SURFACES LEADING TO ANTIMICROBIAL BEHAVIOR

Introduction

Polymer surfaces, particularly aliphatic polyethylene (PE) and polypropylene (PP), in contact with biological systems are susceptible to adverse macroscopic processes among which antimicrobial film formation represents a serious problem. Simple devices, such as catheters, tubing, or other devices can potentially lead to infections, which account for over 100,000 deaths annually in the U.S. alone. One approach to alleviate this situation is to chemically modify surfaces of commodity polymers in a manner that makes them safe in biological environments and also prevents deadly infections. Although non-covalent and covalent surface modifications have been utilized to attain desirable surface properties while maintaining bulk attributes, the majority of physico-chemical approaches, such as layer-by-layer deposition,\textsuperscript{1} chemical etching,\textsuperscript{2} or radiation grafting,\textsuperscript{3} offer relatively limited antimicrobial effectiveness. In the layer-by-layer approach, multiple layers are deposited onto surfaces by dipping substrates in different polyelectrolyte solutions, with the multi-layers held together by opposite electrostatic charges. However, the resulting films are mechanically unstable and often exhibit chemically heterogeneous surfaces.\textsuperscript{4} Chemical etching alters the surface’s hydrophobicity via oxidation and morphology changes; radiation grafting uses sources such as infrared, visible light, ultraviolet, and $\gamma$ radiation as well as high energy electrons to generate reactive groups on surfaces in the presence of monomer to graft species to or from the surface.\textsuperscript{2,3} In contrast, microwave plasma surface reactions are a fast,
solventless, and sterile method for covalently attaching –COOH groups to almost any polymer substrate. Here, excited ionized gas is utilized to create reactive groups in the presence of a desirable monomer, thus providing controllable conditions for covalently attaching desirable chemical entities. Typical reaction times are 0.5 – 10 seconds. When maleic anhydride is used as a monomer, resulting surface modifications are the formation of –COOH groups covalently attached to a polymer backbone.

For aliphatic polymer substrates, such as PE and PP, the presence of –COOH surface groups provides an opportunity for further reactions. Although previous studies demonstrated that various surface entities can be covalently attached, the reaction yields decreased as more layers were added. Click chemistry is a highly efficient (>95%) synthetic route that achieves high reaction yields in relatively short times and under mild conditions. Reactions that meet “click” chemistry criteria but are not limited to, include Huisgen 1,3-dipolar cycloaddition, Diels-Alder reactions, nucleophilic substitution with epoxy and aziridine compounds, Sharpless dihydroxylation, and thiol-yne reactions. Most notably, the Huisgen 1,3-dipolar cycloaddition involves a copper(I)-catalyzed 1,2,3-triazole formation from azides and alkyne functionalities with complete specificity of reactants. In terms of surface reactions, cycloaddition click reactions yielding high efficiencies with Cu, Au, Si, glass, and even carbon nanotube surfaces have shown promising results, such as the biofunctionalization of Si surfaces with “clicked” biotin and glucose were successful. These unique attributes of click chemistry also broke new grounds for selective reactions with complex dendrimers, hydrogels, vesicles, and nanoparticles. In this work, both microwave plasma reactions and click chemistry approaches will be combined to create antimicrobial PE and PP surfaces.
Although click chemistry has been utilized in a variety of model studies, their practicality has been limited by their inability to react to inert polymeric surfaces. In view of previous studies utilizing microwave plasma reactions to functionalize aliphatic polymer surfaces with −COOH groups, this study outlines a series of simple and clean surface reactions that can modify almost any polymer surface without adversely affecting polymer bulk properties. Figure 8 schematically depicts a sequence of surface reactions using two simple steps: microwave plasma reactions to form −COOH groups, followed by covalent attachment of alkyne moieties to create polymeric surfaces whereby any azide containing molecule may be “clicked.” Selected examples include peptides utilized in stem cell adhesion, fluorophores for labeling hydrogels, polyhedral oligomeric silsequioxane (POSS), biotin, bacteriophages, or DNA. In these studies the focus is on the attachment of antibiotics, such as ampicillin (AMP) which exhibits antimicrobial behavior.

Experimental

All reagents were purchased from Sigma-Aldrich and used as received unless otherwise specified. Medical grade ultra-high molecular weight polyethylene (PE) and polypropylene (PP) were purchased from McMaster-Carr Supply Co. (Atlanta, GA), cut into 1 x 1 cm squares, washed in isopropanol, and dried at room temperature in a desiccator before use. To obtain −COOH terminated PE and PP surfaces, microwave plasma reactions were conducted in the presence of maleic anhydride (MA) under open reactor conditions, as described elsewhere. Several approaches were used in the next step to determine the optimal method of alkyne functionalization. Initially, PP with −COOH surface functionalities were placed with 0.1 mmol diglycidyl ether polyethylene
glycol (PEG) ($M_n = 526$) and 1 – 2 drops Et$_3$N in 10 ml DMF and stirred at 50 °C for 24 h. Samples were rinsed in DMF followed by immersion into 5 ml anhydrous DMF with 1 g propargylamine. One drop of Et$_3$N was added to the solution which was then stirred under N$_2$ atmosphere for 120 h. The surfaces were rinsed in DMF followed by washing with DI water and stored in a desiccator. Additional PP surfaces exhibiting –COOH groups were reacted with propargylamine via carbodiimde coupling chemistry by dissolving 1.3 mM dicyclohexyl-carbodiimide (DCC) coupling agent and 0.25 mM 4-(dimethylamino)-pyridine (DMAP) catalyst in 20 mL methylene chloride and stirred for 16 h. The samples were then removed and placed into 5 ml anhydrous 2-propanol (IPA) with 1 g propargylamine. One drop of Et$_3$N was added to the solution and stirred under N$_2$ atmosphere for 120 h. The surfaces were rinsed in IPA followed by washing with DI water and stored in a desiccator. Similarly, PE and PP surfaces containing –COOH groups were placed in neat oxalyl chloride for 2 h to create –COCl groups and rinsed in chloroform before immediate immersion into 5 ml anhydrous IPA with 1 g propargylamine. One drop of Et$_3$N was added to the solution and stirred under N$_2$ atmosphere for 120 h. The surfaces were rinsed in IPA followed by washing with DI water and stored in a desiccator.

*Ampicillin Azide Synthesis*

AMP-PEG-N$_3$ (Sigma-Aldrich) was prepared via carbodiimde coupling chemistry by dissolving 1.3 mM 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDAC) coupling agent, 0.25 mM N-hydroxysuccinimide (NHS) catalyst, and 0.15 g of ampicillin (AMP) in 1.5 mL DI water, then mixing the solution with 0.10 g O-(2-aminoethyl)-O'-(2-azidoethyl)pentaethylene glycol (NH$_2$-PEG$_6$-N$_3$). The solution was stirred at room
temperature for 48 h, at which time an aliquot was taken for ATR FT-IR analysis to confirm the formation of AMP-PEG-N\textsubscript{3}.

**Click Reactions of AMP on PE/PP Surfaces**

Aqueous click reactions were carried out using methods outlined elsewhere\textsuperscript{30} between alkyne functionalized PP and PE surfaces and AMP-PEG-N\textsubscript{3}. PE and PP surfaces previously reacted with propargylamine were added to 0.5 mmol AMP-PEG-N\textsubscript{3} and 0.05 mmol sodium ascorbate in 2 mL of DI water. 0.01 mmol of CuSO\textsubscript{4} was added to the mixture and stirred at room temperature for 18 h. The surfaces were then washed twice in DI water and stored in a desiccator prior to use.

