ABSTRACT

GOLI, KIRAN KUMAR. Functional Coatings Based on Denaturation and Adsorption of Proteins. (Under the direction of Jan Genzer and Orlando Rojas.)

We have developed a novel method for the modification and functionalization of hydrophobic polymeric surfaces. This method offers a simple and versatile platform that is alternative to the commonly used flame, corona, plasma and other types of treatments. Specifically, denatured protein molecules were physisorbed on hydrophobic surfaces including flat solid surfaces modified with self-assembled \( n \)-octadecyltrichlorosilane (ODTS) hydrophobic layers and polypropylene (PP) materials through nonspecific interactions followed by cross-linking the adsorbed proteins forming a stable coating. Protein denaturation and properties of adsorbed protein layers were characterized by using analytical techniques including circular dichroism, ellipsometry, contact angle, and Fourier transform infrared spectroscopy in the attenuated total reflection mode. The amount of protein adsorbed on the surface was adjusted by the means of protein denaturation (chemical or thermal), varying pH and protein concentration in the deposition solution, and adsorption time. The inherent hydrophilic amino acid functionalities of the protein layer including hydroxyl and amino groups endowed surfaces with improved wettabilities. The protein primers also provided opportunity to create/generate new functional coatings for selected applications, which involve growing polymeric grafts and attaching silver nanoparticles.

The hydroxyl- and amino-functional groups present on protein-modified surfaces served as reactive/anchoring sites for the attachment of polymerization initiators from which poly (2-hydroxyethyl methacrylate) (PHEMA) polymer grafts are grown directly through “grafting from” via atom-transfer radical polymerization (ATRP). Grafted polymer layers enriched surfaces with functional hydroxyl groups in HEMA units, which served as attachment points for small molecule fluorinated modifiers of different sizes. Anti-fouling properties of the resultant amphiphilic coatings on PP surfaces were tested against the adsorption of fluorescein isothiocyanate-labeled bovine serum albumin as a model fouling
protein. Our work established that these amphiphilic coatings suppressed significantly the adsorption of proteins as compared to PP surfaces or PP surfaces coated with parent PHEMA brushes. Modulation of the surface composition of the top-most layer of the amphiphilic coating and its anti-fouling capability is evident by varying the type of the fluorinated unit grafted to PHEMA.

We have also generated coatings with antibacterial characteristics by attaching with silver nanoparticles to PP fiber surfaces via electrostatic interactions. Specifically, lysozyme protein layer assembled on PP surfaces created numerous cationic charged functional groups depending upon the solution pH conditions. Citrate capped silver nanoparticles were subsequently deposited through ionic interactions on to the protein-modified surfaces. Characterization of the resulting functional surfaces with UV-vis spectroscopy and scanning electron microscopy confirmed the attachment of silver nanoparticles. The concentration of silver nanoparticles on the surface increased with the increasing the amount of protein on the surface. The modified surfaces exhibited excellent antibacterial characteristics against *E. coli.*
Functional Coatings Based on Denaturation and Adsorption of Proteins

by
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DEDICATION

This dissertation is dedicated to my parents, Anjali Devi and Venkata Rao Goli, my brothers Ashok Babu and Leela Krishna and my advisors Dr. Jan Genzer and Dr. Orlando Rojas, for their love, support and encouragement.
BIOGRAPHY

Kiran Kumar Goli was born to Anjali Devi and Venkata Rao Goli in Epurupalem, Andhra Pradesh, India. He completed his high school education from Bharathi Junior College in May 2002. Later, he joined Mumbai University Institute of Chemical Technology, Mumbai, India and completed the Bachelor of Technology program in Fibers and Textile Processing in May 2006. In Fall 2006, he was admitted to the Master of Science program in Textile Chemistry and his dissertation thesis was guided by Dr. Peter Hauser and Dr. Brent smith at North Carolina State University in Raleigh, NC. Upon completion of his Master of Science degree in June 2008, he joined the Ph.D. program in Materials Science and Engineering at the same university, in the area of ‘surface modification of hydrophobic polymeric surfaces’ under the guidance of Dr. Jan Genzer and Dr. Orlando Rojas.
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CHAPTER 1 - INTRODUCTION

Surface modification of polymeric materials is of great interest in materials science and interfacial engineering.¹ Most polymers are inertly hydrophobic in nature, i.e., they possess low surface energy, resulting in poor compatibility with other materials and environment. However, they exhibit superior bulk physical and chemical properties, are processable and inexpensive, which makes them versatile for numerous applications. The surface properties of these materials are equally important for their success in applications involving adhesion, biomaterials, protective coatings, friction and wear, composites, microelectronic devices and thin-films.² In order to improve wettability of most polymeric materials, they must be treated with various surface modification methods. In general, surface properties can be altered and optimized for specific applications by tuning the surface chemistry, topography, surface modulus, charge density, and mobility of surface groups. These treatments improve the performance of a given material by introducing new functionalities thus imparting specific surface characteristics. The characteristics include enhanced surface hydrophilicity, superhydrophobicity, biocompatibility, conductivity, anti-fogging, anti-fouling, grazing, surface hardness, surface roughness, adhesion, lubrication, antistatic properties, and others.¹,³ For instance, many applications of polymers require good bonding with printing inks, paints, coatings, lamination and adhesives to the surfaces.⁴ Hence, introducing polar groups onto hydrophobic surfaces through surface modification techniques improves the adhesion to a greater extent. In addition, the fact that hydrophobic surfaces are more susceptible to bioadhesion than hydrophilic ones results in enhanced “bio-fouling”, i.e., non-specific adhesion of biological species, which is highly undesirable in separation or filtration membranes, biosensors, microfluidics, and coatings that are in contact with biological milieu.⁵ Thus, modification of polymer surfaces has become increasingly important and
useful for many practical applications in industry as it creates materials with tailor-made coating properties.

Many physical and chemical modification methods have been reported in the literature to optimize the surface properties of polymers. Physical modification involves the exposure of surfaces to environments that contain active species such as free radicals, electrons, ions, excited molecules or electromagnetic radiation. These active species attacks and reacts with the polymer surface molecules and generate (typically oxygen-containing) polar and reactive groups on the treated surfaces. The most common physical modification methods include treatments by flame, corona, plasma, UV, ozone, combined UV/ozone (UVO), electron beams, ion beams, and γ-rays. In general, physical treatments do not involve hazardous chemicals; hence these methods are typically environmentally safe, relatively clean, and engage relatively short processing times. Chemical modification techniques include grafting of small hydrophilic units, chemical treatments (such as oxidation by strong acids), deposition of self-assembled monolayers (SAMs), grafting of macromolecules or polymerization processes that are initiated directly on the surface of said material. Alternatively, surface modification can be achieved by the adsorption of surface active amphiphiles (i.e., copolymers or surfactants) or by pre-mixing (melt-blending) polymer precursors with surface-active agents (i.e., alkyl alcohol ethoxylates) during manufacturing processes, which segregate to the surface of the initially hydrophobic films or fibers. In many cases, a combination of physical and chemical methods must be employed to achieve desired coatings. Such reactions involve grafting polymer chains on the substrate surface pre-activated via plasma or glow discharge, corona discharge or UV light.

The inert nature of the hydrophobic materials demands the use of harsh treatments or severe reaction conditions resulting in polymer degradation. However, a successful surface modification technique that endows materials with desired surface properties without sacrificing bulk properties and eliminates the additional need to redesign the bulk materials to obtain a tailor-made performance is over-due. Hence, experimental conditions must be optimized carefully to convert cheaper materials into valuable finished products.
The main objective of this Ph.D. thesis is to devise a novel and versatile surface modification technology to impart hydrophilic and functional characteristics by denaturation and adsorption of proteins on hydrophobic polymer surfaces. Flat hydrophobic $n$-octadecyltrichlorosilane (ODTS) and PP nonwoven substrates are used to immobilize the denatured protein molecules. In general, proteins in aqueous solutions adopt folded “micelle-like” structures with hydrophobic amino acid residues found predominantly in their interior, while the hydrophilic amino acid residues are found on the surface of folded proteins. Denatured protein molecules obtained by adding urea exposed more hydrophobic amino acids (which are protected in the interior of the core of native proteins) to the exterior provided more sites for binding of proteins onto hydrophobic surfaces. Adsorption of these unfolded protein molecules on ODTS surfaces occurs preferentially with hydrophobic amino acid residues partitioning on hydrophobic surfaces while the hydrophilic amino acid residues are present on the periphery of the protein layer. The inherent hydrophilic amino-acid functionalities of the protein layer including hydroxyl and amino groups as well as the charged groups on the surface are subsequently utilized to introduce desired functionalities by anchoring functional polymer grafts, amphiphilic polymer grafts or depositing silver nanoparticles for application ranging from improved wettabilities to antibacterial properties.

The initial part of the study presented in Chapter 2 is focused on hydrophilizing flat hydrophobic substrates featuring self-assembled ODTS and PP nonwovens surfaces through the adsorption of denatured protein molecules from aqueous solutions. The hydrophobic polymer of interest in this study is polypropylene (PP), since it is the most popular and widely used material in the nonwoven industry owing to its good mechanical strength, excellent chemical resistance, low density, thermal stability, and low cost. Those attributes make PP a promising candidate for various applications involving functional textiles, filtration devices, medical implants, and many others. The market share of PP in the nonwoven industry is increasing steadily due to the development of numerous surface modification techniques and in 1999 it accounted for approximately 62% of the man-made fibers market.$^{12,13}$
Proteins represent an interesting class of coating materials to create hydrophilic surfaces because they adsorb through non-specific interactions on hydrophobic surfaces without involving any harsh treatments. Denaturation of protein molecules is desired to make the hydrophobic residues available for adsorption on hydrophobic surfaces. This can be accomplished by chemical (i.e., using urea) or thermal means in the bulk solution, and can be monitored by various methods, such as circular dichroism. The amount of urea and heat-denatured proteins adsorbed is monitored for depositions at varying conditions, including, protein concentration in solution, pH, adsorption time, and temperature. Adsorbed protein molecules are subsequently used as primers to graft polymer brushes by surface-initiated atom-transfer radical polymerization of selected monomers. The resultant properties of protein and polymer layers such as thickness, wettability, surface chemical changes and surface morphology are explored and the results are discussed.

In Chapters 3 and 4 of this Thesis, protein-coated primers on hydrophobic ODTS and PP non-woven substrates are utilized to engineer surface properties towards the creation of desired functional products required for current industrial, institutional, and hygienic applications. Specifically, Chapter 3 summarizes our efforts aimed at the formation of amphiphilic coatings to impart anti-fouling characteristics. The surface modification comprises a three-step process. In the first step, the protein layer is deposited that provided active sites for “grafting from” polymerization of poly(2-hydroxyethyl methacrylate) (PHEMA), which is carried out in the next step. Finally, amphiphilic grafts are generated by fluorinating PHEMA chains with selected fluorinating agents. The changes in surface characteristics are studied by using various analytical tools. The activities of the resultant amphiphilic coated surfaces against protein fouling are investigated. Chapter 4 illustrates the work conducted to attach silver nanoparticles to the protein-coated PP surfaces. The synthesized nanoparticles are characterized by UV-Vis spectroscopy and transmission electron microscopy. The antibacterial properties of the silver nanoparticle decorated PP fiber surfaces pre-coated with protein molecules are determined by testing against gram
negative bacteria, *Escherichia Coli*. **Chapter 5** proposes potential approaches and insights towards the advancement of this facile and robust technology to diverse applications.

### 1.1. Surface modification techniques

This section offers a brief overview of the most conventional and current surface modification techniques used to alter surface properties of polymers. This section also points out the reasons and motivation for the development of a novel, facile and robust surface modification technology.

#### 1.1.1. Flame treatment

Flame treatment was developed in the 1950s to improve the wetting and adhesion properties of polyolefin films.\(^{14}\) A schematic diagram for a flame treatment set-up is shown in **Figure 1.1**. In this method, one side of the polymer film to be treated is exposed to the burner while the backside is cooled by water-cooled backing roll. The burners are fed with a mixture of natural gas and air flame in controlled ratios to oxidize the polymer surfaces. Free radicals, such as OH and atomic oxygen, present in the flame primarily attack the tertiary carbon atom of the PP parent/backbone leading to the formation of polymer-radicals which would react with O, -OH and O\(_2\) species. This treatment results in the generation of oxygen-containing species such as hydroxyls, carboxyls, and carbonyl functionalities on the polymer surfaces. Flame treatment is used extensively to treat paperboard or thick polyolefin materials, such as blow-molded bottles by increasing the number of burners. Typical exposure times of the films to the flames must be less than 1 s. The difficulty in controlling the chemical composition of fuel and the distance between the tip of the flame and the object results in inconsistent treatments\(^{8,14,15}\)

#### 1.1.2. Corona discharge

Exposure of the polymer surface to corona discharge has been one of the common methods used to improve adhesion and printability. Corona discharge emerged as the
primary method of treating films in comparison to flame treatments due to the safety concerns related to open flames in industrial environments.\cite{14} A schematic of corona treatment system is depicted in Figure 1.2. High frequency electrical energy is used to generate the electrical discharge in a gap between two electrodes under atmospheric pressure. The electrical field generated excites the gas molecules (air) and dissociates some of them thus generating ions, radicals, electrons, and ozone, which reacts with the surface polymer molecules (of the film that is passed on the roll during the treatment processes) forming radicals. These radicals react rapidly with the atmospheric oxygen as shown in Figure 1.3, leading to the introduction of polar oxygen atoms (mainly, peroxide or carboxylic groups).\cite{4,8} The exposure times of the films to the corona were fractions of seconds depending upon the energies supplied.\cite{15} Corona pre-treated PE films can be grafted with various water polymers such as poly(acrylic acid) to introduce new functional properties as reported in previous works.\cite{3} Elsabee et al.\cite{16} activated PP films with corona-discharge to generate peroxide and carboxylic acids which were used to attach chitosan, thus imparting antimicrobial properties.

The equipment set-up of both flame and corona treatments are simple, cost-effective and can be used in continuous operations at high processing speeds. However, since both the treatments are carried out under ambient conditions, the resultant treatment may be inconsistent due to the difficulty in controlling temperature, humidity or contaminants in the atmosphere. In addition, prolonged exposures lead to the degradation of polymers. Another limitation is that the enhanced surface properties are short-lived, therefore further applications or bonding processes such as printing, adhesion or sealing must be carried out immediately.\cite{8,14,15}

1.1.3. Plasma treatment

Plasma treatment is the most widely industrially used method for enhancing wettability, printability, dyeability, sealability, and adhesion properties of polymer due to the versatility of the method.\cite{17} Plasma is broadly referred to as a partially ionized gas that contains charged species. In general, plasma treatments are carried out at low gas pressures and low
temperatures, in which gas species are ionized by the glow discharge producing ions, electrons, radicals and excited molecules. The polymer surfaces that come in contact with gas plasma are bombarded by this energetic species generating various functional groups such as –COOH, –OH, and –NH$_2$ depending upon the nature of treatment gases used. The most commonly used non-polymerizable treatment gases are air, oxygen, argon, hydrogen, ammonia, argon/ammonia and fluorine. In comparison to conventional flame and corona treatments, plasma treatments offer several advantages listed below: 8,18,2

1. Can be confined to the top surface layer with depths typically ranging between 0.005 to 0.05 µm, thereby not damaging the bulk properties.
2. Introduce diverse chemical functionalities onto polymer surfaces by variation of the treatment gas used.
3. Modify polymer surfaces, irrespective of the structure or chemical reactivity of the surfaces.
4. Operate at lower temperatures (relative to corona or flame treatments), which helps in modifying heat-sensitive polymeric materials.
5. Relatively long-lived (in comparison to flame and corona).
6. Provide fairly uniform modification throughout the substrates.
7. Do not involve any harmful byproducts; it is an environmentally safe and dry process.

Since, plasma treatment is carried out at low gas pressures, this technique is best suited for batch processes which increase production times, operational costs and may thus not be economical. To overcome these limitations, plasma treatments are operated at atmospheric pressures. Figure 1.4 shows a schematic of atmospheric pressure plasma modification. Because atmospheric pressure plasma has poor control over low-pressure plasmas due to the difficulty in controlling the composition of air mixed with carrier gas and treatment gases, it is difficult to achieve effective and reproducible treatment due to the complexity of plasma processes. The major disadvantage of these processes is that targeted surface properties produced on plasma-treated films are not stable and gradual ‘hydrophobic recovery’ takes place. Depending upon the environment, this can result in undesirable surface reconstruction over time. Hence, plasma-treated samples must be used immediately for further
functionalization or must be stored in a sealed container before they can be treated with further functionalization.\textsuperscript{18,19} Petasch \textit{et al.}\textsuperscript{20} reported on a considerable improvement in bond strengths of low-density polyethylene (LDPE) and PP and poly(ethylene terephthalate) (PET) films after treatment with O\textsubscript{2} plasma due to the generation of polar functional groups at the surface layer. O’Kell \textit{et al.}\textsuperscript{21} explored the effect of air and nitrogen plasma-treatments on peel strengths of aluminum/PE/aluminum laminates. They reported a significant increase in peel strength of PE/aluminum interface for air plasma-treated samples as compared to nitrogen plasma-treated substrates. This is most likely due to the better adhesion provided by the oxygen-containing functional groups added to the PE surface during the air-plasma modification. In addition to generating functional groups on polymer surfaces, cross-links can be created on the surface to improve the barrier properties of polymers. A relevant work of Rossi \textit{et al.}\textsuperscript{22} on Ar plasma-modified LDPE films showed an increase in O\textsubscript{2}, CO\textsubscript{2} and N\textsubscript{2} barrier properties due to the cross-linking induced over the polymer surfaces. Guruvenket \textit{et al.}\textsuperscript{23} modified the polystyrene and polyethylene films using oxygen and argon plasmas. They observed a significant improvement in wettabilities of oxygen and argon-treated polymers. In the former case, higher degrees of surface hydrophilicities were evident due to the occurrence of simultaneous processes. Atomic oxygen and other active species from the plasma reacted with surface carbon atoms of the polymer surface resulted in the formation of volatile reaction products and oxygen containing functional groups. However, in the latter case, the active species from argon plasmas abstracted hydrogen atoms from the polymer surface and generated free radicals. These radicals interacted with other radicals and formed cross-links and unsaturated groups with chain scission resulting in surface modification.

Plasma polymerization or grafting monomers or formation of a thin polymer film on polymer substrate surfaces or initiating graft polymerization on polymer substrates by utilizing the polar groups introduced on plasma treated samples represents an alternative surface modification route to impart additional functional surface properties or increase the polar components. In general, plasma polymerization can be carried out by using organic monomer molecules such as CH\textsubscript{4}, C\textsubscript{2}H\textsubscript{4}, C\textsubscript{2}H\textsubscript{6}, C\textsubscript{2}F\textsubscript{4} or C\textsubscript{2}F\textsubscript{6} as treatment gases. Glow
discharge ionizes these monomers followed by their deposition on polymer surfaces. Plasma polymerization is considered to be a very effective technique to grow a thin layer of a surface coating in a single-step process that may endow polymer surfaces with a permanent finish. However, the structure resulting from plasma-polymerized films is not well-defined as it differs from the structure produced by conventional polymerization schemes using the same monomer. In addition, the control over surface chemistry of the produced coating is challenging due to its dependency on the many factors such as the design of the reactor, input power, monomer flow rate, substrate temperature, and frequency. Yuan et al. functionalized PE films with N-vinyl-2-pyrrolidone and allyl alcohol through plasma polymerization. They observed an increase in the surface energy as well as oxygen barrier properties of the treated material. Kim et al. demonstrated an improvement in water repellency of plasma-treated linear low-density polyethylene (LLDPE) films using CF₄ fluorinated compound as a plasma gas. Zhao et al. reported on grafting of poly[3-(methacryloylamino)propyl]-dimethyl(3-sulfopropyl) ammonium hydroxide polymers on PP nonwovens through O₂ plasma pre-treatment and subsequent polymerization with UV irradiation. Plasma pretreatment produces necessary oxygen-containing polar peroxide groups needed for free radical polymerization. Zhao and co-workers observed that the modified surfaces exhibited excellent resistance to platelet adhesion and protein adsorption. The work by Yang et al. explored generation of anti-fouling coatings on PP micro porous membranes pre-activated with plasma with subsequent formation of cross-linked poly(N,N-dimethylaminoethyl methacrylate) layers. Liu et al. grafted poly(γ-stearyl-l-glutamate) peptide onto the surface after activating PP surfaces via ammonia-plasma treatment for 20 min followed by aminolysis with γ-(aminopropyl)triethoxy-silane.

