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MOLECULAR DESIGN AND PATTERNING OF BIOSURFACES ON
POLY(TETRAFLUOROETHYLENE) (PTFE)

by

Nattharika Aumsuwan

Abstract of a Dissertation
Submitted to the Graduate School
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ABSTRACT

MOLECULAR DESIGN AND PATTERNING OF BIOSURFACES ON POLY(TETRAFLUOROETHYLENE) (PTFE)

by Nattharika Aumsuwan

May 2010

This dissertation describes the design, synthesis, and development of biocompatible poly(tetrafluoroethylene) (PTFE) surfaces that exhibit anti-microbial, anti-coagulant, and dual functional surface properties. It consists of two parts: (1) design, synthesis, and analysis of antimicrobial and anti-coagulant PTFE surfaces, and (2) controllable micropatterning of anti-microbial and anti-coagulant species on the surface. PTFE was modified by Ar microwave plasma reactions in the presence of maleic anhydride, which upon hydrolysis creating COOH groups. These COOH primers were utilized as a platform for further surface reactions to attach polyethylene glycol (PEG) spacers, and penicillin (PEN) or ampicillin (AM) onto the PTFE surfaces. The use of a PEG spacer facilitates enhanced antimicrobial effectiveness of the antibiotics by increasing their mobility, allowing easier contact with the bacteria. Utilizing spectroscopic analysis combined with scanning electron microscopy these studies showed for the first time covalent attachment of PEN or AM leading to antimicrobial activities against Gram (+) and/or Gram (-) bacteria. Such chemically and morphologically modified PTFE surfaces showed effectiveness against Gram (+) Staphylococcus aureus and AM-modified PTFE surface are effective against Gram (+), Staphylococcus aureus, Bacillus thuringiensis, and Enterococcus faecalis, and Gram (-), Escherichia coli, Pseudomonas putida, and Salmonella enterica bacteria. Anti-coagulant PTFE properties
were achieved by covalent attachment of alternating multilayers (CAM) of heparin (HP) and PEG, with homogenous coverage and enhanced hemocompatibility, as manifested by a 75±1% decrease of the platelet adhesion and a 60±5% decrease of platelet activation. Finally, dual functional PTFE surfaces were generated by the simultaneous inkjet printing of biotinylated ampicillin (B-AM) and biotinylated heparin (B-HP) on streptavidin (STR) functionalized surfaces. Using this bioconjugation approach dots with a spatial resolution of 20 µm were printed side-by-side and in an alternating stripe pattern. The micropatterned surface showed antimicrobial activity against *S. aureus* bacteria.
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INTRODUCTION

This dissertation is concerned with new advances in creation and enhancement of biocompatibility of poly(tetrafluoroethylene) (PTFE) surfaces. The goal is to design and synthesize PTFE surfaces with anti-microbial and anti-coagulant properties. The last part of this work will also highlight the creation of a dual functional antimicrobial and anticoagulant PTFE surface using inkjet printing.

Chapter I focuses on a review of the recent advances of polymers in biomedical applications and significant reasons for surface modifications of polymeric materials that enhance antifouling, anticoagulant and antimicrobial properties. Protein absorption (biofouling), blood coagulation (thrombosis), and microbial growth (biofilm formation) are complex biological events that are closely related to one another, and it is imperative that each of these events be minimized for improved biocompatibility of polymer surfaces. Based on the mechanism of occurrence, a number of specific strategies have been taken to deal with these problems.

Chapter II describes the covalent attachment of poly(ethylene glycol) (PEG) and penicillin (PEN) to expanded polytetrafluoroethylene (ePTFE). This was accomplished by microwave plasma reactions in the presence of maleic anhydride (MA) to create COOH functional groups on ePTFE surfaces, followed by esterification reactions with 200 and 600 molecular weight linear PEG. Such surfaces were utilized for further reactions with PEN through etherification reactions to create anti-microbial surfaces. These reactions resulted in surface morphological changes, and spectroscopic analysis using attenuated total reflectance Fourier transform infrared spectroscopy (ATR FT-IR) revealed the formation of ester linkages resulting from reactions between PEN and PEG.
functionalities. Antibacterial activities were evaluated by a series of experiments where PEN-modified ePTFE specimens were immersed in a liquid *Staphylococcus aureus* (*S. aureus*) culture and the bacteria growth was quantified by measuring % absorbance of the suspension at 600 nm wavelength. The lowest absorbance was observed for the solution containing PEN-PEG-MA-ePTFE specimens, thus showing highly effective anti-bacterial activity toward gram (+) *S. aureus* bacteria.

Chapter III involved a quantitative of the PEN-modified ePTFE surfaces, evaluating the effectiveness of the PEN surfaces against the proliferation of microbes. Using variable angle attenuated total reflectance (ATR-FTIR) spectroscopy, the volume concentration changes of PEN was determined as a function of depth from the ePTFE surface. At depths ranging from 0.2 µm to 1.2 µm from the surface, PEN concentration levels decrease from 8.85 µg/m³ to 3.33 µg/m³. Assessments of concentration levels of the colony forming units (CFUs) of *S. aureus* bacteria as a function of contact time with the PEN-PEG-MA-ePTFE surfaces showed profound effectiveness of PEN in preventing microbial proliferation. Hydrolytic stability tests of PEN-PEG-MA-ePTFE surfaces revealed that even with a 32 % loss of PEN due to the cleavage of the ester linkages between PEN and PEG spacer, anti-microbial activity is still maintained.

Chapter IV addresses the attachment of a broad spectrum antibiotic, ampicillin (AM), to expanded poly (tetrafluoroethylene) (ePTFE) surfaces to form antimicrobial surfaces effective against gram (+), *Staphylococcus aureus*, *Bacillus thuringiensis*, and *Enterococcus faecalis*, and gram (-), *Escherichia coli*, *Pseudomonas putida*, and *Salmonella enterica* bacteria. These ePTFE surface modifications were accomplished by utilization of microwave maleic anhydride (MA) plasma reactions leading to the
formation of acid groups, followed by amidation reactions of heterofunctional NH₂/COOH-terminated polyethylene glycol (PEG). The final step, the attachment of AM to the PEG spacer, was achieved by amidation reactions between COOH-terminated PEG and NH₂ groups of AM. This approach protects the COOH-AM functionality and diminishes the possibility of hydrolysis of the antimicrobial active portion of AM. These studies also show that approximately 90% of AM molecules are still covalently attached to PEG-MA-ePTFE surfaces after exposure to the bacteria solutions, and even after a 24 hour period, the AM volume concentration at the surface changes only from 2.25 to 2.04 μg/m³. Depending upon the bacteria type, the bacteria suspensions containing AM-PEG-MA-ePTFE specimens retain 85-99 % of their initial optical density, indicating retardation of bacterial growth.

Chapter V reports on the synthetic approach of producing anticoagulant surfaces on poly(tetrafluoroethylene) (PTFE) substrates by covalently attaching multilayers (CAM) of heparin (HP) and poly(ethylene glycol) (PEG). While HP facilitates anticoagulant properties, the presence of PEG reduces non-specific adsorption of proteins. In an effort to achieve desirable surface properties, sequential covalently bonded layers were reacted to COOH-modified PTFE surfaces. The latter was facilitated by microwave plasma reactions in the presence of maleic anhydride which, upon hydrolysis, results in covalent bonding of COOH to the polymer surface. Spectroscopic and morphological analysis of the CAM surfaces showed that alternating layers of PEG and HP were covalently attached to COOH-PTFE surfaces. The volume concentration and surface density of PEG and HP on the PTFE surface achieved by the CAM process were 7.02-6.04 x 10⁻³ g/cm³ (2.1-1.8 x 10⁻⁷ g/cm²) and 9.3-8.7 x 10⁻³ g/cm³ (2.8-2.6 x 10⁻⁷ g/cm²).
g/cm$^2$), respectively, which exceeded minimum levels required to achieve anticoagulant properties. Such CAM functionalized PTFE surfaces were in-vitro tested for blood biocompatibility using ovine blood and showed that 75% decrease in platelet deposition and a 60% decrease in platelet activation.

Chapter VI illustrates the formation of multifunctional assemblies containing streptavidin (STR) - biotin (Bio) bioconjugates on poly(tetrafluoroethylene) (PTFE) surfaces. STR was conjugated to a Bio-functionalized PTFE surface was generated by grafting biotinylated PEG (B-PEG) to COOH modified PTFE (MA). Inkjet printing of biotinylated ampicillin (B-AM) and biotinylated heparin (B-HP) was used to immobilize these species on the PTFE surface in a spatially controlled patterning. Spectroscopic and morphological analysis showed that B-PEG was covalently bounded and that the STR-Bio conjugation occurred at the PTFE surface. Quantitative spectroscopic analysis showed the surface density of the COOH was 2.94x10$^{-7}$ g/cm$^2$, whereas the densities of the B-PEG and STR were 9.2x10$^{-8}$ g/cm$^2$ and 3.5x10$^{-8}$ g/cm$^2$, respectively. A 20 μm spatial resolution of inkjet printing was achieved. Chemical attachments and spatial distribution were detected using Internal reflection IR imaging (IRIRI). These experiments showed for the first time the effective creation of dual antimicrobial and anticoagulant functional PTFE surfaces using inkjet micro-patterning.
CHAPTER I

MOLECULAR DESIGN AND PATTERNING OF POLYMER BIOSURFACES

Introduction

The widespread utilization of polymers in biomedical field has significantly impacted methods of treatment of numerous medical conditions and ailments. This is attributed to tunable physical and chemical properties of polymers which have offered a broad spectrum of applications in implants,\textsuperscript{1-3} tissue engineering,\textsuperscript{3-6} medical devices,\textsuperscript{1, 3, 7} and drug delivery systems.\textsuperscript{1, 8, 9} More importantly, these materials can be integrated into biological environments due to inertness, biocompatibility and/or biodegradability. For that reason many polymers have been explored for biomedical use among which ultra-high molecular weight poly(ethylene) (UHMWPE), poly(methylmethacrylate) (PMMA), poly(ethyleneterephthalate) (PET), expanded polytetrafluoroethylene (ePTFE), polypropylene (PP), polyurethanes (PUR), poly(lactic acid) (PLA), poly(caprolactone) (PCL), and poly(lactide-co-glycotide) (PLGA) play a key role.

To achieve a high impact strength, low friction coefficient, and resistance to abrasion, UHMWPE\textsuperscript{3} is often used as a hip and knee replacement as well as artificial joint and spine implants. It is also self-lubricating with low moisture absorption.\textsuperscript{3} On the other hand, PMMA has been utilized in implants for replacement of intraocular lenses due to its transparency and UV resistance as well as for orthopedic surgery for bone cement and dentures.\textsuperscript{10} In contrast, due to non-toxicity and excellent chemical and thermal resistance, PET and ePTFE can be employed in implant vascular grafts.\textsuperscript{10} Due to porous morphologies the growth of tissues concatenated into the ePTFE matrix.\textsuperscript{6} The entanglement of the tissue with ePTFE networks prevents the living tissue from
recognizing the polymer as a foreign object. This polymer is particularly useful in heart patches and stapes prosthesis.

The need for hemodialysis membranes and mesh for hernia repairs requires materials with good physical strength and excellent impact resistance. It turns out that this combination of properties is offered by PP. In contrast, catheters and vascular prostheses require a combination of flexibility and strength, and PURs offer these attributes. In other applications, however, biodegradability is necessary, and therefore, PLA, PCL, and PLGA are often used in sutures, stents, and scaffolding materials for tissue engineering, where biodegradation kinetics, good mechanical properties, low immunogenicity and non-toxic are essential properties.

In view of these considerations the inert nature of polymeric materials combined with their excellent physical properties offer a number of specific applications. However, these materials are not exempt from absorption of non-specific proteins, platelet adhesion and activation or attack of microorganisms, such as bacteria and fungi which ultimately can lead to infections. Thus, surface functionalizations of polymers with bioactive molecules is one of the approaches to prevent the occurrence of these undesired events. Ideally, one would like to obtain polymers with multi-functional surfaces that exhibit tunable and stimuli-responsive properties.

Polymer Surface Reactions

The key aspect in determining biocompatibility of biopolymers in a given biological environment are surface properties. While some applications may require only a monolayer of bioactive molecules to be effective, there is often a desire to maximize the amount of bioactive molecules per unit area. Among the variety of surface modifications,
the most common approaches to immobilize bioactive molecules on polymeric surfaces is by either non-covalent or covalent bonding. While non-covalent bonding is accomplished by physisorption, electrostatic and hydrophobic interactions, ligand-receptor recognition, or H-bonding, covalent bonding is achieved by chemical reactions. Figure 1.1 illustrates selected examples of surface modifications, where (A) physisorption, (B) electrostatic interaction, (C) ligand-receptor recognition represent non-covalent approaches, and D-F are covalent approaches, where (D) photochemical reactions, (E) chemical reactions, and (F) high energy radiation.

Adsorption of bioactive molecules onto polymer surfaces is the simplest method of enhancing biopolymer surface properties. As an example, gentamicin is used to soak polymers for implants before surgery in order to prevent infections.\textsuperscript{13} Along the same lines, fibronectin and collagen are often applied to PLA surfaces in order to enhanced cell adhesion and proliferation.\textsuperscript{14} Layer-by-layer (lbl) surface modification is one of the non-covalent bonding approach that utilizes electrostatic interactions.\textsuperscript{15, 16} This process involves dipping of a charged substrate into a dilute aqueous solution of polyelectrolyte with opposite charges allowing adsorbing of alternating reverse charges on a substrate. Since multilayer films are constructed by sequential adsorptions of anionic and cationic polyelectrolytes, their stability is questionable.\textsuperscript{16} An example of lbl surface modification is multilayer thrombresistant thin films containing poly(ethylenimine) (PEI), dextran sulfate (DS), and heparin (HP), deposited on poly (vinyl chloride) (PVC) surfaces as illustrated in Figure 1.2.A.\textsuperscript{17} Aside from electrostatic interactions, one can take advantages of ligand-receptor recognitions through biotin-streptavidin/avidin which is the strongest reported non-covalent bond with disassociation constant $K_d$ of $10^{-15}$ M$^{18}$ where
specific affinity for biotin with four receptor sites lead to orientation specific immobilization on surfaces. Figure 1.2.B illustrates a deposition process of biotinylated RGD peptides on PLA surfaces through avidin-biotinylated ligands to generate a PLA cell-adhesive surfaces. Another example shown in Figure 1.2.C illustrates the ligand-receptor binding used for cell adhesion and signal transduction on polymer surfaces; (A) multivalent binding to protein target and (B) multivalent binding to cell surface receptors. Non covalent bonding offers opportunity for binding biomolecules to polymer surfaces, however in an effort to achieve significantly more stable and durable surfaces, covalent attachment is highly desirable.

UV radiation leading to photografting have been extensively used to graft bioactive molecules to polymer surfaces with the aid of photoinitiators and photosensitizer. Different functionalities can be introduced to the surface depending upon the chemical structure of the grafted polymer. The process begins with the UV light exposure of surface photoinitiator. For example, benzophenone (BPO) can be either coated on the surface or applied from a solution, which upon UV excitation extracts hydrogen atoms from a polymer substrate creating free radical reactive sites. Photografting onto biopolymer surfaces to enhance biocompatibility can be also achieved by the attachment of N-vinylpyrrolidone to PP surfaces to create an antimicrobial surfaces or the fabrication of DNA arrays onto PMMA with UV treatment. Aside from photografting, surface initiated polymerization, such as controlled living polymerizations including reversible-addition fragmentation chain transfer (RAFT) and atom transfer radical polymerization (ATRP) have gained significant interest because they provide controllable chain lengths as well as higher grafting density. The methods of
grafting-to (end-functionalized polymer chain are grafted to the substrate) and grafting-from (grafting reaction proceed by polymerization from the substrate) are illustrated in Figure 1.3.A. Examples of surface initiated polymerization are ATRP grafted dimethylamino-ethylemethacrylate from PP surfaces to create antimicrobial properties or grafting poly(poly(ethylene glycol) methyl ether monomethacrylate) (PPEGMA) to poly(dimethyl siloxane) (PDMS) as illustrates in Figure 1.3.B. Aside from ATRP, triblock copolymer grafting onto UV radiated PDMS surface to manipulate the albumin adsorption via RAFT polymerization was investigated.

Chemical modifications or wet chemistry can be utilized to modify polymer surface without specialized equipment requirement. The process involves the use of liquid reagents that treated the polymer surfaces to create reactive groups include aminolysis and alkaline, acidic hydrolysis, and hydrogen peroxide treatment. Examples of chemical modification are the submersion of PE into aqueous solution of chromium trioxide and sulfuric acid to generate COOH groups, or amine formation on PMMA surfaces by aminolysis of 1,6-hexanediame. In general, this approach offer a simple way to modify polymer surface, but it provides hazardous chemical waste and may lead to undesired surface etching. In addition this technique is nonspecific and cannot be repeatable between polymers with different molecular weight, crystallinity, or tacticity.

High energy radiation including electron beam radiation and plasma reactions is another efficient and potent method for modifying the surface properties of biopolymers without affecting their bulk properties. In particular microwave plasma offers a clean, solventless route, and the surface reactions can be performed in a matter of seconds.
Plasma reactions require gas environments, typically argon (Ar), ammonia (NH₃), oxygen (O₂), carbon dioxide (CO₂), or nitrogen (N₂) make vacuum. Upon glow discharge plasma, gases are ionized to generate highly reactive species within the reactor. These reactive species interact with the polymer surface in the reactor, resulting in both chemical and physical alternations at the surface. The plasma induced changes at the substrate surface can reach depths of several hundred angstroms up to 10 microns. Several studies have been employed plasma reactions for modifying biopolymer surfaces including patterning of PDMS followed by the ammonolysis with amoxicillin to create antimicrobial surfaces. The sequence of events leading to the formation of patterned PDMS is illustrated in Figure 1.3.C. Another example is immobilization of fibroblast growth factor on plasma treated PLGA surfaces to improve cell adhesion.

There are significant reasons for surface modifications of polymeric materials, especially regarding biomedical applications, are enhancement of antifouling, anticoagulant and antimicrobial properties. Protein absorption (biofouling), blood coagulation (thrombosis), and microbial growth (biofilm formation) are complex biological events that are closely related to one another, and it is imperative that each of these events be minimized for improved biocompatibility. Based on the mechanism of occurrence, a number of specific strategies have been taken to prevent these problems.