**Surface Characterization**

Attenuated total reflectance Fourier transform infrared (ATR FT-IR) spectra were acquired using a Bio-Rad FTS-6000 FT-IR single-beam spectrometer set at a 4 cm\textsuperscript{-1} resolution, equipped with DTGS detector and a 45º face angle Ge crystal. Each spectrum represents 200 co-added scans ratioed against a reference spectrum obtained by recording 200 co-added scans of an empty ATR cell. All spectra were corrected spectral distortions using Q-ATR software.\textsuperscript{31}

Raman spectra were obtained using a Renishaw Raman microscope-spectrometer equipped with a computer controlled three-axis encoded motorized stage, a RenCam CCD detector, and a Leica microscope (DMLM series). The 785 nm diode laser provided an excitation source with a maximum power output of 300 mW. Raman spectra were for each sample at 30 mW laser power at an acquisition time of 10 sec.
Results and Discussion

Figure 9 illustrates the two-step process whereby MA was reacted to PE and PP (A)\textsuperscript{5,29} to form –COOH groups, followed by their conversion to acid chloride. The second step relies on reactions of propargylamine (B) to obtain alkyne functionalized polymeric surfaces that are reactive with any azide containing molecule.

Figure 10-(a)-A, B, C, and D illustrates structural features that develop as a result of the reactions depicted in steps A and B, Figure 9. Structural features resulting from these reactions labeled as A, B, and D were confirmed using FT-IR spectroscopy, whereas the formation of C was confirmed by Raman spectroscopic measurements. Traces A in Figure 10-b and 10-c correspond to PE and PP substrate spectra, respectively, with the 1712 cm\textsuperscript{-1} band in Traces B of each Figure corresponding to the C=O vibrations of –COOH groups due to the addition and hydrolysis of maleic anhydride. Traces C in Figure 10-b and 10-c represent the spectra recorded after amidation reactions of PPA with modified PE and PP. Here the C=O bands at 1660 and 1530 cm\textsuperscript{-1} represent amide I and II formation due to C=O and N-H functionalities, respectively. Click reactions with azide functionalized AMP are illustrated in Figure 10-a, and the spectra of those surfaces are shown in Traces D of Figure 10-b and 10-c, with the β-lactam moiety of ampicillin exhibiting a band at 1770 cm\textsuperscript{-1} due to C=O stretching vibrations.

Figure 11 illustrates a series of Raman spectra recorded after each step leading to the formation of alkyne moieties. Trace A of Figure 11 represents the reference spectrum of PP, Trace B the Raman spectrum of PP after MA attachment, Trace C the Raman spectrum of PP-MA surfaces after reaction with diglycidyl ether PEG having terminal epoxide groups which reacted with PPA. Note, no alkyne bands are observed. Trace D
shows the spectrum of PP-MA whereby PPA was reacted via carbodiimide coupling chemistries using DCC. Again, no alkyne bands are observed. Trace E shows the spectrum of PP-MA with PPA attached by converting the surface –COOH groups to highly reactive –COCl using oxalyl chloride (OC) prior to addition of PPA. The band at 2127 cm\(^{-1}\) (Trace E) corresponds to C≡C groups present on the polymer surface.

Although spectroscopic analysis can identify molecular entities reacted after each step of the sequence, an ultimate goal of these studies was to examine the effectiveness of AMP covalently attached to polymer surfaces against \textit{Staphylococcus aureus} bacteria. Therefore, PE-MA-PPA-AMP and PP-MA-PPA-AMP surfaces were tested for antimicrobial activity. The results are shown in Figure 12; the colony forming units (CFU) per cm\(^2\) were enumerated using a drop plate method for PE/PP (a), PE-MA/PP-MA (b), PE-MA-PPA/PP-MA-PPA (c), and PE-MA-PPA-AMP/PP-MA-PPA-AMP (d) surfaces. The data indicates that clicked AMP on both PE and PP greatly enhances the antimicrobial activity, manifested by a drop of CFUs from 4000-5000 for non-AMP modified specimens to 10-100 for PE-MA-PPA-AMP and PP-MA-PPA-AMP specimens.

**Conclusion**

In this work a simple method for clicking molecules onto aliphatic polymeric surfaces via microwave plasma surface reactions and further functionalization with alkyne species was developed allowing facile reaction with any azide containing molecule. In order to justify the efficacy of these chemistries, a model system was examined whereby azide functional AMP was synthesized and clicked onto PE and PP surfaces. These AMP clicked surfaces exhibit highly efficient antimicrobial activity against \textit{S. aureus}, with a 97-99.8% decrease of bacterial growth.
Figure 8. Schematic diagram of ‘clickable’ polymeric surfaces exhibiting alkyne functionalities for ‘clicking’ any azide containing molecule.

Figure 9. Surface reactions leading to the formation of alkyne surface groups; (A) Microwave plasma reactions in the presence of MA; (B) and alkyne functionalization using propargylamine.
Figure 10. (a) Reaction sequences on PE and PP substrates leading to AMP clicked surfaces; (b) ATR-FTIR spectra of A: PE, B: PE-MA, C: PE-MA-PPA, D: PE-MA-PPA-AMP; (c) ATR-FTIR spectra of A: PP, B: PP-MA, C: PP-MA-PPA, D: PE-MA-PPA-AMP.
Figure 11. Raman spectra of A: PP; B: PP-MA; C: PP-MA-epoxide-PPA; D: PP-MA-DCC-PPA; and E: PP-MA-OC-PPA.
Figure 12. Antimicrobial activity against *S. aureus* of (a) PE (blue) and PP (red) surfaces; (b) PE-MA and PP-MA surfaces; (c) PE-MA-PPA and PP-MA-PPA surfaces; (d) PE-MA-PPA-AMP and PP-MA-PPA-AMP surfaces.
REFERENCES


Modifying polymeric surfaces provides an opportunity for altering their interactions with the environment while retaining their useful bulk properties. This is particularly critical in biomaterials which, in order to coexist with biological systems, must maintain bulk characteristics and also exhibit desirable surface and interfacial properties that do not interfere with multi-faceted bioactive functions. Although numerous studies have focused on modifying polymer surfaces, recent advances in surface and interfacial chemistry have resulted in the development of a new class of stimuli-responsive materials. For example, by creating switchable surfaces using polyelectrolytes, followed by functionalizing the end groups of these polyelectrolytes, a given surface may adapt to environmental pH changes. Taking this concept a step further leads to the creation of functional surfaces with engineered surface responsiveness to an array of external stimuli.

The majority of surface reaction chemistries have focused on silicon and gold surfaces. However, reactions on polymer substrates are challenging due to the inert nature of polymer surfaces and their morphological heterogeneities. This is particularly important if responsiveness to external stimuli, such as temperature, ionic strength, UV radiation, or pH are required. These studies focus on the development of stimuli-responsive polymeric surfaces “decorated” with cationic and anionic...
polyelectrolytes terminated by –COOH or –NH₂ groups. The advantages of oppositely charged polyelectrolytes include the ability to form hydrophilic surfaces on hydrophobic polymer substrates, as well as introduce surface dynamic properties as a function of pH. Specifically, when cationic segments extend, anionic tethers collapse, and vice versa. Figure 13-A illustrates the overall scheme of the coupling reactions used to attach poly(2-vinyl pyridine) (P2VP) and poly(acrylic acid) (PAA). Because P2VP and PAA chain ends are terminated with –NH₂ and –COOH groups, respectively, the scope of these studies is further expanded to include the attachment of heparin (HEP) on P2VP and ampicillin (AMP) on PAA (Figure 13-B) to achieve antimicrobial and anticoagulant pH responsive polymeric surfaces.