Even though plasma surface modification is the most successful method employed in industry, the complexity involved in its process has led to non-uniform treatments and changes in surface topography due to etching and surface degradation of parent material associated with overexposure to plasma. In addition, the functional layer generated on the polymer substrate is not firm; in some cases the generated functional layer can be easily
removed by rinsing with aqueous or polar solvents.\textsuperscript{27} Hence, there has been a long-standing demand to develop an alternative technique to impart permanent functionalities on hydrophobic surfaces.

1.1.4. UV light treatment

UV light treatments provide polymer surfaces with similar modifications as reported for plasma treatments. In general, exposure of polymer surfaces to UV-light results in photo-crosslinking or photo-oxidation in air, or photochemical reactions in reactive atmosphere. The advantage of UV irradiation as compared to other methods is that wide areas as well as small and localized areas can be treated and the cost of the energy source is lower as compared to ionizing radiation sources. In addition, photo irradiation affects properties either at the outer surface or deeper into the bulk polymers. In contrast to plasma, the power intensity of UV can be well controlled by using continuous wave (CW) UV-lamps.\textsuperscript{2} In general, UV-treatments are carried out between wavelength ranges of 200-400 nm. However, treating polymers that contain pigments or coatings results in uncontrolled surface alterations due to the partial absorption and scattering of UV radiations by pigments. In addition, exposure of UV light to the workplace must be avoided by proper safe shielding installations.\textsuperscript{8}

Geetha \textit{et al.}\textsuperscript{29} modified LLDPE, medium-density polyethylene (MDPE) and high-density polyethylene (HDPE) films by exposing them to UV-light at wavelengths greater than 250 nm in air. The resultant treated films, especially LLDPE and MDPE, exhibited an improvement in elongation at break. However, prolonged exposure times adversely affected the mechanical properties of the treated films. Swanson \textit{et al.}\textsuperscript{30} observed an improvement in bonding strength of UV-light modified HDPE films to each other with cyanoacrylate-based adhesive as compared to untreated films.

In addition to adding oxygen-containing polar groups onto polymer surfaces, polymers can be surface-grafted onto UV irradiated surfaces with or without photo-initiators. When polymer surfaces containing chromophores are irradiated with UV light, the molecules on
polymer surfaces jump to an excited state, resulting in generation of reactive free-radicals directly on polymer backbone through bond dissociation. If a monomer is supplied to this polymer surface with active groups, graft polymerization occurs resulting in polymer brushes. However, if the UV irradiation does not generate the necessary active groups on polymer surface, photo-initiators or photo-sensitizers can be used to produce the active sites. For instance, benzophenone is used as a photoinitiator to promote the formation of free radicals. Upon UV irradiation ($\lambda = 365$ nm) benzophenone excites to a singlet state and then jumps to a more stable triplet state, which facilitates the abstraction of a hydrogen atom from the polymer surface creating grafting sites on the surface.\(^5\,\text{and}\,\text{Jang et al.}\)^{31} Jang et al.\(^{32}\) graft polymerized 2-hydroxyethyl methacrylate (HEMA) onto PP fabrics in the presence of benzophenone initiator to improve the dyeability of PP surfaces. Zhao et al.\(^{26}\) modified PP nonwoven membranes by activating via oxygen-plasma pretreatment followed by grafting [3-(methacryloylamino)propyl]-dimethyl(3-sulfopropyl) ammonium hydroxide (MPDSAH) polymers using UV-irradiated technique. Recently, Ma et al.\(^{33}\) reported a novel two step approach to modify PP membranes with acrylic acid. In the first step, benzophenone was covalently bonded to the PP surface under UV-light to produce benzpinakol grafts. In the second step, benzpinakol grafts were photo-cleaved using far-UV irradiation producing graft polymers. Huang et al.\(^{31}\) demonstrated a novel method to graft PDMAEMA by living radical polymerization with benzophenonyl 2-bromoisobutyrate initiator to improve the antibacterial properties. UV irradiation might trigger photo-degradation of polymer surfaces depending upon the UV energy employed.\(^8\)

### 1.1.5. Other surface treatments

In general, UV-light treatments are ineffective over PP surfaces as compared to PET surfaces due to the insufficient absorption of UV radiation by PP.\(^{15}\) Hence, ozone treatment was coupled with UV to produce efficient surface oxidation, in which case UV light decomposes $\text{O}_3$ and $[\text{O}]$ to generate active species to promote the surface treatment. Ozone generates reactive species such as $\text{O}^\cdot$ or $[\text{O}]$, which will attack the polymer surface layer resulting in surface oxidation. Plasma surfaces can be modified under atmospheric pressures
using ozone. The treatment efficiencies of UV, O₃ and UV/O₃ are smaller than those of plasma treatments. Kumagai et al. modified PE films by exposing the films to ozone at temperatures of 25 and 65°C. The results showed that the ozone oxidation was more efficient at higher temperatures. The work by Wang et al. reported on surface-initiated polymerization of grafting of N-vinyl-2-pyrrolidone (NVP) monomer onto PP nonwovens pretreated with ozone to generate oxygen functional groups especially peroxide groups which were subsequently reduced chemically by potassium iodide to generate hydroxyl groups homogeneously which assisted in immobilization of ATRP initiators. Strobel et al. compared the surface oxidation processes for flame, corona discharge, remote air plasma, ozone and combined UV/ozone treatments. They reported that polymer surfaces can be readily oxidized by flame, corona and plasma treatments. In contrast, UV and UV/ozone treatments required more reaction times to obtain similar surface oxidation levels.

Other surface treatments include using high energy sources such as γ-ray, electron beam, and ion beam irradiation. All these treatments alter polymer surfaces either through the formation of free radicals sites on the surface induced by photons or the bombardment of high energy electrons or ions on polymer surfaces leading to reduction or oxidation or cross-linking. In addition to these techniques, hydrophobic surfaces can be modified by subjecting to wet chemical treatments such as exposing the polymer surfaces to strong bases or acids. Bamford et al. grafted acrylamide monomers onto PP surfaces pretreated with aqueous potassium peroxydisulfate at 80°C to generate hydroxyl groups. Acids, such as fuming sulfuric acid and chlorosulfonic acids, were reported to oxidize polyethylene surfaces. In addition chromic acid in the presence of sulfuric acid acts as an efficient chemical etchant to hydroxylate PE surfaces. Zhang et al. improved the antifouling characteristics of poly(dimethylsiloxane) (PDMS) substrates by treating with piranha solution (H₂SO₄/H₂O₂) to generate surface hydroxyl groups which were subsequently utilized to form dendronized PEG methacrylate brushes by SI-ATRP. A relevant work by Chaudhury et al. described the formation self-assembled monolayers on PE surfaces in a number of
steps where PE film was oxidized using plasma oxidation followed by the formation of a thin silicate layer and then exposed to the vapors of alkyltrichlorosilanes.

Although all of the aforementioned treatments alter the surface properties of the materials, they degrade the polymer surfaces, which may compromise their mechanical and optical properties. Hence, there is a need to develop alternative and versatile surface modification technology to impart consistent, uniform and durable hydrophilicities/functionalities. This should involve less harsh treatments without damaging the bulk properties.

1.2. Surface modification by physical deposition of monolayers or thin films

1.2.1. Formation of polyelectrolyte multilayers by layer-by-layer (LbL) self-assembly

A simple technique developed by Decher generally involves the sequential deposition of oppositely charged polyelectrolytes onto a substrate. The versatility of this method helps in introducing numerous functional groups on a broad spectrum of charged polymer substrates. However, only a few papers have reported on the formation of layer-by-layer (LbL) films on neutral hydrophobic surfaces due to experimental limitations. Park et al. modified PDMS and ODTS hydrophobic surfaces by adsorbing poly(allylamine-hydrochloride) (PAH) and poly(styrene sulfonate) (PSS) polyelectrolytes sequentially. PAH, a hydrophobic weak polyelectrolyte, was used as the first adhesion layer on neutral hydrophobic surfaces. However, this method is subjected to the limitation as it requires sequential/multiple adsorption steps and suffers from interlayer penetration and stability dependence on solution pH and ionic strength.

1.2.2. Formation of thin films by Langmuir-Blodgett (LB) technique

The LB technique can be used to deposit highly ordered and ultrathin layered structures onto planar substrates. In the first step, amphiphilic molecules are spread on the surface of water in a trough and then the surface area of the molecules is compressed by reducing the
distance between the barriers on the trough forming highly oriented molecules. In the next step, the molecules are transferred onto the substrate using a horizontal or vertical deposition method. The formation of a monolayer on the substrate depends upon many factors such as the direction and speed of the substrate, surface pressure, composition, temperature and pH of the sub-phase. In addition, surface chemical structures of the Langmuir-Blodgett (LB) films are unstable depending upon the surrounding medium. Habibi et al. formed smooth and stable thin film of cellulose nanocrystals on hydrophobic surfaces using horizontal-lifting also known as Langmuir-Schaeffer (LS) technique.

1.2.3. Formation of block copolymer thin films

A thin layer of polymer coating can be formed on polymer surfaces by the adsorption of block copolymers. Liu et al. adsorbed Pluoronic® (P-105, EO₃₇PO₅₆EO₃₇), a symmetric triblock nonionic polymer comprising ethylene oxide (EO) and propylene oxide (PO) blocks, onto PP substrates to improve the wettability. The segregation of triblock copolymers on hydrophobic surfaces took place with hydrophobic PO blocks acting as anchoring points while the hydrophilic EO blocks dangled to the surrounding environment.

In summary, many of the aforementioned treatments may either have detrimental effects on the final properties of the fiber surface or they are non-reproducible; they can be difficult to control, are not long-lived or can add additional cost to the final product. In this study, we offer a very cost-effective, robust and facile approach for tailoring the surface properties of any hydrophobic surfaces including fiber materials through the adsorption of protein molecules. The next section will give an overview about the protein molecules.

1.3. Functionalization based on denaturation and adsorption of proteins

1.3.1. Protein adsorption

Adsorption of proteins on interfaces or solid surfaces can be either an advantage or a setback. On the one hand, proteins are adsorbed for a specific purpose in the areas of
biosensors, in vitro immune assays, protein chromatography, tissue and cell culturing and drug delivery systems. On the other hand, protein adsorption is less desirable in biocompatible materials for surgical implants, to prevent adverse host responses such as surface-induced thrombosis or blood coagulation. Adsorption of proteins also results in marine fouling and contamination of processing equipment. In the latter cases protein adsorption should be minimized.

Our approach towards modifying hydrophobic substrates is inspired by the known affinity of proteins to solid surfaces (both hydrophobic and hydrophilic) as a result of non-specific interactions. The formation of an interface at two different phases results in a higher standard free energy at the interface relative to bulk phase. This provides the driving force for the adsorption of any species other than solvent molecules from bulk phase onto the interface to attain thermodynamic stability. The substances might change their orientation and conformation during adsorption or after the adsorption, depending on the solid substrate, substance and solvent systems. Protein adsorption is one of such particular phenomena observed in various fields. This adsorption technique can offer a very cost-effective, robust and flexible technique for tailoring the surface properties of the polyethylene (PE), polypropylene (PP), poly(ethylene terephthalate) (PET) and nylons to impart hydrophilicity.

1.3.2. Proteins

Interaction of proteins with interfaces is a dynamic phenomenon. To this end, studying protein size and structure is vital in optimizing protein interactions with surface. Proteins are large and complex amphipathic biomolecules produced by the step reaction polymerization of amino acids. A vast number of proteins can be created with diverse properties by a small change in the sequence of the twenty available common amino acids. A common amino acid consists of an α-carbon atom at center, to which an amino group, a carboxyl group, a hydrogen atom, and a side chain (R) with a particular functionality, which can change the molecular structure of the protein dramatically. Based on the functionality of the side
groups, amino acids are categorized into non-polar, polar, negatively-charged and positively-charged moieties. The pH of the system determines the charge of an amino acid. At a certain pH, referred to as the isoelectric point (pI), amino acids contain equal amounts of positive and negative charges on an amino and carboxyl group forming zwitterions. Primary, secondary, tertiary and quaternary structures are the important levels of protein architectures. These structures arise from primary structure of proteins due to numerous intra- and inter-molecular protein interactions.

1.3.3. Protein structures

Primary structure of protein refers to the linear sequence of amino acids linked by peptide bonds. The localized hydrogen interactions between amino acid units lead to bending, folding and curling of the peptide chains within the localized domains results in the formation of the secondary structure of a protein. These structures are composed of areas of ordered regions in the protein chain. Two main structures, α-helix and the β-pleated sheets, are the most thermodynamically stable secondary structures. The content of the secondary structures in proteins varies considerably from very small to a large percentage for different proteins. Generally, most globular proteins are composed of 40-80% of secondary structures buried mainly in the interior of the proteins. The tertiary structure of proteins results from spontaneous interactions between amino acids and between amino acids and water under aqueous environments. Protein folding owing to these interactions generates three-dimensional structure with little order. Interactions between amino acid side chains are the dominant forces involving in tertiary structure formation. These interactions include van der Waals (between leucine or phenylalanine amino acids), hydrogen (between polar serine or tyrosine amino acids), ionic (between positively charged lysine or arginine with negatively charged aspartic acids or glutamic acid), and covalent (between cysteine residues) bond formations. In addition, amino acid residues either repel or attract depending on the sequence of the amino acids and the type of functionality present on them. These repulsions or attractions twist and fold the proteins thus minimizing unfavorable interactions and forming a likely three dimensional protein structure. The strengths of
different bonds, which govern the proteins tertiary structure, are shown in Table 1.1. The bonds with weaker strengths (van der Waals and hydrogen bonding forces) are the most important binding interactions that govern the tertiary structure for majority of the proteins rather than higher strength covalent bonds. This is because the disulfide bond is the only covalent bond possible in the tertiary structure created by the oxidation of two cysteine amino acids. However, eight amino acids can contribute towards the formation of van der Waals and hydrogen bond interactions individually in contrast to ionic bond interactions (four amino acids are capable of forming ionic bonds).

The structure of proteins in aqueous media is also influenced by the solvent environment. Highly polar Water forms hydrogen and ionic interactions with the hydrophilic amino acids, whereas the hydrophobic amino acids are repelled by water forming an inner core. Thus most of the hydrophilic groups form a corona around the inner hydrophobic core that interacts strongly with water. The hydrophobic groups inside the core interact with one another thus stabilizing the tertiary structure. The interior core contains regions of polar groups stabilized through hydrogen bonding or salt bridge associations, while the exterior corona contains some regions of non-polar groups. The inner region of protein is packed very closely comparable to a protein crystal with loosely packed reactive sites providing accessibility for chemical reactions. Different amino acid chains each with its primary, secondary and tertiary structures come together forming quaternary structures. Quaternary structures are also controlled by the hydrophobic and hydrophilic interactions existing between primary, secondary, and tertiary structures. Depending on strong and weak internal cohesions, proteins are classified as hard and soft proteins respectively. Hard proteins possess high internal stability; they undergo fewer structural changes while the soft proteins experience reasonable surface-induced structural changes with lower internal stability. Examples of selected hard and soft proteins are tabulated in Table 1.2.
1.3.4. Protein-surface interactions

When soluble proteins come into contact with a solid surface, protein adsorption takes place instantaneously by altering the protein conformation or unfolding the protein structure. The continual change in conformation of the proteins changes physical-chemical properties of surfaces. Adsorption alters the interfacial boundary layer by replacing water, ions and other solutes with proteins. Most proteins exhibit inherent surface activity because of the amphiphatic nature and adsorbs readily onto the interfaces.\textsuperscript{48,52,59} Thus interaction of proteins on solid surfaces is a dynamic and complex phenomenon, quite difficult to understand and control. The adsorbed protein surface can act as primer for the creation of new functional properties by generation of amphiphilic polymer grafts or attachment of silver nanoparticles or adhesion of eukaryotic cells.\textsuperscript{52}

A monolayer of proteins adsorbs onto the solid surfaces within a time span of seconds to minutes from soluble protein solution. Surface concentration of proteins adsorbed on a solid surface is typically in the range of 1 \( \mu \text{g/cm}^2 \), which is often more concentrated than in the bulk phase. Once the solid surface is saturated by proteins, protein adsorption reaches a maximum and remains constant irrespective of the higher initial bulk protein concentrations. Protein adsorption is in most cases irreversible and results in protein layers bound relatively tightly to the substrate. Adsorbed proteins can be removed only under harsh treatments by using detergents such as sodium dodecyl sulfate (SDS).\textsuperscript{54}

The nature of the proteins as well as the physical and chemical properties of the solid surface that affect protein adsorption processes are tabulated in Tables 1.3 and 1.4. Solution conditions such as pH, ionic strength, addition of denaturants and temperature also affect the interaction of proteins to a significant extent.\textsuperscript{50,60} Figure 1.5 shows that a large protein molecule adsorbed on to the surface with more contact points in comparison to the small protein molecules. Thus a large molecule binds strongly to the substrate and cannot rotate freely and remain in a preferred orientation due to these multiple contacts or binding sites.\textsuperscript{58,60} At the isoelectric point (pI), the global number of positive and negative charges in a protein is balanced. When these charges are distributed in a uniform manner over the
protein molecule, intramolecular electrostatic interactions are attractive resulting in shrinkage of the protein molecule. Hence, a large amount of protein is allowed to bind onto the surface at the pI.\textsuperscript{58} Hydrophobic dehydration and electrostatic interactions are important driving forces for protein adsorption at solid surfaces. In the case of apolar surfaces, hydrophobic dehydration dominates the electrostatic interaction and proteins adsorb readily onto the hydrophobic surface thus overcoming electrostatic repulsion. As discussed earlier, the globular protein structure in an aqueous environment is governed by the intramolecular hydrophobic interactions at the core shielded by the polar parts exposed to water. When a protein comes closer to the solid surface, water on one side of the protein is replaced by the solid surface. As a result, apolar interactions within the interior of the dissolved protein are outweighed by exposing its apolar groups to the (hydrophobic) surface forming newer hydrophobic interactions. The extent of formation of these hydrophobic interactions between protein and the surface depends upon the degree of the hydrophobicity of the surface. However, at polar surfaces, hydrophobic dehydration forces are not available and the protein nature plays a pivotal role in governing the adsorption. Some proteins come into contact with polar surfaces through hydrogen bonding. Soft proteins undergo structural/conformational changes due to their low stability resulting in the loss of secondary ordered structure, as shown in Figure 1.6.\textsuperscript{58} Due to this perturbation, the peptide units experience partial unfolding and become less restricted; as a result some structural ordering is lost. Subsequently, the amino acid units may form hydrogen bonds with the polar surface. The conformational entropy gained in the disruption of this internal structure is significant to promote the adsorption process against electrostatically unfavorable conditions. In contrast, hard proteins do not undergo significant surface-induced structural changes upon adsorption and bind to the surface only under electrostatically favorable conditions.\textsuperscript{58}

Norde\textsuperscript{58} reported that the surface-induced structural changes result in heterogeneous monolayer formation of the proteins on the surface in the form of native (N) and relaxed/perturbed (P) conformations. The adsorbed protein molecules attempt to relax onto the surface with time forming many bonds per molecule with the surface. However, the
molecules can relax or unfold only if they find sufficient unoccupied surface area on the substrate. An increased concentration of proteins on surface does not allow perturbation/relaxation of the native molecule due to the lack of space available for relaxation of the protein molecule resulting in adsorption of a heterogeneous population of both native and relaxed proteins. The P/N ratio can be increased by increasing the hydrophobicity of the solid surface or by decreasing the surface coverage of the proteins.

Protein unfolding/relaxation is very important since it can expose or increasing the number of contacts with the surface while the numbers of interactions are less for folded proteins and could not bind strongly to the surface. The unfolding/denaturation of the proteins can be promoted by chemical and thermal means. Chemical denaturants, including, urea, guanidine hydrochloride and dimethyl sulfoxide, interact with protein molecules by breaking their molecular interactions which stabilize the native structure of proteins. Thermal denaturation is brought about by an increase in temperature which can break certain molecular interactions. At lower bulk concentrations, the amount of surface available per molecule is higher, which allows more spreading or relaxation of the protein molecule onto the surface. In contrast, at higher bulk concentrations the surrounding area available for protein decreases resulting in less unfolding as shown in Figure 1.7.