Antifouling Surfaces

The initial event when a polymer comes into contact with a biological environment is the adsorption of proteins. The adsorbed protein layers will influence the subsequent biological reactions including platelet adhesion and activation. The process is very complicated and depends on several factors including surface energy,
electrostatic interactions between the protein and the surface, protein concentration levels, all of which influence by protein structure.\textsuperscript{19,30} Protein adsorption on the surface has been studied for many decades and two modes of interaction illustrated in Figure 1.4.A\textsuperscript{31} may be distinguished. The primary adsorption (i) mode occurs at the polymer brush-substrate interface, caused by small proteins that can penetrate through the polymer brush and adsorb at the grafting surface or secondary (ii) mode causes by larger proteins occurs at the brush-solvent interface,\textsuperscript{10} which is dictated by van der Waals attraction\textsuperscript{32}. A number of approaches have been proposed to prevent non-specific protein adsorption, and most of them rely on the attachment of poly(ethylene glycol) (PEG) to the surface due to its non-ionic, hydrophilic, low toxicity, and immunogenicity.\textsuperscript{30,33} The solubility in water of PEG results in preferred polar gauche conformations as well as hydrogen bonding with water via its two hydrogen bond acceptors. This is shown in Figure 1.4.B\textsuperscript{32} where the conformation causes the extensive hydration of PEG in aqueous environments and due to high chain mobility and flexibility, a steric exclusion repels proteins\textsuperscript{34}. Functionalized PEG and PEO onto polymer surfaces such as PTFE,\textsuperscript{25} PE,\textsuperscript{11} PU,\textsuperscript{33} PDMS,\textsuperscript{20} PP,\textsuperscript{19} PMMA,\textsuperscript{6} PVDF,\textsuperscript{26} as well as copolymer\textsuperscript{27} using different surface modification approaches which are plasma reactions, photo-irradiation, and surface initiated polymerization have been reported. Figure 1.5.A illustrates the schematic diagram of Ar plasma treated PVDF membrane with PEG to obtain antifouling surface.\textsuperscript{26}

Polymer containing zwitterionic molecules, which are phosphorylcholine (PC) and polybetaine such as carboxybetaine (CB) and sulfobetaine (SB) are also utilized as antifouling polymers due to their high hydration capacity as well as electroneutrality.\textsuperscript{35,36} The chemical structures of zwitterionic PC, CB, and SB are shown in Figure 1.5.B.
Polymer surfaces grafted with zwitterionic polymers have been reported\textsuperscript{37} and representative examples are the grafting of zwitterionic PC onto PDMS surfaces via photo-induced polymerization which exhibit antifouling properties\textsuperscript{35} and grafted 2-methacryloyloxyethyl phosphorylcholine (MPC) to PE membrane to resist cell adhesion.\textsuperscript{29}

**Anticoagulant Surfaces**

It is well established that adsorption of non-specific proteins will lead to platelet adhesion and blood coagulation known as thrombus. A key protein leading to platelet aggregation is fibrinogen that exhibit bridging the platelets.\textsuperscript{29} Thrombosis or blood clotting within minor wounds is essential for hemostasis and schematically is illustrated in Figure 1.6.A.\textsuperscript{38} The first step begins with the adhesion of platelets forming a plug at the site of a wound within a blood vessel, referred to as primary hemostasis. The second stage involves clotting factor proteins in the blood plasma that activate the prothrombin to form thrombin. The thrombin latter being responsible for converting fibrinogen into fibrin, strengthening and bridging the platelet plug. This fibrin mesh along with the platelets and other trapped red blood cells pull the hole in vessel together to allow healing. After the clot has completed, it is dissolved in a process called fibrinolysis.\textsuperscript{39, 40}

To prevent thrombosis amino acids such as hirudin and bivalirudin are used.\textsuperscript{41} Many studies have involved covalent immobilization these amino acids to polymer surfaces such as PET,\textsuperscript{42} PLGA,\textsuperscript{43} and poly(carbonate)urethane (PCUR)\textsuperscript{44} via carboxylic acid groups to inhibit the formation of thrombus. Other species such as heparin, a linear polysaccharide consisting of uronic acid-(1,4)-\textit{D}-glucosamine repeating disaccharide subunits,\textsuperscript{45} known as HP are also utilized. Its anticoagulant activity is achieved by
binding to a thrombin inhibitor, which is known as antithrombin III (AT-III), through lysine sites leading to conformational changes that accelerate and covalently bond to the clotting enzymes.\textsuperscript{45, 46} As illustrated in Figure 1.6.B, heparin dissociates from the complex and can be reutilized.\textsuperscript{46} The unique heparin pentasacharide sequence binding to antithrombin is shown in Figure 1.6.C.\textsuperscript{45} Heparin coagulation activity can vary and its anticoagulant properties are influenced by the chain length of the molecule. A number of studies have involved immobilization of heparin to polymer surfaces including PTFE,\textsuperscript{47, 48} PU,\textsuperscript{10} and PET\textsuperscript{47} via lbl surface modification with the aim of developing hemocompatible devices useful in variety of biomedical applications such as dialysis membranes, balloon catheters, coronary stents, and vascular prostheses.\textsuperscript{10, 41, 49}

Acetyl salicylic acid, also known as aspirin, and dipyridamole are also utilized as antiplatelet agents. Their anticoagulant activities involve the inactivation of clotting enzyme that facilitate the thrombus formation. Asparin have been incorporated into polymer matrix such as PLCA\textsuperscript{50} and PVA\textsuperscript{51} to enhance the blood compatibility or the covalently attached of dipyridamole onto PUR surface via photomodification.\textsuperscript{52}

Minimization of biofouling and blood coagulation at the surface through various surface modifications greatly enhances biocompatibility of polymeric materials; however, infections may develop postsurgically on implant or device surfaces. The number of deaths resulting from infection continues to increase annually, and approximately 64% of infections acquired at hospitals can be attributed to attachment of viable bacteria to medical devices and implants.\textsuperscript{15} This problem is critical and escalating, and many of these infections could be avoided by taking the appropriate preventative measures. The
introduction of antimicrobial agents onto the surface of medical devices and implants will
discourage bacteria attachment as well as prevent biofilm formation.

Antimicrobial Surfaces

From the staining point of view, bacteria are classified into two types. Gram
positive (Gram (+)) bacteria such as *S. aureus* exhibit an outermost multilayer
peptidoglycan cell wall, embedded with teichoic acid polymer, which is an inner cell
membrane that can include ion channels and protein receptors. Gram negative (Gram (-)) bacteria such as *E. coli* exhibit a single peptidoglycan layer between a liposacharide
rich inner layer as shown in Figure 1.7.A. The adhesion of bacteria cells to polymer
surfaces and interfaces is the first step in bacterial colonization and biofilm formation. A
biofilm is an aggregate of microorganisms in which cells are stuck to each other and
adhere to the surface. Three stages of biofilm development and the biofilm formed on a
catheter surface are illustrated in Figure 1.7.B. The first stage involves attachment of
free-floating bacteria to the surface, followed by its adhesion. This process can be
affected by many factors among which surface morphology, electrostatic charges, and/or
hydrophobicity play a key role. Upon attachment, an extracellular polymer matrix (EPS)
is produced which consists of poly-N-acetylglucosamine (PNAG), also known as
polysaccharide intercellular adhesion (PIA). PNAG acts as a glue that sticks the bacteria
cells together by electrostatics due to the bacteria cell surface is negatively charged
and PNAG carries a positively charge. After the biofilm has developed, it will form a
complex three-dimensional mushroom-like or tower structure, this is known as stage II. Biofilms can propagate through detachment of small or large clumps of cells (stage III)
which allow the bacteria to attach to other surfaces.
Several attempts have been made to render biomaterials with the ability to resist microbial film formation. These have included adding antimicrobial agents into the polymer during processing or immobilizing the agents onto the polymer surfaces.\textsuperscript{13} Antimicrobial agents include, for example, quaternary ammonium (or phosphonium) salts (QAS), chitosan, antimicrobial peptides (AMPs), silver ions (Ag\textsuperscript{+}), and antibiotics. These species exhibit various mechanisms of inhibiting bacterial growth.

QAS is a positively charge polyions with the structure of NR\textsubscript{4}\textsuperscript{+} where R is an alkyl group, and some examples of QAS are shown in Figure 1.8.A.\textsuperscript{13,57} QAS kill bacteria via the interaction with their cell walls, causing in intracellular contents to leak out, leading to cell death. QAS have been immobilized to polymer surfaces such as PP\textsuperscript{58} via photo-induced polymerization and show bactericidal properties against \textit{S. aureus} and \textit{E. Coli} bacteria.\textsuperscript{58} The covalent attachment of poly(vinyl-N-hexylpyridinium) onto HDPE and PET surfaces also show bactericidal against \textit{S. aureus} and \textit{E. Coli}.\textsuperscript{59}

Along the same lines, chitosan, a cationic polysaccharide obtained from the deacetylation of chitin, is also a known antimicrobial agent.\textsuperscript{60} Interaction between positively charged chitosan molecules and the negatively charged microbial cell membrane lead to the alternation of cell permeability causing leakage of intracellular contents, leading to cell death.\textsuperscript{60} Chitosan has been grafted to PET via Ar plasma reaction as illustrated in Figure 1.8.B\textsuperscript{13} and the antimicrobial activity against \textit{S. aureus} has shown high growth inhibition.\textsuperscript{13} AMPs are cationic small peptides that share similar mechanism of action with QAS and chitosan. Cationic AMPs will bind to microbe cell surfaces which will cause microbe membrane disruption.\textsuperscript{61-63} The attachment of AMPs to polymer surface have been studied including covalent grafted AMPs to PS surface, and the growth
of *E. Coli, L. monocytogenes, S. aureus, P. fluorescens*, and *K. marxianus* were suppressed.\(^{64}\)

These antimicrobial agents that display positive charges share the mode of action of penetrating the cell membrane or inducing cation exchange that disrupts the membrane integrity, leading to cell lysis. However, Ag\(^+\) which also utilized as antimicrobial agent shows a different mode of action.\(^{65}\) Ag\(^+\) binds to electron donor groups containing sulfur, oxygen, or nitrogen, which are present in biological molecules as thio, amino, imidazole, carboxylate and phosphate groups.\(^{66}\) By displacing other important ions such as Ca\(^{2+}\) and Zn\(^+\), the Ag\(^+\) effectively interrupts a number of cellular transport and oxidation processes.\(^{65,66}\) Ag\(^+\) have been incorporated to PET film by lbl surface modification with PEI and PAA as illustrates in Figure 1.8.C and shows the biocidal against *S. aureus* and *E. Coli*.\(^{67}\)

Antibiotics also provide antimicrobial properties with different mechanisms of action. The two common classes are aminoglycosides and \(\beta\)-lactams. Aminoglycosides, such as gentamicin (GEN) and streptomycin (STM), inhibit bacteria protein synthesis through irreversible binding to the 30S bacterial ribosome causing the ribosome to misread the genetic code and interrupt the initiation of protein synthesis.\(^{68,69}\) On the other hand, \(\beta\)-lactams such as penicillin (PEN) and ampicillin (AM), inhibit bacteria peptidoglycan cell wall synthesis by inhibiting the final crosslinking transpeptidation.\(^{68}\) This is achieved through binding with transpeptidases and disrupting cell wall synthesis, leading to cell lysis. The chemical structures of selected examples of antibiotics are shown in Figure 1.9.A where (a) GEN, (b) STM, (c) PEN, and (d) AM.
The bacteria cell wall, also known as peptidoglycan, is composed of repeating disaccharides with two sugar subunits, \(\text{N-acetylglucosamine (NAG)}\) and \(\text{N-acetylmuramic acid (NAM)}\). The crosslinking of the chains takes place in the final stages of cell wall synthesis and involves the bacterial enzymes transpeptidase. The serine of transpeptidase attacks the carbonyl of the penultimate D-Ala of peptidoglycan precursor, leading to the cleavage of the D-Ala terminate and formation of an acyl-enzyme intermediate. An amine from the side chain of another peptidoglycan then reacts with the ester of the acyl-enzyme intermediate to obtain the crosslinked matrix,\(^6\) as illustrated in Figure 1.9.B.

\(\text{\beta-lactam antibiotics mimic the conformation of the acyl-D-Ala-D-Ala of peptidoglycan as shown in Figure 1.10.A. Similarity in structure allows the antibiotics to act as inhibitory substrates. When the serine of peptidoglycan reacts with the antibiotic, the enzyme no longer functions, and as a result, transpeptidation is inhibited and the bacterial cell dies. Figure 1.10.B illustrates the reaction of PEN with the serine transpeptidase that terminates the transpeptidation process.}^7\) A number of studies have involved incorporation of antibiotics on polymer surface and within the polymer matrix to enhance antimicrobial properties. For example, GEN was immobilized to PP surface using a hydrogen peroxide aqueous solution\(^7\) or Ibl deposition of GEN with PEI and PSS polyelectrolyte effectively prevent proliferation of \(\text{S. aureus}\) bacteria.\(^7\) Along the same lines, both GEN and PEN were utilized to modify poly(amide) (PA) fibers via reaction with poly(acrylic acid) that was grafted on the PA surface. As a result, PA showed strong biocidal effects on \(\text{S. aureus and E. coli.}^7\)
In summary, surface modifications of polymeric materials for biomedical applications is of significant importance because they will eliminate undesired events occurring at the polymer surface leading to biofouling, thrombosis, infection, and/or death. These events can be minimized with the appropriate surface treatments without altering the bulk polymer properties. The adsorption of non-specific proteins on polymer surfaces has primarily been dealt with by the immobilization of PEG or zwitterionic polymers which become hydrated in biological environments and repel proteins. However, minimization of adsorbed proteins is only the first step in enhancing biocompatibility; further treatment is required to avoid thrombosis and infection. Thrombosis has been prevented by immobilization of anticoagulant agents such as heparin which will bind to a thrombin inhibitor and deactivate the clotting factor enzymes, inhibiting thrombosis formation. However, these anticoagulant agents mostly are attached to the surface by electrostatic interaction, which may easily washed away in a short period of time. Finally, in order to improve overall biocompatibility it is desirable to immobilize agents for prevention of infection. Several approaches have involved antimicrobial agents being physisorped or covalently immobilized onto polymer surfaces. However those agents, in particular ones containing metals, may render the polymer dangerous for implantation due to potential leaching of toxic materials into the bloodstream. As an alternative, covalent attachment of antibiotics results in long term stability as well as the ability to kill a broad spectrum of microbes. In addition, the use of a spacer can enhance the effectiveness of antibiotic by increasing mobility and allowing easier contact with microbes. The great need for materials that fight infection has given incentive for discovery and use of antimicrobial polymers.
Figure 1.1. Polymer surface modification approaches; (A) physisorption, (B) electrostatic interaction, (C) ligand-receptor recognition, (D) photochemical reactions, (E) chemical reactions, and (F) high energy radiation.
**Figure 1.2.A.** Schematic diagram of multilayered structure on PVC surface.\(^{17}\)

**Figure 1.2.B.** Schematic diagram of biotinylated RGD deposited on PLA surface through avidin-biotin recognition.\(^{18}\)

**Figure 1.2.C.** Schematics of multivalent binding between ligands and receptors; (A) multivalent binding to protein target, (B) multivalent binding to cell surface receptors.\(^{9}\)
Figure 1.3.A. Surface-initiated polymerization with (A) grafting-to and (B) grafting-from methods.\textsuperscript{22}

Figure 1.3.B. Surface modification of PDMS with PPEGMA brush via ATRP.\textsuperscript{23}

Figure 1.3.C. PDMS surface patterning by microwave plasma reactions.\textsuperscript{27}
Figure 1.4.A. Two modes of adsorption of a particle on a polymeric layer grafted to a substrate: (i) primary and (ii) secondary adsorption.\textsuperscript{31}

Figure 1.4.B. Trans and gauche conformations of PEG and their hydrogen bonding with water.
**Figure 1.5. A.** Schematic diagram of antifouling PVDF membrane with PEG chain via Ar plasma treatment.

**Figure 1.5.B.** Chemical structures of zwitterionic (a) PC, (b) CB, and (c) SB.
Figure 1.6.A. The mechanism of blood clotting in hemostasis.\textsuperscript{38}

Figure 1.6.B. Inactivation of clotting enzymes by heparin.\textsuperscript{46}

Figure 1.6.C. The unique antithrombin III-binding pentasaccharide sequence of heparin (anticoagulant moiety).\textsuperscript{45}
**Figure 1.7.A.** Structures of Gram (+) and Gram (-) bacteria.\(^{15}\)

**Figure 1.7.B.** Schematic diagram of biofilm development: stage I-attachment, stage II-maturation, stage III-detachment; and the S.aureus biofilm formed on the catheter surface.\(^{54}\)
Figure 1.8.A. Chemical structure of (a) QAS glycidyl methacrylate (GMA)-1,4 divinylbenzene and (b) QAS hydrantoinyl/quat siloxane copolymers.\textsuperscript{13, 57}

Figure 1.8.B. Schematic diagram of chitosan-grafted PET via O\textsubscript{2} plasma treatment.\textsuperscript{13}

Figure 1.8.C. Schematic diagram of the lbl of PET film consisted of PEI, AgNO\textsubscript{3}, and PAA.\textsuperscript{67}
Figure 1.9.A. Chemical structures of (a) GEN, (b) STM, (c) PEN, and (d) AM.
Figure 1.9.B. Biosynthesis of bacterial peptidoglycan cell wall synthesis.
Figure 1.10.A. The chemical structure of penicillin and D-Ala-D-Ala.