Experimental Section

Covalent Attachment of Multilayers (CAM)

All reagents used were purchased from Sigma-Aldrich and used as received unless otherwise specified. Medical grade poly(tetrafluoroethylene) (PTFE) and ultra-high molecular weight polyethylene (PE) were purchased from McMaster-Carr Supply Co. (Atlanta, GA), cut into 1 x 1 cm squares, washed in isopropanol, and dried at room temperature in a desiccator before use. Silicon (Si) wafers were purchased from Ted Pella Inc. and washed in isopropanol before use. To obtain –COOH terminated PTFE surfaces, microwave plasma reactions were conducted in the presence of maleic anhydride (MA) under open reactor conditions, as described elsewhere. In the next step, –COOH exhibiting surfaces were placed in oxalyl chloride solution for 2 h in order to create –COCl groups followed by immediate immersion into undiluted ethylenediamine (EDN) for 4 h. α-amino-ω-carboxy-terminated heterobifunctional poly-
2-vinylpyridine (P2VP; Mn = 10,000; Polymer Source Inc.) and α-ω-dicarboxy-terminated bifunctional poly-β-butyrlacrylate (PtBA; Mn = 10,000; Polymer Source Inc.) were used as received. Initially, the –COOH terminal ends of 0.5 μM PtBA were activated in 10 mL oxalyl chloride for 2 h. The –NH₂ functionalized PE and PTFE surfaces were placed in the activated PtBA solution for 16 h. Upon removal, the specimens were washed in chloroform and then washed in deionized (DI) water for 30 min, and dried in a desiccator. The terminal –COOH groups of 0.5 μM –COOH-NH₂ terminated P2VP were converted to –COCl groups by initially dissolving in 10 mL chloroform then adding 10 mL oxalyl chloride to the solution for 2 h before PtBA modified polymer surfaces were added to the solution and stirred for 16 h.

Surface modifications were carried out to attach ampicillin (AMP) to PtBA tethered chains and heparin (HEP) to P2VP tethered chains. For AMP terminated surfaces, AMP was attached via carbodiimide coupling chemistry. PtBA and P2VP tethered surfaces with terminal –COOH were placed in 15 mL methylene chloride with 1.3 nM dicyclohexyl-carbodiimide (DCC) (Fluka) coupling agent and 0.25 mM 4-(dimethylamino)-pyridine (DMAP) catalyst. 0.1 g of AMP was dissolved in 5 mL methylene chloride which was then added to the DCC/DMAP flask containing the surfaces. The specimens were stirred in the mixture for 6 h in an ice water bath, followed by washing in methylene chloride and then in DI water for 30 min and dried in a desiccator. HEP was attached via carbodiimide coupling chemistry by dissolving 1.3 mM 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC) and 0.25 mM N-hydroxysuccinimide (NHS), as well as 0.5 mM HEP in 10 mL acetate buffer (pH 4.6). P2VP and PtBA tethered surfaces were added to the mixture and stirred for 16 h,
followed by washing in DI water for 30 min and drying. Once terminal chain ends of PtBA were functionalized, trifluoroacetic acid ≥99% in methanol was used to remove the t-butoxy group leading to the formation of PAA.

Dilute solutions of sodium hydroxide and hydrochloric acid in DI water at pH 2, 4.9, and 10 were prepared using a Thermo Orion pH meter model 350 with a glass combination electrode (Orion 9202 BN). The PTFE and PE surfaces presenting both PAA and P2VP were placed in the pH specific solutions for 1 h followed by rapid drying at 50 °C to lock in the extended or collapsed configuration.

Surface Characterization

Attenuated total reflectance Fourier transform infrared (ATR FT-IR) spectra were collected using a Bio-Rad FTS-6000 FT-IR single-beam spectrometer set at a 4 cm\(^{-1}\) resolution equipped with DTGS detector and a 45° face angle Ge crystal. Each spectrum represents 200 co-added scans ratioed against a reference spectrum obtained by recording 200 co-added scans of an empty ATR cell. All spectra were corrected spectral distortions using Q-ATR software\(^{12}\).

Atomic force microscopy (AFM) measurements were conducted on a Nanoscope IIIa Dimension 3000 scanning probe microscope, Digital Instruments. A silicon probe with 125 μm long silicon cantilever, nominal force constant of 40 N/m and resonance frequency of 275 kHz was used in a tapping mode, allowing assessment of surface topography. AFM data were analyzed using WSxM software\(^{13}\) to obtain RMS values, 3-D AFM images, and surface profile measurements.
Antimicrobial Testing of Ampicillin Tethered Surfaces

To determine anti-microbial activity of AMP functionalized polyelectrolyte tethered surfaces, each specimen was exposed to cultures of *Staphylococcus aureus* (*S. aureus*) (RN 4420). *S. aureus* bacteria were incubated for 4 hours at 37 °C in Triptic Soy Broth (TSB). Bacterial growth was determined by optical density, measured from absorbance at 600 nm using a UV-vis spectrometer. A 0.1 mL solution of *S. aureus* at a concentration of 10 µg/mL in TSB was placed on the sample surface and incubated at 37 °C for 4 h. After incubation, 10 µL of solution was removed from the surface and used to make serial dilutions. The dilutions were plated on Triptic Soy Agar (TSA) plates using a drop-plate method and the bacterial colonies were counted.

Anticoagulant Testing of Heparin Tethered Surfaces

To determine anticoagulant activity, lagomorph blood was collected by venipuncture and transferred to a syringe containing 10 µL/mL heparin. The use of laboratory animals followed the NIH guidelines. Heparin attached samples and controls were placed into 2.5 mL vials without any additive and filled with heparinized lagomorph blood followed by incubation at 37°C for 2 h on a hematology mixer (Fisher Scientific, Pittsburgh, PA). Samples were rinsed with DI water to remove any non-adhered blood from the surfaces. Raman spectra were obtained using a Renishaw Raman microscope-spectrometer equipped with a computer controlled three-axis encoded motorized stage, a RenCam CCD detector, and a Leica microscope (DMLM series). The 785 nm diode laser provided an excitation source with a maximum power output of 300 mW. The samples, as well as, blood reference were placed on a gold slide, and each Raman spectra was collected at 30 mW laser power at an acquisition time of 10 sec. Raman imaging was
carried out on each sample at 3 mW laser power for 1 min and tuned to 1620 cm\(^{-1}\) for detecting the presence of blood on each surface.

Results and Discussion

Figure 13 depicts the general theme to achieve pH responsive anticoagulant and antimicrobial surfaces, whereas Figure 14 illustrates the reaction sequences that formed covalently attached multilayers (CAM) tethered to Si, PE, and PTFE surfaces.

In the first step (1), MA is reacted to a substrate\(^{10,16}\) followed by hydrolysis to create –COOH groups, which are then converted to acid chloride. In the second step (2), EDN is reacted with –COCl groups resulting in –NH\(_2\) terminated surfaces. The next step (3) involves the attachment of dicarboxy terminated-PrBA to the –NH\(_2\) terminated surfaces via amide linkages, followed by the attachment of P2VP (4), carried out by reacting amide linkages between –COOH groups on P2VP and the –NH\(_2\) groups. Upon terminal functionalization with desired bioactive species, PrBA is hydrolyzed under mild conditions to form PAA polyelectrolyte (5). This choice of sequences with polyelectrolyte chain ends containing –NH\(_2\) and –COOH terminal groups achieved two objectives: the ability to attach bioactive species and to facilitate pH sensitive expansion or collapse responses. These studies are organized into the following sections: –COOH terminated CAM reactions; –NH\(_2\) terminated CAM reactions; switchable CAM on Si, PE, and PTFE surfaces; and bioactive CAM functionalized surfaces.

-**COOH Terminated CAM Reactions**

Following reaction sequences depicted in Figure 14, Figure 15-a illustrates a sequence of key functional groups formed by CAM reactions that lead to –COOH terminal entities. The first step involves MA reactions to a substrate, followed by EDN
and PrBA (PAA) functionalization. In an effort to identify species responsible for the attachment of each subsequent layer, ATR-FTIR spectra were recorded after each step, and are illustrated in Figure 15-b, c, and d for Si, PE, and PTFE, where Traces A, B, C, and D correspond to the reaction sequences shown in Figure 15-a.