Desorption of proteins from the surface is less likely, but an exchange of proteins between the surface and solution can take place. In a multicomponent solution, composition of the adsorbed protein species on solid surface changes with time until a pseudo-dynamic equilibrium state is achieved. The protein molecule with a large diffusion coefficient reaches the surface faster than the molecule with smaller diffusion coefficient although affinity of proteins for surface is higher for the latter than the former. This adsorption is transitory and with time proteins having greater affinity to adsorb onto the surface by replacing the already-adsorbed lower-affinity proteins. This dynamic protein exchange process continues until the surface is occupied with proteins which have higher affinity for it. This effect is known as the “Vroman” effect. This exchange of the proteins also takes place when excess protein molecules are present relative to the available surface area. However, the structural stability
of the molecule after transitory adsorption depends on the nature of proteins, surfaces, and solution conditions.\textsuperscript{48,60}

1.3.5. Thermodynamics of protein adsorption

Regardless of the kinetics and mechanism of protein adsorption process, protein adsorption takes place spontaneously if the change in the Gibbs free energy of the system is negative at a constant pressure and temperature.\textsuperscript{60,62} Thus, it is important to determine the corresponding changes in thermodynamic parameters such as Gibbs free energy ($\Delta_{\text{ads}} G$), enthalpy ($\Delta_{\text{ads}} H$) and entropy ($\Delta_{\text{ads}} S$) during adsorption:

$$\Delta_{\text{ads}} G = (\Delta_{\text{ads}} H - T\Delta_{\text{ads}} S) < 0 \text{ (for spontaneous adsorption)}$$ (1.1)

$\Delta_{\text{ads}} G$ depends upon numerous interactions existing between and within the system components involving the sorbent surface, the protein molecules, the solvent and the presence of any other solutes. Specifically, intermolecular interactions, such as forces between proteins and the surface, solvent and surface, or protein and solvent and intramolecular interactions, such as forces between protein molecules and within the protein molecules, contribute towards change in Gibbs free energy. Lifshitz-van der Waals forces (dispersion, orientation and induction forces), Lewis acid-base forces (hydrogen bond forces), electrostatic forces (including ion pairing), hydrophobic forces (entropically-based), and internal packing (steric/excluded-volume) restrictions are a few of examples of such interactions.\textsuperscript{63} Adsorption data are generally represented in the form of adsorption isotherms. The amount of protein adsorbed ($\Gamma$) is monitored with an increase of protein concentration in solution at a constant temperature. Norde\textsuperscript{62} studied the thermodynamics of bovine serum albumin (BSA) adsorption on glass and obtained the adsorption isotherm. The adsorption isotherm revealed that the process was irreversible since the variation of $\Gamma$ (curves) with increasing and decreasing concentrations did not coincide. Generally, adsorption of proteins is irreversible with the exception of hard proteins adsorbing on the surface with counter charge electrostatic interactions.
Adsorption of proteins increases with increasing bulk concentration and reaches a saturation point. In some cases, adsorption is enthalpically unfavorable depending upon the nature of the proteins and interfaces. Heats of adsorption for selected proteins on specific surfaces were determined and are summarized in Table 1.5. The resulting positive values of enthalpy observed for spontaneous adsorption suggest that the protein adsorption process is dominated strongly by changes in the entropy of a system. The entropy gain arises from the dehydration of the sorbent surface and structural changes taking place at the interior of the protein molecule.50,52,54,64

1.3.6. Kinetics of protein adsorption

Adsorption of proteins to solid surfaces is distinguished into two parts: An initial phase with rapid kinetics controlled by diffusion followed by a sluggish phase before steady state is reached. In the initial phase, a linear increase is observed for the amounts of proteins adsorbed versus square root of time, a characteristic of diffusion-controlled process. Initially, proteins adsorb quickly on to the surface due to readily available surface sites. With increasing time, adsorption slows down because it becomes increasingly difficult to find a suitable position on the surface that can fit the incoming proteins.54 Different models were developed to explain the kinetics of a single protein adsorption. All of these models are based on the hypothesis that the adsorption of the process is surface-reaction controlled. Figure 1.8(a) depicts the reversible Langmuir adsorption model. However, protein adsorption is irreversible in most cases. The model developed by Lundström is shown in Figure 1.8(b). Protein adsorbs on to the surface in the native form (state 1) with a rate constant, ka. After adsorption, with time some of the native molecules undergo denaturation with structural changes at a rate constant k1c (state 2). Beissinger’s and Leonard’s model (Figure 1.8(c)) assumes reversible adsorption and desorption of protein molecules in their native as well as in their denatured conformation. Soderquist and Walton (Figure 1.8(d)) believe that the conformational changes proceed to a complete denaturation of proteins with time via a rate constant of k2c (state 3). At this stage, proteins will not desorb from the surface. However, this model assumes that the adsorbed protein molecules at stage 1 & 2 are
Since protein adsorption is a dynamic phenomenon, considerable research studies were carried out to develop a better model.

1.3.7. Characterization of surface properties

Characterization of pre-modified and protein-modified substrates is necessary in order to understand the complex protein adsorption process. Surface parameters can be characterized by different (often complementary) experimental tools, some of which are tabulated in Table 1.6. The process of adsorption and the conformations of the adsorbed protein layer can also be studied by using the techniques summarized in Table 1.7, which can offer an in-depth knowledge of the kinetics and thermodynamics of the protein adsorption process.

1.3.8. Characterization of denatured proteins

Circular dichroism (CD) provides information about the conformational structure of proteins in solutions. As will be discussed in Chapter 2, the effects of adding urea and increasing the protein solution temperature on the structural changes of proteins was monitored by using CD. The principle of CD is based on the differential absorption of left (A<sub>L</sub>) and right (A<sub>R</sub>) circularly polarized components (ΔA=A<sub>L</sub>-A<sub>R</sub>), as the light passes through an optically active environment containing chiral molecules. Most of the CD measurements are reported in degrees of ellipticity (θ) by using a simple relation, θ = 32.98 ΔA. The CD spectrum is obtained by measuring the ellipticity as a function of wavelength. In general, proteins possess chiral structures because of the chirality in amino acid building blocks, making them suitable candidates for characterization using CD. The ellipticities (i.e., the difference in absorbance between left and right circular polarized components of the incident radiation, ΔA) obtained for the biological samples are very small, typically on the order of 10 mdeg. Hence, it is important to monitor the experimental conditions to obtain representative data. To this end, the concentration of proteins in solution must be selected such that the total absorbance must be less than one for the wavelength range of interest to reduce the spectral noise. A typical protein concentration for obtaining CD spectra in the far UV region
ranges in between 0.2 and 1 mg/mL. In addition, buffers, supporting electrolytes and solvents absorb the radiation and often interfere with the CD signal where structural features exhibit characteristic absorption bands. Hence, it is important to use buffers at low molar concentrations to minimize solvent absorption. Using cells with shorter path lengths also minimizes solvent absorption.\textsuperscript{65,66}

The dichroism of protein molecules in the spectral region (far-UV, 190-250 nm) results from the absorption of chromophores mainly from peptide bonds. Typically, CD spectrum in the far UV region gives information about the structural contents of secondary (α-helix and β-sheet) and unordered conformations. The characteristic ellipticities at 208 and 222 nm arise from the α-helix structure of the proteins. Specifically, ellipticity measurements at 222 nm are considered to be the hallmark of protein conformation as at this wavelength α-helix has maximal optical activity. The presence of a significant UV signal is a good indication of a folded and well-defined protein structure. The dichroism of proteins in the spectral region (near-UV, 250-310 nm) is sensitive to contributions due to the tertiary structure and results from the absorption of aromatic amino acid side chains and disulfide bonds including tryptophan, tyrosine and phenylalanine. These aromatic amino acids are placed in the chiral environment resulting from the tertiary structure of the protein. Tryptophan is signaled by a characteristic peak at 292 nm with a fine structure between 290 and 305 nm. Tyrosine residues are predominant in the region between 275 and 282 nm while the ellipticities between 255 and 270 nm are attributable to phenylalanine. Studying all these aspects gives information on structural changes of proteins. However, CD provides very limited information about the quaternary structure of proteins. This technique requires only small amounts of the sample and the non-destructive nature of the analysis allows for the possible recovery of the sample.\textsuperscript{65,66,67}

The use of CD by several authors to study the structural changes of proteins resulting from unfolding has been reported. Boninocontro et al.\textsuperscript{68} studied the denaturation of chicken egg-white lysozyme in the presence of 8 M urea (pH 5) at 20 and 65°C. The authors observed that urea partially denatured protein molecules. However, heating protein solutions
in the presence of urea induced significant changes in the protein’s secondary and tertiary structures. Anionic amphiphiles such as sodium dodecyl sulfate (SDS) induces the unfolding of protein molecules through a) the binding of negatively charged sulfate groups of SDS with positively charged amino acid side chains, and b) interaction between the alkyl chains of SDS with aliphatic side chains. Cationic and zwitterionic surfactants partially unfold protein structures under certain conditions. Previous studies indicated that SDS unfolds \( \alpha \)-lactalbumin molecules by perturbing secondary and tertiary structures through the binding of surfactant molecules to amino acid side chains in multiple steps. Fernandez-Sousa et al.\(^ {67} \) reported that lysozyme experienced significant loss of the secondary structure in the presence of 4 % sodium dodecylsulfate solution. Chen et al.\(^ {70} \) revealed that heating bovine fibrinogen proteins at different temperatures between 40 and 110\( ^\circ \)C resulted in a two-state transition. This lead to an irreversible denaturation accompanied by a loss of the \( \alpha \)-helix structure at higher temperatures.

1.4. Applications of denatured proteins

Several studies have demonstrated the functionalization of hydrophobic surfaces through the formation of polymer grafts prior to their pre-activation with various aggressive afore-mentioned techniques including corona-discharge,\(^ {16} \) plasma\(^ {26} \) and ozone.\(^ {34} \) The increased use of fine fiber-based materials in filtration materials demands the development of surface treatments that do not degrade bulk properties of the base substrates. Adsorption of the denatured protein molecules provides a versatile surface modification technology for hydrophobic surfaces alternative to the existing plasma and corona discharge techniques without involving harsh treatments. In addition, an adsorbed protein layer enriches or activates the hydrophobic surfaces with numerous inherent amino acid functionalities of proteins. We have used these varieties of functionalities to attach initiator molecules followed by subsequent deposition of synthetic polymers.

Numerous studies have been reported on synthesizing protein-polymer conjugates through the chemical reaction of reactive side chains of protein residues including lysine or
cysteine with active sites of polymers.\textsuperscript{71} These hybrid protein-polymer conjugates deliver unique properties for specific biological applications resulting from the combination of two different worlds.\textsuperscript{72} In general, PEGylation of proteins, \textit{i.e.}, attachment of PEG to proteins, is extensively employed to improve the pharmacological profiles of proteins including, enhancing stabilities, solubilities and circulation half-life and reducing renal clearance, immunogenicity and antigenicity and preventing proteolytic degradation.\textsuperscript{71,72} For instance, Abuchowski \textit{et al.}\textsuperscript{73} conjugated poly(ethylene glycol) (PEG) polymer to bovine serum albumin (BSA) to reduce the immunogenicity while increasing their blood circulation. The work of Maeda \textit{et al.}\textsuperscript{74} demonstrated about the preferential accumulation of neocarzinostatin (NCS, antitumor protein)-styrene-maleic anhydride copolymer conjugates in tumor sites as a result of enhanced permeability and retention (EPR) effect. Various drugs including Pegintron, Pegasys, Neulasta, and Mircera are currently available in the market to treat diseases namely, Hepatitis C, and cancer.\textsuperscript{72} Alternatively, protein-polymer conjugates were synthesized via surface-initiated polymerization from the surface of a protein/peptide to increase the yield alternative to post-polymerization conjugation. In this approach, polymerization initiator was chemically linked through reactive amino-acid functional group of protein, followed by subsequent in situ growth of the polymer. However, inhomogeneous distribution of reactive residues on protein surfaces resulted in the conjugation of polymers at undefined (random) sites leading to poor stoichiometry and poor yields.\textsuperscript{71,72} Hence, different strategies were developed to chemically attach initiator molecules at specific sites such as N or C terminal amino acids of the proteins. Gao \textit{et al.}\textsuperscript{71} reported on surface-initiated atom transfer radical polymerization (ATRP) of oligo(ethylene glycol) methyl ether methacrylate (OEGMA) from the C terminus of green fluorescent protein (GFP). Peeler \textit{et al.}\textsuperscript{75} demonstrated incorporation of initiators at specific sites of GFP to initiate polymerization of oligo(ethylene oxide) monomethyl ether methacrylate monomer via ATRP. The work by Depp and coworkers\textsuperscript{76} reported on modification of chymotrypsin with various polymers including poly(2-methacryloyloxyethyl phosphorylcholine), poly(N-2-hydroxypropylmethacrylamide), poly(monomethoxy-polyethyleneglycol-methacrylate) by ATRP. To our knowledge, polymerization of various synthetic polymeric materials from the
surface of denatured protein layer pre-adsorbed on hydrophobic surfaces was not reported. In our approach, 2-bromopropionyl bromide (2-BPB) initiators are incorporated on to the surface of denatured LYS and FIB protein layer pre-adsorbed on model ODTS hydrophobic and PP nonwoven surfaces. Polymer brushes are then grown from these surfaces by ATRP polymerization of 2-hydroxyethyl methacrylate (HEMA) and N-isopropylacrylamide (NIPAAm). Generation of these polymer brushes improves the wettability of modified surfaces due to the enrichment of polar hydroxyl groups and amide linkages coming from grafted PHEMA and PNIPAAm brushes. In addition, hydroxyl groups of grafted PHEMA from the surfaces facilitate the introduction of new functional properties desired for anti-fouling coatings which were discussed in Chapter 3. In general, hydrophilic surface are more biocompatible than hydrophobic surfaces. Numerous studies have reported that substrates coated with ethylene glycol, phosphazene or zwitterionic containing materials are not always effective against bio-fouling of materials. This is because of the broad spectrum of bio-organisms which are amphiphilic in nature. For example, some bio-organisms, i.e., Ulva, preferentially adhere to hydrophobic surfaces while other species, i.e., Navicula, prefer to adhere on hydrophilic surfaces. Hence, it is important that the material designed to resist bio-adhesion must minimize the adhesion of multiple bio-species using heterogeneous chemistries. To serve this purpose, multiple coatings based on chemistries comprising ethylene glycol and fluorinated chemistries were developed. For instance, our group has previously developed amphiphilic co-polymers grafted onto silica surface and studied their activity against fibrinogen. We reported that the coating’s generated on the silica surface minimized the adhesion of fibrinogen to a greater extent. Here, hydroxyl groups of PHEMA brushes grafted on hydrophobic and PP nonwoven surfaces were utilized to react chemically with fluorinating agents of different chain lengths generated amphiphilic copolymers. Multiple steps involved during the formation of the amphiphilic copolymers onto these inert surfaces do not involve any harsh pre-activation mechanism steps.

Another application of these protein-coated surfaces involves the introduction of antimicrobial characteristics which are discussed in Chapter 4. The increasing use of
polypropylene in medical, institutional, and hygienic applications demands for a material with antimicrobial properties. The most challenging task is to incorporate or deposit antimicrobial agents onto polymeric materials, especially on hydrophobic surfaces. Many studies have reported on the application of antimicrobial finishes onto PP surfaces. Abdou et al. \cite{78} deposited chitosan onto PP nonwovens pre-activated with plasma irradiation and demonstrated effective antimicrobial activity. Huang et al. \cite{31} grafted quaternary polymer brush, poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) onto PP surfaces via surface-initiated ATRP providing strong antibacterial activity against *E.coli*. Yao et al. \cite{79} reported on grafting of block copolymer brushes of poly(ethylene glycol) monomethacrylate (PEGMA) and 2-(dimethylamino)ethyl methacrylate (DMAEMA) (PEGMA-*b*-PDMAEMA) on to PP hollow fiber membranes via surface-initiated ATRP. The resultant surfaces exhibited permanent antibacterial activity. However, as explained earlier, the disadvantage of these methods stems from surface damage due to etching. In our approach, protein pre-coated surfaces provide numerous accessible sites on the periphery of the protein layer that facilitate easy fixation of silver nanoparticles either through electrostatic interactions or inherent affinity of thiol groups of protein with silver nanoparticles. Specifically, LYS molecules present on the surface of fiber surface assists in the adhesion of silver nanoparticles; this adhesion originates from electrostatic interactions between positively-charged LYS coatings (at pH<11 LYS will be positively charged) and negatively charged Ag particles. The negative charge on the silver nanoparticles is generated during the particle synthesis through the attachment of citrate groups that act as stabilizers in minimizing particle aggregation in solution.

In summary, this chapter introduces general issues encountered in modification of hydrophobic surfaces of polymers. Unlike most widely used conventional techniques including flame, corona, plasma and other types of treatments discussed in this chapter, we have developed a new technique based on nonspecific adsorption of protein molecules. Specifically, denatured protein molecules were assembled on hydrophobic surfaces, followed by cross-linking the adsorbed proteins forming a stable coating. This protein-coating
technology offered a robust platform which can be utilized to created new functional coatings. In this Ph.D. thesis we will discuss several applications developed from these protein primers which involve growing polymeric grafts and attaching silver nanoparticles.
1.5. References


Table 1.1. Strengths of different bonds in tertiary protein structure.$^{54}$

<table>
<thead>
<tr>
<th>Bond type</th>
<th>Strength (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Covalent (S-S)</td>
<td>250</td>
</tr>
<tr>
<td>Ionic</td>
<td>20</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>7-40</td>
</tr>
<tr>
<td>van der Waals</td>
<td>1.9</td>
</tr>
</tbody>
</table>
Table 1.2. Protein classification based on internal structure stability.\textsuperscript{51,58}

<table>
<thead>
<tr>
<th>Soft proteins</th>
<th>Hard proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>Lysozyme</td>
</tr>
<tr>
<td>Human serum albumin (HSA)</td>
<td>$\alpha$-chymotrypsin</td>
</tr>
<tr>
<td>Immunoglobulin (IgG)</td>
<td>Ribonuclease (RNase)</td>
</tr>
<tr>
<td>$\alpha$-lactalbumin</td>
<td>$\beta$-lactoglobulin</td>
</tr>
<tr>
<td>$\beta$-casein</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td></td>
</tr>
<tr>
<td>Phytase</td>
<td></td>
</tr>
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</table>
Table 1.3. Protein adsorption based on nature of proteins.²⁶⁰

<table>
<thead>
<tr>
<th>Feature</th>
<th>Effect on adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>Larger size proteins are held on the surface by a larger number of contact points</td>
</tr>
<tr>
<td>Charge</td>
<td>Adsorption is highest at the isoelectric point (pI)</td>
</tr>
<tr>
<td>Structure stability</td>
<td>Less stable proteins unfold to a greater extent resulting in establishing more contact points with the surface</td>
</tr>
<tr>
<td>Unfolding rate</td>
<td>Proteins that unfold rapidly interact more quickly with the surface</td>
</tr>
</tbody>
</table>
Table 1.4. Protein adsorption based on nature of surfaces.\textsuperscript{60}

<table>
<thead>
<tr>
<th>Feature</th>
<th>Effect on adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Topography</td>
<td>Greater surface area promotes more interactions with proteins</td>
</tr>
<tr>
<td>Composition</td>
<td>Material surface chemistry determines the driving forces and the nature of interactions that governs protein adsorption</td>
</tr>
<tr>
<td>Hydrophobicity</td>
<td>Increasing hydrophobicity enhances protein adsorption</td>
</tr>
<tr>
<td>Potential</td>
<td>Surface potential greatly influences the adsorption process especially for hydrophilic surfaces</td>
</tr>
</tbody>
</table>
Table 1.5. Enthalpy of protein adsorption on different surfaces.\textsuperscript{54}