Figure 1.10.B. Schematic diagram of serine transpeptidase enzyme reacts with penicillin.
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CHAPTER II

ANTIMICROBIAL SURFACES ON EXPANDED POLY(TETRAFLUOROETHYLENE): PENICILLIN ATTACHMENT

Introduction

Ongoing search for materials that could be either utilized as implants or other devices in contact with a human body continues to be a major challenge. Although various polymers have found diversified biomedical applications, it is apparent that there is virtually no polymeric material that requires no surface modifications. By modifying surfaces one may achieve a number of desirable properties which range from blood clotting prevention to controllable drug release, and other applications, while maintaining useful bulk polymer properties. A variety of polymers are being utilized in these applications; for example, poly(vinyl chloride) (PVC) serves in cardiac catheters, surgical tapes, artificial hearts, blood pumps and artificial limbs,\(^1\)\(^{-5}\) and requires different surface modifications than poly(methyl methacrylate) (PMMA) utilized for contact lenses, bone cement, artificial teeth, and dental fillings.\(^1\)\(^{,6-8}\) Along the same lines, poly(dimethyl siloxane) (PDMS) is used for contact lenses, artificial skin, oxygenators, and drug delivery system,\(^1\)\(^{,9-14}\) thus necessitating different surface modifications than expanded poly(tetrafluoroethylene) (ePTFE) utilized for vascular graft prostheses, heart patches, or stapes prosthesis.\(^1\)\(^{,15-20}\)

Regardless of specific surface modifications, all biomaterials are susceptible to bacterial attacks, which may have detrimental effects. Thus, many efforts have been made to generate polymeric surfaces with desirable bio-properties that exhibit antimicrobial activity, and recent studies showed PDMS-amoxicillin surface modifications.\(^{14}\)
Since e-PTFE is a nonreactive and non-toxic flouro-containing polymer, multiple medical devices ranging from vascular grafts to mitral valve tendon replacements, or orthopedic surgeries and soft tissue in plastic and reconstructive surgeries,\textsuperscript{21-23} have been developed. However, its surfaces, just like other polymeric materials implanted into biological environments are not exempt from the bacterial attacks.\textsuperscript{24, 25} Staphylococcus aureus bacteria is particularly important because it causes suppurative infections and toxinoses in humans as well as superficial skin lesions such as styes, boils and furunculosis.\textsuperscript{26}

In view of these considerations, one possible solution is to attach antibiotic molecules to polymeric surfaces in order to inhibit the growth of bacteria. This study focuses on surface modifications of ePTFE that lead to the attachment of penicillin which was selected because of its known ability to inhibit gram-positive bacteria growth.\textsuperscript{27} This paper describes for the first time simple surface modifications using microwave plasma and chemical reactions which effectively prevent the growth of Staphylococcus aureus bacteria on ePTFE surfaces which is schematically illustrated in Figure 2.1, where the employment of surface microwave plasma reactions in the presence of maleic anhydride, followed by surface hydrolysis provides the platform for generating acid groups, followed by esterification reactions employing polyethylene glycol (PEG), and reactions with penicillin (PEN).

Experimental

ePTFE specimens were purchased from Philips Sci Inc. (Rock Hill, SC), cut to 7 x 7 mm squares, followed by washing with acetone in an ultrasonic washer, and dried at room temperature under vacuum conditions before use. Plasma reactions were conducted using open reactor conditions, as described elsewhere.\textsuperscript{28} The ePTFE substrate and 100 mg
of solid maleic anhydride (MA) (Aldrich Chemical Co.) were placed into the microwave reactor chamber and spaced 8.5 cm apart of each other. In a typical experiment, the reactor was evacuated to 150 mtorr, followed by purging it with Ar gas to reach a steady state pressure of 250 mtorr at a flow rate of 2.96 ml/min. At this point, microwave radiation at 600 W of power with an output frequency of 2.45 GHz was turned on to induce plasma formation (Figure 2.1, step 1). Under these conditions, the reaction chamber pressure increases continuously during the microwave plasma discharge. In an effort to maintain plasma environment during longer exposure times, a vacuum was applied continuously to maintain pressure conditions during the experiment. Since monomeric and polymeric forms of MA are water soluble, in an effort to determine stability of the surface treatments and to ensure that the newly formed species are not physisorbed on the surface, the samples were boiled in water for 30 min (Figure 2.1, step 2). After drying, specimens were stored in a desiccator under ambient conditions.

In order to modify MA-ePTFE surfaces with penicillin (PEN) (Sigma Inc.) we utilized an esterification reaction using 4-(dimethylamino)-pyridine (DMAP) and dicyclohexyl-carbodiimide (DCC) coupling agent. Polyethyleneglycol (PEG) (Aldrich) was used as a spacer between modified ePTFE surfaces and PEN. Acid groups on ePTFE surfaces were first converted into acid chloride using thionyl chloride under reflux conditions at 65°C for 6 hours (Figure 2.1, step 3). The sample was removed from the flask and washed with chloroform in order to eliminate excess thionyl chloride. The acid chloride ePTFE surfaces were then placed into a chloroform solution of PEG containing 1:1 molar ratio of linear PEG 200 and 600 molecular weight. The esterification reaction was carried out in a sealed flask at room temperature for 18 hours.
A small amount (1-2 drops) of triethylamine was added into the reaction flask at the onset of the reaction in order to neutralize hydrochloric acid that was generated during the reaction (Figure 2.1, step 4). The sample was washed with chloroform several times to remove un-reacted PEG, followed by the final wash with distilled water for 2 hours.

Reactions of PEG-MA-ePTFE with PEN were conducted using esterification process (Figure 2.1, step 5). The K salt of penicillin V (PEN V) (1.5 mmol) was dissolved in a small volume of water, cooled, and acidified with 0.1 N HCl. Precipitated PEN V was filtered and dried in a vacuum oven at room temperature for 1 hour. PEG-MA-ePTFE specimens and DMAP (0.25 mmol) were placed into a 100 ml flask with 20 ml of methylene chloride. In the next step, dried PEN V was added to the mixture, then stirred and cooled in an ice-water bath. DCC (1.3mmol) was added and the mixture was continuously stirred for 4 hours. Upon removal, all specimens were washed in methylene chloride sequentially for 2 hours, dried for 24 hours, and analyzed.

In order to determine anti-microbial activity of PEN-PEG-MA-ePTFE surfaces, Staphylococcus aureus (RN 6390) and Pseudomonas aeruginosa (ATCC, Rockville, MD) were allowed to grow overnight in LB broth and King’s medium, respectively. A series of specimens (ePTFE, MA-ePTFE, PEG-MA-ePTFE, PEN-PEG-MA-ePTFE and PEN-ePTFE) were immersed into freshly incubated cultures of each bacteria and incubated at 37°C 3 to 4 hours. Anti-microbial activity was determined by measuring the absorbance at 600 nm using UV-VIS spectrometer (Beckman DU-600).

A scanning electron microscope (SEM) Quanta FEI series 200 FEG was used to evaluate surface morphologies. All specimens were sputter coated with gold and analyzed at a 45° angle with a scanning electron beam. 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 a deuterated triglycine sulphate (DTGS) detector and a 45° face angle Ge crystal. Each spectrum represents 400 co-added scans ratioed against a reference spectrum obtained by recording 400 co-added scans of an empty ATR cell. All spectra were corrected spectral distortions using Q-ATR software.³²

Results and Discussion

Figure 2.1 illustrates a schematic diagram of surface reactions employed in order to obtain anti-microbial ePTFE surfaces. As indicated in the Introduction, the effective method of generating acid groups on polymeric surfaces is the employment of surface microwave plasma reactions in the presence of maleic anhydride, followed by surface hydrolysis.²⁸ These reactions are illustrated in Figure 2.1, Steps 1 and 2. In an effort to confirm that indeed these reactions have occurred on the ePTFE surface we analyzed the surfaces. Figure 2.2, Traces A and B, illustrate ATR-FTIR spectra recorded from the surface of ePTFE before and after reactions, respectively. As expected, there are no bands in the 1900-1500 cm⁻¹ region for ePTFE (Trace A). In contrast, as illustrated in Trace B, the bands at 1781, 1852 and 1710 cm⁻¹ are detected as a result of microwave plasma reactions (Figure 2.1, Steps 1 and 2). These bands are attributed to anhydride C=O and acid C=O stretching vibrations³³-³⁵ and their presence indicates that indeed ePTFE surfaces were chemically modified through a C=C bond opening of the maleic anhydride ring and its hydrolysis.²⁸

One of the significant differences between PTFE and ePTFE polymers is surface morphology. As shown in Figure 2.3.A, SEM images of ePTFE show mesh-like network
morphology, which is functional in bio-environments as it exhibits the ability for body tissues to network with and grow into it. As shown in Figure 2.3, B and C, as a result of microwave plasma surface reactions without and in the presence of maleic anhydride, respectively, the morphology remains virtually the same, as manifested by SEM images, and the only difference is the formation of whitish particle-like sparkles. However, ATR-FITR measurements illustrated in Figure 2.2 clearly show that COOH modifications have occurred. It should be also noted that these reactions result in water contact angle changes from 125° for ePTFE to 95° for COOH-modified ePTFE.

The presence of COOH groups on the ePTFE surfaces is useful because such entities may serve for further reactions. Since our interest is the attachment of penicillin (PEN) that would effectively prevent the growth of bacteria, it is necessary to provide suitable surface functionality and morphology. Although acid groups are capable of reactions with PEN, in order to enhance anti-bacterial effectiveness of PEN, we introduced PEG flexible spacer between the COOH-functionalized surface and PEN molecules. The choice of PEG was dictated by its non-toxicity, biocompatibility, and the ability to swell in aqueous environments. Furthermore, in order to enhance antimicrobial surface activity, non-uniform surface morphology was facilitated by varied lengths of PEG spacers. The premise behind this is that by introducing random esterification reactions between acid chloride and hydroxyl groups of PEG with 200 and 600 MW molecular roughness is introduced, which is capable of enhancing antimicrobial functionality. The latter is believed to be attributed to the ability of PEN-terminated PEG to inhibit the growth of the bacteria as they deposit on the surface. Thus, the enhanced surface roughness obtained by varying PEG chain lengths will increase the effective
surface area in contact with the bacteria. This is illustrated in Figure 2.4, A, which schematically shows the attachment of PEG, followed by reactions with PEN (B), and anticipated retardation of the bacteria in contact with the modified surface (C). However, in order for the surface groups to be more reactive, COOH groups were first converted to acid chloride functionalities using SOCl₂ solvent. This is schematically illustrated in Figure 2.1, Step 3. This approach creates more reactive groups for further reactions with OH functionalities on PEG. Similarly to the previous experiments, we utilized ATR-FTIR to determine the extent of surface reactions. Figure 2.5.a and b, Traces A, B, and C show ATR-FTIR spectra of ePTFE (A), MA-ePTFE (B), and PEG-MA-ePTFE (C) in the 1300-1000 (a) and 1850-1680 (b) cm⁻¹ spectral region, respectively. While Traces A and B serve as references, Trace C illustrates the presence of the 1105 and 1734 cm⁻¹ bands due to C-O-C stretching and C=O ester vibrations resulting from esterification reactions. These spectra were normalized to the C-C stretching vibrations at 1177 cm⁻¹. SEM images shown in Figure 2.6.A, B, and C illustrate surface morphologies of ePTFE, MA-ePTFE, and PEG-MA-ePTFE, respectively, which are significantly altered as a result of the surface reactions and exhibit fewer voids.

As illustrated in Figure 2.1, Step 5, the final step of the process involves reactions of PEG-MA-ePTFE with PEN. For that reason we utilized esterification reactions with DCC as the coupling reagent and DMAP as the catalyst. These reactions were carried out in one step process and do not require prior activation of the reactants. As a result, PEN was attached to modified ePTFE surfaces via ester linkages. In order to illustrate that indeed this reaction occurred, ATR-FTIR analysis was performed and Figure 2.7 shows the results. Again, for reference purposes, Traces A and B represent the spectra of ePTFE
and PEN, respectively, while Trace E represents the spectrum of PEN-PEG-MA-ePTFE. As seen, the presence of the C=O vibrations at 1680, 1734 and 1780 cm$^{-1}$ which are attributed to amide, ester, and β-lactam C=O stretching vibrations, respectively, is detected and indicates that the β-lactam ring, which is the bio-active group on PEN, remains intact during the coupling reactions. In summary, spectroscopic and morphological data illustrate that PEN was chemically attached to the ePTFE surface, and the next question is how effective this approach is in the presence of *Staphylococcus aureus* bacteria.

In an effort to demonstrate the effectiveness of these surface reactions, a series of experiments was conducted where modified and unmodified ePTFE specimens were placed into bacterial cultures. Figure 2.8 illustrates a photograph that depicts turbidity differences in *Staphylococcus aureus* cultures as a result of ePTFE exposure. While test tubes #1, 2, and 3 represent three controls of bacterial growth in the presence of neat ePTFE, MA-ePTFE, and PEG-MA-ePTFE, test tube #4 shows a bacterial cultures growth with a PEN-PEG-MA-ePTFE specimen. Only the PEN-PEG-MA-ePTFE specimen was able to retard bacterial growth, as seen by the lack of turbidity in the growth medium. In contrast, the cloudiness of the solutions containing ePTFE, MA-ePTFE, and PEG-MA-ePTFE (test tubes #1, 2, and 3) indicates bacterial growth. Another control that was utilized is exposure of a bacterial culture to the PEN-ePTFE specimen. For that purpose direct ePTFE modification with PEN only (Steps 1-4 in Figure 2.1 were skipped) was attempted. As seen in Figure 2.8, the solution in test tube #5 is turbid and supports bacterial growth indicating that the reaction of PEN to unmodified ePTFE surfaces did not occur, thus ePTFE surfaces not capable of retarding the bacteria growth.
In order to quantify the antimicrobial effectiveness of surface-attached PEN the absorbance at 600 nm was measured for the solutions illustrated in Figure 2.8, 1-5. Figure 2.9, A illustrates the results of these experiments and shows that the lowest relative absorbance is detected for the solution exposed to PEN-PEG-MA-ePTFE specimen, thus demonstrating the antibacterial activity of this specimen. It should also be noted the above experiments were conducted using *Staphylococcus aureus* which is gram positive. The same series of experiments conducted using the gram negative *Pseudomonas aeruginosa* showed that the solution containing PEN-PEG-MA-ePTFE are turbid and show high relative absorbance values for all solutions including test tube #4 containing PEN-PEG-MA-ePTFE specimen. This is illustrated in Figure 2.9, B. As anticipated, theses experiments showed that PEN attached to modified ePTFE surfaces is effective for gram positive bacteria.

Conclusions

These studies show that maleic anhydride and carboxylic acid groups can be chemically bonded to ePTFE surfaces when microwave plasma radiation is utilized. Maleic anhydride reacts with ePTFE surfaces through a C=C bond opening of the maleic anhydride ring and its hydrolysis results in chemically attached carboxylic acid groups. Using esterification reactions in the presence of PEG spacer, PEN was reacted onto such ePTFE surfaces, which subsequently exhibits highly effective antimicrobial activity towards gram positive *Staphylococcus aureus* bacteria. This approach may serve as a general surface modification process for the development of polymeric surfaces with anti-microbial properties.
Figure 2.1. Schematic diagram of surface reactions on ePTFE: step 1 - Ar microwave plasma reaction; step 2 - hydrolysis of MA-ePTFE; step 3 - conversion of acid groups; step 4 - PEG reaction; step 5 - PEN reaction.
Figure 2.2. ATR-FTIR spectra of: A- ePTFE; B- maleic anhydride plasma reacted ePTFE (MA-ePTFE).
Figure 2.3. SEM images of: A-ePTFE; B-plasma reacted ePTFE; C – MA plasma reacted ePTFE (MA-ePTFE).
Figure 2.4. Representation of ePTFE surface modifications: A- PEG-MA-ePTFE; B- PEN-PEG-MA-ePTFE; C- retardation of bacteria by PEN-PEG-MA-ePTFE.
Figure 2.5. ATR-FTIR spectra of PEG-MA-ePTFE in (a) 1300-1000 cm\(^{-1}\) and (b) 1900-1600 cm\(^{-1}\). A- ePTFE; B- MA-ePTFE; C- PEG-MA-ePTFE.
Figure 2.6. SEM images of: A- ePTFE; B- MA-ePTFE; C- PEG-MA-ePTFE.
Figure 2.7. ATR-FTIR spectra in the 1900-1650 cm\(^{-1}\) region of: A- ePTFE; B- PEN; C- MA-ePTFE; D- PEG-MA-ePTFE; E- PEN-PEG-MA-ePTFE.
Figure 2.8. Photographs of test tubes containing the following specimens in *Staphylococcus aureus* cultures: ePTFE (1), MA-ePTFE (2), PEG-MA-ePTFE (3), PEN-PEG-MA-ePTFE (4) and PEN-ePTFE (5).
Figure 2.9. % Absorbance plotted for liquid (A) *Staphylococcus aureus* and (B) *Pseudomonas aeruginosa* bacterial cultures growth in the presence of ePTFE (1), MA-ePTFE (2), PEG-MA-ePTFE (3), PEN-PEG-MA-ePTFE (4) and PEN-ePTFE (5).
References


CHAPTER III

THE EFFECTIVENESS OF ANTIBIOTIC ACTIVITY OF PENICILLIN ATTACHED TO EXPANDED POLY(TETRAFLUOROETHYLENE) SURFACES:
A QUANTITATIVE ASSESSMENT

Introduction

It is well known that adhesion of bacteria to surfaces of medical implants and devices results in the formation of biofilms and often lead to the outbreak of detrimental infections. According to the US Centers for Disease Control (CDC), approximately 90,000 people annually die in the US alone from infections unrelated to their medical conditions.¹ To alleviate this serious problem, one approach is to disrupt the proliferation of microbes on surfaces and a number of studies²⁻⁷ have investigated and developed surface modifications with the goal of eliminating harmful microbial films. The recent Roundtable on Biomedical Engineering Materials and Applications (BEMA) at the National Academy of Sciences (NAS) manifests the need for further advances in this field.⁸

Although a number of polymer surface modification reactions have been offered ranging from grafting-from⁹⁻¹³ and grafting-to¹⁴⁻¹⁶ approaches, considering the fact that majority of polymeric substrates are hydrophobic and inert, it is often difficult to develop a universal surface modification that would provide a platform for further reactions of a variety of species leading to antimicrobial, antifouling, or anticoagulating surface properties. To add to this effort we developed solventless, clean, and ultra-fast microwave plasma surface reactions that utilizes maleic anhydride, which upon hydrolysis, forms COOH groups on poly(dimethylsiloxane) (PDMS)¹⁷⁻²⁰, poly(vinylidene
fluoride) (PVDF)\textsuperscript{21}, and expanded poly(tetrafluoroethylene) (ePTFE).\textsuperscript{22} The presence of COOH groups serves as a platform for further surface reactions shown in Figure 3.1, whereby the attachment of penicillin (PEN) was one of the recent developments that tested positive in proliferation of microbes.\textsuperscript{22} It should be also noted that the effectiveness of these surface reactions in preventing microbial film formation is not only accomplished by the presence of PEN, but one of the key components is a polyethylene glycol (PEG) spacer\textsuperscript{23} that was placed between COOH surface groups and terminal PEN. This approach facilitates the mobility of PEN molecules to become effective in preventing the proliferation of microbes, which we believe is a critical component to achieve effectiveness against infections. Thus a crucial component is the length of a PEG spacer and its lengths dispersity, which results in sub-nano level surface roughness resulting in anti-microbial activity as well as minimized protein adsorption.