Traces A of Figure 15-b, c, and d represent the reference spectra of Si, PE, and PTFE substrates, Traces B show the band at 1707 cm\(^{-1}\) corresponding to the C=O stretching vibrations of –COOH groups. Traces C and D in Figure 15-b, c, and d show spectra recorded after amidation reactions on Si, PE, and PTFE, respectively. The appearance of the bands at 1660 and 1530 cm\(^{-1}\) are attributed to C=O vibrations of Amide I and II formation. PrBA attachment is confirmed by the band at 1727 cm\(^{-1}\) due to the C=O stretching vibrations of acrylate pendant groups along the polymer chain before their conversion to –COOH groups via hydrolysis reactions to obtain PAA. The built-in pH sensitivity enables these surface chemistries to reversibly collapse as a function of pH in the range 3.2 < pH < 14. In addition, the presence of terminal –COOH groups serves as a site for further reactions with bioactive species containing –NH\(_2\) moieties.

-\(\text{NH}_2\) Terminated CAM Reactions

The first step involves MA reactions with the substrate, followed by EDN and P2VP attachment. Traces A, B, C, and D correspond to the CAM sequence shown in Figure 16-a. Reactions of P2VP to polymer surfaces facilitates pH responsiveness in the pH range of 1 < pH < 6.7 as well as providing tethers with –NH\(_2\) functionalities for further reactions. Figure 16-a illustrates a sequence of CAM reactions leading to –NH\(_2\) terminal groups, with spectroscopic evidence for their formation on Si, PE, and PTFE substrates provided in Figure 16-b, c, d, respectively.
Traces A in Figure 16-b, c, and d represent Si, PE, and PTFE substrate spectra, respectively, whereas the 1707 cm\(^{-1}\) band in Traces B of each Figure corresponds to the C=O vibrations of –COOH groups due to maleic anhydride reactions and its subsequent hydrolysis. Traces C and D in Figure 16-b, c, and d represent the spectra recorded after amidation reactions on Si, PE, and PTFE surfaces, respectively. The C=O bands at 1660 and 1530 cm\(^{-1}\) are characteristic of Amide I and II formations, and covalent attachment of P2VP is represented by the band at 1620 cm\(^{-1}\) due to aromatic amine pendant groups along the polymer chain.

*Switchable CAM on Si, PE, and PTFE Surfaces*

Simultaneous presence of PPA and P2VP creates switchable surfaces that respond to variable pH values. Whereas the formation of –COOH and –NH\(_2\) terminal groups allows attachment of other entities, the switchability of the PAA-P2VP CAM is attributed to the presence of side groups capable of protonation and deprotonation. Controlled by pH values, the response will deviate from the isoelectric point at pH 4.9\(^2\), thus causing the PAA and P2VP surface chains to extend or collapse in response to solution ionic strength changes. That is why PAA and P2VP polyelectrolytes were reacted with PTFE-MA-EDN surfaces and their surface responsiveness was measured as a function of pH. ATR-FTIR spectra of PAA and P2VP tethered surfaces recorded as a function of pH are shown in Figure 17.

Trace A shows the ATR-FTIR spectrum of the PTFE-MA-EDN-PAA/P2VP surface at pH 10. The presence of bands at 1595 and 1402 cm\(^{-1}\) indicate deprotonated –COO\(^{-}\)H\(^+\) species. At pH = 2 (Trace B), these bands are no longer observed. The band at 1707 cm\(^{-1}\) due to C=O stretching vibrations of –COOH groups increases under acidic
conditions, consistent with protonation of the PAA chains. Trace C displays the spectrum of the surface acquired at pH 4.9, the isoelectric point. The band at 1735 cm\(^{-1}\) due to C=O stretching vibrations of residual acrylate ester groups of unhydrolyzed PtBA is observed at all pH values, evidence that the PtBA to PAA hydrolysis is incomplete. The bands at 1660 and 1530 cm\(^{-1}\) due C=O stretching and N-H bending modes of Amide I and II groups, respectively, remain intact under all pH conditions, indicating their insensitive nature to pH variations.

AFM was employed to confirm the physical switchability of PAA and P2VP polyelectrolyte containing polymeric surfaces and to correlate spectroscopic changes with surface morphologies of CAM modified surfaces that develop during pH exposure. Figure 18 illustrates 3-D AFM phase images for Si (A), PE (B), and PTFE (C), along with a series of images that depict the switchability of the tethered polyelectrolyte surfaces at pH values of 2 and 10, respectively. Figure 18, A1, B1, and C1 show AFM images of Si, PE, and PTFE, respectively, whereas Figure 18, A`1, B`1, and C`1 represent the surface profiles obtained from AFM images. The first reaction step involved MA attachment via microwave plasma reactions; the AFM images of the reacted surfaces are depicted in Figure 18, A2, B2, and C2. Upon functionalization with P2VP and PAA polyelectrolyte chains, the same surfaces exposed to variable pH conditions change morphologies. However, as illustrated in the AFM images in Figure 3.6, A3, B3, and C3, upon exposure to pH 2, for Si-MA-P2VP, PE-MA-P2VP, and PTFE-MA-P2VP surfaces, the P2VP segments extend due to the protonation of the pyridine pendant groups on the polyelectrolyte chains. This behavior is reversed upon exposure to pH 10, which causes the P2VP chains to collapse. This is manifested by the
AFM images in Figure 18, A4, B4, and C4. AFM images in Figure 18, A5, B5, and C5 for Si-MA-PAA, PE-MA-PAA, and PTFE-MA-PAA show collapsed PAA surface chains at pH 2, because the –COOH groups of PAA are protonated. Figure 18, A6, B6, and C6 depict AFM images of the same surfaces exposed to solutions of pH 10; here the PAA chains extend due to the deprotonation of the –COOH groups to form carboxylate ions. To further illustrate the magnitude of surface morphological changes resulting from pH changes, Figure 18, A′, B′, and C′ show the surface profiles of the respective AFM images which correspond to the height of the surface features. As seen, the magnitude of extension or collapse varies from a few to 40 nm which corresponds to the polyelectrolyte chains with Mn = 10,000.

*Functionalization of CAM Surfaces with Bioactive Species*

As stated previously, –COOH and -NH₂ terminal groups on PE-MA-EDN-PAA, PTFE-MA-EDN-PAA, PE-MA-EDN-P2VP, and PTFE-MA-EDN-P2VP surfaces provide a site for further reactions. Because antimicrobial and anticoagulant properties are highly critical in many applications, AMP was reacted to PE-MA-EDN-PAA and PTFE-MA-EDN-PAA, and HEP was reacted to PE-MA-EDN-P2VP and PTFE-MA-EDN-P2VP surfaces. Figure 19 illustrates the results of spectroscopic analysis of these reactions. Figure 19-a depicts the sequence of reactions, whereas Figures 19-b and 19-c show ATR-FTIR spectra of surfaces after each modification step for PE and PTFE, respectively. All spectra in Figure 19-b and -c exhibit bands for amide linkages represented by the C=O band at 1660 cm⁻¹ and the N-H band at 1530 cm⁻¹ indicating Amide I and II formations. The β-lactam moiety of ampicillin is present, based on the band at 1768 cm⁻¹ due to C=O stretching vibrations. The HEP attachment shown in
Figure 19-b’ and c’ is confirmed by the appearance of the bands at 1608 cm$^{-1}$ due to –OH groups of HEP.

**Bioactivity of Functionalized CAM Surfaces**

PE-MA-EDN-PAA-AMP and PTFE-MA-EDN-PAA-AMP surfaces were tested for antimicrobial activity against *Staphylococcus aureus* and compared to non-pH sensitive surfaces. Figure 20 illustrates the colony forming units (CFU) per cm$^2$ enumerated using a drop plate method for PE and PTFE (a), PE-MA and PTFE-MA (b), PE-MA-EDN-PAA and PTFE-MA-EDN-PAA (c), and PE-MA-EDN-PAA-AMP and PTFE-MA-EDN-PAA-AMP (d) surfaces. Although the data indicates that PE exhibits better antimicrobial resistance than PTFE, it is apparent that covalent attachment of AMP significantly enhances antimicrobial activities. This is illustrated in Figure 20-d which shows a significant decrease in CFUs from 4000-5000 to 10-40, indicating > 99% decrease in bacterial growth.