<table>
<thead>
<tr>
<th>Protein</th>
<th>Surface</th>
<th>Enthalpy change of adsorption (mJ/m\textsuperscript{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse serum albumin</td>
<td>(\alpha)-Fe\textsubscript{2}O\textsubscript{3})</td>
<td>+1.9 at pH=5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+7.0 at pH=7</td>
</tr>
<tr>
<td>RNase</td>
<td>Negatively charged polystyrene with high surface charge density</td>
<td>+4 at pH=5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-2 at pH=11</td>
</tr>
<tr>
<td>Property</td>
<td>Technique</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>---------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Surface composition</td>
<td>X-ray photoelectron spectroscopy</td>
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</tr>
<tr>
<td></td>
<td>Infrared spectroscopy</td>
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</tr>
<tr>
<td>Surface energy</td>
<td>Contact angles</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Critical surface tension</td>
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</tr>
<tr>
<td></td>
<td>Dispersion-polar components of the surface energy</td>
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<tr>
<td>Electrochemical properties</td>
<td>Streaming potential (zeta-potential for surfaces)</td>
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<tr>
<td></td>
<td>Electrophoresis (zeta-potential for particles)</td>
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<tr>
<td></td>
<td>Potentiometric titration</td>
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</tr>
<tr>
<td>Surface structure</td>
<td>Light microscopy</td>
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</tr>
<tr>
<td></td>
<td>Electron microscopy (SEM, TEM)</td>
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</tr>
<tr>
<td></td>
<td>Atomic force microscopy</td>
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Table 1.7. Characterization techniques for studying the adsorption process and the protein-coated surfaces.52

<table>
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<tr>
<th>Property</th>
<th>Technique</th>
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<tbody>
<tr>
<td>Thickness</td>
<td>Ellipsometry</td>
</tr>
<tr>
<td></td>
<td>Hydrodynamic techniques</td>
</tr>
<tr>
<td></td>
<td>Light scattering</td>
</tr>
<tr>
<td>Conformation of adsorbed Proteins</td>
<td>Infrared spectroscopy</td>
</tr>
<tr>
<td></td>
<td>Raman spectroscopy</td>
</tr>
<tr>
<td></td>
<td>Circular dichroism</td>
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<tr>
<td></td>
<td>Immunological methods</td>
</tr>
<tr>
<td>The rate of exchange and surface diffusion of protein</td>
<td>Fluorescence recovery after photobleaching (FRAP)</td>
</tr>
<tr>
<td>The interaction between layers of adsorbed proteins</td>
<td>Surface-force measurements</td>
</tr>
<tr>
<td>Heat of adsorption</td>
<td>Microcalorimetry</td>
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Figure 1.1. Schematic diagram of flame treatment device. (adapted from reference 15)
Figure 1.2. Schematic diagram of corona discharge manifold. (adapted from reference 80)
Figure 1.3. Schematic representation of corona modified polymeric PP surfaces. (adapted from reference 4)
Figure 1.4. Schematic set-up of atmospheric pressure plasma treatment device. (adapted from reference 18)
Figure 1.5. Schematic illustration depicting the interaction of protein molecules of varying sizes with a surface. (adapted from reference 60)
Figure 1.6. Schematic depicting the behavior of soft and hard proteins on electrostatically repelling surface. (adapted from reference 58)
**Figure 1.7.** Effect of protein concentration in the bulk solution on conformational changes of protein structures on surfaces. (adapted from reference 60)
Figure 1.8. Models depicting protein adsorption according to a) Langmuir, b) Lundström, c) Beissinger and Leonard, and d) Soderquist and Walton; $k$ is the rate constant, subscript $a$, $d$, $1c$ and $2c$ are adsorption, desorption, conformation changes during transformation from state 1 to state 2 and conformational changes during transformation from state 2 to state 3. (drawn based on reference 50 & 52)
CHAPTER 2 - GENERATION OF FUNCTIONAL COATINGS ON HYDROPHOBIC SURFACES THROUGH DEPOSITION OF DENATURED PROTEINS FOLLOWED BY GRAFTING FROM POLYMERIZATION

2.1. Abstract

Hydrophilic coatings were produced on flat hydrophobic substrates featuring n-octadecyltrichlorosilane (ODTS) and synthetic polypropylene (PP) nonwoven surfaces through the adsorption of denatured proteins. Specifically, physisorption from aqueous solutions of α-lactalbumin, lysozyme, fibrinogen, and two soy globulin proteins (glycinin and β-conglycinin) after chemical (urea) and thermal denaturation endowed the hydrophobic surfaces with amino- and hydroxyl- functionalities yielding enhanced wettability. Proteins adsorbed strongly onto ODTS and PP through non-specific interactions. The thickness of adsorbed heat-denatured proteins was adjusted by varying the pH, protein concentration in solution, and adsorption time. In addition, the stability of the immobilized protein layer was improved significantly after interfacial cross-linking with glutaraldehyde in the presence of sodium borohydride. The amino and hydroxyl groups present on the protein-modified surfaces served as reactive sites for the attachment of polymerization initiators from which polymer brushes were grown by surface-initiated atom-transfer radical polymerization of 2-hydroxyethyl methacrylate. Protein denaturation and adsorption as well as the grafting of polymeric brushes were characterized by circular dichroism, ellipsometry, contact angle, and Fourier transform infrared spectroscopy in the attenuated total reflection mode.
2.2. Introduction

Modification of hydrophobic polymer surfaces is of great interest in current materials research. Activities in the field involve the development of coatings with applications that range from enhancing wettability or adhesion to protecting surfaces from bio-fouling. Polyolefins, such as polyethylene (PE) and polypropylene (PP), possess desirable properties including good mechanical strength, chemical resistance, thermal stability, and low cost. These attributes make them promising candidates for various applications involving functional textiles, filtration devices, medical implants, and many others. Unfortunately, the major drawback of PE and PP is their low surface energy, which results in inherent hydrophobicity and poor biocompatibility. For example, materials designed for surgical implants must be biocompatible and resist adsorption of biospecies to avoid blood agglutination. The greater susceptibility of hydrophobic surfaces to bioadhesion compared to hydrophilic surfaces has been generally recognized, and numerous studies have been dedicated to the hydrophilization of surfaces using various physical and chemical methods. Grafting of macromolecules to surfaces is particularly attractive because it allows for precise control of the chemical composition of the interface by adjusting the chemistry of the coating. However, the lack of functional groups in polyolefins and their high chemical resistance make grafting a difficult task without employing harsh and aggressive pre-treatments, which may affect adversely their bulk-properties.

To date, several methodologies have been reported that provide routes for pre-activation of hydrophobic surfaces. These approaches involve typically surface modification, including plasma treatment, irradiation with UV light, electron beam, ozone, or γ-rays, or adsorption of surface active amphiphiles (i.e., copolymers or surfactants). Alternatively, surface modification can be achieved during the manufacturing process by pre-mixing (melt-blending) polymer precursors with surface-active agents (i.e., stearyl alcohol ethoxylates) that segregate to the surface of the initially hydrophobic films or fibers. Relevant work includes that of Wang et al. who reported on grafting of poly(N-vinyl-2-pyrrolidone) onto ozone pre-treated PP nonwovens to enhance the hydrophilicity and antifouling properties of PP nonwovens. Lavanant et al. grafted poly(poly(ethylene glycol)
methacrylate) brushes onto polyolefin surfaces pre-modified by photo-bromination. The work by Desai et al.\textsuperscript{23} demonstrated that alkyl bromide groups generated on polypropylene through photo-bromination could be used to grow N-isopropyl acrylamide brushes by atom transfer radical polymerization (ATRP). While these methods are relatively efficient in endowing surfaces with functional groups, involving such harsh treatments might compromise the mechanical properties of the substrates or supports.\textsuperscript{9} This issue is particularly problematic in the case of relatively fine fiber-based materials.\textsuperscript{24} Our approach towards modifying hydrophobic substrates is inspired by the known affinity of proteins to solid surfaces (both hydrophobic and hydrophilic) as a result of non-specific interactions.\textsuperscript{25}

Proteins are macromolecules composed of amino acids; they adopt characteristic micelle-like conformations with characteristics that depend on the hydrophobicity/hydrophilicity of the surrounding medium.\textsuperscript{7,26} For example, in aqueous solutions the hydrophobic amino acids tend to be concentrated inside the core of coil conformations, whereas the hydrophilic residues are present on the periphery. This arrangement results from the tendency of proteins to minimize the exposure of the more hydrophobic residues to water. However, a small portion of hydrophilic and hydrogen-bond forming residues may also be present in the core and a fraction of hydrophobic amino-acids can be located in the corona.\textsuperscript{27,28}

Proteins adsorb readily on both hydrophilic as well as hydrophobic surfaces. The driving force for protein adsorption on hydrophilic surfaces originates from the tendency of the hydrophilic groups to contact the hydrophilic substrate via hydrogen bonding or electrostatic interactions. In contrast, adsorption on hydrophobic surfaces is driven primarily by hydrophobic and van der Waals interactions among the hydrophobic protein domains and the surfaces while the hydrophilic amino acid moieties, which are not in a close proximity to the adsorbing hydrophobic domains, are allowed to dangle to the exterior and interact with the aqueous environment.\textsuperscript{8,26,29-30} Because the hydrophobic residues are present predominantly inside the protein coil, protein partitioning on hydrophobic surfaces involves generally dramatic conformational changes that may result in coil unfolding and permanent destabilization, \textit{i.e.}, denaturation of the proteins.\textsuperscript{7,26,31-32} While in most instances adsorption
of denatured proteins is considered to be a nuisance, primarily because proteins often lose their biological function, in this work we take advantage of this phenomenon to generate functional surfaces on hydrophobic supports.26,32,33

An effective coating of proteins on hydrophobic surfaces is achieved when a sufficient amount of protein is adsorbed from solution and van der Waals and other non-specific interactions between the protein and the substrate is maximized.7,34 Adsorption of proteins is a complex process that depends strongly on multiple variables, including those related to the deposition solution (e.g., pH, ionic strength, temperature), the protein (e.g., primary and secondary structure, which determine the charge density and distribution and structural stability), and the substrate (e.g., surface energy, charge, topography, mobility of surface groups).28,30,32,35 Depending on the solution pH, protein molecules may either contain an effective positive or negative charge. At the isoelectric point (pI), the net charge of the molecule is zero, although pockets of negative or positive charges may be present locally. Under these conditions, the amount of protein adsorbed onto the surface is maximized because electrostatic repulsion among protein molecules adsorbing from solution is minimized.28,32 The density of hydrophobic contacts between the protein and the surface can be further maximized by not only relying on substrate-induced denaturation, but also by denaturing the protein in solution prior to protein adsorption. This denaturation can be accomplished by adding well-known denaturants, such as urea or guanidine hydrochloride, or by heating the protein solutions to high temperatures.34,36,37

Denatured proteins attach to hydrophobic substrates via relatively weak physical van der Waals and other non-specific interactions.26 Although the number of contacts between the surface and the protein molecules is relatively high, these contacts may not be strong enough to hold the adsorbed protein in place permanently. To the best of our knowledge, surface coating with denatured protein and the enhancement of their stability by cross-linking on hydrophobic substrates have not yet been considered. More specifically, we propose to impart protein layer stability by chemically coupling neighboring adsorbed protein molecules with a cross-linker, such as glutaraldehyde (GA).38-39 Our approach has the advantage that
even a single layer of protein will alter the surface properties considerably and thus will be an enabling platform to introduce new functionalities. For instance, the affinities of various functional groups in proteins can be utilized (even after denaturation) to bind or scavenge metals from solution. Metal nanoparticles, *i.e.*, silver-containing, can be attached to impart antibacterial properties, and polymers or amphiphilic macromolecules can be installed to impart antifouling or other properties. Furthermore, surface modification with proteins is simple and in contrast to traditional methods such as layer-by-layer (LbL) deposition of polyelectrolytes, it does not require sequential adsorption steps. In addition, it is not subject to limitations such as interlayer penetration and stability dependence on solution pH and ionic strength, which otherwise may exist in LbL modification.40-41

This paper focuses on the formation of hydrophilic functional coating layers on model hydrophobic ODTS (*n*-octadecyltrichlorosilane) surfaces by adsorbing proteins such as *α*-lactalbumin (LALBA), lysozyme (LYS), fibrinogen (FIB) and soy globulins (glycinin and *β*-conglycinin). Prior to adsorption on the ODTS surfaces, proteins were denatured using urea or by heating to elevated temperatures. After adsorption, the immobilized protein layer was cross-linked with GA in the presence of NaBH₄. These protein coatings were used as primers for further chemical modification. Specifically, we decorated the outer surfaces of the protein coatings with polymerization initiators which served as active sites for “grafting from” polymerization. Concurrently, we prepared hydrophilic coatings on PP nonwoven surfaces by replicating the technological steps used on the flat ODTS surfaces. As proof-of-principle, we demonstrated this process by employing atom transfer radical polymerization (ATRP) of 2-hydroxyethyl methacrylate (HEMA) to form dense PHEMA brushes; however, this technology is applicable to various other polymerization methods involving a wide range of monomers.
2.3. Materials and Methods

2.3.1. Materials

Deionized water (DIW) (resistivity > 16 MΩ cm) was produced using a Millipore water purification system. Silicon wafers (orientation [100]) were supplied by Silicon Valley Microelectronics. ODTS was obtained from Gelest (Morrisville, PA). PP nonwoven fiber sheets were obtained from The Nonwovens Institute pilot facilities at NC State University and were cleaned with isopropanol prior to use. Lysozyme (from chicken egg white, $M_n = 14.3$ kDa, $pI = 11.3$), fibrinogen (from human plasma, $M_n = 340$ kDa, $pI = 5.5$), CuCl ($99.99\%$), CuCl$_2$ ($99.99\%$), 2,2'-bipyridine (bpy, 99\%), 2-bromopropionyl-bromide (2-BPB), 2-hydroxyethyl methacrylate (HEMA, 98\%), glutaraldehyde (GA), sodium borohydride (NaBH$_4$), and poly(allylamine-hydrochloride) (PAH, $M_n = 56$ kDa) were obtained from Sigma-Aldrich and were used as received. Poly(styrene sulfonate) (PSS, $M_n = 75$ kDa) was obtained from Polysciences, Inc. $\alpha$-lactalbumin (from bovine milk, $M_n = 14.2$ kDa, $pI = 4.3$) was received from Davisco Foods International Inc. Soy proteins were supplied by Archer Daniels Midland Company and glycinin (11S) and $\beta$-conglycinin (7S) were obtained by standard fractionation methods. Phosphate buffered saline (PBS) solution, HPLC-grade methanol, and toluene were obtained from Fisher Scientific and used as received. Tetrahydrofuran (THF) and triethylamine were distilled from Na/benzophenone and calcium hydride, respectively.

2.3.1. Hydrophobic ODTS layers assembled on silicon wafers

ODTS layers were prepared on silicon wafers as described in our previous work. The ellipsometric thickness of the obtained ODTS films were $\approx 25 \pm 2$ Å, which is consistent with the theoretical length of a covalently bound ODTS molecule of 26.2 Å. In addition, ODTS film thicknesses reported in the literature were between 20 and 29 Å depending upon the tilt angles of the layers. Thus, we believe that our ODTS layers were approximately a monolayer. Wettability measurements using DIW revealed that the contact angle was 108±2°.
2.3.2. Denaturation and adsorption of proteins

α-lactalbumin (LALBA, pI=4.3), lysozyme (LYS, pI=11.3) and fibrinogen (FIB, pI=5.5) solutions were denatured using either urea treatment or thermal denaturation and adsorbed on surfaces as detailed below.

For chemical denaturation, urea was added to PBS buffer (0.137 M NaCl, 0.0027 M KCl, and 0.0119 M phosphates) and the solutions were then filtered through a 0.2 µm filter followed by protein addition to obtain final concentrations of 0.01, 0.1 or 1 mg/mL. The solutions were allowed to stand at room temperature for 6 h for complete solubilization. Protein adsorption was carried out at the isoelectric point of each protein (i.e., pH 4.3 for LALBA, 11.3 for LYS and 5.5 for FIB); the solution pH was adjusted by adding either 0.1 N HCl or NaOH drop-wise. Sodium azide (NaN₃, 0.2%) was also added to the buffer solution to prevent the growth of bacteria during adsorption. ODTS-coated wafers were used as model hydrophobic surfaces; they were assumed to mimic the physico-chemical characteristics of PE and PP in a flat geometry. ODTS-modified wafers and PP nonwoven sheets were immersed in the chemically denatured protein solutions for 12 h at room temperature.

For thermal denaturation, LYS solutions of various concentrations (i.e., 0.01, 0.1 and 1 mg/mL) were prepared in PBS buffer solution at different pHs of 7.4, 9 and 10. The solutions were allowed to stand at room temperature for 6 h to solubilize the proteins and then kept in an oven pre-heated to 85 °C for 3 min before immersing the ODTS substrates for 15 and 30 min. Control experiments were run by immersing ODTS substrates in protein solutions without heating for the same duration. In addition, solutions of soy protein 7S and 11S fractions of various concentrations (i.e., 0.01, 0.1 and 1 mg/mL) were prepared at pH 7.4. Substrates were immersed in these solutions while heating at 85 °C for 15 min. The substrates were then rinsed in PBS and DI water and blow-dried with nitrogen gas after removal from protein solution. The resultant flat ODTS substrates were characterized by ellipsometry and water contact angle measurements in order to determine the thickness and wettability, respectively, of the substrates with adsorbed protein coatings.
2.3.3. Circular dichroism of protein solutions

Circular Dichroism (CD) spectra for LYS and FIB proteins were measured by using JASCO J-815 spectropolarimeter, equipped with 150 W Xenon lamp. LYS and FIB were immersed into a quartz cell (path length 0.1 cm) at room temperature with constant nitrogen gas purge. The CD spectra were collected at a wavelength range between 195 and 250 nm with a band width of 2 nm, using steps of 0.2 nm, and a scanning speed of 50 nm/min. Each spectrum reported here represents an average of at least three different scans. In all cases, the background contribution from urea and buffer was subtracted.

Proteins were treated with urea or heat for chemical and physical denaturation, respectively. For denaturation using urea, LYS and FIB protein solutions were prepared at concentrations of 0.05 mg/mL at their respective isoelectric points in PBS buffer. Different amounts of urea (2, 4, and 8 M) were added to the resultant protein solutions. The resultant protein solutions were analyzed using CD to determine the extent of protein denaturation. For thermal denaturation 0.05 mg/mL LYS and FIB solutions at pH 7.4 (PBS buffers) were heated in-situ from 40 to 90°C with a heating rate of 2°C/min while monitoring CD ellipticity at 222 nm, which is representative of secondary α-helix structures.47

2.3.4. Cross-linking of adsorbed protein coatings and their stability.

Protein-coated ODTS substrates and PP nonwovens were immersed in a solution containing 1 % (w/v) cross-linker GA and 0.01 % NaBH₄ (w/v). The interfacial cross-linking was allowed to proceed at room temperature for 6 h at pH 9, followed by rinsing with PBS buffer and DI water and blow-drying with nitrogen gas. Others have reported on using GA to cross-link PEI and PAH, however, under different conditions.48,49 The stability of the LYS protein coatings (1 mg/mL, thermally denatured) was investigated by exposing the protein-coated ODTS substrates (featuring either uncross-linked or cross-linked layers) to the following environmental tests: 1) drying the specimens at 85°C for 12 h, 2) sonicating in DI water for 5 min, 3) incubating in THF solution for 12 h at room temperature, or 4) sonicating in 0.1 mg/mL non-ionic Triton X-100 surfactant for 2 min.
2.3.5. Formation of polyelectrolyte layers.