In view of the previous finding and our limited knowledge regarding quantitative effectiveness of anti-microbial activity of PEN-PEG-MA-ePTFE surfaces this study extends the scope of the previous findings and focuses on a quantitative assessment of antibiotic effectiveness of these surfaces.

Experimental

The sequence of quantitative assessments of the antimicrobial activity of PEN-PEG-MA-ePTFE surfaces as a function of time is outlined in Figure 3.2. In the experiment A-1, 20 µl of the \textit{S. aureus}. overnight culture (label L-A) was combined with 15 ml of fresh Luria-Bertani (LB) broth. This culture is labeled L-A-1. Next, a 2 µl of the liquid culture from L-A-1 was extracted and mixed with a 3 ml of LB broth. This culture is labeled A-1-1. A 10 µl of the A-1-1 liquid culture was extracted and diluted 5 times in
order to reduce the amount of bacteria. This culture was spread over an agar plate to grow colonies (time=0). Colony forming units (CFUs) at time = 0 is determined by counting the colonies formed after incubating the agar plate for 16 hours. The concentration at time = 0 is used as an initial concentration. In the experiment A-2, a 2 µl of the liquid culture from L-A-1 was placed into six test tubes containing 3 ml of fresh LB broth. The content of the test tube A-2-1 was the positive control, whereas solutions A-2-2 through A-2-6 solutions contained ePTFE, MA-ePTFE, PEG-MA-ePTFE, PEN-PEG-MA-ePTFE, and ePTFE exposed in PEN solution (PEN-ePTFE), respectively. Typical size of each polymer specimen was 7x7x1.5 mm. The reason of exposing ePTFE in PEN solution was to examine if PEN physisorbs on the surface of ePTFE and becomes effective in anti-microbial activity. After ePTFE immersion in PEN, ePTFE was washed in methylene chloride and dried in a desiccator. All test tubes were incubated at 37 °C for 3, 6, 9, 12, and 24 hours. In experiment A-3 shown in Figure 3.2 aliquots of the liquid culture from each test tube were extracted at 3, 6, 9, 12, and 24 hours time intervals, diluted 6, 7, 8 and 9 times, respectively, and spread on agar plates to grow the colonies. After incubating the plates for 16 hours, CFUs in each aliquot were determined. Each data represents an average of three measurements.

Hydrolytic stability of PEN-PEG-MA-ePTFE surfaces was conducted by immersing a specimen in a phosphate-buffered saline (PBS) solution and incubating it at 37 °C. After 0, 1, 2, 3, 6, 9, 12, 18, and 24 hours exposure times, each specimen was dried and analyzed using ATR-FTIR spectroscopy.

Another set of experiments was performed to determine stability of PEN attached to ePTFE. This is depicted in Figure 3.3. In the experiment B-1, PEN-PEG-MA-ePTFE
specimens were immersed in the LB broth and PBS buffered solution for 2, (labeled B-1-A and B-1-B in Figure 3.3), 12 (labeled B-2-A and B-2-B in Figure 3.3), and 24 hrs (labeled B-3-A and B-3-B in Figure 3.3). After exposure, all specimens were dried in a desiccator for 24 hours, and LB that was used to immerse all specimens LB-B-1 (2 hrs), LB-B-2 (12 hrs), and LB-B-3 (24 hrs), were used again in experiment B-2. In this case, 2 \( \mu \)l solution of the \textit{S. aureus}. culture that was incubated for 16 hours, (L-A-2) was placed into four separate test tubes labeled A, B, C, and D. Test tube A contained fresh LB, whereas B contained LB-B-1, LB-B-2, and LB-B-3, tube C contained fresh LB and specimen B-1-A from experiment B-1, and test tube D contained fresh LB and the specimen B-1-B from experiment B-1. All test tubes were incubated for 4 hours, followed by solution optical density measurements using a UV-VIS spectrometer (Beckman DU-600).

Modified ePTFE surfaces (PEN-PEG-MA-ePTFE) were prepared as previously described.\textsuperscript{22} Attenuated total reflectance Fourier transform infrared (ATR FTIR) spectra were collected using a Bio-Rad FTS-6000 FT-IR single beam spectrometer set at a 4 cm\(^{-1}\) resolution equipped with a deuterated triglycine sulfate (DTGS) detector and a 45\(^{\circ}\) face angle Ge crystal. Each spectrum represents 400 co-added scans ratioed against a reference spectrum obtained by recording 400 co-added scans of an empty ATR cell. All spectra were corrected for spectral distortions using Q-ATR software.\textsuperscript{24} Variable angle ATR was employed to determine the volume concentration of newly formed species after each step of the reaction as a function of depth by using both Ge and KRS-5 50 x 20 x 3 mm crystals and angles varying from 35\(^{\circ}\) to 60\(^{\circ}\).
Since quantitative ATR-FTIR depth profiling requires knowledge of the extinction coefficient for each of these bands, a series of PEG/chloroform and PEN/methylene chloride solutions of known concentrations were prepared, and plots of the absorbance of the 1105 cm\(^{-1}\) C-O-C and 1780 cm\(^{-1}\) C=O stretching bands as a function of concentration were generated. The extinction coefficients of the bands due to PEG and PEN are 778.58 L/mol-cm. and 944.68 L/mol-cm, respectively, whereas the 1710 cm\(^{-1}\) extinction coefficient of C=O vibrations of COOH groups was previously determined to be 544.32 L/mol-cm.\(^{17, 21}\) Using double Kramers-Kronig transformation (KKT) and previously developed algorithm for quantitative analysis using ATR-FTIR spectroscopy,\(^{24}\) concentration levels of COOH groups resulting from the microwave plasma modifications, PEG reactions, and PEN attachment were determined.

**Results and Discussion**

As a first step in this study, we used ATR-FTIR spectroscopy to quantitatively analyze the volume concentration levels of surface species resulting from MA, PEG, and PEN reactions on ePTFE surfaces. Details regarding surface measurements were published elsewhere.\(^{17, 21}\) Here we focused on the characteristic IR bands at 1710, 1105, and 1780 cm\(^{-1}\), which were used to determine COOH, PEG and PEN concentrations as a function of depth from the surface, respectively. Figure 3.4, A-C illustrate volume concentration changes for COOH, PEG, and PEN as a function of depth. As seen, in all cases there is a decay in concentrations while probing from approximately 0.2 to 1.2 \(\mu m\) into the bulk. The volume concentration of the COOH groups decreases from 23.6 to 9.71\(\mu g/m^3\) when going from 0.2 to 1.2 \(\mu m\) and similar trends were observed for PEG and PEN, with the decrease from 12.8 to 7.84\(\mu g/m^3\) and 8.85 to 3.33\(\mu g/m^3\), respectively. An
estimated accuracy of the volume concentration measurements are $\pm 0.1 \mu g/m^3$. These data will be used later on to determine anti-microbial effectiveness of these reactions after a fraction of PEN molecules were hydrolyzed.

While one aspect of quantitative analysis is determination of surface concentration levels, another aspect is the response of bacteria solutions in contact with PEN-modified surfaces. To determine how each step of surface modification alters bacterial growth solution L-A-1 was utilized to conduct a series of experiments A-2 in Figure 3.2. Antimicrobial effectiveness of PEN-PEG-MA-ePTFE surfaces was examined by exposing ePTFE, MA-ePTFE, PEG-MA-ePTFE, PEN-PEG-MA-ePTFE, and PEN-ePTFE to *S. aureus*/LB broth liquid culture, followed by determination of the number of colony forming units (CFUs) as a function of time (Experiment A-3). While CFU represents the number of live bacteria in the culture, Figure 3.5.A illustrates photographs of agar plates with the cultures from A-2-1 through A-2-6 specimens after incubation for 3 hrs (Experiment A-2 and A-3). As seen, photograph A-2-1 is a positive control, whereas A-2-2, A-2-3, A-2-4, A-2-5, and A-2-6 represent the bacteria growth after immersion of ePTFE, MA-ePTFE, PEG-MA-ePTFE, PEN-PEG-MA-ePTFE and PEN-ePTFE, respectively. As seen, only when PEN-PEG-MA-ePTFE is exposed into the culture (A-2-5), formation of bacteria colonies is inhibited, as manifested by low number of colonies shown in photograph A-2-5.

In order to estimate CFU concentration levels in each of the experiments discussed above (Experiment A-3) the colonies were counted and their CFU/ml are plotted as a function of the exposure time. This is illustrated in Figure 3.5.B. As seen, all cultures except the one containing PEN-PEG-MA-ePTFE (A-2-5) the bacteria continue to
multiply. In the presence of PEN-PEG-MA-ePTFE, the bacteria population diminishes and after 24 hours no live bacteria are presented.

While these data undeniably show the effectiveness of PEN-PEG-MA-ePTFE surface modifications, one question that needs to be addressed is the stability of PEN-PEG linkages to hydrolysis. This is particularly important in view of the fact that these surfaces are exposed to aqueous environments and PEN is attached to the PEG spacer via an ester linkage known to have limited hydrolytic stability (Figure 3.1). To determine hydrolytic stability of this linkage we exposed PEN-PEG-MA-ePTFE specimen to PBS buffered solution and monitored the intensity changes of the C=O group (circled) as a function of exposure time. Figure 3.6.a illustrates a series of ATR-FTIR spectra in the C=O region and show that the ester linkage between the PEG spacer and PEN diminishes. However, after 24 hours of exposure, the band is still detected. The plot illustrated in Figure 3.6.b (a) shows the % absorbance loss (curve A) of PEN as a function of time. As seen, approximately 30 % decrease of the C=O bond intensity is observed after 24 hours of incubation at 37°C. In order to illustrate the actual concentration levels after 24 hours of exposure we utilized quantitative surface analysis shown in Figure 3.4 and converted the C=O intensity changes after hydrolysis to the actual PEN volume concentration changes at the surface of ePTFE. This is illustrated in Figure 3.6.b (Curve B) which shows that the actual volume concentration loss of PEN at 0.2 μm from the surface is 32 % with respect to its initial concentration.

These data prompted us to conduct further experiments in order to determine the effectiveness of the remaining PEN attached to PEG-MA-ePTFE surfaces. The sequence of experiments conducted in this part of the study is shown in Figure 3.2 and Figure 3.7.a
illustrates photographs of the A, B, and C series of solutions containing cultures of *Staphylococcus aureus* exposed to PEN-PEG-MA-ePTFE that have been immersed in PBS and LB broth for 2, 12, and 24 hours, respectively. Solutions A, A’, and A’’ shown in Figure 3.7.a are positive controls containing fresh LB and *Staphylococcus aureus* culture, whereas solutions B, B’, and B’’ are LB-B-1, LB-B-2, LB-B-3 and *Staphylococcus aureus* culture. However, solutions labeled C, C’, and C’’ are fresh LB, *Staphylococcus aureus* culture contain B-1-A, B-2-A, B-3-A specimens, respectively. Solutions D, D’, and D’’ are also fresh LB, *Staphylococcus aureus* culture containing B-1-B, B-2-B, B-3-B specimens, respectively. As seen in Figure 3.7.a, solutions A, A’, and A’’ are turbid, which is the indicative of the bacteria growth. In contrast, solutions B’, B’, and B’’ are clear and similar to those of C, C’ and C’’ and D, D’, and D’’, thus manifesting that the bacteria proliferation has been minimized even after about a 32% PEN loss during prior exposure and results from the hydrolysis of the ester linkage. To quantify these data, optical density of each culture was measured at 600 nm, which is illustrated in Figure 3.7.b, and plots the normalized optical density for each solution. As seen, B and C series give low relative absorbance values compared to A, which is a positive control, indicating that the effectiveness of anti-microbial activity for PEN-PEG-MA-ePTFE specimens still remains significant, even after 32% loss of the PEN molecules from the surfaces. It should be noted that PEN is effective toward gram positive bacteria and paralleled experiments conducted in the presence of gram positive and gram negative bacteria confirmed its effectiveness toward gram positive. This is illustrated in Figure 3.8 as the inhibition zone was surrounded the sample with *S. aureus* bacteria only.
The final set of experiments involved the analysis of the remaining active β-lactam ring of PEN in the surface of PEN-PEG-MA-ePTFE. After exposure to the *S.aureus* bacteria culture at 37°C for 24 hours a PEN-PEG-MA-ePTFE specimen was washed with PBS buffered solution three times, dried in a desiccator and ATR-FTIR analysis was conducted. Figure 3.9 shows ATR-FTIR spectra recorded from the PEN-PEG-MA-ePTFE surfaces before and after contact with the bacteria. As expected, the band at 1780 cm\(^{-1}\) attributed to the C=O of β-lactam ring disappears after contact with bacteria, which is attributed to the ring opening of β-lactam ring in reactions that inhibits cell wall synthesis preventing the cross linking of peptide chains in the peptidoglycan layer.\(^{27, 28}\)

Conclusions

These studies illustrate that antibacterial effectiveness of PEN-PEG-MA-ePTFE modified surfaces remains significant even after 24 hrs of exposure to *S.aureus*, gram positive bacteria. In spite of the loss of a 32% of PEN volume concentration these surfaces are still effective in preventing proliferation of microbes and the weakest link responsible for the loss of PEN from the surface is the ester linkage between PEN and PEG molecules. Using variable angle ATR-FTIR, volume concentration of PEN attached to the surface decreases from 8.85µg/m\(^3\) at 0.2 µm to 3.33x10\(^{-3}\) µg/m\(^3\) at 1.2 µm.
Figure 3.1. Molecular structure of PEN-PEG attached to the surface of MA-ePTFE.
Figure 3.2. Schematic diagram of experimental sequences to determine colony-forming units (CFUs).
**Figure 3.3.** Schematic diagram of experiments examining stability of PEN-PEG-MA-ePTFE surfaces.
Figure 3.4. The volume concentration plotted as a function of depth from the surface for: A- MA-ePTFE; B- PEG-MA-ePTFE; and C- PEN-PEG-MA-ePTFE.
Figure 3.5.A. Photographs of colonies form units (CFUs) of *S. aureus*/LB broth liquid culture (A-2-1), *S. aureus*/LB broth liquid culture containing ePTFE (A-2-2), *S. aureus*/LB broth liquid culture containing MA-ePTFE (A-2-3), *S. aureus*/LB broth liquid culture containing PEG-MA-ePTFE (A-2-4), *S. aureus*/LB broth liquid culture containing PEN-PEG-MA-ePTFE (A-2-5), and *S. aureus*/LB broth liquid culture containing PEN-ePTFE (A-2-6).
Figure 3.5.B. Concentration plotted as a function of time for the experimental sequence depicted in Figure 3.2: control (A-2-1), ePTFE (A-2-2), MA-ePTFE (A-2-3), PEG-MA-ePTFE (A-2-4), PEN-PEG-MA-ePTFE (A-2-5), and PEN-ePTFE (A-2-6).
Figure 3.6.A. ATR-FTIR spectra in the C=O region recorded during 0-24 hours exposure to PBS buffer solution: A-0; B-1 hour; C-2 hours; D-3 hours; E-6 hours; F-9 hours; G-12 hours; H-18 hours; and I-24 hours.

Figure 3.6.B. (A) - Percent absorbance loss of the C=O ester plotted as a function of exposure time of PEN-PEG-MA-ePTFE to PBS solution; (B) - Percent loss of PEN volume concentration as a function of exposure time of PEN-PEG-MA-ePTFE to PBS solution.
Figure 3.7.A. Photographs of solutions containing cultures of *S.aureus* exposed to PEN-PEG-MA-ePTFE for 2, 12, and 24 hours: A, A’, and A” are solutions of positive control containing fresh LB and *S.aureus* culture; B, B’, and B’’ are LB-B-1, LB-B-2, LB-B-3 and *S.aureus* culture; C, C’, and C”’ are fresh LB, *S.aureus* culture, and B-1-A, B-2-A, B-3-A specimens; D, D’, and D”’ are fresh LB, *S.aureus* culture and B-1-B, B-2-B, B-3-B specimens.
Figure 3.7.B. Relative Optical Density plotted as a function of time for A, B, C series solutions.
Figure 3.8. Antimicrobial activity of PEN-PEG-MA-ePTFE against (A) gram (+) *Staphylococcus aureus*, and (B) gram (-) *Pseudomonas aeruginosa* bacteria.
Figure 3.9. ATR-FTIR spectra of the PEN-PEG-MA-ePTFE surface: A-before and B-after exposure to *S.aureus* culture for 24 hours.
References

CHAPTER IV
ATTACHMENT OF AMPICILLIN TO EXPANDED
POLY(TETRAFLUOROETHYLEN): SURFACE REACTIONS LEADING TO
INHIBITION OF MICROBIAL GROWTH

Introduction

The ability of polymeric materials to function in a variety of biomedical devices is attributed to their unique physico-chemical properties as well as inherent polymer inertness against biological systems. While these characteristics allowed developments of many life saving devices that do not interfere and often work in harmony with biological functions, surfaces of plastic implants or other devices in contact with blood are not exempted from bacterial attacks, thus leading to undesirable infectious. According to the U.S. Centers for Disease Control (CDC), over 90,000 people annually die in the U.S. alone due to infections. Although administration of antibiotics is a common practice to alleviate this severe problem, it is well known that bacteria mutation may inhibit antibiotic’s effectiveness in preventing the growth of microbial biofilms. Furthermore, typically antibiotics are administered after infection has spread, and in order to eliminate the growth of bacteria, significant quantities of antibiotics are needed.