To determine the longevity of surface antimicrobial activity of pH sensitive PE-MA-EDN-PAA-AMP and PTFE-MA-EDN-PAA-AMP surfaces, antimicrobial activity was tested against *S. aureus* and compared to non-pH sensitive PE-MA-PEG-AMP and PTFE-MA-PEG-AMP surfaces. Figure 21-a, c, and e illustrates the results of bacterial count represented by CFU/cm$^2$ for pH-sensitive PE-MA-EDN-PAA-AMP and PTFE-MA-EDN-PAA-AMP surfaces. Each specimen was exposed to *S. aureus* for 4 hours at 37°C (Figure 21-a), followed by washing and two additional exposures to *S. aureus* for 4 hours (Figure 21-c and e), respectively. For comparison, Figure 21-b, d, and f illustrates the results of bacterial count for non-pH sensitive PE-MA-PEG-AMP and PTFE-MA-PEG-AMP surfaces. Although initially both pH-sensitive and non-pH sensitive
specimens are highly effective, it is apparent that pH sensitive surfaces are even more
effective. It should be pointed out that the controls shown in Figure 20 are at a range of
4000-5000 CFU/cm\(^2\), whereas the data in Figure 21 are in the range of 0-400 CFU/cm\(^2\).

Anticoagulant activity of PE-MA-P2VP and PTFE-MA-P2VP surfaces
functionalized with HEP was determined using Raman imaging in the presence of whole
lagomorph blood. Raman spectra (Appendix B) were collected before and after the
exposure to whole blood, and the band at 1620 cm\(^{-1}\) due to N-H stretching vibrations of
hemoglobin protein constituents\(^1\) was used to determine the degree of coagulation. In an
effort to identify surface coagulation across the entire surface, Raman imaging of a 10 x
10 µm area was obtained by tuning to the 1620 cm\(^{-1}\) band. Data for the specimens
obtained from incubating each surface in lagomorph blood are illustrated in Figure 22, in
which PE (a), PE-MA (b), PE-MA-EDN-P2VP (c), and PE-MA-EDN-P2VP-HEP (d) are
Raman images of the substrates before blood exposure. The color scale bars indicated
that the red color represents high intensity of the 1620 cm\(^{-1}\) band, corresponding to a high
degree of coagulation. Decreased coagulation is represented by color changes whereby
the black color ideally represents no coagulation. Upon exposure to blood, PE (a’) and
PE-MA (b’) specimens do not exhibit anticoagulant activity. PE-MA-EDN-P2VP (c’)
shows some decrease in coagulation, thought to be due to antifouling properties imparted
by the presence of the polyelectrolyte surface tethers. PE-MA-EDN-P2VP-HEP (d’)
greatly minimizes clotting. For comparison, Figure 22-e shows the Raman image of
dried lagomorph blood at 1620 cm\(^{-1}\). Similarly, Figure 23 illustrates Raman images of
PTFE (a), PTFE-MA (b), PTFE-MA-EDN-P2VP (c), and PTFE-MA-EDN-P2VP-HEP
(d) substrates before blood exposure. As shown, PTFE (a’), PTFE-MA (b’), and PTFE-
MA-EDN-P2VP (c’), do not exhibit anticoagulant activity, whereas PTFE-MA-EDN-P2VP-HEP (d’) minimizes clotting. For comparison, Figure 23-e shows the Raman image of dried lagomorph blood at 1620 cm\(^{-1}\). The effectiveness of HEP covalently attached to PE-MA-EDN-P2VP and PTFE-MA-EDN-P2VP surfaces against blood coagulation is apparent.

Conclusion

In this work stimuli-responsive PE, PTFE, and Si surfaces were developed using covalent attachment of multilayers (CAM) for tethering pH-responsive “switching” polyelectrolytes consisting of poly(2-vinyl pyridine) (P2VP) and poly(acrylic acid) (PAA) terminated with NH\(_2\) and COOH groups. These surfaces upon pH changes are able to collapse or extend. When pH < 2.3, P2VP segments became protonated and extended, whereas PAA segments collapsed. At pH > 5.5, PAA segments became extended, whereas P2VP segments collapsed. The presence of terminal NH\(_2\) and COOH moieties facilitated the attachment of bioactive species such as AMP and HEP, resulting in antimicrobial and anticoagulant properties manifested by bacterial growth inhibition and the prevention of blood clotting. Compared to static surfaces, the presence of built-in pH sensitive surface tethers also enhance antimicrobial and anticoagulant properties which is believed to be attributed to continuous surface morphology changes and the ability of AMP and HEP molecules to actively intercept microbial film formation and blood coagulation at early stages.
Figure 13. (A) Schematic diagram of dual stimuli-responsive polyelectrolyte surfaces terminated with –NH₂ and –COOH moieties; (B) Structures of (a) HEP and (b) AMP, with the moieties available for attachment circled.
Figure 14. Reaction sequences on polymeric substrates that form stimuli-responsive polyelectrolyte surfaces terminated with –COOH and –NH₂ functionalities and exhibiting bioactive molecules.
Figure 15. (a) Reaction sequences (circles represent reaction sites) on Si, PE, and PTFE surfaces leading to terminal –COOH groups; (b) ATR-FTIR spectra of A: Si, B: Si-MA, C: Si-MA-EDN, D: Si-MA-EDN-PtBA; (c) ATR-FTIR spectra of A: PE, B: PE-MA, C: PE-MA-EDN, D: PE-MA-EDN-PtBA; (d) ATR-FTIR spectra of A: PTFE, B: PTFE-MA, C: PTFE-MA-EDN, D: PTFE-MA-EDN-PtBA.
Figure 16. (a) Reaction sequences (circles represent reaction sites) on Si, PE, and PTFE surfaces leading to terminal –NH₂ groups (circled); (b) ATR-FTIR spectra of A: Si, B: Si-MA, C: Si-MA-EDN, D: Si-MA-EDN-P2VP; (c) ATR-FTIR spectra of A: PE, B: PE-MA, C: PE-MA-EDN, D: PE-MA-EDN-P2VP; (d) ATR-FTIR spectra of A: PTFE, B: PTFE-MA, C: PTFE-MA-EDN, D: PTFE-MA-EDN-P2VP.
Figure 17. ATR-FTIR spectra of PTFE-MA-EDN-PAA/P2VP switching behavior observed at (A) pH 10, (B) pH 2, and (C) pH 4.9 (isoelectric point).
Figure 19. (a and a’) Chemical structures from reactions with AMP to PE-MA-EDN-PAA and PTFE-MA-EDN-PAA and HEP to PE-MA-EDN-P2VP and PTFE-MA-EDN-P2VP (circles represent reaction sites); (b, b’, c, and c’) ATR-FTIR spectra of PE-MA-EDN-PAA-AMP, PTFE-MA-EDN-PAA-AMP, PE-MA-EDN-P2VP-HEP, and PTFE-MA-EDN-P2VP-HEP.
Figure 20. Antimicrobial activity against *S. aureus* of (a) PE (blue) and PTFE (red) surfaces; (b) PE-MA and PTFE-MA surfaces; (c) PE-MA-EDN-PAA and PTFE-MA-EDN-PAA surfaces; (d) PE-MA-EDN-PAA-AMP and PTFE-MA-EDN-PAA-AMP surfaces.
Figure 21. Antimicrobial activity against *S. aureus* of PE-MA-EDN-PAA-AMP (blue) and PTFE-MA-EDN-PAA-AMP (red) surfaces (a, c, and e); PE-MA-PEG-AMP and PTFE-MA-PEG-AMP surfaces (c, d, and f) after first (1), second (2), and third (3) 4 hour exposure to *S. aureus*. 
Figure 22. Raman images of the 1620 cm\(^{-1}\) band before/after exposure to whole blood to (a and a’) PTFE; (b and b’) PTFE-MA; (c and c’) PTFE-P2VP; (d and d’) PTFE-P2VP-HEP; (e) blood reference. Each image represents a 10 x 10 µm area.
Figure 23. Raman images of the 1620 cm\(^{-1}\) band before/after exposure to whole blood to (a and a’) PE; (b and b’) PE-MA; (c and c’) PE-P2VP; (d and d’) PE-P2VP-HEP; (e) blood reference. Each image represents a 10 x 10 µm area.
REFERENCES


CHAPTER V
PHAGE-BACTERIUM WAR ON POLYMERIC SURFACES;
CAN SURFACE-ANCHORED BACTERIOPHAGES
ELIMINATE MICROBIAL INFECTIONS?