FIB-modified substrates were placed in polycation solutions (0.02 M PAH, 0.5 M NaCl, pH 7) for 30 min, followed by rinsing in DIW and blow-drying with nitrogen gas to form the first layer of PAH. The resultant PAH-functionalized substrate was subsequently placed in a solution of PSS polyanion (0.02 M PSS, 0.5 M NaCl, pH 7) for 30 min; this treatment provided a surface rich in negatively-charged sulfonate groups. A second PAH layer was deposited by dipping the PSS-functionalized substrate in PAH solution. In case of LYS-modified surfaces, PSS was deposited as the first layer followed by the subsequent deposition of the PAH layer. The build-up of polyelectrolyte layers was carried out according to the procedure as described by Balachandra and coworkers.\textsuperscript{50}

2.3.6. Immobilization of ATRP initiator on protein-coated surfaces.

Initiator molecules were attached to amine and hydroxyl groups of protein-coated ODTS supports via amide and ester linkages. The substrate was immersed initially in a solution of 15 mL of dry THF containing 0.363 g TEA. In another vial, 0.648 g of 2-BPB initiator was added to 15 mL dry THF. Both solutions were cooled to 0 °C prior to the addition of initiator solution. Initiator solution was added drop-wise to the TEA solution under continuous stirring with the substrate present. The reaction was terminated after 2 min and the substrate was moved to a vial containing fresh THF followed by rinsing with ethyl acetate, ethanol, and DIW and blow-drying with nitrogen gas. TEA neutralizes the acid that would be released during the reaction between 2-BPB and the amine and hydroxyl groups.\textsuperscript{50} A similar procedure was used to immobilize initiator molecules over PP nonwoven surfaces.

2.3.7. Preparation of PHEMA brushes on modified surfaces via ATRP.

A typical “grafting from” polymerization of 2-hydroxyethylmethacrylate (HEMA) included dissolving 9.09 mL (75 mM) of HEMA in a mixture of 9.09 mL (225 mM) methanol, 1.82 mL (101 mM) DI water and 0.54 g (3.39 mM) bpy. This mixture was degassed by three freeze-thaw cycles to remove oxygen, followed by the addition of 0.172 g
CuCl (1.74 mM) and 0.013 g CuCl₂ (0.095 mM) under nitrogen gas atmosphere. The molar ratios of the various reactants were [HEMA]:[bpy]:[CuCl]:[CuCl₂] = 790 : 36 : 18 : 1. The resultant mixture was subjected to an additional freeze-thaw cycle. The initiator-immobilized ODTS substrate and PP nonwoven surfaces were immersed in this polymerization solution. The polymerization reaction was allowed to proceed for 9 h at room temperature. After polymerization, the substrates were removed from the reaction mixture, promptly rinsed with methanol and deionized water, and dried gently with nitrogen gas.

2.3.8. Ellipsometry

The thickness of bare ODTS films before and after the formation of protein coating was determined by variable angle spectroscopic ellipsometry (VASE, J.A. Woollam Co.). In addition, ellipsometry measurements were carried out to monitor the “dry” thickness (H) of protein coatings, polyelectrolyte films, and PHEMA brushes. Ellipsometric data were collected at an incidence angle of 75° for SAMs and protein coatings or 70° for PHEMA brushes using wavelengths ranging from 400 to 1100 nm in 10 nm increments. For each coating layer, thickness values were determined for at least three different spots and then averaged. The thicknesses were calculated using a Cauchy layer model assuming the index of refraction as 1.5 for ODTS and 1.54 for protein coatings. A refractive index of 1.465 was assumed for all polyelectrolyte layers. The thickness of PHEMA brushes on protein coatings was calculated using the method used by Arifuzzaman et.al.

2.3.9. Contact angle measurements.

Contact angles (CAs) were measured with Ramé-Hart CA goniometer (model 100-00) using deionized water (DIW) as the probing liquid at room temperature to determine the wettability of protein coatings, polyelectrolyte films, and PHEMA brushes. Static DIW contact angles were recorded by releasing 8 µL droplets of DIW on the surface. DIW contact
angle measurements were carried out on at least three different positions over the surface and then averaged.

2.3.10. Fourier transform infrared spectroscopy.

The Fourier transform infrared (FTIR) spectra for ODTS and modified PP nonwoven surfaces were recorded using a Bio-Rad-Digilab FTS-3000 Fourier transform infrared spectrometer equipped with crystalline ZnSe in an attenuated total reflection (ATR) mode with a continuous nitrogen gas purge. The nonwoven fiber was pressed against the crystal under a uniform pressure of ≈700 psi using the micrometer pressure clamp. The spectra reported represent an average of 5 accumulations of 64 scans with a resolution of 4 cm⁻¹. The data were analyzed using the Bio-Rad Win IR Pro software.

2.3.11. Atomic force microscopy.

Surface topography after protein deposition on ODTS substrates was examined using Asylum Research MFP3D atomic force microscope (AFM). The AFM was operated in tapping mode in AC mode using Al-backside-coated Si cantilevers with a force constant of ≈5 N/m and a resonance frequency in the range of 120-180 kHz. During imaging, care was taken to keep the tip in the repulsive mode. The root-mean-square (RMS) surface roughness was calculated from height images using the software provided by Asylum Research.

2.4. Results and Discussion

2.4.1. Urea and thermal denaturation of proteins.

Protein denaturation (thermal or chemical) and adsorption is illustrated schematically in Figure 2.1; the schematic indicates the disruption of the native protein conformation by breaking hydrogen bonds that stabilize the protein’s secondary structure (thus unfolding the α-helix and/or β-sheet sections of the protein molecule).²⁷,⁵⁴
Figures 2.2a and 2.2b show the far-UV CD spectra for native and urea-denatured LYS and FIB solutions at three different urea concentrations. Data reported here refers to the wavelength range 200-250 nm. The addition of urea prevents CD spectra measurements below 210 nm for 2 and 4 M urea and below 215 nm for 8 M urea due to the high absorbance of the urea-solvent mixture. The CD spectra of native LYS and FIB are characterized by two minima located at 208 and 222 nm, which correspond to the proteins’ secondary structures.\textsuperscript{47,55} The CD spectra of native protein obtained are in good agreement with previous reports.\textsuperscript{56-57} In general, molar ellipticity measurements at 222 nm are considered to be the index of protein denaturation and at this wavelength, $\alpha$-helix has maximal optical activity.\textsuperscript{47,56-58} To put these data into context, ellipticity measurements at 222 nm for a range of urea concentrations are replotted in Figure 2.2c. The monotonous increase of negative ellipticities towards zero with increasing urea concentrations indicates that both proteins under investigation exhibited a reduction in the $\alpha$-helix content due to the progressive unfolding of LYS and FIB; the degree of unfolding is most pronounced at urea concentrations of 8 M.

Analogous results were reported by Barnes \textit{et al.}\textsuperscript{37} for BSA protein denatured with 8 M urea. Fernandez-Sousa \textit{et al.}\textsuperscript{59} also observed similar alterations in ellipticity values for LYS (from insect eggs) proteins, denatured with 4% sodium dodecysulfate. The differences noticed in CD spectra for FIB and LYS are attributed to structural stability characteristics as well as different amounts of secondary structures present in the individual proteins; the $\alpha$-helix contents present in LYS and FIB reported in the literature are 42 and 30-35 \%, respectively.\textsuperscript{60,28,61,62}

We also unfolded protein structures using thermal denaturation, which involves heating protein solutions. Thermal denaturation leads often to permanent conformational unfolding of the proteins, in contrast to denaturation using urea (since in the latter case protein can fold back partially with time).\textsuperscript{38,54,63} As can be observed from Figure 2.3, the negative ellipticities of FIB and LYS protein solutions decrease with increasing temperature thus implying unfolding of proteins by breaking the $\alpha$-helices. Analogous reductions in ellipticity
values were observed for FIB protein solutions heated from 40 to 110 °C by Chen et al.\textsuperscript{61} The results clearly suggest that protein denaturation increases with increasing protein solution temperature.

2.4.2. Adsorption of urea-denatured proteins.

The adsorption of LALBA, LYS, and FIB proteins on ODTS substrates was followed by determining the dry thicknesses of protein layers. Proteins have been reported to adsorb onto surfaces as monolayers, sub-monolayers, or multilayers depending on the protein type and experimental conditions.\textsuperscript{7} The dry thickness was calculated and expressed as a “fractional surface coverage”, and is summarized in Figure 2.4 (top) (details on the fractional surface coverage calculation are provided in Appendix A). At low protein concentrations (0.01 mg/mL), the surface coverages of LALBA and LYS are comparable and significantly larger than that of FIB, indicating that the adsorption of proteins on hydrophobic surfaces depends on protein molecular weight and conformation. Haynes et al.\textsuperscript{60} reported that LALBA and LYS possess similar sizes, shapes, and specific densities. Unlike the smaller, globular LALBA and LYS, the complex and elongated FIB protein structure forms large aggregates and prevents close-packing. These properties might result in relatively low FIB surface coverage. Considering the molecular dimensions of native LALBA (25 × 32 × 37 Å\textsuperscript{3}),\textsuperscript{27} LYS (30 × 30 × 45 Å\textsuperscript{3}),\textsuperscript{7} and FIB (60 × 60 × 450 Å\textsuperscript{3})\textsuperscript{7} proteins, one must note that the observed thicknesses are smaller than those predicted for end-on or side-on protein adsorption (that is, smaller than the respective protein size), indicating protein denaturation. On the basis of the above results, these thicknesses correspond to the length of a few amino acids that form loose structures that do not pack ideally, leading to fractional surface coverage smaller than unity.

The surface coverage increases with increasing concentration of protein in solution. The adsorbed amount (\textit{i.e.}, surface coverage) of LALBA seems to nearly saturate the surface at 0.1 mg/mL concentration. This observation is in contrast to the case of LYS and FIB; here, surface coverage increases progressively with protein concentrations increasing from 0.01 to
1 mg/mL. The reasons for these pronounced differences warrants further investigation, for example, by using the notion of “soft” (LALBA) \(^7\) and “hard” (LYS & FIB)\(^{28,64}\) proteins. LALBA, being a “soft” protein, adsorbs to a larger extent even at lower concentrations, undergoing considerable structural changes upon adsorption onto the surface as compared to “hard” proteins, which undergo limited structural rearrangements due to stronger internal cohesion. At 1 mg/mL concentrations, it appears that all proteins have adsorbed in a compact manner.

Further insight into the structure of the adsorbed protein layers can be gained from static water contact angles. As summarized in **Figure 2.4** (bottom), DIW contact angle values decreased from 108±2° (for ODTS) to 61±3° after adsorption of LALBA, LYS and FIB proteins, regardless of protein type and concentration. Our results are in agreement with the findings of Wösten et al.\(^{65}\) who reported that adsorption of hydrophobin protein on a Teflon surface resulted in the reduction of DIW contact angles from 108 to 63°. The DIW contact angles of ≈61° observed in this study suggest that protein adsorption on ODTS considerably reduced the initially hydrophobic ODTS surface by exposing many hydrophilic (as well as some hydrophobic) amino acid residues to the exterior environment. Since the molecular structure of the outer layer of any denatured protein used here is not known, we cannot comment on which sections of each protein are likely to be exposed to the outer surface and which parts are likely to be in contact with the underlying hydrophobic substrate. While the contact angles of adsorbed LALBA, LYS, and FIB are all ≈60°, Wösten and co-workers mention that high wettabilities with contact angles as low as 22° are known for hydrophobin protein coatings;\(^{65}\) unfortunately, a detailed discussion about the wettability behavior upon the structure of amino-acids was not provided in the aforementioned publication. As we will discuss later in the paper, the soy protein fractions exhibit wettabilities that are slightly better than those seen for LALBA, LYS, and FIB. Clearly, the chemical composition of the protein, its conformation and coverage on the surface (including any effects associated with topographical variation due to protein adsorption) play a role in determining the final wettability. Although, it is important to understand and address the microscopic structure of
the outer protein layer, this topic is outside the scope of this paper. As we demonstrate later in the paper, each of the protein coatings provides a sufficient number of anchoring points, which can be employed for attaching the initiator molecules.

2.4.3. Adsorption of thermally-denatured proteins.

The degree of protein unfolding depends on the amount of thermal energy delivered. With prolonged heating at elevated temperatures, more unfolding occurs and can potentially be irreversible. The disruption of the secondary or higher order structures exposes internal hydrophobic groups to the aqueous environment. At this stage, hydrophobic parts of the protein molecules adsorb onto the hydrophobic surface through dehydration. However, far away from the hydrophobic interface, denatured proteins may tend to aggregate due to the formation of intermolecular interactions, primarily consisting of van der Waals and hydrophobic forces.

In our work, we carried out protein adsorption with and without heating for different adsorption times, pHs and concentrations. Away from the isoelectric point of LYS, specifically at pH 7.4, protein coverage did not depend on protein concentration, the adsorption time, or the degree of denaturation (cf. Figure 2.5, left ordinate). This behavior is due to charges present over the protein molecules that minimize adsorption as well as aggregation. Lu et al. has also observed similar behavior during the adsorption of LYS at room temperature away from the isoelectric point. As the solution pH approaches the isoelectric point, the global net charge of proteins becomes neutral, which results in an increase in protein adsorption. In addition, we observed that denatured proteins, which contain a higher surface density of hydrophobic groups, adsorbed more extensively as compared to native proteins.

For protein bulk concentrations of 0.01 mg/mL and in some cases of 1 mg/mL, protein coverage is less than unity. In contrast, protein coverage values exceed that of a single monolayer for proteins adsorbed from solutions under the conditions of a) pH closer to pI, b) 1 mg/mL concentrations, and c) adsorption times of 30 min at temperatures 85°C (cf. Figure
2.5). These conditions allow the proteins to aggregate in solution due to minimization of electrostatic repulsions and higher probability of protein-protein contact. Similarly, Treuheit et al.\textsuperscript{67} reported that protein aggregation was enhanced with increased protein concentration in bulk solution. In addition, they observed that unfolded proteins were more vulnerable to aggregation than native proteins. Under our experimental conditions, protein molecules adsorb as multilayers either due to the adsorption of aggregates formed in solution or due to adsorption of individual protein molecules to surfaces tempered with previously adsorbed proteins.

Corresponding DIW contact angle measurements (cf. Figure 2.5, right ordinate) suggest that denatured protein layers possess higher wettabilities as compared to undenatured protein layers. This effect might be attributed to higher concentrations of the hydrophilic protein residues present on the denatured protein coating surfaces. As observed with the urea-denatured protein coatings, the DIW contact angle of the absorbed LYS decreased to 63±3° (relative to 108±2° measured for ODTS). The only exceptions are the native LYS protein layers and denatured protein layers formed with protein concentrations of 0.01 mg/mL at pH 7.4. We also observe that the contact angle increases once the fractional coverage is greater than one, where multilayers are present. The multilayers may consist of a mixture of hydrophobic and hydrophilic patches present on the surface, unlike monolayers, which are primarily composed of hydrophilic groups oriented preferentially to the surrounding medium. Overall, the DIW contact angle measurements indicate that the wettabilities of protein coatings on surfaces do not depend on the mechanism of denaturation (at least for proteins investigated in this study).

The application of protein coatings depends on the availability and cost effectiveness of the protein molecules. 7S (β-conglycinin, 140-170 kDa) and 11S (glycinin, 340-375 kDa) represent two major fractions of globulins, constituting about 75% of the total soy protein. Furthermore, 7S and 11S globulins have been the subject of work for modification of hydrophilic surfaces (silica and cellulose).\textsuperscript{42} In the context of the present investigation they are expected to be good candidates for modification of hydrophobic substrates. To serve this
purpose, thermally denatured soy proteins were adsorbed onto ODTS surfaces to provide viable alternatives compared to relatively expensive functional proteins such as LALBA, LYS and FIB. The denaturation temperatures of 7S and 11S are \( \approx 77.1 \) and \( \approx 93.3 \) °C, respectively, as reported in the literature. \(^{69}\). 7S and 11S solutions were heated to temperatures of \( \approx 85 \) °C for 15 min at pH 7.4. No visible precipitation of protein molecules was observed since the pH (=7.4) of protein solutions was away from the isoelectric points of the soy proteins (pI \( \approx 4.5 \)). Proteins were adsorbed on ODTS substrates while maintaining protein solution temperatures at 85°C at pH 7.4. The resultant properties of soy protein coatings are summarized in Figure 2.6. The coverage and DIW contact angle values of ODTS surfaces after adsorption of 7S and 11S proteins are generally similar to those of LYS coatings. At low protein bulk concentrations of 0.01 mg/mL, the fractional coverage of 7S and 11S are smaller than those of LYS, while at higher protein bulk concentrations, the protein coverage of 7S and 11S are comparable to that of LYS. Wettability measurements reveal that while at low protein coverage, the DIW contact angle values are closer to 70±3°, the DIW contact angle values reduce to \( \approx 52±5° \) with increasing protein coverage. The latter values are lower than those observed in LALBA, LYS, and FIB. These results reiterate our earlier statements that the actual wettability depend on the local molecular features of the protein layer that are difficult to assess. Further investigation is needed to shed more light into this phenomenon.

The adsorption of soy proteins was demonstrated to confirm that functionalizing the hydrophobic surfaces is not limited to a specific protein. We have selected FIB and LYS proteins for further studies investigating protein layer stability and functionalization using polymer brushes, as discussed in detail in the next sections.

2.4.4. Cross-linking and stability of protein coatings

Adsorbed protein molecules were cross-linked in order to improve the stability of the adsorbed layers. Cross-linking of proteins in solutions was assessed by using SDS-PAGE (see details in the Appendix A). After cross-linking with GA, the thickness increased by 3±1 Å due to the incorporation of GA as it reacted with the hydroxyl and amine groups of the protein. Cross-linking the protein layer also resulted in a small increase in DIW contact
angles. The stability of the resultant protein layers (1 mg/mL LYS, pH=9, thermally denatured) on ODTS substrates was investigated by exposing the substrates to conditions similar to those that may be encountered in commercial applications. Specifically, LYS protein coatings were selected for the stability tests since LYS does not form an inherent network structure, unlike FIB, which forms networks even without cross-linking. The results, summarized in Figure 2.7, reveal that cross-linking the LYS layer greatly improved the coating’s stability. The experiments indicate that the fractional coverage and DIW contact angle did not change when the cross-linked protein was exposed to these conditions. The uncross-linked protein layers showed a reduction in coverage and increased DIW contact angle, especially in the presence of organic solvent and surfactant. The limited stability of uncross-linked protein coatings may be attributed to the lack of in-plane cross-links among adsorbed protein molecules. In contrast, penetration of surfactant and solvent molecules and their ability to displace protein coatings at the hydrophobic support will be limited in cross-linked systems.

2.4.5. Deposition of polyelectrolyte layers and surface-initiated ATRP of polymer brushes.

Figure 2.8 shows schematically three methods employed based on FIB protein primers as substrates for surface-initiated polymerization of HEMA. A stable cross-linked protein coating on top of a hydrophobic substrate was employed as a primer for attaching polymerization initiator, 2-BPB, by reaction with hydroxyl and amine groups of protein molecules as represented in Method (i). In addition, single (i.e., PAH) or multilayers (i.e., PAH/PSS/PAH) of polyelectrolytes were formed through electrostatic interactions on top of which the 2-BPB initiator was attached, as shown in Method (ii) and Method (iii), respectively. The reason for depositing polyelectrolyte layers was to create a more uniform and homogenous distribution of functional groups throughout the substrate, to anchor the 2-BPB initiator. We considered that the usage of PAH as the outermost layer (instead of protein molecules) might make the composition of functional –NH₂ groups more uniform, as compared to the protein-only primer, which possesses a heterogeneous distribution of –OH and –NH₂ groups. Furthermore, the addition of polyelectrolytes may create stronger surfaces
with fewer defects as well as improved chemical and/or mechanical stability.\textsuperscript{70} The adsorption of each polyelectrolyte layer was monitored by ellipsometry and contact angle measurements.

The adsorption of polyelectrolytes was conducted at pH 7, where FIB is negatively charged (pI=5.5), PAH is positively charged (pK\textsubscript{a}=8.5) and PSS is negatively charged (pK\textsubscript{a}=1). The data in Figure 2.9 (top row) show thickness increases and contact angle measurements associated with the formation of LbL polyelectrolyte assemblies as well as polymer brushes on FIB-coated ODTS surfaces. The amount of adsorbed polyelectrolytes depends strongly on the type of the underlying surface. In particular, the adsorbed amount of PAH\textsubscript{1} (9 Å) on top of FIB layer was less than the PAH\textsubscript{2} (16.4 Å) adsorbed on a 16.4 Å thick PSS layer. These findings are attributed to the greater anionic charge density over a PSS film compared to that for FIB at pH 7. Although FIB acquires anionic charge globally at the experimental pH conditions employed, it contains some positive charges locally. Lvov et al.\textsuperscript{71} reported that the direct assembly of oppositely charged proteins is difficult. This is because the electrostatic attraction between globular proteins is not optimal due to the patchy nature of protein. In contrast, the electrostatic interactions between polyions are optimal due to uniform distribution of charges and flexible nature of synthetic polyelectrolytes. Hence, we presume that the electrostatic interactions between protein and a polyelectrolyte are less than optimal. One can take advantage of the sensitivity of contact angle measurements to monitor surface changes effectively. The addition of a PAH\textsubscript{1} layer on top of FIB protein layer resulted in an increase of DIW contact angles from 59 to 70°. In general, DIW contact angles measured from the respective individual PAH and PSS layers alternate depending on the topmost layer. Our results indicate that the PAH-terminated layers exhibit DIW of \approx70°, while PSS-terminated layers are more hydrophilic (\approx53°). While it is possible to grow or add more polyelectrolyte layers onto ODTS surfaces modified with pre-adsorbed proteins, we limited ourselves to only a few polyelectrolyte assemblies, as explained above.