In an effort to inhibit microbial biofilm formation several approaches have been proposed among which addition of the biocide during polymer during processing or developments of coatings with antimicrobial agents, such as silver, quaternary ammoniums, quaternary phosphoniums, sulfoniums, or chitosan, were the primary advances. Another approach proposed by our group was to modify polymeric surfaces with drugs that inhibit proliferation of microbes, and recent studies demonstrated
that penicillin (PEN) molecules can be effectively attached to polymeric surfaces. Two key factors in this development were microwave plasma reactions in the presence of maleic anhydride (MA), which formulated a platform leading to the formation of carboxylic acid groups on polymeric surfaces, and the attachment of a molecular spacer. While microwave plasma reactions in the presence of maleic anhydride (MA) offer the universal and clean, solventless approach which served to modify surfaces of poly(dimethylsiloxane) (PDMS)\textsuperscript{14-17} and other polymeric surfaces\textsuperscript{18-20} including low surface energy expanded poly(tetrafluoroethylene) (ePTFE),\textsuperscript{18, 19} the presence of a molecular spacer between the surface acid groups and active antibiotic species is crucial in providing sufficient surface mobility to the activity of the attached antibiotic. When penicillin was attached to such modified surfaces of ePTFE, proliferation of the gram positive \textit{Staphylococcus aureus} (\textit{S. aureus}) bacteria was inhibited.\textsuperscript{18} It should be noted that in these experiments the amount of antibiotics attached to polymer surfaces is significantly smaller compared to antibiotics administered in other forms, but such surfaces are highly effective in inhibiting localized bacteria growth due the mobility of antibiotic molecules attached to the end of the molecular spacer and their antibiotic strength. Consequently, when bacteria come in contact with the surface and attempts to grow, the peptidoglycan cell wall synthesis is immediately interrupted by antibiotic molecules, thus preventing the microbe biofilm formation.

While the attachment of PEN to ePTFE constituted an important first step, this antibiotic, although widely used, is only effective against gram-positive bacteria. In an effort to develop antimicrobial polymeric surfaces that are effective against a wider range of bacteria proliferation, the spotlight of these studies is the attachment of ampicillin
(AM), a broad spectrum gram-positive and negative antibiotic. An ultimate goal is to determine its surface effectiveness against *Staphylococcus aureus* (*S. aureus*), *Bacillus thuringiensis*, (*B. thuringiensis*), *Enterococcus faecalis* (*E. faecalis*), *Escherichia coli* (*E. coli*), *Pseudomonas putida* (*P. putida*), and *Salmonella enterica* (*S. enterica*). Ampicillin with the structure shown in Figure 4.1, is often used to treat urinary and respiratory tract infections\(^{21-24}\) and commonly known as a broad-spectrum aminopenicillin type which interferes with the cell wall peptidoglycan synthesis, thus inactivating of inhibitors of autolytic enzymes.\(^ {25}\)

**Experimental**

Microwave plasma modified ePTFE surfaces (MA-ePTFE) were prepared as previously described.\(^ {18}\) To modify MA-ePTFE surfaces with AM (Sigma Inc.) COOH/NH\(_2\) terminated heterobifunctional polyethylene glycol (PEG) 2000 g/mol molecular weight. (JenKem Tech. USA) was used as a flexible spacer between maleic anhydride modified ePTFE surfaces and AM molecules. The first step involved conversion of COOH groups on the ePTFE surfaces into acid chloride (COCl) using thionyl chloride (SOCl\(_2\)) under reflux conditions at 65\(^0\)C for 9 hours. Upon completion, the sample was removed from the flask and washed for 1 hour with chloroform to eliminate an excess of thionyl chloride. The COCl-ePTFE modified surface films were then placed into a 0.5 M chloroform solution of heterobifunctional PEG for further reactions. The next step involved amidation reactions that were carried out in a sealed flask at room temperature for 18 hours. A small amount (1-2 drops) of triethylamine was added into the reaction flask at the onset of the reaction in order to neutralize hydrochloric acid that was generated during the reaction. Upon completion, the sample
was washed with chloroform several times to remove unreacted PEG. Reactions of PEG-MA-ePTFE with AM were conducted using again amidation reactions in which COOH-terminated PEG groups were first converted into acid chloride using thionyl chloride (SOCl₂) under reflux conditions at 65°C for 9 hours. Upon sample removed from the flask, a specimen was washed for 1 h with chloroform in order to eliminate excess thionyl chloride. The COCl₂-PEG-MA-ePTFE surface modified specimens were then placed into a 0.2 M chloroform solution of AM in a sealed flask and reacted at room temperature for 18 hours. A small amount (1-2 drops) of triethylamine was added into the reaction flask at the onset of the reaction in order to neutralize hydrochloric acid that was generated during the reactions. The sample was washed with chloroform several times to remove unreacted AM, dried for 24 h, and analyzed. Figure 4.2 illustrates all reactions involved in surface modifications.

In order to determine anti-microbial activity of AM-PEG-MA-ePTFE surfaces, gram positive bacteria *S. aureus* (RN 6390), *B. thuringiensis* (ATTC 10792), *E. faecalis* and gram negative bacteria *E. coli* (DH5α), *P. putida*, and *S. enterica* (LT2) were allowed to grow overnight in *Luria-Bertani* (LB) broth. A series of specimens, ePTFE, MA-ePTFE, PEG-MA-ePTFE, and AM-PEG-MA-ePTFE, and positive control were immersed into freshly incubated cultures of each bacterium and incubated at 37°C for 5 hours. Anti-microbial activity was determined by measuring the optical density (OD) of the suspensions at 600 nm using UV-VIS spectrometer (Beckman DU-600).

Hydrolytic stability of PEN-PEG-MA-ePTFE surfaces was conducted by immersing a specimen in a phosphate-buffered saline (PBS) solution and incubating it at
37°C. After 0, 1, 2, 3, 6, 12, 18, and 24 hour of exposure times, each specimen was dried and analyzed using ATR-FTIR spectroscopy.

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 a DTGS detector and a 45° face angle Ge crystal. Each spectrum represents 400 co-added scans ratioed against a reference spectrum obtained by recording 400 co-added scans of an empty ATR cell. All spectra were corrected spectral distortions using Q-ATR software.²⁶ A scanning electron microscope (SEM) Quanta FEI series 200 FEG was used to evaluate surface morphologies. All specimens were sputter coated with gold and analyzed at a 45° angle with a scanning electron beam.

Internal reflection IR imaging (IRIRI) experiments were conducted on a Varian Stingray system with a Ge internal reflection element allowing spatial resolution of about 1 μm or better.²⁷ This system consists of a Varian FTS 7000 spectrometer, an UMA 600 FT-IR microscope with a focal plane array (FPA) image detector, and a semi-spherical Ge crystal. IRIR images were collected using the following spectral acquisition parameters: under sampling ratio of 2, rapid scan speed of 5 kHz, and 8 cm⁻¹ spectral resolution. Image processing was performed using the Environment for Visualizing Images (ENVI) software (Research Systems, Inc., version 3.5). When appropriate, baseline correction algorithms were applied to compensate for baseline deviations which were accomplished by built-in application software supplied by GRAMS/AI v7.02 (Galactic Ind.).²⁷
Results and Discussion

As stated above, the primary objective of these studies is to develop effective surface modifications on highly inert ePTFE surfaces that inhibit proliferation of gram-positive and gram-negative bacteria. The first step shown in Figure 4.2 (Step A) involves the attachment of maleic anhydride (MA) which upon hydrolysis generates COOH groups. Similarly to the previous studies,\textsuperscript{14-17, 20} in order to confirm the COOH formation, ATR-FTIR spectroscopy was utilized and the spectra were recorded from the surface of ePTFE before and after plasma reactions are illustrated in Figure 4.3.a. For reference purposes, Trace A shows the spectrum of ePTFE, whereas Trace B represents the spectrum of ePTFE after microwave plasma reactions and hydrolysis (MA-ePTFE). As seen, the bands at 1852, 1781, and 1710 cm\(^{-1}\) are detected and attributed to anhydride C=O and acid C=O stretching vibrations,\textsuperscript{28, 29} respectively. Although one could take advantage of the presence of the amine groups of AM and react these species directly with the surface COOH groups, this approach will anchor and thus immobilize AM species on the surface, thus making it ineffective against microbial film formation.\textsuperscript{30, 31}

To alleviate this issue which was of a concern when PEN was attached to the surface,\textsuperscript{18, 19} we utilized heterobifunctional NH\(_2\) and COOH terminated polyethylene glycol (PEG). This approach allows us to react the NH\(_2\) end of PEG with the COOH groups of MA-ePTFE surface, thus resulting in the COOH terminated flexible spacer for further reactions with AM. This is illustrated in Figure 4.2 (Step B) and Figures 4.3. a and b, Traces A-C, shows ATR-FTIR spectra recorded from the surfaces of ePTFE (A), MA-ePTFE (B), and PEG-MA-ePTFE (C) in the 1800-1450 and 1300-1000 cm\(^{-1}\) spectral regions, respectively. While Traces A and B serve as references, Trace C illustrates the
presence of the 1105, 1650 and 1720 cm\(^{-1}\) bands due to C-O-C stretching, C=O amide, and C=O acid vibrations\(^{28,29}\) resulting from amidation reactions between heterobifunctional PEG and COOH groups on the surface. These data show that heterofunctional PEG is covalently attached to the ePTFE surface through amide linkage, such as show in Figure 4.2 (Step B). The last step of this process involves amidation reactions of PEG-MA-ePTFE with AM which are illustrated in Figure 4.2 (Step C).

Spectroscopic analysis shown in Trace D of Figure 4.3 illustrates that AM was attached to NH\(_2\)-PEG-MA-ePTFE terminated surfaces, as manifested by the presence of 1650 cm\(^{-1}\) band due to amide linkages between AM and PEG, and the bands at 1720, and 1780 cm\(^{-1}\) due to C=O acid and \(\beta\)-lactam stretching vibrations,\(^{28,29}\) respectively.

In an effort to determine if these surface reactions result in stable covalently bonded species and if the above surface grafting is adequate in inhibiting microbial film formation effective against gram-positive and gram-negative bacteria, a series of experiments with two sets of gram-positive and gram-negative bacteria were conducted. Figures 4.4 and 4.5 summarize the results and both figures illustrate a set of photographs A, B, and C, depicting turbidity differences of cultures containing \textit{S. aureus}, \textit{E. faecalis} and \textit{B. thuringiensis} gram positive cultures (Figure 4.4), and \textit{E. coli}, \textit{S. enterica}, and \textit{P. putida} (Figure 4.5) which are gram negative. The results of the optical density (absorbance) measurements are also provided in the inserts A’, B’, and C’ of Figures 4.4 and 4.5. In each of the A/A’, B/B’, and C/C’ series, specimen # 1 is a positive control and specimens # 2, #3, and #4 represent four controls of bacteria growth in the presence of neat ePTFE (#2), MA-ePTFE (#3), and PEG-MA-ePTFE (#4). Specimen # 5 represents a bacterial culture growth in the presence of AM-PEG-MA-ePTFE. As seen, the presence
of AM covalently bonded to the ePTFE surface (AM-PEG-MA-ePTFE) inhibits the proliferation of bacteria, as demonstrated by the lack of turbidity of the solution. Quantitative analysis of antimicrobial effectiveness of AM-PEG-MA-ePTFE surfaces was conducted by measuring optical density (absorbance) intensity at 600 nm for A-C series shown in Figures 4.4 and 4.5, A’, B’, and C’. Again, only specimen # 5 containing AM-PEG-MA-ePTFE shows lowest % relative absorbance values for all gram-positive and gram-negative bacteria whereas the remaining samples do not inhibit the proliferation of bacteria. The absorbance values for AM-PEG-MA-ePTFE varies from 1-15 %, depending upon the bacteria, thus showing excellent inhibition against bacterial proliferation, whereas for the specimens #1-4 the absorbance values remain at almost 100 % levels. These measurements show that the attachment of AM inhibits the growth of microbial films.

It is well-known that unique ePTFE mesh-like morphology provides suitable environments for providing the body tissue to form interwinding network with ePTFE and, the question is how the above surface reactions alter ePTFE. Furthermore, do the above surface reactions result in homogenous surface coverages while maintaining desirable no-uniform morphologies? To address these issues we combined SEM and IRIRI measurements, and analyzed both surface morphologies along with the molecular makeup of surface heterogeneities. Figure 4.6, 1-4, illustrate a series of SEM (A) and IRIRI (B) images as well as the IR spectra of ePTFE (Figure 4.6.1), MA-ePTFE (Figure 4.6.2), PEG-MA-ePTFE (Figure 4.6.3), and AM-PEG-MA-ePTFE (Figure 4.6.4) recorded from different areas of each surface. As seen, there are morphological differences resulting from the surface reactions shown in the SEM images manifested by
the presence of a fewer voids after MA, PEG, and AM surface reactions, thus
demonstrating minor morphological changes. Nevertheless, the mesh-like morphology is
maintained. As the same time, the chemical make up of the surfaces remains
homogenous, as illustrated by tuning IRIRI to the bands characteristic of COOH, C=O,
C-O-C, and C=O (lactam ring) groups, which are detected through out the entire analyzed
surface except the areas where there is no material (mesh) and therefore no bands are
detected in Figure 4.6. Traces a, b, or c represent the IR spectra recorded from the areas a,
b, or c, respectively, and confirm each step of the surface modifications shown in Figure
4.2 (Step A, B, and C).

Often raised question is the stability of the surface species resulting from the
surface reactions shown in Figure 4.2. Of particular interest and importance is the last
step involving the attachment of AM to PEG-MA-ePTFE (Figure 4.2 (Step C)) su-
faces. To determine hydrolytic stability of this linkage, we exposed AM-PEG-MA-ePTFE
specimens to PBS buffered solutions and monitored the intensity changes of the C=O
band attributed to the amide linkage between AM and PEG as a function of the exposure
time. Figure 4.7.a illustrates a series of ATR-FTIR spectra recorded as a function of time
and shows that the amide linkage between the PEG spacer and AM slightly decreases, but
even after 24 h of incubation at 37 °C the band retains approximately 90% of its initial
absorbance. This is plotted in Figure 4.7.b. (line A). In an effort to determine the actual
concentration levels of AM as a function of time, we determined the absorption
coefficient of the C=O group and, utilizing Q-ATR algorithm, determined the
concentration levels of the AM species present at the surface. Using Ge crystal the depth
from which the ATR measurements are capable of probing (excluding the areas where
there is no material due to mesh-like morphologies) is approximately 0.4 μm. As shown in Figure 4.7.b (line B) the initial amount of AM was in 2.25 μg/m³ and diminishes to 2.04 μg/m³ upon exposure for 24 hours. It should be noted that when PEN was attached similar surfaces, even the loss of 30% of PEN molecules resulted in antimicrobial activity. In the case of AM, the loss of 10% from the surface does not alter its effectiveness against gram-positive and gram-negative bacteria.

Conclusions

These studies show for the first time that a broad spectrum AM can be reacted to the ePTFE surfaces while maintaining antibiotic effectiveness against gram-positive and gram-negative bacteria. AM molecules were covalently bonded to chemically modified ePTFE surfaces via amide linkages. During this process, bio-active β-lactam ring remains intacted during the reactions. This approach provides enhanced hydrolytic stability to AM molecules attached to ePTFE surfaces and only around 10% of AM is released after immerse in PBS buffered solution for 24 hours.
Figure 4.1. Chemical structure of ampicillin (AM).
Figure 4.2. Schematic diagram of surface reactions on ePTFE: A-Microwave Ar plasma reactions in the presence of maleic anhydride followed by hydrolysis; B-Amidation reactions between NH$_2$-PEG-COOH and MA-ePTFE; C-Amidation reactions leading to the formation of AM-PEG-MA-ePTFE surfaces.
Figure 4.3.a. ATR-FTIR spectra of: ePTFE (A); MA-ePTFE (B); PEG-MA-ePTFE (C); AM-PEG-MA-ePTFE (D) in the 1800-1450 cm$^{-1}$. 
Figure 4.3.b. ATR-FTIR spectra of: ePTFE (A); MA-ePTFE (B); PEG-MA-ePTFE (C); AM-PEG-MA-ePTFE (D) in the 1300-1000 cm$^{-1}$ regions.
**Figure 4.4.** Photographs of bacterial cultures after exposure to specimens containing gram positive (A) *Staphylococcus aureus*, (B) *Enterococcus faecalis* and (C) *Bacillus thuringiensis* cultures: positive control (#1), ePTFE (#2), MA-ePTFE (#3), PEG-MA-ePTFE (#4), and AM-PEG-MA-ePTFE (#5). Optical density (absorbance) recorded at 600 nm for: (A') *Staphylococcus aureus*, (B') *Enterococcus faecalis* and (C') *Bacillus thuringiensis* cultures: positive control (#1), ePTFE (#2), MA-ePTFE (#3), PEG-MA-ePTFE (#4), and AM-PEG-MA-ePTFE (#5).
Figure 4.5. Photographs of bacterial cultures after exposure to specimens containing gram negative (A) *Escherichia coli*, (B) *Salmonella enterica*, and (C) *Pseudomonas putida* cultures: positive control (#1), ePTFE (#2), MA-ePTFE (#3), PEG-MA-ePTFE (#4), and AM-PEG-MA-ePTFE (#5). Optical density (absorbance) recorded at 600 nm plotted for (A’) *Escherichia coli*, (B’) *Salmonella enterica*, and (C’) *Pseudomonas putida* cultures: positive control: (#1), ePTFE (#2), MA-ePTFE (#3), PEG-MA-ePTFE (#4), and AM-PEG-MA-ePTFE (#5)
**Figure 4.6.1.** SEM (A), IRIRI (B) images, and IR spectra (C) recorded from selected areas a, b, or c of IRIRI images (B) of ePTFE.

**Figure 4.6.2.** SEM (A), IRIRI (B) images, and IR spectra (C) recorded from selected areas a, b, or c of IRIRI images (B) of MA-ePTFE.
Figure 4.6.3. SEM (A), IRIRI (B) images, and IR spectra (C and C’) recorded from selected areas a, b, or c of IRIRI images (B) of PEG-MA-ePTFE.