Introduction

The majority of interactions between biologically active species and synthetic materials are inherently non-favorable. However, formation of biofilms represents an important and unwelcome exception resulting from the attachment of bacteria to a synthetic surface. This leads to the formation of a complex biofilm community that is often encased on a surface of polymeric materials in contact with blood. Microbial biofilms are very resilient communities that resist removal by chemical or physical means because their cells are capable of adhering to a variety of biotic and abiotic surfaces and continue to grow biofilms as long as nutrients become available. In the context of human health, biofilms are responsible for the majority of deadly infections including medical-device associated diseases. Because they often become resistant to antibiotics or host defenses, the use of antibiotics may be ineffective to many pathogens, making conventional therapies troublesome.¹

Therefore, new approaches are needed to address infections on various fronts but in particular surface medical-device associated infections. These may include simple catheters, implants, stents, or monitoring devices. The first logical step has led to the development of novel drugs, however surface modifications with drugs bring another level of challenges associated with their attachment, long-term effectiveness, and maintenance. Among anti-biofilm formation strategies, covalent attachments of
antibiotics or antimicrobial agents to polymeric surfaces have been somewhat successful with relatively longer durations, but long-term activities are still limited.\textsuperscript{2} Therefore, the creation of stimuli-responsive attributes on polymeric surfaces, where a surface remains silent unless external stimulus triggers desirable responses is especially attractive. In essence, the goal is to prevent biofilm formation from developing at its inception.

An alternative approach for fighting microbial wars on polymeric or other surfaces is to utilize bacterial viruses (or bacteriophages) that attach specifically to their target host bacteria, inject their genetic material, reproduce inside the host, kill the host, and release their progeny. Although the first observations of lytic phages to cure infectious diseases go back to the end of the 19\textsuperscript{th} and early 20\textsuperscript{th} Centuries,\textsuperscript{4-6} it was not until 1960s, where prophylaxis and treatment of bacterial infections proved favorable with a remarkable recovery efficacy above 92\%.\textsuperscript{7} This led to using bacteriophages (phages) by physically mixing with or physisorption on polymers (Nylon\textsuperscript{TM}),\textsuperscript{8, 9} glass,\textsuperscript{10} and gold.\textsuperscript{11} The unique attributes of bacteriophages are their host-dependent reproduction; bacteriophages will remain silent until they find a specific bacterium, thus minimizing safety concerns associated with excessive concentration levels. Furthermore, due to evolving with their hosts and host-specificity, numerous bacteriophages exist for each bacterium.

Taking advantage of the ability of bacteriophages to selectively recognize a host bacterium and effectively kill it, T1 and Φ11 phages were covalently attached onto polytetrafluoroethylene (PTFE) and ultra high molecular weight polyethylene (PE) surfaces. The premise behind this approach is the covalent anchoring of the bacteriophages to polymer surfaces while maintaining their biological activity.
Experimental

Phage Farming

T1 and Φ11 bacteriophages were prepared by Plate lysis method. A heavy suspension of bacteria from a 16 h incubated plate was suspended in 2 ml of Tryptic Soy Broth. Then 500 µl of bacterial suspensions, 500 µl of phage stock solution, and 200 µl of cold CaCl$_2$ at 4°C were added to a 15 ml falcon tube followed by adding 5ml of top agar (Tryptic soy broth containing 0.70% agar, cooled to 50°C) and mixing well, then pouring on prepared TSA plates (pre-warmed for 30 min at 37°C). The top agar was allowed to cool and the plates were incubated at room temperature overnight or until clear lyses of the whole plate were observed. The top agar was scrapped gently with a sterile spreader by adding 5-6 ml of 1X PBS. The scrapped top agar from all plates was poured into a 50 ml falcon tube, followed by centrifuging at 10,000 rpm for 10 min at room temperature. The supernatant was collected and pellet was discarded. The supernatant was filter-sterilized using a 0.45 µm syringe filter with 100 µl of phage filtrate being spread on a plain TSA plate and incubated overnight to ensure sterility.

Phage Purification and Concentration by PEG Method

100 g of PEG (MW 10,000) and 6 g of NaCl was mixed with 250 ml of water, autoclaved, and pH adjusted to 7.2 under sterile conditions. A 1:4 volume ratio of PEG:Bacteriophage supernatant was prepared and refrigerated overnight (stable up to 2 weeks at 4°C), followed by centrifuging the tubes at 10,000 rpm for 2 h at 4°C. The supernatant was discarded and the tube was left in an inverted position for 10-20 min. The pellet was resuspended in 0.1 of the original volume of phage suspension using PBS and stored at 4°C until needed.
**Plaque Formation Assay for the Phage Attached Surfaces**

PTFE and PE surfaces containing covalently attached bacteriophages (T1 for *Escherichia coli* and Φ11 for *Staphylococcus aureus* sub spp RN4220) were used for plaque formation assay. In a typical experiment, overnight culture of bacteria (*E. coli* for T1 and Φ11 for *S. aureus*) were diluted at a 1:1,000 ratio in TSB and allowed to grow for 3 h. The cells were normalized up to 0.1 (OD$_{600}$ nm). Two 15 ml falcon tubes containing 500 µL of respective bacteria were prepared. 5 ml of top agar (cooled to 50°C) was added to each tube and poured into thin layered TSA plates pre-warmed at 37°C for 30 min. The final buffer in which the surface was suspended and the surface without the phage attached to it as well as the phage itself were also included as negative and positive controls. The respective surfaces were then stabbed into the top agar before solidification. The plates were incubated at room temperature for 24-48 h and results were observed in the form of clear plaques seen around the surfaces. This experiment was performed independently for each type of surface attached with respective phages. For mixed T1:Φ11 phage attached surfaces, similar assays were performed. Three plates T1, Φ11, and T1 and Φ11 for each phage attached surfaces were tested for plaque formation. Buffers in which the surfaces were suspended were also tested to eliminate any free bacteriophages present. Positive and negative controls were included separately in each assay. Images were taken to record the plaques produced by the phages.

**Phage Attachment**

Medical grade PTFE and UHMWPE (PE) were purchased from McMaster-Carr Supply Co. (Atlanta, GA), cut into 1 x 1 cm squares, washed in isopropanol, and dried at room temperature under vacuum before use. To obtain –COOH terminated PTFE and PE
surfaces, microwave plasma reactions were conducted in the presence of maleic anhydride (MA) (Aldrich Chemical Co.) under open reactor conditions, as described elsewhere.\(^2\)\(^,\)\(^13\) In the next step, PTFE-COOH and PE-COOH surfaces were placed in PBS buffer pH 7.4 (Invitrogen) containing 2.5 mmol of EDAC and 2.5 mmol NHS for 2 h in order to create −COO⁻ groups followed by washing in PBS buffer, then immediate immersion into 10 mL buffer solution containing 500 µL of concentrated T1 phage or Φ11 phage from above for 16 h. Additional PTFE and PE surfaces were reacted with a mixture of T1 and Φ11 phage following the aforementioned process using 500 µL of each phage in 10 mL of PBS buffer. The surfaces were then washed seven times in PBS buffer to remove all non-covalently attached phages.