We also employed the LbL method to prepare functional coatings on top of LYS supports. At pH 7, LYS is positively charged (pI=11.3). Thus, PSS was deposited above the
LYS layer followed by deposition of oppositely charged PAH. This led to two methods to
grow the PHEMA brushes on the LYS: Method (i), where the PHEMA brushes were directly
polymerized on the LYS, and Method (ii), where the PHEMA brushes were grown on
polyelectrolyte multilayers (i.e., PSS/PAH). The resultant thickness and contact angle
measurements are summarized in Figure 2.9 (bottom row). Specifically, the adsorbed
amount of PSS was 7 Å on top of LYS layer and the subsequent PAH was 10 Å. This result
further supports the presumption that interactions between polyelectrolytes and proteins are
not as strong as the interactions between polyelectrolytes. No large variation in DIW contact
angle values (60° for LYS and 57° for PSS layer) was noted after the adsorption of PSS layer
above LYS layer, which is in accordance with the thickness observations.

Coupling of 2-BPB initiator to protein and PAH-terminated surfaces resulted in a 4±1 Å
increase in thickness as measured by ellipsometry. XPS confirmed coupling of the 2-BPB
initiator to the protein substrate (see Appendix A). The growth of PHEMA brushes from 2-
BPB from bare FIB, LYS and their polyelectrolyte modified analogs was confirmed with
ellipsometry measurements. The thickness of the resultant PHEMA brushes produced from
bare FIB and LYS films and their respective polyelectrolyte layers was 440±15 Å. In
addition, control experiments carried out on bare ODTS surfaces without adsorbed (FIB and
LYS) proteins revealed no increase of thickness, indicating that the 2-BPB initiator did not
attach. After the synthesis of PHEMA polymer brushes from the surface, DIW contact angle
decreased to ≈49±2°. Control experiments carried out on bare ODTS resulted in no increase
in dry ellipsometric thickness suggesting no growth of PHEMA brushes. In addition, DIW
contact angle measurements of control substrates remained at ≈99° indicating the absence of
PHEMA brushes above the surface. Thus, adsorption of proteins onto ODTS substrates
provides the foundation for the growth of PHEMA brushes as described. Surprisingly, the
results in Figure 2.9 reveal that the thickness and wettability of the final PHEMA layers
prepared directly on top of the protein primers are comparable to those formed on top of LbL
films.
2.4.6. Morphologies of LYS and PHEMA-coated ODTS layers.

Surface topography of bare, protein- and protein/polymer brush-modified substrates were studied by AFM. In Figure 2.10 we present AFM images of surfaces coated with ODTS only, LYS layers deposited onto ODTS pre-coated substrates, and PHEMA grafts grown from ODTS/LYS-modified surfaces. Our AFM observations reveal that hydrophobic ODTS surfaces possess an RMS roughness of 0.24 nm. Adsorption of LYS proteins results in an increase of surface RMS roughness to 1.89 nm with the appearance of a few structural features. Overall, the protein deposition is uniform across the sample. PHEMA modified samples appear to be relatively smooth on a global scale as judged from the RMS roughness value of 1.11 nm. Though the RMS roughness values of PHEMA modified surface is less than that of the LYS modified surface, these differences are relatively small and can be attributed to experimental variation. On the local scale, the AFM image of the PHEMA samples reveals the existence of local features of in-plane heterogeneity. These structural features are not very large, i.e., the variation between the extremes in height is \( \approx 3 \text{ nm} \), which is less than \( \approx 7\% \) of the total brush height. This thickness heterogeneity may originate from multiple sources, including, inhomogeneous deposition of the initiator or inaccessibility of all initiating sites for polymerization. In order to understand the detected roughness effects one has to monitor the overall modification process in situ that can give some insight about the possible rearrangements of the functional primers after (or even before) initiator attachment or even during polymerization. Unfortunately, direct observation of LYS deposition and the preceding phenomena is a very challenging task and it is outside the scope of this paper. Additional work would have to be done to shed more light onto these phenomena.

2.4.7. Modification of hydrophobic fibers.

In order to further verify the adsorption of FIB protein molecules and growth of PHEMA brushes over hydrophobic surfaces, similar experiments of protein adsorption and polymer brush growth were repeated on PP nonwoven fiber surfaces. The deposition of protein coating and PHEMA layers onto the fiber supports was characterized by using FTIR-ATR. In Figure 2.11 we plot, IR spectra of: (a) PP non-woven surfaces, (b) FIB-modified PP fibers.
and, (c) PP non-woven surfaces with PHEMA brushes. The appearance of new peaks located between 1700 - 1550 cm\(^{-1}\) corresponds to the presence of amide I (C=O stretching) and amide II bands (C-N stretching and N-H bending). In addition the emergence of amide A (N-H stretching) and O-H stretching between 3420 - 3250 cm\(^{-1}\) confirms the attachment of protein molecules to PP supports. Since protein molecules contain several amino-acids, the bands appeared to be featureless and broadened in nature.\(^{72-73}\) PHEMA growth is evident from the appearance of a strong carbonyl peak at 1720 cm\(^{-1}\). The peaks at 1250 and 1080 cm\(^{-1}\) are assigned to C-O stretching and O-H deformation of the C-O-H groups respectively. The characteristic hydroxyl stretching vibration extended to lower frequencies with significant increase in its intensity as well as broadness between 3500-3100 cm\(^{-1}\) also confirms the growth of PHEMA brushes.\(^{3,51,74}\) In addition, the wetting experiments indicate clearly that the surface properties of PP are altered after each modification step. Specifically, water droplets placed on top of unmodified PP nonwoven sheets “roll away”. In contrast, the protein-modified PP nonwoven exhibits an improved wettability as shown by the behavior of the water droplet, which does not “roll away”. Finally, considerable increase in wicking was noticed after the growth of PHEMA brushes, due to the presence of polar hydroxyl groups on the surface.

2.5. Conclusions

Denatured \(\alpha\)-Lactalbumin (LALBA), lysozyme (LYS) and fibrinogen (FIB) were used to alter the physico-chemical properties including surface chemistry (functional groups) and surface wettability of hydrophobic ODTS and PP nonwoven surfaces though physical deposition after denaturation. The results demonstrated that the wettability of the protein coatings formed on hydrophobic surfaces improved significantly regardless of protein type and the denaturation method employed. Surface modification of hydrophobic surfaces by protein molecules is very appealing because it imparts numerous inherent amino-acid functionalities of proteins without involving any harsh treatments. In addition, the properties of soy proteins (glycinin and \(\beta\)-conglycinin) were similar to those of the expensive functional
model proteins, suggesting the application of the former ones as potential alternative surface modifiers. Surfaces functionalized with denatured protein layers were subsequently employed as supports for surface-initiated polymerization of HEMA. The properties of the resulting PHEMA grafts are similar to those grown from model flat surfaces indicating a high density and stability of polymeric grafts produced on top of protein-modified hydrophobic surfaces. Our surface functionalization approach is very versatile and can be employed to alter any kind of hydrophobic material. Selected applications of these functional fibers that might be explored include capture of metals or other contaminants from waters, prevention of protein adsorption, attachment of metallic nanoparticles and many others.
2.6. References


(56) Bomboi, F.; Bonincontro, A.; La Mesa, C.; Tardani, F. *J. Colloid Interface Sci.* 2011, 355, 342-347.


Figure 2.1. Schematic representation of protein denaturation in solution and its subsequent deposition on a hydrophobic substrate. The latter process results in the formation of a coating layer whose surface is comprising predominantly hydrophilic moieties present originally in the corona section of a native protein.
Figure 2.2. CD spectra of urea-denatured protein solutions collected in far UV region for LYS (a) and FIB (b) proteins. The colored spectrum lines represent the native protein (black), protein with 2 M urea (red), protein with 4 M urea (green), and protein with 8 M urea (blue). CD spectra of LYS and FIB (c) with increasing urea concentration at 222 nm.
Figure 2.3. Ellipticity at 222 nm determined from CD spectra of thermal denatured LYS and FIB protein solutions as a function of increased temperature.
Figure 2.4. Ellipsometry (top) and DIW contact angle (bottom) profiles of urea-denatured protein coatings on ODTS surfaces at various bulk concentrations of denatured proteins. The horizontal dotted line in the bottom spectrum denotes the wettability of ODTS.
Figure 2.5. Ellipsometry (left) and DIW contact angle (right) profiles of parent/native (black squares) and thermally-denatured (red circles and green triangles) LYS protein coatings on ODTS surfaces at various bulk concentrations.
Figure 2.6. Ellipsometry (top) and DIW contact angle (bottom) profiles of thermally denatured LYS and soy (7S & 11S) protein coatings on ODTS surfaces at various bulk concentrations.
Figure 2.7. a) Ellipsometry (top) and b) DIW contact angle (bottom) profiles of thermally denatured LYS layers on ODTS surfaces after stability studies. The DIW contact angle of ODTS is 108°.
Figure 2.8. Schematic depicting built-up of protein and polyelectrolyte layers followed by subsequent ATRP polymerization of PHEMA brushes. In method (i) the 2-BPB initiator is attached directly to the cross-linked FIB protein layer. In methods (ii) and (iii), 2-BPB gets anchored to substrates comprising a single (i.e., PAH₁) or a multilayer (i.e., PAH₁/PSS/PAH₂), respectively, of polyelectrolytes deposited by the LbL method on top of protein primer.
Figure 2.9. (top row) Dry thickness (left) and DIW contact angle (right) of PHEMA brushes grown from functional layers deposited on top of flat ODTS/FIB-coated silica substrates. The layer comprising denatured FIB was cross-linked with GA/NaBH4. (bottom row) Dry thickness (left) and DIW contact angle (right) of PHEMA brushes grown from functional layers deposited on top of flat ODTS/LYS-coated silica substrates. The layer comprising denatured LYS was cross-linked with GA/NaBH4. Data corresponding to fabrication methods (i)-(iii) denoted pictorially in Figure 8 are shown in the top row. The data corresponding to LYS protein primer are shown in the bottom row. For clarity the order in the DIW data have been reversed relative to that of the thickness.
Figure 2.10. AFM images, $3 \times 3 \ \mu m^2$, of control ODTS (left), LYS-coated ODTS (middle) and PHEMA-coated ODTS (right) surfaces.
Figure 2.11. FTIR spectra collected in transmission from (from bottom to top) PP nonwoven sheet (black), PP-sheet coated with denatured fibrinogen (FIB) layer (olive) and PP-FIB fiber with PHEMA brushes (orange).
CHAPTER 3 - FORMATION AND ANTIFOULING PROPERTIES OF AMPHIPHILIC COATINGS FORMED ON POLYPROPYLENE FIBERS

3.1 Abstract

We describe the formation of amphiphilic polymeric assemblies via a three-step functionalization process applied to polypropylene (PP) nonwoven surfaces and to reference hydrophobic self-assembled n-octadecyltrichlorosilane (ODTS) monolayer surfaces. In the first step, denatured lysozyme (LYS) and fibrinogen (FIB) proteins are adsorbed onto the hydrophobic PP and ODTS surfaces followed by cross-linking with glutaraldehyde (GA) in the presence of sodium borohydride (NaBH₄). The hydroxyl and amine functional groups present on the proteins permit the attachment of initiator molecules, from which poly (2-hydroxyethyl methacrylate) (PHEMA) polymer grafts are grown directly through “grafting from” atom-transfer radical polymerization (ATRP). The terminal hydroxyls of HEMA’s pendent groups are modified with fluorinating moieties of different chain lengths resulting in amphiphilic brushes. A palette of analytical tools, including ellipsometry, contact angle goniometry, Fourier transform infrared spectroscopy in the attenuated total reflection mode, and x-ray photoelectron spectroscopy is employed to determine the changes in physicochemical properties of the coatings after each modification step. Anti-fouling properties of the resultant amphiphilic coatings on PP are analyzed by following the adsorption of fluorescein isothiocyanate-labeled bovine serum albumin as a model fouling protein. Our results suggest that amphiphilic coatings suppress significantly adsorption of proteins as compared to PP fibers or PP surfaces coated with PHEMA brushes. The type of fluorinated chain grafted to PHEMA allows modulation of the surface composition of the top-most layer of the amphiphilic coating and its anti-fouling capability.
3.2 Introduction

Tailoring surfaces with biocompatible and non-fouling characteristics represents one of the outstanding challenges in today’s surface science. Most material surfaces in use are prone to non-specific adsorption of biomolecules; a widespread polymer such as polypropylene (PP) is not exempt from such phenomenon. Owing to its good mechanical strength, low density, chemical resistance, thermal stability, and low cost, PP is widely used in textile and filtration industries, among others. Unfortunately, inherent hydrophobicity and poor biocompatibility of PP limit its use in biological applications including bio-filtration and bio-separation. In general, when synthetic surfaces come into contact with biological milieu, adsorption of platelets and proteins occurs nearly instantaneously leading to undesired effects, such as thrombus and plaque formation on medical implants, teeth, and dental restoratives, contamination of food products, etc. Likewise, fouling of textile surfaces, contact lenses, kidney dialysis membranes or bi-processing equipment is ubiquitous. This phenomenon compromises the efficiency of the product and its functions. In particular, during bio-filtration, proteins from blood and body fluids interact strongly with hydrophobic surfaces causing pore blocking and biofouling of PP supports. In turn, fluid flux is reduced which leads to high trans-membrane pressures, operational cost, and eventually device deterioration. Hence, controlling bio-adhesion of hydrophobic materials, including PP, to enhance bio-compatibility is an important prerequisite in their deployment. To this end, numerous studies have been carried out to tailor the surface properties of materials after chemical and physical modifications. It has been established that grafting surfaces with macromolecules of tailored chemical composition, length, and surface density is paramount in achieving biocompatibility. In addition, polymer grafting provides numerous opportunities to introduce new functional groups that can further enhance the functionality of coating layers.

A generalized approach to prevent fouling of surfaces is restricting initial protein adsorption, which necessitates the installation of protein-repellent functionalities. Surfaces grafted with ethylene glycol, i.e., oligo- or poly-ethylene glycol (OEG or PEG) are used
nearly exclusively as benchmarks for protein-repellent coatings. However, temperature instability as well as sensitivity toward oxidation or auto-oxidation catalyzed by transition metals limit the widespread application of such coatings. In addition, OEG or PEG chains contain only a limited number of accessible hydroxyl groups, which restrict further chemical modifications. These limitations have triggered the development of novel protein-repellent materials. 

In particular, 2-hydroxyethyl methacrylate, sulfobetaine methacrylate, carboxybetaine methacrylate, 2-methacryloyloxy-ethyl phosphorylcholine, 3-sulfopropylmethacrylate and 2-(tert-butylamino)ethyl methacrylate have been tested and proposed as efficient protein-repellent coating polymers.

Numerous surface grafting methods have been employed to generate polymeric coatings. Among the most efficient ones are anchoring ex situ synthesized polymers to the surface of interest (so-called “grafting to” method) or generating polymeric grafts directly from surface-bound polymerization initiators (so-called “grafting from” method). In both cases, the grafts are anchored to the surface by either naturally present functional moieties on the surface or by those that have been generated by surface pre-activation. The pre-activation methods typically involve irradiation by UV, electron beam, and ozone. Some surfaces can also be modified by using flame, plasma and corona treatments. While relatively efficient in endowing the surfaces with reactive groups, such “harsh” physical treatments might compromise the mechanical properties of the substrates or supports. This is particularly problematic in the case of relatively thin fiber-based materials. In order to overcome this limitation, we have proposed a novel route for achieving surface functionalization of hydrophobic materials (flat supports and polyolefin-based textile fibers) primed with denatured proteins as a primary step for further modification with functional groups as described in Chapter 2.

Specifically, we decorate the outer surfaces of protein coatings with polymerization initiators, which serve as active sites for “grafting from” polymerization of protein-repellent poly(2-hydroxyethyl methacrylate) (PHEMA). Amphiphilic grafts are then generated by fluorinating PHEMA chains with selected fluorinating agents. The motivation of the post-
polymerization via fluorination reaction comes from our recent study demonstrating that a coating with a single chemistry, such as ethylene glycol, is not sufficient to prevent the adsorption of a broad spectrum of proteins and bio-organisms. Moreover, silicon substrates carrying amphiphilic coatings based on ethylene glycol and fluorinated chemistries act in concert to minimize protein adsorption. For instance, they are effective in preventing adhesion of biospecies such as *Ulva* and *Navicula*, which possess affinity for hydrophobic and hydrophilic supports, respectively. Concurrently with the functional PP fibers, we employ the same methodologies on reference flat surfaces after hydrophobization of silicon wafers via self-assembled monolayer (SAM) of n-octadecyltrichlorosilane (ODTS). The latter experiments enable facile characterization of the individual fabrication steps using sensitive analytical methods. We employ fluorescently-tagged bovine serum albumin probes to demonstrate that amphiphilic PHEMA-fluorinated coating layers installed on the pre-adsorbed protein layer endows PP fibers with effective antifouling characteristics.

### 3.3 Materials and Methods

#### 3.3.1 Materials

Deionized water (DIW) (resistivity >16 MΩ cm) was produced using the Millipore water purification system. Silicon wafers (orientation [100]) were supplied by Silicon Valley Microelectronics. Phosphate buffered saline (PBS) solution, HPLC-grade methanol, and toluene were purchased from Fisher Scientific and used as received. ODTS was obtained from Gelest (Morrisville, PA). Lysozyme (from chicken egg white, $M_w = 14.3$ kDa, isoelectric pH or pI=11.3), fibrinogen (from human plasma, $M_w=340$ kDa, pI=5.5), fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA, $M_w=66$ kDa), pyridine, CuCl (99.99%), CuCl$_2$ (99.99%), 2,2’-bipyridine (99%), 2-bromopropionylbromide (2-BPB), 2-hydroxyethyl methacrylate (HEMA, 98%), trifluoroacetic anhydride (C$_4$O$_3$F$_6$, F1), heptafluorobutyryl chloride (C$_7$F$_{15}$COCl, F3), pentadecafluoro-octanoyl chloride (C$_7$F$_{15}$COCl, F7), methanol, dichloromethane, glutaraldehyde (GA), and sodium borohydride (NaBH$_4$), were all purchased from Sigma-Aldrich and were used as received.
Tetrahydrofuran (THF) and triethylamine were purchased from Sigma-Aldrich and Fluka and distilled from Na/benzophenone and calcium hydride, respectively.

3.3.2. Preparation of protein solution and deposition on surfaces

Urea (8 M concentration) was added to PBS buffer solutions and filtered through a 0.2 µm filter followed by the addition of protein to obtain lysozyme (LYS) and fibrinogen (FIB) concentrations of 1 and 0.1 mg/mL, respectively. The solutions were allowed to stand at room temperature for 6 h to solubilize the proteins completely. Protein adsorption was carried out at the isoelectric point (pI) of each protein (i.e., pH 11.3 or 5.5 for LYS and FIB, respectively); the solution pH was adjusted by adding either 0.1 N HCl or NaOH drop-wise. Sodium azide (NaN₃, 0.2%) was added to the buffer solution to prevent the growth of bacteria during the adsorption process.