Figure 4.6.4. SEM (A), IRIRI (B) images, and IR spectra (C) recorded from selected areas a, b, or c of IRIRI images (B) of AM-PEG-MA-ePTFE.
Figure 4.7.a. ATR-FTIR spectra in the C=O region of the amide linkage recorded as function of exposure of AM-PEG-MA-ePTFE to PBS buffered solution for: (A) 0 h; (B) 1 h; (C) 2 h; (D) 3 h; (E) 6 h; (F) 12 h; (G) 18 h; (H) 24 h.

Figure 4.7.b. Curve A - % absorbance losses of the C=O amide band plotted as a function of exposure time of AM-PEG-MA-ePTFE specimen to PBS buffered solution; Curve B - AM volume concentration losses plotted as a function of exposure time of AM-PEG-MA-ePTFE specimen to PBS buffered solution.
References


CHAPTER V

COVALENT ATTACHMENT OF MULTILAYERS (CAM) OF HEPARIN AND POLY(ETHYLENE GLYCOL) ON POLY(TETRAFLUOROETHYLENE) (PTFE) SURFACES

Introduction

Although many polymeric materials serve in biomedical applications ranging from implants\textsuperscript{1-3} to artificial organs,\textsuperscript{4, 5} or tissue regeneration\textsuperscript{2, 6, 7} to drug delivery,\textsuperscript{7, 8} their biocompatibility continues to be an ongoing challenge.\textsuperscript{9} Considering the fact that the number of patients with cardiovascular diseases resulting from thrombosis continues to increase annually in the U.S. and worldwide,\textsuperscript{2} there is an ongoing need for materials that inhibit thrombosis. One of the common approaches to minimize thrombosis is to modify polymer surfaces in contact with biological agents. For example, layer-by-layer (lbl) deposition processes\textsuperscript{10-14} have been employed, but the stability and consequently the effectiveness of physisorption is limited by electrostatic interactions. Other approaches, including covalent attachments using UV surface grafting\textsuperscript{2} or endcapped of anticoagulants molecules to polymers,\textsuperscript{6} were also employed, but achieving controllable biochemical and morphological features is not obvious. While the covalent attachment\textsuperscript{2, 5}.\textsuperscript{15-19} of molecules or macromolecules to polymer that exhibit antithrombotic characteristics offer significant advantages, inertness of polymeric substrates along with low surface energy imposes other limitations. Ideally, one would like to covalently attach multi-layered structures that are stable and exhibit suitable effectiveness against thrombosis.
The approach developed in this study deviates from the previous attempts and utilizes a covalent attachment of multilayers (CAM) of antithrombotic and anticoagulant species by reacting alternating layers of heparin (HP) and poly(ethylene glycol) (PEG) species while maintaining useful polymer functionalities. These reactions will be accomplished by a sequential covalent bonding of PEG and HP, a highly sulfated anionic polysaccharide which consists of repeating disaccharides of (1,4) linked glucosamine and uronic acid residues, to a polymeric surface of interest. For that reason we will utilize previously developed microwave plasma reactions in the presence of maleic anhydride (MA) capable of generating COOH groups on poly(vinyl chloride) (PVC), poly(propylene) (PP), poly(tetrafluoroethylene) (PTFE), and poly(vinylidene fluoride) (PVDF). This approach, combined with the development of CAM process in this study will offers the formation of alternating layers of PEG and HP. The premise behind these experiments is to engineer and test in-vitro modified PTFE surfaces which, in contact with blood, will retain useful anticoagulant and antifouling attributes for extended periods of time.

Experimental

PTFE specimens were purchased from McMaster-Carr (Atlanta, GA), cut to 7x7 mm squares, washed with a 1:1 mixture of acetone and isopropanol, and dried at room temperature. Plasma reactions were conducted using open reactor conditions, as described elsewhere. The PTFE substrate and 1.5 g of solid maleic anhydride (MA) (Sigma-Aldrich) were placed into the microwave reactor chamber and spaced 8.5 cm apart of each other. In a typical experiment, the reactor was evacuated to 150 mtorr, followed by purging it with Ar gas at a flow rate of 3.0 ml/min to reach a steady state.
pressure of 250 mtorr. At this point, microwave radiation at 600 W of power with an output frequency of 2.45 GHz was turned on to induce plasma formation for 7 seconds. Under these conditions, the reaction chamber pressure increases continuously during the microwave plasma discharge. In an effort to maintain the plasma environment during longer exposure times, a vacuum was applied continuously to maintain pressure conditions during the experiment. To ensure the covalent attachment of MA to the PTFE surface and that all the MA were converted to COOH groups, the specimens were washed in DI water for 30 min, followed by boiling in water for 20 min. After drying, the specimens were kept in the desiccator under ambient conditions.

The CAM process involves covalent attachment of alternating HP and PEG layers. To attach the first PEG layer (PEG-MA-PTFE), the COOH surfaces were converted to acid chloride (COCl) using thionyl chloride (SOCl₂) (Sigma Aldrich) under reflux conditions at 65 °C for 12 hours. The sample was removed from the flask and washed with chloroform in order to eliminate excess thionyl chloride. The acid chloride PTFE surfaces were then placed in a chloroform solution of either α, ω–hydroxyl (Mw 600 g/mol) or amine terminated PEG (Mw 2000 g/mol). The reactions were carried out in a sealed flask at room temperature for 24 hours. A small amount (1-2 drops) of triethylamine was added to the reaction flask in order to neutralize hydrochloric acid generated during the reaction. The samples were washed with chloroform several times to remove unreacted PEG, dried and kept in the desiccator. The attachment of HP onto PEG-MA-PTFE surface was carried out by activating the COOH groups of the 1% w/v HP with 0.05M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sigma Aldrich) in a pH 4.6 buffer solution for 3 hours, then PEG-MA-PTFE specimen was reacted with
the activated HP for 24 hours. The specimen was then remove from the reaction, washed
with the buffer solution for 20 min, rinsed with DI water, dried and kept in a desiccator.
The next PEG and HP layers were attached to the HP-PEG-MA-PTFE surface by
repeating the reactions described above.

Spectroscopic analysis of HP immobilized PTFE surfaces was conducted using
attenuated total reflectance Fourier transform infrared (ATR FT-IR) spectroscopy. 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 a deuterated triglycine sulphate (DTGS) detector
and a 45° face angle Ge crystal. Each spectrum represents 400 co-added scans ratioed
against a reference spectrum obtained by recording 400 co-added scans of an empty ATR
cell. All spectra were corrected for spectral distortions using Q-ATR software.\(^{25}\) A
scanning electron microscope (SEM) Quanta FEI series 200 FEG was used to evaluate
surface morphologies. All specimens were sputter coated with gold and analyzed at a 45°
angle with a scanning electron beam. Internal reflection IR imaging (IRIRI) experiments
were conducted on a Varian Stingray system with a Ge internal reflection element
allowing spatial resolution of about 1 \(\mu\)m or better. This system consists of a Varian FTS
7000 spectrometer, an UMA 600 FT-IR microscope with a focal plane array (FPA) image
detector, and a semi-spherical Ge crystal. IRIR images were collected using the following
spectral acquisition parameters: under sampling ratio of 2, rapid scan speed of 5 kHz, and
8 cm\(^{-1}\) spectral resolution. Image processing was performed using the Environment for
Visualizing Images (ENVI) software (Research Systems, Inc., version 3.5). When
appropriate, baseline correction algorithms were applied to compensate for baseline
deviations which were accomplished by a built-in application software supplied by
Variable angle ATR was employed to determine the volume concentration of newly formed species after each step of the reaction as a function of depth by using Ge (50 x 20 x 3 mm) crystals and angles varying from 35° to 60°. Since quantitative ATR-FTIR depth profiling requires knowledge of the extinction coefficient for each of these bands, various concentration standards of heparin were prepared, and plots of the absorbance of the 1620 cm⁻¹ OH bending bands as a function of concentration were generated. The extinction coefficients of the bands due to HP is 6778.6 L/mol-cm, whereas the 1710 cm⁻¹ extinction coefficient of C=O vibrations of COOH groups and 1080 cm⁻¹ of C-O-C of PEG were previously determined to be 544.32, and 778.58 L/mol-cm, respectively. Using a double Kramers-Kronig Transformation (KKT) and previously developed algorithm for quantitative analysis using ATR-FTIR spectroscopy, concentration levels of COOH groups resulting from the microwave plasma modifications, PEG reactions, and HP attachment were determined.

Ovine blood was collected directly by jugular venipuncture and transferred into a syringe containing heparin (3.0 or 6.0 U/mL for 1 and 2.5 h blood contacting experiments) using an 18 gauge 1.5” needle with the first 3 mL discarded. The use of laboratory animals followed the NIH guidelines. CAM modified samples and PTFE control were placed into Vacutainer blood collection tubes (BD Biosciences, Franklin Lakes, NJ) without any additive filled with heparinized ovine blood and then incubated at 37°C for a specified time on a hematology mixer (Fisher Scientific, Pittsburgh, PA).

The morphologies of the CAM surfaces after contact with heparinized ovine blood (3U/mL) for 2 h under continuous rocking were observed by SEM (JSM-6330F, LEOL USA, Inc., Peabody, MA). The adherent platelet coverage (%) on the samples
after contact with ovine blood (heparin 3 U/mL) was calculated by an Image-J program (N=6) and quantification of the % of activated ovine platelets in the bulk phase of blood during surface contact was obtained by flow cytometric measurements of Annexin V binding. Data are presented as a means with standard deviation. Statistical significance between sample groups was determined using ANOVA followed by post-hoc Newman-Keuls testing and accepted at p<0.05.

Results and Discussion

The primary objective of these studies is to create long lasting hemocompatible PTFE surfaces containing HP and PEG. Figure 5.1 depicts a schematic diagram of the process which involves the covalent attachment of multilayers (CAM) of HP and PEG layers. To ensure covalent attachment of the first PEG layer, MA was reacted to the PTFE surface using microwave plasma conditions (step 1), followed by its hydrolysis producing COOH-PTFE surfaces. The resulting groups were converted to acid chloride by reactions with thionyl chloride (SOCl₂) (step 2), followed by grafting either α-ω-OH or NH₂-PEG (step 3 and 3’), generating first a₁ and a₁’ PEG layers. The remaining distal ends of the PEG bearing OH or NH₂ groups were then reacted with heparin in the presence of EDC and NHS (step 4 and 4’) resulting in the b₁ and b₁’ HP layers. Using this approach, two layers composed of PEG (a and a’) and HP (b and b’) were attached to the PTFE surface. This process was repeated three times to create six alternating PEG and HP layers that are chemically anchored to the PTFE surface and/or to each other.

Figure 5.2.A and B, shows covalent linkages (circled) between each layer (left) and the resulting ATR FT-IR spectra (right) obtained after the covalent immobilization of each layer. The first MA layer is obtained by COOH-functionalization of PTFE
manifested by the band at 1715 cm$^{-1}$ due to C=O of COOH groups. As shown in Figure 5.2.A, the reactions of COOH functionalized PTFE with OH-PEG lead to ester formation (C=O), and the alternating layers of HP and OH-PEG also produce ester linkages. ATR FT-IR spectrum of the first PEG layer ($a_1$) shows the band at 1720 cm$^{-1}$ due to ester linkages$^{30,31}$ resulting from COOH-PTFE and OH-PEG entities. The band at 1070 cm$^{-1}$ due to C-O-C of PEG was also detected which is shown in Figure A.1 of the Appendix A. The first HP layer ($b_1$) was attached in the next step which is manifested by the presence of the band at 1720 cm$^{-1}$ due to ester linkages that result from reactions of COOH of HP and OH of PEG as well as the band at 1590 cm$^{-1}$ due to OH bending modes of HP. These results confirm the covalent attachment of OH-PEG and HP layers on the COOH-PTFE surface. Since the ester bands of the second ($a_2$ and $b_2$) and third layers ($a_3$ and $b_3$) are identical to those produced in the first layer ($a_1$ and $b_1$), enhanced intensities of the bands at 1720 cm$^{-1}$ of ester linkages (PEG and HP reactions) and at 1590 and 1620 cm$^{-1}$ due to OH bending vibrations of heparin are also detected, confirming the covalent attachment of OH-PEG and HP layers to COOH-PTFE surface.

An alternative path of reactions to produce CAM is shown in Figure 5.2.B, where reactions of COOH-PTFE and NH$_2$-PEG were employed to give amide formation (C=O-NH), also producing the alternating layers of HP and NH$_2$-PEG amide linkages. ATR FT-IR spectra shown in Figure 5.2.B illustrate the presence of the band at 1710 cm$^{-1}$ due to C=O of COOH entities in the first COOH-PTFE layer (MA) resulting from the microwave plasma reactions, and at 1670 cm$^{-1}$ due to amide linkages$^{30,31}$ produced from the reactions of COOH-PTFE and NH$_2$-PEG ($a_1'$). The band at 1080 cm$^{-1}$ due to C-O-C of PEG (not shown) is detected. The next reacted layer is HP ($b_1'$), which is also
manifested by the bands at 1650 cm\(^{-1}\) due to NH\(_2\) linkages resulting from the reactions of COOH of HP and NH\(_2\) of PEG, and the 1590 cm\(^{-1}\) band attributed to OH bending vibrations of HP, confirming covalent attachment of NH\(_2\)-PEG and HP layers. Again, as subsequent layers are reacted, the band intensities increase, indicating that the multiple layers of NH\(_2\)-PEG and HP were covalently attached to the COOH-PTFE surfaces. Hydrolytic stability of the surfaces produced by the CAM process containing OH- and NH\(_2\)-PEG was determined by exposing each specimen to boiling water for 20 min while stirring. The IR analysis shown in Figure A.2 of Appendix A show that the C=O bands due to ester and amide linkages before and after boiling are undistinguishable, thus indicating chemical stability of the CAM layers.

Since reactions leading to CAM formation were conducted in solutions, it is important to determine concentration levels of each layer on the PTFE surface. Figures 5.3.A and B, illustrate the summary of the quantitative analysis obtained by measuring the volume concentrations of each layer of OH- and NH\(_2\)-PEG and HP multilayers shown in Figure 5.2.A and B. As in the past, we employed ATR FT-IR analysis which allows us to quantify surface concentrations by measuring intensities of the bands of interest after covalent immobilization of each layer. While details regarding surface measurements were published elsewhere,\(^{12,24,25}\) concentration levels of the species of interest is expressed by the mass of a given species per volume (g/cm\(^3\)) or the mass of monolayer per unit surface area (g/cm\(^2\)). In these studies, we utilized the bands at 1715, 1080, and 1620 cm\(^{-1}\) which are characteristic of COOH, C-O-C, and O-H bands of MA, PEG, and HP layers, respectively.
Figure 5.3 A illustrates surface volume concentration changes of COOH-PTFE, OH-PEG, and HP after each step in the CAM process shown in Figure 5.1. The volume concentration of the COOH layer is $5.65 \times 10^{-3} \text{g/cm}^3$ ($1.7 \times 10^{-7} \text{g/cm}^2$), whereas for the first OH-PEG and HP layers are $3.08 \times 10^{-3} \text{g/cm}^3$ ($9 \times 10^{-8} \text{g/cm}^2$) and $4.57 \times 10^{-3} \text{g/cm}^3$ ($1.4 \times 10^{-7} \text{g/cm}^2$), respectively. As anticipated, ATR FT-IR spectra obtained after layer attachment shows increased intensities of the bands at 1100 and 1620 cm$^{-1}$, because in these ATR FT-IR depth profiling experiments a Ge crystal was employed which gives the penetration depth of ~280 nm. Consequently, each subsequent volume represents the summation of the last and previously deposited layers. After attaching six alternating layers, the overall volume concentrations of HP are $9.37 \times 10^{-3} \text{g/cm}^3$ ($2.8 \times 10^{-7} \text{g/cm}^2$). Similar trends are observed for NH$_2$-PEG, as illustrated in Figure 5.3, B, where the volume concentrations of the initial NH$_2$-PEG and HP layer are $4.62 \times 10^{-3} \text{g/cm}^3$ ($1.4 \times 10^{-7} \text{g/cm}^2$) and $4.28 \times 10^{-3} \text{g/cm}^3$ ($1.3 \times 10^{-7} \text{g/cm}^2$), respectively. After attaching all subsequent layers, the volume concentrations of the HP are $8.65 \times 10^{-3} \text{g/cm}^3$ ($2.6 \times 10^{-7} \text{g/cm}^2$). It should be noted that the HP concentrations levels at the surface for OH- and NH$_2$-PEG are retained at sufficient levels. Recent literature data indicates that the minimum surface concentrations levels should be $1 \times 10^{-7} \text{g/cm}^2$.

The CAM and unmodified PTFE surfaces were subjected to contact with anticoagulated ovine blood for 2 h at 37$^\circ$C and such surfaces were analyzed by SEM in triplicate (N=3). As shown in Figure 5.4 A-D, substantial improvement of blood compatibility of the CAM modified PTFE surfaces are shown as manifested by heavy platelet deposition on neat PTFE (A) compared to MA modified PTFE (MA) (B). Furthermore, upon CAM reaction producing multilayers of OH-PEG and HP (OH-HP)
and NH$_2$-PEG and HP (NH$_2$-HP) is further reduced, as shown in Figure 5.4.C and D. These data illustrate that platelet deposition decreases dramatically when CAM PTFE surfaces are exposed to ovine blood environments. The adherent platelet coverage (%) on the specimens after contact with ovine blood with heparin 3 U/mL for 2 h under continuous rocking were determined and the results are illustrated in Figure 5.5.A. CAM surfaces OH-HP and NH$_2$-HP showed a dramatic 70-75 % decrease in the adherent platelet coverage compared to neat PTFE and MA. A similar trend was shown in Figure 5.5.B, that a significant reduction of 45-65% in platelets binding annexin V was shown for CAM surfaces OH-HP and NH$_2$-HP compared to neat PTFE and MA modified PTFE. In summary, significant improvement of blood compatibility of PTFE surfaces was obtained by utilizing CAM process.