**Analysis**

Attenuated total reflectance Fourier transform infrared (ATR FT-IR) spectra were collected using a Bio-Rad FTS-6000 FT-IR single-beam spectrometer set at a 4 cm\(^{-1}\) resolution equipped with DTGS detector and a 45° face angle Ge crystal with a depth of penetration of 0.37 µm. Each spectrum represents 200 co-added scans ratioed against a reference spectrum obtained by recording 200 co-added scans of an empty ATR cell. All spectra were corrected spectral distortions using Q-ATR software.\(^14\)

Atomic force microscopy (AFM) measurements were conducted on a Bruker Dimension icon scanning probe microscope with ScanAssist, Digital Instruments. A silicon probe with 125 µm long silicon cantilever, nominal force constant of 40 N/m and resonance frequency of 275 kHz were used in a ScanAssist Air tapping mode, allowing assessment of surface topography. Quantification of bacteriophages covalently attached
to Si surfaces was performed by using ImageJ software (NIH)\textsuperscript{15} to analyze surface particles.

**Results and Discussion**

Figure 24, A-D, depicts a sequence of steps leading to the formation and subsequent destruction of the bacteria attempting to form biofilms on the phage-modified polymeric substrates. The first step (Figure 24, A) involves the formation of reactive acid groups on polymeric substrates\textsuperscript{13} accomplished by simple and clean microwave plasma reactions in the presence of maleic anhydride, followed by covalent attachment of T1 phages via acid-amine reactions leading to amide linkages. Figures 25 and 26 provide spectroscopic evidence for these reactions.

When bacteria attempt to adhere to the surface of the phage-modified polymer substrate, the phage attaches specifically to the external structure of the bacterium (e.g. lipopolysaccharide or protein) and injects its genetic material into its target bacteria (Figure 24, C). Upon completion of this process, the phage DNA is replicated by the bacterial host machinery, several capsid proteins are produced and assembled, phage DNA is packaged and the bacteriophage progeny are released by lysis of the bacterial host. Depending on the particular bacteriophage, there can be up to 200 progeny phages for each individual infection. Perhaps the most significant advantage of using bacteriophages comes from the fact that each member of the progeny is capable of infecting more bacteria and releasing a progeny of its own. This amplification effect continues until all bacteria cells are killed. However, potential challenges exist which are associated with control of the phage population, especially when utilized in therapy, which needs to be carefully adjusted.
Verification of chemical reactions leading to T1 and Φ11 covalent attachments is shown in Figures 25 and 26, respectively. Traces A, B, C, and D of Figures 25 and 26 show ATR-FTIR spectra recorded from PE (a) and PTFE (b) surfaces, respectfully.

Traces B depict the spectra of maleic anhydride plasma modified PE-MA and PTFE-MA surfaces. Traces C show that spectra obtained when T1 or Φ11 phages are reacted to PE-MA and PTFE-MA surfaces. Two characteristic bands at 1662 and 1550 cm$^{-1}$ due to Amide I and II bands characteristics of the T1 and Φ11 outer functionalities are detected in Figure 25, which are due to covalent attachment of bacteriophage T1 to –COOH functionalized PE and PTFE surfaces. In the same manner, Figure 26, (a) and (b), illustrates ATR-FTIR spectra of PE (a) and PTFE (b) surfaces, with two bands at 1655 and 1545 cm$^{-1}$ due to Amide I and II bands characteristic of Φ11 functionalities. For comparison, Traces D in Figures 25 and 26, (a) and (b), depict FTIR spectra of T1 and Φ11 phages.

The experiments were also conducted on Si surfaces. The spectroscopic results of these experiments confirm covalent attachment of T1 and Φ11 phages and are summarized in Appendix C. The presence of T1 and Φ11 phages on Si surfaces was visually assessed by AFM images collected after each step. Figure 27, A, B, C and D shows AFM height images collected from Si wafer surfaces after each step. Figure 27, A depicts the height image of the Si wafer surface, whereas B shows the height image after covalent attachment of maleic anhydride via microwave plasma reaction. Figure 27, C and D confirm the presence of T1 and Φ11 phages on surfaces, respectfully. The inserts of C and D visually show the shapes corresponding to T1 and Φ11 morphologies and size. AFM data shown in Figure 27 was used to quantify the mean number of phages per
µm² using image analysis. The mean average number of T1 and Φ11 phages per µm² is 5.8 ± 1.7 and 10.8 ± 1.0, respectively.

The antimicrobial effectiveness of T1 and Φ11 phages covalently attached to PTFE and PE surfaces against *E. coli* and *S. aureus* was confirmed using plaque formation assays. Figure 28, A and B show PE and PTFE surfaces with covalently attached T1 phages which kill *E. coli* bacteria, as manifested by the formed plaques (clear zone) around each substrate. Figure 28, E and F, show the PE and PTFE surfaces with covalently attached Φ11 phages that kill *S. aureus* bacteria; also detected as the clear zone surrounding each substrate. To achieve simultaneous dual antimicrobial protection, a 1:1 mixture of T1 and Φ11 phages were covalently attached to PE and PTFE surfaces. The efficacy of these surfaces against bacteria are illustrated in Figure 28, C and D, for PE and PTFE with T1 and Φ11 phages against *E. coli*, and Figure 28, G and H, for the surfaces against *S. aureus* bacteria. As shown, the effectiveness of T1 and Φ11 phages covalently attached to PE and PTFE surfaces is apparent.

**Conclusion**

These studies established a universal approach for covalent attachment of bacteriophages to inert PE and PTFE polymeric surfaces. These reactions can be conducted on almost any surface. Although the limiting factor can be the maintenance of biological activity of phages, this work shows that covalent attachment of T1 and Φ11 does not adversely affect the phages biological activities. Multiple venues for polymer surface modifications combined with over a thousand individual phage species, with hundreds of different hosts, provide endless possibilities of creating surfaces capable of killing specific bacteria. Several synthetic approaches to combat biofilm formation were
exploited, however the use of bacteriophages to kill human pathogens anchored to synthetic surfaces show a promising and effective means of combating antibiotic-resistant infections.
Figure 24. A: Covalent attachment of acid groups to polymeric surfaces; B: Reactions of NH₂ groups of T1 with polymer surface acid groups; C: Attachment of phages to bacteria and injection of DNA; D: Replication of DNA and destruction of bacteria.
Figure 25. A: ATR-FTIR spectra of PE (a) and PTFE (b) surfaces; B: after plasma reactions on PE and PTFE surfaces in the presence of maleic anhydride; C: after T1 phage covalent attachment to MA modified surfaces; D: Reference spectrum of T1 phage (at 20% scale).
Figure 26. A: ATR-FTIR spectra of PE (a) and PTFE (b) surfaces; B: after plasma reactions on PE and PTFE surfaces in the presence of maleic anhydride; C: after Φ11 phage covalent attachment to MA modified surface; D: Reference spectrum of Φ11 phage (at 20% scale).
Figure 27. A: AFM height image of Si wafer surface; B: Height image of Si-MA surface; C: AFM height image of T1 bacteriophages attached to Si-MA surfaces; D: Height image of Si-MA surfaces with covalently attached Φ11 phages.
Figure 28. Plaque formation assays for covalently attached T1 phages on A: PE and B: PTFE surfaces against *E. coli*; C: PE and D: PTFE with covalently attached 1:1 mixture of T1 and Φ11 phages against *E. coli*. Plaque formation assays for covalently attached Φ11 phages on E: PE and F: PTFE surfaces against *S. aureus*; G: PE and H: PTFE with covalently attached 1:1 mixture of T1 and Φ11 phages against *S. aureus*. 
REFERENCES


11. Li-Mei C. Yang; Phillip Y. Tam; Benjamin J. Murray; Theresa M. McIntire; Cathie M. Overstreet; Gregory A. Weiss; Penner, R. M., Virus Electrodes for Universal Biodetection *Analytical Chemistry* **2006**, *78*, 3265.