PP nonwoven sheets were obtained from the Nonwovens Institute (North Carolina State University) and were cleaned with isopropyl alcohol prior to use. Self-assembled monolayers (SAMs) of n-octadecyltrichlorosilane (ODTS) on silicon wafers were prepared according to the procedure developed by Liu et al.24 The ODTS SAMs were employed to mimic the physico-chemical characteristics of PP in a flat geometry. PP nonwoven and ODTS wafers were immersed in protein solutions for 12 h at room temperature, then removed, rinsed with PBS and DIW and blow-dried with nitrogen gas. The resultant flat ODTS substrates were characterized by ellipsometry and contact angle measurements in order to determine the thickness and wettability, respectively, before and after protein adsorption. Cross-linking of the resultant protein layers on ODTS substrates and PP nonwovens were carried out using the experimental conditions adopted from our previous work (i.e., Chapter 2).22 Cross-linking of proteins in solutions was assessed by using SDS-PAGE as described in Appendix A.

3.3.3. Formation of amphiphilic polymer brush layers

The accessible hydroxyl and amine groups of the protein’s hydrophilic fragments present over the free surface of the cross-linked protein layer were used to attach 2-BPB by
following the conditions described earlier.\textsuperscript{25} ATRP of HEMA following the “grafting from” scheme from the surface-bound 2-BPB initiator centers was carried out at 25 °C for 9 h according to the method described by Arifuzzaman\textsuperscript{23} et al. The PHEMA brushes were functionalized chemically by employing post-polymerization modification (PPM) reactions to obtain P(HEMA-co-fHEMA) amphiphilic grafts. Various fluorocarbons were used, including, trifluoroacetic anhydride (C$_4$O$_3$F$_6$, F1), heptafluorobutyryl chloride (C$_3$F$_7$COCl, F3) and pentadecafluoro-octanoyl chloride (C$_7$F$_{15}$COCl, F7). The experimental conditions for the PPM were adopted from our earlier work.\textsuperscript{23} Because the length of the PHEMA brushes are much smaller than the radius of curvature of the fiber surfaces (diameter = 5.15 ± 2.17 µm), we assumed that the physico-chemical properties of the brushes and its fluorinated counterparts were comparable to those grafted on flat substrates, \textit{i.e.}, curvature effects were assumed to be negligible.

3.3.4. Ellipsometry

The thickness of bare ODTS SAMs before and after the formation of protein coated layers was determined using variable angle spectroscopic ellipsometry (VASE, J.A. Woollam Co.). Specifically, ellipsometry was used to determine the thickness of the ODTS SAM and PHEMA brushes before and after the fluorination reaction in order to establish the “degree of fluorination”. For each coating, the thicknesses were measured in the dry state at three or more spots on each specimen and then averaged. Ellipsometric data were collected at an incidence angle of 75° for SAMs and protein coating layers and at an angle of 70° for PHEMA and amphiphilic polymers, using wavelengths ranging from 400 to 1100 nm in 10 nm increments. The thicknesses were estimated using a Cauchy layer model assuming the index of refraction as 1.5\textsuperscript{26} for ODTS SAMs and 1.54\textsuperscript{23} for protein layers on top of the ODTS SAMs. The thickness of PHEMA and P(HEMA-co-fHEMA) on protein coating layer was calculated using the method developed by Arifuzzaman\textsuperscript{23} et al.
3.3.5. Contact angle measurements

Contact angles (θ) were measured with Ramé-Hart contact angle goniometer (model 100-00) using deionized water (DIW) as the probing liquid at room temperature. Static contact angles were recorded by releasing an 8 µL droplet of DIW on the surface. The advancing and receding contact angles, θ_A and θ_R, respectively, were measured by imaging the droplet at the tip of the syringe and adding (θ_A) or withdrawing (θ_R) 4 µL DIW. Contact angle measurements were carried out on at least three different positions over the surface and then averaged. In addition to wettabilities, contact angle hysteresis (CAH), taken as θ_A - θ_R, was evaluated to provide information about chemical heterogeneity of the substrates. A CAH of < 10° indicated that the substrate coating was relatively uniform.

3.3.6. X-ray photoelectron spectroscopy

A Kratos Analytical AXIS ULTRA DLD x-ray photoelectron spectroscopy (XPS) instrument employing monochromated Al Kα radiation with charge neutralization was utilized to determine the chemical composition of fluorinated PHEMA random copolymer grafts, i.e., P(HEMA-co-fHEMA). Survey and high-resolution spectra were collected with pass energies of 80 and 20 eV, respectively, using both electrostatic and magnetic lenses for single angle spectra collection. The depth-dependent distribution of the fHEMA units in P(HEMA-co-fHEMA) coatings was assessed by angle-resolved XPS (AR-XPS), which was conducted at 90 and 30° take-off angles using electrostatic lens in order to achieve enhanced angular resolution. The take-off angle, defined as the angle between the sample and the detector probes allowed penetration depths of ≈ 90 and ≈ 45 Å at 90 and 30°, respectively. The resultant data were analyzed using the CasaXPS software package.

3.3.7. Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy spectra for PP nonwoven surfaces before and after functionalization were recorded using a Bio-Rad-Digilab FTS-3000 FTIR spectrometer equipped with crystalline ZnSe in the ATR mode with continuous nitrogen purging. During the spectral
collection, the nonwoven was pressed against the crystal under a uniform pressure of \( \approx 700 \) psi using the micrometer pressure clamp. The spectra reported represent an average over 5 accumulations of 64 scans with a resolution of 4 cm\(^{-1}\). The data were analyzed using the Bio-Rad Win IR Pro software.

3.3.8. Atomic force microscopy (AFM)

The surface topography of ODTs substrates before and after functionalization was examined using Asylum Research MFP3D atomic force microscope (AFM). The AFM instrument was operated in tapping mode in AC mode using an Al-backside-coated Si probe with a force constant of \( \approx 5 \) N/m and a resonance frequency in the range of 120-180 kHz. During AFM imaging, care was taken to keep the tip in the repulsive mode. The root-mean-square (RMS) surface roughness was calculated from the height images using the MFP-3D software.

3.3.9. Confocal Microscopy

To visualize the antifouling properties of P(HEMA-co-fHEMA) coatings on PP nonwovens, the specimens were incubated with 0.1 mg/mL fluorescently-labeled bovine serum albumin (FITC-BSA) in PBS buffer at its pI of 4.9. After 12 h, the substrates were removed from the protein solution, washed thoroughly with PBS buffer followed by rinsing with DIW and dried in a stream of nitrogen gas. Images were taken using a laser scanning confocal microscope (Zeiss, LSM710, magnification 20X). All samples were exposed to an excitation source of argon laser at 488 nm, while the emission was collected in the range of 492-632 nm.
3.4. Results and Discussion

3.4.1. Adsorption of denatured proteins onto ODTS and PP surfaces

Figure 3.1 depicts schematically the formation of functional coatings on PP fibers. Similar procedures were applied to flat ODTS reference surfaces. Detailed description of the experimental procedures leading to such coatings is provided in the Experimental Section. Lysozyme (LYS, $M_n=14.3$ kDa, pI=11.3$^{27}$) and fibrinogen (FIB, $M_n=340$ kDa, pI=5.5$^{27}$) were chosen to prime the surface because of their different molecular weights and isoelectric points. The layer comprising adsorbed denatured proteins was characterized by ellipsometry and contact angle measurements. The dry thicknesses (H) of LYS and FIB layers over the ODTS substrate were 22.7 and 31.5 Å, which corresponded to protein coverages of 0.79 and 0.38, respectively. The coverage is defined here as the mass fraction of protein compared to that for a perfect monolayer ($H_o$), i.e., coverage=$H/H_o$ with $H_o=\left[M_o/(\rho N_A)\right]^{1/3}$; $M_o$ is the molecular mass or molecular weight of the protein, $\rho$ is the density of the protein (assumed 1 g/cm$^3$), and $N_A$ is Avogadro’s number.$^{28}$ The sizes of native, globular LYS and FIB proteins are 30 x 30 x 45 Å$^3$ and 60 x 60 x 450 Å$^3$, respectively. The thickness of the protein coating layer adsorbed on hydrophobic ODTS is less than that for end-on or side-on orientations of native proteins. Therefore, it may be concluded that the denaturation process leads to the rupture (disruption/unfolding) of protein’s native structure.$^{11,29}$ In fact, the thickness of denatured/unfolded protein coating formed is found to be equivalent to the length of a few amino acid side chains that form loose random structures on the surface with hydrophobic amino acids interacting with the hydrophobic surface and the hydrophilic residues dangling into the outer medium.$^{22}$ The lower coverage of FIB relative to LYS cannot be explained entirely by the difference in deposition solution concentrations.$^{22}$ Hence, the difference also may be attributed to the complex elongated structure of FIB, which forms large aggregates that prevents close packing of protein molecules.$^{22}$ In contrast, LYS is smaller than FIB and can pack more readily on the surface.

As reported in our previous work (i.e., Chapter 2), protein coating stabilities were improved significantly by introducing cross-links among the adsorbed protein molecules.
using well-known cross-linking agent, GA in the presence of NaBH₄. The cross-linking reaction resulted in an increase in protein coating thickness of 3±1 Å due to the incorporation of GA resulting from the reaction of hydroxyl and amine groups of protein with carbonyl groups of GA. The presence of a protein layer was further confirmed by contact angle measurements. The static contact angle measured by deionized water (DIW) on the bare ODTS substrate was 108±2°. After adsorption of FIB (or LYS), the contact angle decreased to 59±3°, suggesting that hydrophilic amino acids were exposed to the outer environment, while hydrophobic amino acids resided primarily at the ODTS surface. Water droplets placed on top of unmodified PP nonwoven sheets rolled away whereas the protein-modified PP nonwoven exhibited improved wettability; the wettability of the fibers indicated the attachment of protein layers to PP that renders the surface hydrophilic. Cross-linking with GA did not affect the wettability of the denatured protein layer as deduced from the contact angle measurements.

### 3.4.2. Formation of amphiphilic polymeric brushes

Polymerization initiators, 2-bromopropionylbromide (2-BPB), were grafted to the cross-linked protein coatings that served as reaction centers for the ATRP of 2-hydroxyethyl methacrylate (HEMA). The experimental details pertaining to the grafting and polymerization of HEMA are described in the Materials and methods section. The resulting PHEMA macromolecular grafts grown from the initiators attached to LYS and FIB protein coatings on flat ODTS SAMs had thickness of 63.0±1.5 and 63.5±1.5 nm, respectively. Contact angle measurements performed after the polymerization revealed that the wettability was reduced to 48±2°, confirming the presence of added hydrophilic moieties. Water droplets also wetted PP nonwoven sheets grafted with PHEMA due to the presence of polar hydroxyl groups on the surface. An important attribute of PHEMA chains is that the hydroxyl terminus in each HEMA monomer can be further modified chemically. In our work, we have derivatized PHEMA chains using post-polymerization modification (PPM) protocols with various fluorination agents, including, trifluoroacetic anhydride (C₄O₃F₆, F1), heptafluorobutyryl chloride (C₃F₇COCl, F3) and pentadecafluoro-octanoyl chloride
(C$_7$F$_{15}$COCl, F7), to produce random copolymers of P(HEMA-co-fHEMA), where fHEMA denotes the HEMA segment fluorinated with the respective fluorination agent (cf. Figure 3.2).

We employed Fourier-transform infrared spectroscopy measurements in the attenuated total reflectance mode (FTIR-ATR) to verify the chemical composition of the coatings after each modification step. Figure 3.3 presents the IR spectra for PP non-woven sheets following their modifications with FIB and amphiphilic polymer brushes. Table 3.1 summarizes the characteristic IR vibrations found in PP and the functionalized PP fibers. The presence of FIB on the PP surface is verified by the appearance of peaks located between 1700-1550 cm$^{-1}$ assigned to amide I (C=O stretching), amide II bands (C-N stretching and N-H bending) and between 3420-3250 cm$^{-1}$ that originate from amide A (N-H stretching) and O-H stretching. The observed bands are featureless and broad, due to the overlap of various underlying component bands of proteins.$^{31,32,33}$ In PHEMA-grafted PP nonwoven mats, a new peak appears at 1720 cm$^{-1}$ that corresponds to the C=O stretching vibration of the ester group present in PHEMA. In addition, peaks at 1250 and 1080 cm$^{-1}$ are attributed to C-O stretching and O-H deformation of the C-O-H groups respectively. The peak at 1150 cm$^{-1}$ also arises from the C-O-C stretching of the PHEMA. The significant increase in the intensity and broadness of the band at 3500-3100 cm$^{-1}$ is attributed to the characteristic –OH stretching of PHEMA which verifies the grafting of PHEMA to PP surface. The disappearance of hydroxyl stretches at 3500-3100 cm$^{-1}$ in specimens containing PHEMA-F1, PHEMA-F3, and PHEMA-F7 attached to the FIB-modified PP nonwovens is associated with the attachment of F1, F3 and F7 to PHEMA. The derivatization of hydroxyl groups of PHEMA with fluorinated groups is further evidenced by the appearance of a characteristic stretch at 1800 cm$^{-1}$ corresponding to formation of the carbonyl group CH–COO–CF (fluorinated ester) and the characteristic -CF$_2$- stretches at 1214 and 1150 cm$^{-1}$.\(^{23,30,25}\)

Fluorination of PHEMA brushes leads to increase of the dry thickness of the grafted layer built on ODTM flat supports with primer protein layer. A non-uniform PPM reaction may affect the surface topography; therefore, atomic force microscopy (AFM) was used to image
the surface of samples before and after derivatization with fluorinating agents. **Figure 3.4** presents typical AFM scans of LYS-grafted PHEMA (top row) and FIB-grafted PHEMA (bottom row) before and after coupling reactions. The PHEMA surface appears relatively smooth on both protein supports revealing that ATRP of HEMA produces uniformly distributed PHEMA grafts. Derivatization of PHEMA with fluorinated agents results in an increased surface roughness. In addition, the surfaces become rougher with increasing size of the fluorinated agent. While roughness may affect the characterization of surfaces using methods that are suited for flat samples, we put the effect of roughness aside temporarily and return to it after presenting the results of ellipsometry, angle-resolved XPS (AR-XPS), and contact angle measurements.

The top portion of **Figure 3.5** summarizes the thickness data of PHEMA-based films prepared on LYS- and FIB-based coatings, on ODTS SAMs before and after fluorination with various agents. The ellipsometry data indicate that the absolute thickness after fluorination increases with increasing size of the fluorinating agent. The thickness increase due to fluorination was used to obtain information about the amount of fHEMA in the P(HEMA-co-fHEMA) copolymer by using a simple model developed by Arifuzzaman et al. (see detailed model in the Appendix B). The resulting chemical compositions in the P(HEMA-co-fHEMA) copolymers are plotted in the bottom portion of **Figure 3.5**. The data in **Figure 3.5** reveal that the amount of F3 inside the PHEMA brush is highest while that of F7 is the lowest. Several factors can contribute to the observed size dependence, including, i) larger steric hindrance imposed on the F7 molecules relative to F3, and ii) higher tendency of F7 to cluster due to strong interactions leading to larger complexes that may not penetrate deep inside the brush. We attribute the lower loading of F1 inside the brush relative to F3 due to the different reaction conditions employed in coupling F1 relative to attaching F3 or F7 to PHEMA (see Materials and methods section).

Although ellipsometry offers insight into the overall loading of fluorinated agents inside the brush it does not provide information about their spatial distribution throughout the PHEMA grafts. Contact angle and AR-XPS measurements can reveal key information on the
distribution of fluorinated agent at the surface and in the sub-surface region of the P(HEMA-co-fHEMA) brush. Therefore, in order to investigate the spatial distribution of fHEMA in P(HEMA-co-fHEMA) developed on LYS-coated ODTS we employed AR-XPS at two different take-off angles, 90° and 30°, whose probing depths are ≈90 and ≈45 Å, respectively. The discrete points in Figure 3.6 summarize atomic concentrations of carbon (C), oxygen (O), fluorine (F) and silicon (Si) as a function of take-off angles for PHEMA brushes produced on LYS proteins before and after modification with fluorinated agents. The solid lines in Figure 3.6 represent the predicted elemental concentrations corresponding to uniform distributions of fHEMA fluorinating agents, i.e., assuming that all hydroxyl groups of HEMA are coupled uniformly with the respective fluorinated agents. The agreement between the predicted and measured elemental compositions for both angles reveals that the distribution of the fluorinated agents inside the PHEMA brush is relatively uniform within the first 90 Å.

Contact angle measurements provide information about the physico-chemical properties of the surface with the amphiphilic coatings. Figure 3.7 summarizes the changes in the advancing (θA, squares) and receding (θR, circles) DIW contact angles of PHEMA brushes grown from LYS and FIB supports after PPM with fluorinating agents. The DIW contact angle over a PHEMA grafted surface is ≈48°, while the contact angles of –CF2- and densely packed –CF3 groups are ≈114° and ≈119°, respectively.23,34 From the data, coupling fluorinating agents to PHEMA units results in an increase of DIW contact angles with increasing the size of the fluorinating modifier. There is no considerable effect of the protein support layer on the contact angle data. Using the Cassie-Baxter model, an estimate of the expected contact angle can be obtained for a system featuring uniformly distributed fluorinated units within HEMA building blocks, θmix.35 By assuming that the areal fractions of HEMA and –CF3 are approximately equal, one arrives at a value of θmix≈82~85°. From the data, the θA of PHEMA-F1 is very close to the expected value corresponding to a uniformly distributed HEMA and F1 units. In contrast, PHEMA-F7 samples display θA in the range of ≈110~115°, which may be attributed to the surface segregation of fluorinated
molecules at the top of PHEMA brush. However, AR-XPS experiments rule out considerable surface segregation of the fluorinated moieties on the surface. One possible reason for the increased contact angle can be associated with the roughness on the surface. This is supported by the observed increase in roughness of the samples with increasing size of the fluorinating agent. Furthermore, roughness affects wettability on both the macro and microscales. On the macroscale, the wettability is described by the Wenzel law. Here roughness contributes to wettability in a non-monotonous manner; wettability increases with increasing roughness for $\theta \approx <90^\circ$ while it decreases for $\theta \approx >90^\circ$. Concurrently, the contact angle hysteresis (CAH) also increases. From the data in Figure 3.7, it is unlikely that macroscopic roughness affects the wettability in our specimens. On the microscale, increasing roughness leads to decreasing wettability; this effect is often accompanied by a decrease of CAH. While it is tempting to relate the wettability data and the surface roughness alone, molecular orientation (including possible surface reconstruction) of chemical moieties present on the surface may also contribute to the observed behavior.

Wang and coworkers\textsuperscript{36} demonstrated that the surface energy of surfaces comprising semifluorinated moieties depends on the in-plane organization of those groups, which, in turn, varies with increasing molecular packing. Longer fluorocarbon “fingers” featuring a larger number of $\text{–CF}_2$- repeat units packed better and produced surfaces with lower surface energies and lower wettabilities (i.e., higher DIW contact angles). Specifically, Wang \textit{et al.} reported that surfaces with longer fluorocarbon side chains, \textit{i.e.}, $-(\text{CF}_2)_6-$ or longer, produced highly oriented smectic B structures whose top layer consisted mainly of $\text{–CF}_3$ units.\textsuperscript{36} Based on these findings we suggest that while there is very little orientation of the fluorinated moieties in PHEMA-F1 and PHEMA-F3, PHEMA-F7 may feature some degree of self-organization among the F7 modifiers on the surface. \textbf{Figure 3.8} presents pictorially the proposed molecular structure of the P(HEMA-\textit{co}-fHEMA) brushes. We thus propose that in addition to surface roughness the observed decrease in wettability of PHEMA-F7 compared to PHEMA-F1 and PHEMA-F3 may also be associated with increasing fraction of $\text{–CF}_3$ units and their better packing on the surface of P(HEMA-\textit{co}-fHEMA) grafts. Experiments involving tilting samples after water drop deposition provide further evidence that surfaces
featuring the F1 and F3 moieties exhibit a relatively uniform distribution of the fluorinating agents (drop pinning). In contrast, coatings prepared by PPM using F7 have a large concentration of –CF₃ groups on the surface resulting in water droplets rolling off of the surface. The molecular orientation of the fluorinating agents on the surfaces will affect the performance of such amphiphiles, as will be demonstrated below.