Conclusions

These studies showed for the first time that the CAM process on surfaces of PTFE result in the formation of stable alternating PEG and HP layers that inhibit platelet deposition and enhanced blood compatibility. This was achieved by creating COOH “primer” groups on the surface of PTFE using microwave plasma reactions in the presence of MA, followed by subsequent reactions of alternating layers of PEG and HP. The results of the quantitative analysis of volume concentration levels have shown that the volume concentration of HP is 9.3-8.7 x 10$^{-3}$ g/cm$^3$ (2.8-2.6 x 10$^{-7}$ g/cm$^2$). The unique feature of these surface modifications is that stability and enhancement of hemocompatibility, as manifested by a 75±1% decrease of the platelet adhesion and a 60±5% decrease of platelet activation.
Figure 5.1. Schematic diagram of CAM process; covalent attachment of alternating OH-PEG (a1-a3), OH-HP (b1-b3), NH2-PEG (a1’-a3’), and NH2-HP (b1’-b3’) layers.
Figure 5.2. (A)- CAM process of modifying COOH-PTFE surfaces using OH-PEG: a₁-PEG; b₁-HP; a₂-PEG; b₂-HP; a₃-PEG; b₃-HP, (A’)- ATR FT-IR spectra of PTFE, MA, PEG (a₁ layer), HP (b₁ layer), PEG (a₂ layer), HP (b₂ layer), PEG (a₃ layer), and HP (b₃ layer). Circles indicate covalent linkages.
Figure 5.2. (B)- CAM process of modifying COOH-PTFE surfaces using NH$_2$-PEG: a$_1$'-PEG; b$_1$'-HP; a$_2$'-PEG; b$_2$'-HP; a$_3$'-PEG; b$_3$'-HP, (B’)- ATR FT-IR spectra of PTFE, MA, PEG (a$_1$’ layer), HP (b$_1$’ layer), PEG (a$_2$’ layer), HP (b$_2$’ layer), PEG (a$_3$’ layer), and HP (b$_3$’ layer). Circles indicate covalent linkages.
Figure 5.3. Quantitative representation of a number of alternating HP and PEG layers plotted as a function of their respective volume concentrations: (A) OH-PEG process (Figure 5.2, A-A’); (B) NH2-PEG process (Figure 5.2, B-B’). The volume concentration was determined by the Beer-Lambert law using the extinction coefficient and the absorbance obtained from the experiment.
Figure 5.4. SEM images of (A) PTFE, (B) MA modified PTFE (MA), (C) OH-PEG and HP (OH-HP), and (D) NH₂-PEG and HP (NH₂-HP) after contact with ovine blood (heparin 3U/mL) for 2 h at 37 °C. The measurements were repeated in triplicate (N=3).
Figure 5.5. (A) % platelet coverage rate of PTFE, MA, OH-HP and NH$_2$-HP; (B) activated platelets of PTFE, MA, OH-HP and NH$_2$-HP.
References


CHAPTER VI
MICRO-PATTERNING OF STREPTAVIDIN-BIOTIN ON POLY(TETRAFLUOROETHYLENE) (PTFE) SURFACES

Introduction

The ability to attach bioactive molecules to synthetic polymer surfaces enhancing biocompatibility provides a number of opportunities for new biosensor technologies, implant developments, tissue engineering, and others. Numerous methods have been introduced to enhance cell adhesion, blood compatibility, anti-microbial properties, and anti-fouling properties of polymeric surfaces. Furthermore, many naturally occurring proteins and polysaccharides have been utilized to self-assemble mono-layers (SAMs), layer-by-layer deposition (lbl), photo-grafting, or microwave plasma reactions. Regardless of the approach, the primary objective of these studies was to increase biocompatibility of polymer surfaces by attaching either biologically active species, or creating surfaces that would not adversely affect biological functions in contact with polymers.

In many approaches, non-covalent attachments driven primarily by H-bonding, van der Waals forces, or dipole-dipole interactions have lead to relatively weak layers with a limited lifetime. Ideally, one would like to covalently attach surface species or selectively chemisorb them to surfaces in order to create a buffer layer with enhanced biocompatibility. One of these unique interactions is the formation of streptavidin (STR)-biotin (Bio) conjugates via a well-defined strong ligand-receptor force manifested by the strongest known non-covalent bonding with four receptor sites which exhibits $K_d$ of $10^{-15}$ M. Since selectivity and strength of this non-covalent bonding is comparable
to the strength of covalent attachment, combining these interactions into one engineered polymer surface may lead to highly selective and sustainable surfaces.

While previous studies have shown that the covalent attachment of penicillin,\textsuperscript{23} ampicillin,\textsuperscript{13} and heparin with alternating PEG layers to inert PTFE surfaces utilizing microwave plasma reactions result in antimicrobial and anticoagulant properties, these approaches were limited to a single functionality. In these studies we demonstrate a novel approach of creating multi-functional surfaces by the simultaneous, spatially resolved attachment of ampicillin and heparin onto a PTFE surface via STR-Bio conjugates. In an effort to achieve horizontal and vertical control of surface distribution inkjet micropatterning of biotinylated ampicillin (B-AM) and biotinylated heparin (B-HP) onto STR functionalized PTFE surfaces and biotinylated PEG (B-PEG) will be used.

**Experimental**

Medical grade PTFE specimens (McMaster-Carr, Atlanta, GA) were cut to 1x1 cm squares, cleaned in an ultrasonic washer with a mixture of acetone and isopropanol for 30 min, dried, and kept in the dessicator. Plasma reactions were conducted using open reactor conditions, as described elsewhere. The PTFE substrate and powdered maleic anhydride (MA) (Aldrich Chemical Co.) were placed into the microwave reactor chamber and spaced 8.5 cm apart of each other. In a typical experiment, the reactor was evacuated to 150 mTorr, followed by purging it with Ar gas to reach a steady-state pressure of 250 mTorr at a flow rate of 3 mL/min. At this point, microwave radiation at 600 W of power with an output frequency of 2.45 GHz was applied to the reactor to induce plasma formation for 7 seconds. Under these conditions, the reaction chamber pressure increased continuously during plasma discharge. After the reactions, specimens were washed in
boiling water for 30 min to ensure that the newly formed species were not physisorbed on the surface and all maleic anhydride (MA) surface groups were converted to COOH groups. The carboxylic acid primed PTFE surfaces (MA-PTFE) were stored in a dessicator at ambient temperature.

EZ-link® amine-PEG-biotin (B-PEG) (Thermo Scientific, Rockford, IL) was attached to the MA-PTFE surfaces by amine reactions with surface carboxylic acid groups. The PTFE was placed in 0.1 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sigma Aldrich) for 4 h, then the EDC activated surface was reacted with 1 mM B-PEG for 18 h. Such B-PEG-MA-PTFE specimens were removed, and washed with DI water for 20 min and dried in a dessicator. STR was immobilized on the surfaces by incubation of the B-PEG-MA-PTFE specimens overnight in 30 μg/mL STR (Thermo Scientific, Rockford, IL) in phosphate-buffered saline (PBS) pH 7.4. The specimens with immobilized STR (STR-B-PEG-MA-PTFE) were subsequently rinsed in PBS and store at 4 °C.

B-AM was prepared by dissolving 0.06 g of ampicillin (AM) in 30 mL of a 1:1 mixture of dimethyl sulfoxide (DMSO) and water, then mixing the solution with excess 0.03 M N-hydroxysuccinimide ester of biotin (NHS-biotin) (Sigma Aldrich). The mixture was gently stirred for 6 h at room temperature. The reaction was stopped by adding Affi-Gel 102 (Bio-Rad Laboratories, Richmond, CA) in excess with stirring for 3 h. B-AM was separated from the solution by centrifugation and freeze dried under a vacuum for 2 days. The B-AM was always freshly prepared before use. The B-AM formation was confirmed by ATR FT-IR and NMR in Figure B.3 of Appendix B.
The patterned arrays of B-AM and B-HP (Sigma Aldrich) were generated with inkjet printing to create 100-20 μm diameter dots. 10μg/mL of B-AM and B-HP aqueous solution were utilized as an ink for printing on the STR-B-PEG-MA-PTFE surfaces. After printing, the specimens set overnight, followed by rinsing with DI water, and drying in a dessicator.

Inkjet printing was conducted on a piezoelectric drop on demand (DOD) inkjet printer (Jetlab4) manufactured by Microfab Technologies Inc (Plano, TX). The printing area was approximately 70 x70 mm with an adjustable height (Z). The sample was held on the stage and polymer ink was printed through the 60 and 20 μm printhead orifice.

The modification of AFM tip was carried out by soaking the Si₃N₄ cantilever (Veeco Probes, CA) with 1 μg/mL of biotinylated bovine serum albumin (B-BSA) solution (Thermo Scientific, Rockford, IL) overnight at 37°C. The cantilever was rinsed with PBS solution and dried on a glass slide.²⁴

A scanning electron microscope (SEM) Quanta FEI series 200 FEG was used to evaluate the AFM tip before and after B-BSA immersion. All specimens were sputter coated with gold and analyzed at a 45° angle with a scanning electron beam.

To determine anti-microbial activity of multifunctional surfaces containing B-AM and B-HP (B-AM/B-HP-STR-B-PEG-MA-PTFE), each specimen was contacted with cultures of Staphylococcus aureus (S. aureus) (RN 6390). S. aureus bacteria was allowed to grow for 4 hours at 37 °C in Triptic Soy Broth (TSB). The growth of bacteria was determined by the optical density (OD), measured from the absorbance at 600 nm using a UV-vis spectrometer. A 50 μL of S. aureus culture was combined with 20 mL of fresh TSB and 0.7% of Triptic Soy Agar (TSA). This culture was then spread over individual
TSA plates containing specimens from each reaction step to grow colonies. The typical size of each polymer specimen was 20 x 0.9 x 1.5 mm. PTFE, MA-PTFE, B-PEG-MA-PTFE, STR-B-PEG-MA-PTFE were used as controls. B-AM and B-HP solutions were printed in two distinct regions of the same specimen as well as using an alternating checkerboard patterning. Two additional controls printed only with B-AM or B-HP solutions were proceeded in order to determine the antimicrobial activity of the individual species. After incubating the TSA plates at 37 °C for 16 h, the antimicrobial activity of each specimen was determined.

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 a deuterated triglycine sulphate (DTGS) detector and a 45° face angle Ge crystal. Each spectrum represents 400 co-added scans ratioed against a reference spectrum obtained by recording 400 co-added scans of an empty ATR cell. All spectra were corrected for spectral distortions using Q-ATR software.

¹H Nuclear magnetic resonance (NMR) spectra were acquired using the Varian Mercury 300 MHz NMR spectrometer. Samples were prepared (5 mg/mL) in chloroform (CDCl₃), and spectra were recorded at room temperature. Typical acquisition parameters were a 45° pulse, 5 s relaxation delay, and 2 s collection with 256 repetitions.

Atomic force microscopy (AFM) measurements were analyzed on a Nanoscope IIIa Dimension 3000 scanning probe microscope, Digital Instruments. A silicon probe with 125 µm long silicon cantilever, nominal force constant of 60 N/m and resonance frequency of 200 kHz were used in a tapping mode, allowing estimation of surface topography and roughness.
Internal reflection IR imaging (IRIRI) experiments were conducted on a Varian Stingray system with a Ge internal reflection element allowing spatial resolution of about 1 μm or better. This system consists of a Varian FTS 7000 spectrometer, an UMA 600 FT-IR microscope with a focal plane array (FPA) image detector, and a semi-spherical Ge crystal. IRIR images were collected using the following spectral acquisition parameters: under sampling ratio of 2, rapid scan speed of 5 kHz, and 8 cm⁻¹ spectral resolution. Image processing was performed using the Environment for Visualizing Images (ENVI) software (Research Systems, Inc., version 3.5). When appropriate, baseline correction algorithms were applied to compensate for baseline deviations which were accomplished by built-in application software supplied by GRAMS/AI v7.02 (Galactic Ind.).

Results and Discussion

Using inkjet printing with a spatial resolution of 20 μm we deposited sequential layers of B-AM and B-HP onto a COOH modified PTFE surface. In the first step of creating controllable micropatterned multi-functional PTFE surfaces we reacted a surface anchor in the form of COOH groups, followed by a sequence of surface reactions of B-PEG, STR, B-AM and B-HP. Figure 6.1.a, Steps A-E, illustrate a schematic diagram of the sequence of steps which are as follows: A- microwave plasma reactions were utilized to covalently attach COOH groups onto a PTFE surface; B- B-PEG was reacted to COOH; C- STR was immobilized by Bio-STR conjugate formation; D and E –B-HP (D) and B-AM (E) were attached to a Bio-STR conjugate. Figure 6.1.b illustrates ATR FT-IR spectra corresponding to each step shown in Figure 6.1.a. While Trace A illustrates the spectrum of the PTFE substrate, formation of COOH groups on the PTFE surfaces (MA-
PTFE) is shown in Trace B, where the new band at 1720 cm\(^{-1}\) due to C=O (acid)\(^{25}\) is detected. The covalent attachment of B-PEG to the COOH-PTFE surfaces (B-PEG-MA-PTFE) with the aid of a carbodiimide is manifested in Trace C by the presence of the band at 1650 cm\(^{-1}\) due to amide linkages\(^{25}\) resulting from the reactions of surface COOH with NH\(_2\) groups of B-PEG. The bands in Trace C at 1715 cm\(^{-1}\) due to the C=O of biotin ureido ring\(^{25}\) and the 1560 cm\(^{-1}\) due to amide N-H represent the immobilization of B-PEG onto MA-PTFE surface. The band at 1080 cm\(^{-1}\) due to C-O-C of PEG is also detected in the 1300-1000 cm\(^{-1}\) region shown in Figure B.1 of the Appendix B. The biotinylated surfaces were then immobilized in STR solutions, resulting in the STR-Bio binding to form STR-B-PEG-MA-PTFE surface complexes. The bands in Trace D at 1660, 1635 and 1535 cm\(^{-1}\) due to the C=O (amide I) and N-H (amide II) bands of the STR protein\(^{25}\) are detected, confirming the presence of STR on the surface. The last steps, D and E, result in the formation of B-HP and B-AM, respectively, on the STR-B-PEG-MA-PTFE surface which is confirmed by the presence of the band at 1630 cm\(^{-1}\) due to OH bending of heparin (Trace E) as well as the band at 1775 cm\(^{-1}\) due to C=O (\(\beta\)-lactam) of B-AM (Trace F).

In an effort to determine if these surface reactions result in stable, covalently bonded species, after each reaction step the specimen was boiled in water for 30 min to eliminate all physisorbed species. The spectroscopic analysis after boiling confirmed the covalent attachment after each step of the reaction and IRIR images in Figure B.2 of the Appendix B show that identical IR spectra were obtained before and after boiling. Also, to determine surface density, quantitative analysis performed after each step using double Kramers-Kronig transformation (KKT) and the previously developed algorithm for
quantitative with ATR-FTIR spectroscopy\textsuperscript{26,27} for the bands at 1715, 1080, and 1640 cm\textsuperscript{-1} due to COOH, PEG, and STR was conducted.\textsuperscript{20,23} The surface density of COOH, B-PEG and STR layers was found to be 2.94x10\textsuperscript{-7} g/cm\textsuperscript{2}, 9.2x10\textsuperscript{-8} g/cm\textsuperscript{2}, and 3.5x10\textsuperscript{-8} g/cm\textsuperscript{2}, respectively. This is illustrated in Figure 6.1.c.

While the above results show the covalent attachment of multilayers, another objective of these studies was to create patterned surfaces which exhibit horizontal patterned morphologies. The motivation behind this approach is to generate surfaces not only with accessible anticoagulant and antimicrobial features in order to be effective against micron size or larger biological species in order to effectively inhibit their growth or adhesion, but also to control surface morphologies. For that reason B-AM and B-HP were inkjet printed onto the PTFE surface to obtain multi-patterned, multi-functional surfaces, such as illustrated in Figure 6.2.a. After printing, optical images shown in Figure 6.2.a’ were obtained, but to confirm the controllability of the patterning process, polymer ink B-AM and B-HP were printed onto STR-B-PEG-MA-PTFE surface and IRIR images were recorded. While Figure 6.2.a” illustrates the side-by-side B-AM and B-HP patterns, Figures 6.2.b, b’, c, and c’ show IRIR images as well as IR spectra recorded from the printed B-AM and B-HP surfaces. As seen in Figure 6.2.b’, upon tuning to the band characteristic of B-AM at 1780 cm\textsuperscript{-1}, the spherical dark areas A, B, and C in Figure 6.2.b show higher intensities compared to the areas D and E. Similarly, the spectra shown in Figure 6.2.c’ exhibit the highest intensities collected from the areas A’, B’, C’, and D’ (Figure 6.2.c), where the B-HP species are present, as we tuned to the band characteristic of B-HP at 1630 cm\textsuperscript{-1}. These results confirm that B-AM and B-HP were printed in rows with a controllable horizontal side-by-side fashion.
Figure 6.2.a” illustrates an alternating stripe pattern, where alternating B-AM and B-HP dots were printed. As seen in Figure 6.2.d, tuning to the band characteristic of B-HP at 1630 cm\(^{-1}\) results in the spherical dark spots, with areas labeled B and D showing enhanced intensities compared to the spectra collected from areas A and C. In contrast, the spectra collected from the areas A and C exhibit enhanced intensities of the bands at 1670 and 1590 cm\(^{-1}\) attributed to C=O (amide) and C-C aromatic of B-AM, respectively. These results confirm again that B-AM and B-HP were printed in an alternating stripe pattern on the surface. It should be noted that the band at 1785 cm\(^{-1}\) was also detected in the spectra collected from the areas B and D where B-HP were printed, indicating the pattern overlap or a likely hood of the mixing of B-AM and B-HP spots.

In an effort to further advance understanding of surface morphologies and forces governing the attachment of B-HP and B-AM we conducted a series of AFM experiments in which dynamic recognition force mapping of the STR-B-PEG-MA-PTFE surface with a biotinylated AFM tip was conducted. This is shown in Figure 6.3.1. In these studies we immobilized biotinylated bovine serum albumin (B-BSA) on the Si\(_3\)N\(_4\) AFM tip. The B-BSA protein was chosen in these model experiments in place of B-AM and B-HP due to its sufficiently stronger bond to the AFM tip\(^{24}\). Figure 6.3.1.a and b, illustrate SEM images of the AFM tip before and after immobilization of B-BSA. As seen in Figure 6.3.1.b, immobilization of the B-BSA is clearly indicated by the presence of a coating on the AFM tip. The recognition images obtained by simultaneous oscillating AFM tip modified with B-BSA on PTFE and STR-B-PEG-MA-PTFE surfaces are illustrated in Figure 6.3.2.b’ and c’. For unmodified PTFE, no significant features are observed. However, the STR-B-PEG-MA-PTFE recognition between biotin and STR is illustrated
by the dark areas in the AFM image shown in Figure 6.3.2.c’ and is attributed to a
decrease of the oscillation of the AFM tip caused by STR-Bio conjugates formation.