APPENDIX A

SUPPORTING INFORMATION FOR CHAPTER III

Synthesis of AMP-PEG-N₃

Synthesis of AMP-PEG-N₃ was carried out via carbodiimide coupling chemistry between –COOH groups of AMP and –NH₂ groups of NH₂-PEG₆-N₃ (Sigma). Formation of AMP-PEG-N₃ was confirmed by ATR-FTIR spectroscopy as illustrated in Figure A.1. Trace A shows an ATR-FTIR spectrum of NH₂-PEG-N₃ (Sigma-Aldrich), whereas Trace B depicts the synthesized AMP-PEG-N₃ with the presence of the band at 2107 cm⁻¹ corresponding to N=N=N stretching vibrations of the azide moiety, and the 1402 cm⁻¹ band representing C-O-C stretching vibrations of the PEG species. Trace C shows the FTIR spectrum of AMP with the band at 1773 cm⁻¹ representing C=O stretching vibrations of the β-lactam moiety. This band is also visible in the product (Trace B). Additionally, Trace B contains bands at 1649 and 1528 cm⁻¹ indicative of amide I and II linkages, respectfully, confirming the covalent reactions between NH₂-PEG-N₃ and AMP.
Figure A.1. ATR-FTIR spectra of A: NH$_2$-PEG-N$_3$ (Sigma-Aldrich); B: AMP-PEG-N$_3$; and C: AMP reference (reaction sites of A and C are circled).
To determine the effect of attached polyelectrolyte tethers on polymeric surface hydrophobicity, contact angles were calculated using the sessile drop method measured with a contact angle goniometer. Figure B.1 shows contact angle measurements of poly(tetrafluoroethylene) (PTFE) and ultra-high molecular weight polyethylene (PE) substrates before and after the attachment of maleic anhydride (MA) and polyelectrolytes poly-2-vinylpyridine (P2VP) and polyacrylic acid (PAA) measured at pH values of 2, 4.9 (isoelectric point of dual polyelectrolyte system), and 10. Contact angles significantly decrease with attachment of MA and subsequent attachment of polyelectrolyte tethers indicating that the inherently hydrophobic substrates become more hydrophilic.

A and A’ of Figure B.1 show water droplets from which contact angles are measured on PTFE and PE substrates, respectively, while B and B’ show water droplets on MA attached PTFE and PE substrates. C, C’, D, D’, and E, E’ are images of water droplets on PAA and P2VP polyelectrolyte tethered substrates at pH 2, 4.9, and 10, respectively. Figure B.2 illustrates the plotted contact angles of the substrates pictured in Figure B.1.

Raman Spectroscopy of Lagomorph Blood and Polymer Surfaces

To determine the Raman band necessary for obtaining practical Raman images of coagulated blood on functionalized PTFE and PE substrates, Raman spectra were collected for the polymer surfaces with and without attached polyelectrolyte tethers, as well as a blood reference. The Raman spectra are shown in Figure B.3, with Trace A
corresponding to dried blood and Trace B representing PE substrates with covalently
attached P2VP and heparin (HP). Traces C and D illustrate the Raman spectra of PE and
PTFE substrates, respectively. The band at 1620 cm\(^{-1}\) in the blood reference was chosen
as the band to which the spectrometer was tuned to for Raman Imaging analysis. This
band was selected due to the lack of overlapping bands for the surface chemistries present
in Traces B-D.
**Figure B.1.** Images of water droplets on A: PTFE; B: PTFE-MA; C: PTFE-MA-PAA-P2VP at pH 2; D: PTFE-MA-PAA-P2VP at pH 4.9; E: PTFE-MA-PAA-P2VP at pH 10; A’: PE; B’: PE-MA; C’: PE-MA-PAA-P2VP at pH 2; D’: PE-MA-PAA-P2VP at pH 4.9; E’: PE-MA-PAA-P2VP at pH 10.

**Figure B.2.** Contact angle measurements of substrates A: PTFE and PE; B: PTFE and PE-MA; C: PTFE and PE-MA-PAA-P2VP at pH 2; D: PTFE and PE-MA-PAA-P2VP at pH 4.9; E: PTFE and PE-MA-PAA-P2VP at pH 10.
Figure B.3. Raman spectra of the 2000-500 cm$^{-1}$ region for A: Blood reference; B: PE-P2VP-HP; C: PE and D: PTFE.
**APPENDIX C**

**SUPPORTING INFORMATION FOR CHAPTER V**

Phage Attached Si Surface Spectroscopic Analysis

Figure C.1 shows ATR-FTIR spectra recorded from Si surfaces. Traces A depict the spectra for the Si wafer surfaces, whereas Traces B represent the spectra of maleic anhydride plasma modified Si-MA surfaces. Traces C show that spectra obtained when T1 or Φ11 phages are reacted to Si-MA surfaces. Two characteristic bands at 1653 and 1543 cm\(^{-1}\) are detected due to Amide I and II linkages characteristic of T1 or Φ11 outer functionalities as well as covalent attachment of bacteriophages to –COOH functionalized Si surfaces. For comparison, Traces D in Figure C.1, (a) and (b) depict ATR-FTIR spectra of T1 or Φ11, respectively.

Antimicrobial Testing of Phage Attached Si Surface

Biological activity of phages covalently attached to Si surfaces (Figure C.1) was confirmed using plaque formation assays of T1 and Φ11 phages. The results of exposure to their respective bacterial hosts *E. coli* and *S. aureus* are shown in Figure C.2, A and B. As manifested by the formed plaques (clear zone) around each substrate, covalently attached T1 phages (Si-MA-T1) kill *E. coli* bacteria (Figure C.2, A), whereas Si-MA-Φ11 phages kill *S. aureus* bacteria (Figure C.2, B). The effectiveness of T1 and Φ11 phages covalently attached to Si surfaces is apparent.

Plaque Formation Assays of Surface Controls

In contrast to phage attached PE-MA-T1 and PTFE-MA-Φ11 (Chapter V) and Si-MA-T1 and Si-MA-Φ11 (Figure C.2) surfaces, Figures C.3 and C.4 show that PE, PTFE, and Si (Figure C.3) as well as PE-MA, PTFE-MA, and Si-MA (Figure C.4) without the
covalent attachment of T1 and Φ11 phages do not exhibit antimicrobial activity against *E. coli* and *S. aureus*. These results confirm that antimicrobial activity observed for T1 and Φ11 attached PE, PTFE, and Si surfaces (Chapter V) is due to bacteriophage activity.
Figure C.1. A: ATR-FTIR spectra of Si surfaces; B: after plasma reactions on Si surfaces in the presence of maleic anhydride (Si-MA); C: after T1 (a) or Φ11 (b) phage covalent attachment to Si-MA surfaces (Si-MA-T1 and Si-MA-Φ11); D: Reference spectra of T1 (a) or Φ11 (b) (at 20% scale).

Figure C.2. Plaque formation assay on Si-MA-T1 (A) and Si-MA-Φ11 (B) substrates upon exposure to *E. coli* (A) and *S. aureus* (B).
Figure C.3. Plaque formation assay against *E. coli* on PE (A), PTFE (B), Si (C) surfaces and against *S. aureus* on PE (D), PTFE (E), and Si (F) surfaces.
Figure C.4. Plaque formation assay against *E. coli* for plasma reacted maleic anhydride PE-MA (A), PTFE-MA (B), and Si-MA (C), substrates and against *S. aureus* bacteria plasma reacted maleic anhydride on PE-MA (D), PTFE-MA (E), and Si-MA (F) surfaces.