While the results of these model experiments confirm that the STR-B-PEG-MA-
PTFE surface is capable of recognizing biotinylated species such as B-AM and B-HP, the
measurements of molecular recognition forces of STR-Bio obtained from AFM will
provide further information regarding adhesion force. Figure 6.3.2.b” and c” illustrate the
the plot of cantilever deflection (d) as a function of the vertical displacement of the
piezoelectric scanner (z). As seen in Figure 6.3.2.b” only minute deflection is observed
for unmodified PTFE. In contrast, the magnitude of deflection for STR-B-PEG-MA-
PTFE surfaces is 18.17 nm, resulting from the adhesion forces due to recognition of STR-
Bio. This is illustrated in Figure 6.3.2.c”. The cantilever deflection obtained from these
experiments can be converted into adhesion force (F) following the Hooke’s law $F=kd$,
where $k$ is the spring constant, $d$ is the deflection and the adhesion force of B-BSA and
STR-B-PEG-MA-PTFE analysis of the data shown in Figure 6.3.2.c” is 1090 pN.
Recent literature studies reported the unbinding force to rupture a single STR-Bio
interactions is 80-100 pN,$^{28,29}$ thus suggesting that the AFM tip in our experiments
probed approximately 11-13 pairs of STR-Bio conjugates.

The antimicrobial activity of the B-AM and B-HP bound to the STR-B-PEG-MA-
PTFE surface was also evaluated. Figure 6.4.a-h, illustrates the photographs of TSA
plates containing the specimens obtained from each reaction step shown in Figure 6.1
covered with $S. aureus$. While PTFE (a), MA-PTFE (b), B-BEG-MA-PTFE (c), and
STR-B-PEG-MA-PTFE (d) did not exhibit any inhibition to the bacterial growth, when
B-AM was printed on the left and B-HP on the right (e) (Figure 6.2.a”) an inhibition zone
is observed on the B-AM side. Similarly, for an alternating stripe printing of B-AM and B-HP (Figure 6.2.a’’’) (f) the inhibition zone surrounding the specimen confirm that B-AM was effective at killing the bacteria. Additional controls B-AM (g) and B-HP (h) printed patterns were used to determine the effectiveness of B-AM and B-HP by themselves. As seen, for B-AM printed alone (g), the inhibition zone is present around the specimen confirming that bacteria were killed, whereas the cloudy agar around B-HP print alone (h) is observed. In summary, these data show that the horizontal and vertical covalent bonding inhibits the proliferation of microbes.

Conclusions

Controllable micropatterned multi-functional PTFE surfaces were generated by the simultaneous inkjet printing of B-AM and B-HP. These surface reactions were possibly initiated by microwave plasma reactions, generating COOH groups on the PTFE surface, followed by the reactions of B-PEG and STR immobilization. Quantitative surface measurements showed that the surface density of COOH groups was 2.94x10^{-7} g/cm², whereas these values for B-PEG and STR were 9.2x10^{-8} g/cm² and 3.5x10^{-8} g/cm², respectively. 20 µm patterned dots of B-AM and B-HP with side-by-side and an alternating stripe patterns were confirmed by IRIR image experiments and the adhesion force of STR-Bio was found to be 1090 pN, the unbinding of multiple STR-Bio conjugates. The antimicrobial activity was demonstrated for the B-AM printed areas, which effectively killed the *S. aureus* bacteria compared to the control.
Figure 6.1. (a) Schematic diagram of surface reactions on PTFE: (A) Microwave Ar plasma reactions leading to the formation of COOH groups, (B) Attachment of B-BEG to COOH-PTFE surface, (C) Immobilization of STR via STR-Bio conjugates, (D) B-HP binding to STR-B-PEG-MA-PTFE, (E) B-AM HP binding to STR-B-PEG-MA-PTFE; (b) ATR FT-IR spectra in the 1900-1400 cm⁻¹ region: Trace A- PTFE, Trace B- MA-PTFE, Trace C- B-BEG-MA-PTFE, Trace D- STR-B-PEG-MA-PTFE, Trace E- B-HP-STR-B-PEG-MA-PTFE, Trace F- B-AM-STR-B-PEG-MA-PTFE.
Figure 6.1. (c) Surface density obtained from quantitative analysis of COOH, B-PEG, STR.
Figure 6.2. (a) Inkjet printing of B-AM and B-HP onto STR-B-PEG-MA-PTFE surface, (a’) optical images of B-AM and B-HP printed from the inkjet.
Figure 6.2. (a’”) horizontal side by side printing pattern of B-AM and B-HP, (b) IRIRI images of B-AM, (b’) IR spectra recorded from selected areas A, B, C, D, E of IRIR images of B-AM, (c) IRIRI images of B-HP, (c’) IR spectra recorded from selected areas A’, B’, C’, D’, E’ of IRIR images of B-HP.
Figure 6.2. (a’”) alternating stripe printing pattern of B-AM and B-HP, (d) IRIR images of B-AM and B-HP, (d’) IR spectra recorded from selected areas A, C of IRIR images of B-AM and areas B, D of IRIR images of B-HP.
Figure 6.3.1. SEM images of (a) Si$_3$N$_4$ AFM tip before modification, (b) after modification with B-BSA.
Figure 6.3.2. AFM dynamic force recognition of (b’) PTFE, (c’) STR-B-PEG-MA-PTFE; voltage displacement curve of (b”) PTFE, (c”) STR-B-PEG-MA-PTFE.
Figure 6.4. Photographs of TSA plates containing (a) PTFE, (b) MA-PTFE, (c) B-BEG-MA-PTFE, (d) STR-B-PEG-MA-PTFE, (e) B-AM/B-HP-STR-B-PEG-MA-PTFE with side-by-side pattern, (f) B-AM/B-HP-STR-B-PEG-MA-PTFE with checker board pattern, (g) B-AM-STR-B-PEG-MA-PTFE, (h) B-HP-STR-B-PEG-MA-PTFE after incubation with *S. aureus* for 16 h at 37 °C.
References


CHAPTER VII
CONCLUDING REMARKS

Functionalization of polymer surfaces with bioactive molecules offers new avenues in development of polymeric materials utilized in biomedical applications. With the inert nature of polymeric materials combined with their excellent physical properties, these materials are not exempt from absorption of non-specific proteins, platelet adhesion and activation or attack of microorganisms, such as bacteria and fungi which ultimately can lead to infections. PTFE has been used in medical devices and implants due to its non-toxicity and excellent chemical and thermal resistance. Surface modification of PTFE enhances its biocompatibility without altering the bulk properties. In this work, PTFE was modified by Ar microwave plasma reactions in the presence of maleic anhydride, which upon hydrolysis create surface bound COOH groups. These COOH primers were used to attach polyethylene glycol (PEG) spacers, and penicillin (PEN) or ampicillin (AM) onto PTFE surfaces for antimicrobial properties. The use of a PEG spacer facilitates enhanced antimicrobial effectiveness of the antibiotics by increasing their mobility, allowing easier contact with the bacteria. Antimicrobial effectiveness of modified PTFE surfaces with PEN and AM was shown against gram (+) and/or gram (-) bacteria. Utilizing covalent attachment of alternating multilayers (CAM) of heparin (HP) and PEG onto PTFE anti-coagulant PTFE surfaces were created. The CAM modified PTFE showed enhanced hemocompatibility as evidenced by a 75±1% decrease of the platelet adhesion and 60±5% decrease of platelet activation. Finally, dual functional PTFE surfaces were generated by the simultaneous inkjet printing of biotinylated ampicillin (B-AM) and biotinylated heparin (B-HP) on streptavidin (STR) functional
surfaces. Using this approach dots with a 20 $\mu$m spatial resolution were printed side-by-side and in an alternating stripe pattern. The dual functional, micropatterned surfaces showed antimicrobial activities against gram (+) *S. aureus* bacteria.

The patterning of the polymer surfaces with two or more functionalities provides a high degree of control of the surface properties not achievable by most other approaches. Many opportunities and challenges exist for creation of multifunctional polymer surfaces. Smaller spatial resolution and variations on the patterns and shapes may open new avenues for the development of advanced biopolymer surfaces.
APPENDIX A

COVALENT ATTACHMENT OF MULTILAYERS (CAM) OF HEPARIN AND POLY(ETHYLENE GLYCOL) ON POLY(TETRAFLUOROETHYLENE) (PTFE) SURFACES

To confirm covalent attachment of the PEG and HP layers, ATR FT-IR spectroscopy was utilized as illustrated in Figure A.1.a and b. As seen in Figure A.1.a, the first MA layer is obtained by COOH-PTFE showing the band at 1715 cm\(^{-1}\) due to C=O of COOH groups. The reactions of COOH functionalized PTFE with OH-PEG lead to ester formation (C=O). The alternating layers of HP and OH-PEG also produce ester linkages. The ATR FT-IR spectrum of the first PEG layer (a\(_1\)) shows the band at 1730 cm\(^{-1}\) due to ester linkages between the COOH-PTFE and OH-PEG. The band at 1070 cm\(^{-1}\) due to C-O-C of PEG was also detected. The first heparin layer (b\(_1\)) was attached in the next step which is manifested by the presence of the band at 1730 cm\(^{-1}\) due to ester linkages that result from the reactions of COOH of HP and OH of PEG as well as the band at 1600 cm\(^{-1}\) due to the OH bending modes of heparin. These results confirm the covalent attachment of OH-PEG and HP layers on the COOH-PTFE surface. Since the ester band formation of second (a\(_2\) and b\(_2\)) and third layers (a\(_3\) and b\(_3\)) is identical to those produced in the first layer (a\(_1\) and b\(_1\)), higher intensities of the bands at 1730 cm\(^{-1}\) due to ester linkages between PEG and HP layers and at 1620 and 1600 cm\(^{-1}\) due to OH bending vibration of heparin are also detected, confirming the covalent attachment of OH-PEG and HP layers to COOH-PTFE surface.

An alternative path of reactions to produce CAM was to utilize NH\(_2\)-PEG, where reactions of COOH-PTFE and NH\(_2\)-PEG lead to amide formation (C=O-NH), also
producing the alternating layers of HP and NH$_2$-PEG amide linkages. ATR FT-IR spectra shown in Figure A.2.b, illustrate the presence of the band at 1715 cm$^{-1}$ due to C=O of COOH entities in the first COOH-PTFE layer (MA) resulting from the microwave plasma reactions and at 1650 cm$^{-1}$ due to amide linkages produced from the reactions of COOH-PTFE and NH$_2$-PEG ($a_1'$) and again the band at 1080 cm$^{-1}$ due to C-O-C of PEG was detected. The next reacted layer is heparin ($b_1'$), which is also manifested by the bands at 1630 cm$^{-1}$ due to NH$_2$ linkages resulting from the reactions of COOH of HP and NH$_2$ of PEG and the 1600 cm$^{-1}$ band attributed to OH bending vibrations of heparin, confirming covalent attachment of NH$_2$-PEG and HP layers. Again, as subsequent layers are reacted, the band intensities increase, indicating that the multiple layers of NH$_2$-PEG and HP were covalently attached to the COOH-PTFE surfaces.
Figure A.1. ATR FT-IR spectra in the 1900-1000 cm\(^{-1}\) region of each CAM layer; (a) ester linkages of OH-PEG: Trace A- PTFE, Trace B- MA, Trace C- PEG (a\(_1\)), Trace D-HP (b\(_1\)), Trace E- PEG (a\(_2\)), Trace F- HP (b\(_2\)), Trace G- PEG (a\(_3\)), Trace H- HP (b\(_3\)); and (b) amide linkages of NH\(_2\)-PEG: Trace A- PTFE, Trace B- MA, Trace C- PEG (a\(_1\)' ), Trace D- HP (b\(_1\)' ), Trace E- PEG (a\(_2\)' ), Trace F- HP (b\(_2\)' ), Trace G- PEG (a\(_3\)' ), Trace H-HP (b\(_3\)' ).
Hydrolytic stability of the CAM surfaces containing OH- and NH$_2$-PEG was determined by exposing each specimen to boiling water for 20 min while stirring. The IR analysis shown in Figure A.2, a-c, show that the C=O bands at 1730 and 1630 cm$^{-1}$ due to ester and amide linkages before and after boiling are indistinguishable, thus indicating stability of the CAM layers.

Figure A.2. ATR FT-IR in the 4000-1000 cm$^{-1}$ region of the CAM surfaces before and after H$_2$O wash at 100 $^\circ$C for 20 min of (a) OH-HP and (b) NH$_2$-HP.
In an effort to control hemocompatibility performance of heparinized materials, uniformity of PEG and HP modified surface is important. This is primarily related to the fact that the presences of unevenly areas of HP allow unwanted platelet adherence and aggregation. As illustrated in Figure A.3, the SEM images of the unmodified PTFE exhibits a certain degree roughness. After plasma modification (MA-PTFE) and CAM process (OH-HP and NH$_2$-HP) the surfaces become smoother.

![Figure A.3. SEM images of PTFE, MA-PTFE, OH-HP, and NH$_2$-HP surfaces.](image)

The smoothness alone indicated good surface coverage, but does not prove complete coverage. Therefore, IRIRI were conducted. Figure A.4 illustrate IRIRI images and IR spectra of the surface containing (a) OH-PEG, (b) NH$_2$-PEG, (c) OH-HP, and (d) NH$_2$-HP recorded from random areas of the surface. As seen from the spectra, the layer formed on the surface shows chemically homogenous distribution of these species by tuning to the bands characteristic of C-O-C (PEG), and O-H (HP).
Figure A.4. IRIR images and IR spectra recorded from random areas A, B, and C of (a) OH-PEG, (b) NH2-PEG, (c) OH-HP, and (d) NH2-HP surfaces.
APPENDIX B

MICRO-PATTERNING OF STREPTAVIDIN-BIOTIN ON POLY(TETRAFLUOROETHYLENE) (PTFE) SURFACES USING CAM PROCESS

Figure B.1 illustrates ATR FT-IR spectra after each reaction step. PTFE substrate was first microwave plasma reacted with maleic anhydride monomer obtain COOH surface functionality (MA-PTFE). Formation of acid groups on the PTFE surfaces was confirmed by ATR FT-IR analysis. Trace B of Figure B.1 shows the band at 1715 cm\(^{-1}\) due to C=O of COOH formation.\(^1\) was detected. The next step was attachment of biotinylated polyethylene glycol (B-PEG) to COOH-PTFE surfaces with the aid of a carbodiimide. While Figure B.1, Trace D, serves as a reference for B-PEG, the band in Trace C at 1650 cm\(^{-1}\) due amide linkages\(^1\) resulting from the reaction of COOH groups on the surface with NH\(_2\)-groups of B-PEG confirmed a covalent attachment of B-PEG to COOH-PTFE surfaces. The IR bands at 1710 cm\(^{-1}\) due to the C=O of biotin ureido ring and at 1080 cm\(^{-1}\) due to C-O-C linkages of PEG\(^1\) in Trace C were also detected. The biotinylated surfaces were then contacted with STR solution, resulting in biotin-streptavidin binding. Figure B.1, Trace F, shows the spectrum of STR, and the bands in Trace E at 1640 and 1535 cm\(^{-1}\) due to the C=O (amide I) and N-H (amide II) bands of the STR protein\(^1\) are detected, confirming the presence of STR on the surface.
In an effort to determine whether the B-PEG and STR immobilization resulted in uniform surface coverage, IRIRI from the surfaces were collected. Figure B.2.a shows IRIR images tuned to the band at 1080 cm$^{-1}$, which was attribute to C-O-C stretching of PEG after the B-PEG was attached. The highest intensity of the band due to PEG was present on the area C collected from the B-PEG-MA-PTFE surface, confirming attachment of the B-PEG to the COOH-PTFE surface. As randomly recorded spectra from different areas band intensities at major bands were maintained, indicating homogenous distribution of these species on the surface. In the previous studies covalent attachment penicillin$^2$ and ampicillin$^3$ to PTFE surface also resulted homogenous coverages of the species with the difference between the red and the green area intensity at 5%. It should be noted that the band characteristic of C-F for the PTFE surface was close to the band at 1080 cm$^{-1}$, therefore the color intensity was higher than the MA-PTFE surface which has characteristic band at 1715 cm$^{-1}$. After the immobilization of STR, IRIR images were obtained from the same area tuned to the band at 1640 cm$^{-1}$, due
to the amide I of STR as illustrate in Figure B.2.b. Again the highest intensity was found in the area D collected from STR-B-PEG-MA-PTFE and indicated that the STR-biotin recognition occurred at the surface.
Ampicillin was biotinylated with NHS-biotin as shown in Figure B.3.a. The reaction of NHS with the amine from ampicillin produces amide linkage. Figure B.3.b illustrates the ATR FT-IR spectrum of the B-AM. Bands were detected at 1650 cm\(^{-1}\) due to the amide linkage, at 1780 cm\(^{-1}\) due to C=O (β-lactam) and at 1710 cm\(^{-1}\) due to C=O (acid) and C=O (biotin ureido ring), confirming the biotinylation of AM and presence of the antibiotic moieties of AM. Moreover, the disappearance of the bands at 1730 and 1820 cm\(^{-1}\) confirm the reaction has taken place through NHS. Figure B.3.c shows \(^1\)H NMR spectra from the biotinylation of AM. The upfield shift in the resonance (circled) at 2.62 ppm, due to the methylene unit adjacent the NHS moiety of biotin indicated reaction with
the amine moiety of ampicillin. The resonances at 7.14 and 6.5 ppm due to the aromatic protons of ampicillin and the NH protons of biotin, respectively, confirm the presence of biotin and AM in the reaction product after purification.
Figure B.3. (a) Schematic diagram of the biotinylation of ampicillin; (b) ATR FT-IR in the 1900-1300 cm$^{-1}$ region of NHS-biotin, B-AM, and ampicillin; (c) $^1$H NMR of the NHS-biotin, B-AM, and ampicillin.
References