We have recently demonstrated that surfaces made by modifying PHEMA brushes with various fluorinating agents on silica substrates, some of which are presented in this paper, are effective in minimizing the adsorption of proteins.²³ Here we show that PP nonwoven sheets modified with the methodology shown in Figure 3.1 exhibit protein fouling that is smaller than that of bare PP or PHEMA-modified PP. After surface modification, the functionalized PP sheets were incubated in a solution of fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) (0.1 mg/mL concentration, pH 4.3) for 12 h at room temperature. The modified fibers were imaged using laser scanning confocal microscope. Confocal microscopy images of unmodified PP and LYS- and FIB-coated PP nonwoven sheets modified with amphiphilic coatings are shown in Figures 3.9 and 3.10, respectively. The images reveal that control PP, protein-coated PP, and PHEMA-coated PP are stained with FITC-BSA. However, the amphiphilic coatings exhibit a strong reduction in fluorescein intensity indicating a lower deposition of the FITC-BSA and hence better anti-fouling properties. There is relatively little difference among the individual coatings formed from fluorinated agents of different lengths; however, it appears that PHEMA-F7 exhibits visually a larger adsorption of FITC-BSA relative to the other two amphiphiles. This behavior is likely associated with the presence of a large number of hydrophobic –CF₃ groups on the surface of PHEMA-F7, as discussed earlier. While more quantitative studies will have to be performed to quantify any effect of the type of the fluorinated modifier on anti-fouling performance of the coating, our results reveal clearly that combining PHEMA and fluorine-based chemistries leads to coating layers that inhibit significantly adsorption of proteins relative to bare hydrophobic or PHEMA-modified systems.
3.5. Conclusions

We have developed a simple and versatile method of functionalizing hydrophobic surfaces by physisorbing denatured proteins and cross-linking them. We performed experiments with LYS and FIB and demonstrated that the properties of the coating layers were similar for both proteins. This protein primer was subsequently used to prepare amphiphilic polymer brushes by surface-initiated polymerization from the protein coating followed by PPM reactions. Specifically, we prepared PHEMA macromolecular grafts on top and carried out PPM with three fluorinated species. The resulting amphiphilic coatings were tested for their ability to inhibit protein adsorption. The fluorinated PHEMA amphiphilic layers exhibited better anti-fouling characteristics than “bare” PHEMA brushes.

The appeal of this method is its versatility; it can be applied to any hydrophobic surface. In our work we have utilized both flat hydrophobic surfaces as well as PP fibers. The ability to endow PP fibers with functional layers without resorting to harsh physical treatments such as plasma or corona, may be utilized in preparing filtration or separation platforms whose function can be adjusted conveniently by choosing an appropriate chemistry of a polymer brush and eventually enhance it by employing a PPM reaction.
3.6. References


(22) Goli, K. K.; Rojas, O. J.; Özçam, A. E.; Genzer, J. *Biomacromolecules* 2012.
(28) The calculated thicknesses of FIB and LYS monolayers are 82.8 and 28.8 Å, respectively.
Table 3.1. IR vibrations in PP and functionalized PP fiber materials.

<table>
<thead>
<tr>
<th>Wavenumber (cm(^{-1}))</th>
<th>Group / functionality class</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>2990-2850</td>
<td>-CH(_3) and –CH(_2)- in PP</td>
<td>PP</td>
</tr>
<tr>
<td>1380-1370</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1475-1450</td>
<td></td>
<td></td>
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<tr>
<td>1465-1440</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3420-3250</td>
<td>-OH in alcohol and NH stretch (amide A)</td>
<td>FIB, PHEMA</td>
</tr>
<tr>
<td>1700-1550</td>
<td>C=O and –NH(_2) in primary and secondary amides</td>
<td>FIB</td>
</tr>
<tr>
<td>1740-1720</td>
<td>C=O stretch in aldehydes, esters</td>
<td>PHEMA, FIB</td>
</tr>
<tr>
<td>1240-1070</td>
<td>C-O-C in ethers</td>
<td>PHEMA</td>
</tr>
<tr>
<td>1786</td>
<td>C=O in CH-COO-CF</td>
<td>F1, F3 and F7</td>
</tr>
<tr>
<td>1680</td>
<td>C=O in CH-COO-CH</td>
<td>F1, F3 and F7, PHEMA</td>
</tr>
<tr>
<td>1300-1000</td>
<td>C-F in fluoro compounds</td>
<td>F1, F3 and F7</td>
</tr>
</tbody>
</table>
Figure 3.1. Schematic illustration depicting the steps leading to the formation of amphiphilic fiber mats. A polypropylene (PP) nonwoven sheet is exposed to the solution of denatured protein and subsequently cross-linked with glutaraldehyde (GA) and NaBH4. After depositing 2-bromopropinoyl bromide (2-BPB), poly(2-hydroxyethyl methacrylate) (PHEMA) brushes were formed by ATRP of 2-hydroxyethyl methacrylate (HEMA). Subsequent post-polymerization modification (PPM) with fluorinated agents resulted in P(HEMA-co-fHEMA) amphiphilic random copolymer grafts. Similar surface modification sequence was applied to ODTS surfaces.
Figure 3.2. Schematic illustration of chemical modification of PHEMA brushes with trifluoroacetic anhydride (F1), heptafluorobutyryl chloride (F3), and pentadecafluoro-octanoyl chloride (F7) leading to the formation of amphiphilic random copolymers of P(HEMA-co-fHEMA), i.e., PHEMA-F1, PHEMA-F3, and PHEMA-F7, respectively.
Figure 3.3. FTIR spectra collected in transmission mode (from bottom to top) on PP nonwoven sheet (black), PP-sheet coated with denatured fibrinogen (FIB) layer (dark magenta), PP-FIB fiber with PHEMA brushes (orange), PP-FIB-PHEMA modified with various fluorinated agents (green), i.e., F1, F3, or F7, as described in the text (see the Appendix B for FTIR data of LYS).
Figure 3.4. Tapping mode atomic force microscope (AFM) topography images taken from ODTS after functionalization with PHEMA and P(HEMA-co-fHEMA) with three different mesogens in dry state. The PHEMA samples were fabricated utilizing “grafting from” polymerization from denatured lysozyme (top row) and fibrinogen (bottom row) coating on ODTS SAMs using methodological steps described in the text.
Figure 3.5. (top) Ellipsometric dry thickness of parent PHEMA brushes (orange) and the corresponding thickness increase after fluorination of PHEMA with various fluorinating agents (green). The experiments were performed on two types of denatured protein-based substrates: lysozyme (LYS) and fibrinogen (FIB). (bottom) Corresponding mole fraction of the fluorinated HEMA segments in P(HEMA-co-fHEMA) brushes as determined from the ellipsometric data using a simple analytical model described in the Appendix B.
Figure 3.6. XPS atomic surface concentration obtained at two different take-off angles for carbon, oxygen, fluorine and silicon, collected from PHEMA brushes and PHEMA brushes fluorinated with F1, F3, or F7 grown from LYS supports.
Figure 3.7. Advancing (squares) and receding (circles) contact angles measured using deionized water (DIW) from P(HEMA-co-fHEMA) brushes prepared by fluorinating PHEMA brushes with F1, F3 and F7, as described in the text. The experiments were performed on two types of denatured protein-based substrates: lysozyme (LYS) and fibrinogen (FIB).
Figure 3.8. Schematic illustration depicting the spatial distribution of F1, F3, and F7 fluorinating species inside P(HEMA-co-fHEMA) brushes.
Figure 3.9. Merged optical microscopy and fluorescence microscopy images collected from fibers exposed to FITC-labeled BSA solutions. The fibers included bare polypropylene (PP), PP after coating with denatured layer of lysozyme (PP-LYS), PP-LYS fibers after “grafting from” polymerization of HEMA (PP-LYS-PHEMA), and after fluorination of PHEMA with F1, F3, and F7 (PP-LYS-PHEMA-Fx, bottom row).
Figure 3.10. Merged optical microscopy and fluorescence microscopy images collected from fibers exposed to FITC-labeled BSA solutions. The fibers included bare polypropylene (PP), PP after coating with denatured layer of fibrinogen (PP-FIB), PP-FIB fibers after “grafting from” polymerization of HEMA (PP-FIB-PHEMA), and after fluorination of PHEMA with F1, F3, and F7 (PP-FIB-PHEMA-Fx, bottom row).
CHAPTER 4 - GENERATION AND PROPERTIES OF ANTIBACTERIAL COATINGS BASED ON ELECTROSTATIC ATTACHMENT OF SILVER NANOPARTICLES TO PROTEIN-COATED POLYPROPYLENE FIBERS

4.1. Abstract

We present a simple method for attaching silver nanoparticles to the surface of polypropylene (PP) fibers in a two-step process to impart antibacterial properties. Specifically, PP fibers are pre-modified by the adsorption of heat-denatured lysozyme (LYS) from solutions, and cross-linking the protein layers using glutaraldehyde and sodium borohydride. The pre-adsorbed protein layer enriches the surface with numerous charged functional groups at neutral pH conditions. Silver nanoparticles capped with trisodium citrate are subsequently deposited onto the protein-coated PP. The electrostatic interactions between the LYS layer and silver nanoparticles bind the nanoparticles to PP surfaces. The amount of silver nanoparticle deposited on PP depends on the amount of protein adsorbed on the surface. Analytical techniques UV-vis spectroscopy, transmission electron microscopy, Fourier transform infrared spectroscopy and scanning electron microscopy are used to follow all preparation steps and to characterize the resulting functional surfaces. The antibacterial activity of the modified surfaces is tested against gram negative bacteria *E. Coli*. Surfaces coated with silver nanoparticles exhibit excellent antibacterial activity with 100 % killing efficiency.
4.2. Introduction

Antibacterial modification of surfaces is a highly desired in applications that require protective barrier against infection. The extensive use of synthetic polymeric materials in the health and biomedical device industry, food industry, textile industry, packaging and personal hygiene industry thus demands incorporation of biocidal compounds. This is because, most polymers are prone to bacterial adhesion followed by cell growth and colonization resulting in severe infections and transmitting diseases.

The growth of harmful microorganisms on surfaces can be inhibited, reduced and delayed by the incorporation of biocidal agents into the bulk polymeric materials. Coatings based on slow release of these biocides, such as heavy metals, antibiotics, small molecule biocides, halogen species and nitric oxide, render surfaces with effective antimicrobial properties. An alternative strategy is to immobilize cationic polymers with quaternary ammonium or biguanide groups onto various substrates. Since the action of quaternary ammonium compounds against microbes is limited to specific targets, the efficiency of these materials decreases with prolonged exposures. Several studies confirmed that silver nanoparticles possess excellent antimicrobial activity against a broad spectrum of microbes. In addition, silver nanoparticles are less toxic to human cells as compared to other metals. Possible targets for silver inside the microbial cells are numerous and hence the development/evolution of its resistance against silver compounds is limited. Silver nanoparticles owing to its small size, i.e., large surface area, bind efficiently to the microorganisms and provide enhanced antimicrobial properties. Silver nanoparticles interact with sulfur and phosphorous compounds of membrane proteins resulting in changing cell morphology and structure causing cell death. In addition, silver nanoparticles bind to DNA in the interior of the cell resulting in disruption of its replication ability as well as inactivation of cellular proteins. Attaching these antibacterial agents to the surfaces of polymeric materials, especially in case of hydrophobic surfaces, is the most challenging task. The increasing use of polypropylene (PP) nonwovens in medical, institutional, and hygienic applications demands for developing methods that would endow PP materials with
antimicrobial properties. PP has become a choice of numerous applications due to its superior bulk properties including chemical inertness, mechanical characteristics, low density, and low cost.\textsuperscript{14,15} Low surface energy and lack of functional groups restrict the development of desirable coatings on PP surfaces without aggressive pre-activation treatments. Flame,\textsuperscript{16} corona discharge,\textsuperscript{3} plasma,\textsuperscript{17} UV light,\textsuperscript{18} and electron beam\textsuperscript{19} treatments are the most common approaches employed to activate such surfaces to introduce desired chemical groups on PP and other polymer surfaces.\textsuperscript{2,15,20} These surface modification processes convert such inexpensive materials into highly valuable products featuring functional coatings.\textsuperscript{15} Abdou \textit{et al.}\textsuperscript{21} deposited chitosan onto PP nonwovens preactivated with plasma irradiation and demonstrated effective antimicrobial activity. Huang \textit{et al.}\textsuperscript{2} grafted quaternary polymer brush, poly(2-dimethylaminoethyl methacrylate) (PDMAEMA) onto PP surfaces via surface-initiated atom transfer radical polymerization (ATRP) providing strong antibacterial activity against \textit{E.coli}. Yao \textit{et al.}\textsuperscript{4} reported on grafting of block copolymer brushes of poly(ethylene glycol) monomethacrylate (PEGMA) and DMAEMA (PEGMA-\textit{b}-PDMAEMA) on to PP hollow fiber membranes via surface-initiated ATRP. The polymer grafted surfaces exhibited permanent antibacterial activity. However, the disadvantages of these methods involve surface damage due to etching.\textsuperscript{14} This approach is particularly challenging in the case of finer fibers, where harsh physical modification can seriously compromise the mechanical properties.

Recently, we have demonstrated a convenient method to functionalize the hydrophobic and PP nonwoven surfaces through the adsorption of denatured proteins as described in \textbf{Chapter 2}. The protein coating provides the surface with hydrophilic functionalities, which can be further utilized to introduce new surface chemistries and properties. In addition, our surface modification process is less severe, aqueous-based, fast, robust, and facile and can be employed to any hydrophobic surface.\textsuperscript{22} In this paper, we use the protein surface modification technique through the adsorption of denatured lysozyme (LYS) proteins on PP nonwoven sheets. The amino acid functional groups on the periphery of adsorbed protein layer serve as anchoring points for the attachment of silver nanoparticles. Specifically, the
amount of protein adsorbed on PP nonwoven surfaces is altered by varying experimental conditions including pH and concentration of proteins in the adsorption solution. Silver nanoparticles are deposited on the pre-adsorbed protein layers endowing PP nonwovens with efficient antibacterial activity. The antibacterial characteristics are evaluated by a biocidal test against *Escherichia coli* (*E.coli*).

### 4.3. Materials and Methods

#### 4.3.1. Materials

Deionized water (DIW) (resistivity >16 MΩ cm) was produced using a Millipore water purification system. PP nonwoven fiber sheets were obtained from the Nonwovens Institute pilot facilities at NC State University and were cleaned with isopropanol prior to use. Lysozyme (from chicken egg white, Mₐ = 14.3 kDa, pI=11.3), glutaraldehyde (GA), silver nitrate (AgNO₃), sodium borohydride (NaBH₄), sodium citrate tribasic dihydrate (TSC) were purchased from Sigma-Aldrich and were used as received. D/E (Dey/Engley) neutralizing broth with tween was purchased from Neogen Corporation.

#### 4.3.2. Preparation of silver nanoparticles

Silver nanoparticles are prepared by chemically reducing AgNO₃ with NaBH₄ in the presence of TSC according to the procedure described previously by Murphy *et al.* and others. In detail, aqueous solutions of AgNO₃ and TSC in equal molarities are mixed together under vigorous stirring for 5 minutes. To this solution, NaBH₄ pre-dissolved in water was added rapidly in one batch at room temperature. The stirring was stopped after 1 hour. Upon the addition of NaBH₄, the transparent solution was converted to characteristic pale yellow and deep yellow color depending upon the concentration of AgNO₃ demonstrating the formation of silver nanoparticles. Jana and coworkers synthesized silver nanoparticles at the silver nitrate concentrations of 0.25 mM producing nanoparticles having diameters of 3-5 nm. In this work, silver nanoparticle colloids were prepared at four different concentrations of AgNO₃ ranging between 0.25, 0.5, 1 and 2 mM, while keeping the
final molar ratios of AgNO₃, TSC, and NaBH₄ constant. Sample a (xₐg = 0.25 mM) was prepared to obtain final concentration of AgNO₃, TSC, and NaBH₄ as 0.25, 0.25 and 0.3 mM respectively. Sample b (xₐg = 0.5 mM) was prepared to obtain final concentration of AgNO₃, TSC, and NaBH₄ as 0.5, 0.5 and 0.6 mM respectively. Sample c (xₐg = 1.0 mM) was prepared to obtain final concentration of AgNO₃, TSC, and NaBH₄ as 1.0, 1.0 and 1.2 mM respectively. Sample d (xₐg = 2.0 mM) was prepared to obtain final concentration of AgNO₃, TSC, and NaBH₄ as 2.0, 2.0 and 2.4 mM respectively. Silver nanoparticles prepared at molar concentration of 2 mM are represented as AgNPs.

4.3.3. Adsorption of denatured protein solutions on PP nonwoven surfaces

Denatured protein coatings were prepared on PP nonwoven sheets as described in our previous work. Briefly, lysozyme (LYS) protein solutions at required concentrations (i.e., 0.01 and 1 mg/mL) were prepared in PBS buffer solution. The solution pH was adjusted to desired pH levels of 7.4 and 10. The proteins solutions were allowed to solubilize for 6 h followed by subsequent heating for 3 min in an pre-heated oven at 85 °C before incubating PP nonwoven substrates for 15 min. The stability of the protein coatings on PP nonwoven sheets was improved by cross-linking with GA and NaBH₄.

4.3.4. Deposition of silver nanoparticles on PP nonwoven surfaces

Control PP and LYS-coated PP fiber surfaces with different amounts of proteins on its surface were incubated in silver colloid solution with high molar concentration of nanoparticles (xₐg = 2.0 mM) at room temperature overnight. This process results in adsorption of silver nanoparticles on functionalized PP surfaces. The resultant fibers were rinsed in DI water for several times followed by sonication in PBS buffer for 2 minutes to remove loosely bound nanoparticles. The samples were then used for characterization with UV-vis and SEM analysis and antibacterial activity.
4.3.5. Antibacterial activities of silver nanoparticle treated PP nonwovens:

Test method (a): The antibacterial properties of control PP, LYS-coated PP (PP-LYS) and silver nanoparticle treated PP-LYS (PP-LYS-AgNP) fiber surfaces pre-activated with protein primers were analyzed against *E. coli* DH5-α using an ASTM standard: E2180-07 Standard test method for determining the activity of incorporated antimicrobial agents in polymeric or hydrophobic materials. Luria broth (LB) agar slurry (1 mL) containing ≈5 × 10⁶ cells/mL was placed on 3.0 × 3.0 cm² test specimen followed by spreading with a cotton swab forming a thin film. The agar slurry on the test specimens were allowed to gel at room temperature followed by incubation at 37 °C for 18 hours. After incubation the samples were subjected to vigorous mechanical vortexing for 3 min in a neutralizing broth to release the agar slurry from the sample. The resultant suspension containing cells was then diluted with LB medium to the desired concentration followed by plating them on LB agar growth plates. The agar plates were incubated at 37 °C for 18 hrs and the number of viable cells was determined as colony forming units (CFU). After incubation, each surviving cell develops into a distinct colony, and counting these colonies provides us information to measure bacterial activity. The antibacterial effectiveness was represented as the bacterial killing percentage of calculated by using the following equation

\[
Bacterial\; killing\; percentage = \frac{D_{control}N_{control} - D_{sample}N_{sample}}{D_{control}N_{control}} \times 100\% ,
\]

where \(N_{control}\) and \(N_{sample}\) are the numbers of bacterial colonies counted on LB agar plates corresponding to control PP and treated PP nonwovens, respectively. \(D_{control}\) and \(D_{sample}\) are the dilution factors of control PP and treated PP nonwovens, respectively.

Test method (b): The antibacterial activity of the PP nonwoven surfaces before and after treatment with protein and silver nanoparticles were also tested against *E. coli* using a slight modification of AATCC test method 147-1998: Antibacterial activity assessment of textile materials: parallel streak method. The test specimen (nonwoven mat) with dimension of 2 × 2 cm² was placed at the center of the standard Petri dish plates. 5 mL of agar slurry containing approximately 1×10⁵ cfu/mL of *E. coli* were dispensed into the Petri dish with
nonwoven mat and the slurry was spread forming a uniform thin film. The test specimen was in an intimate contact with the bacterial agar slurry. The agar slurry was allowed to solidify at room temperature. The plates were then incubated at 37 °C for 18 hrs. The resultant plates were then examined visually for growth of bacteria around the area of nonwoven mat. The formation of a clear zone surrounding the fabric (i.e., inhibition zone) was measured and the results were reported as the average width of the zone of inhibition (in mm) extending beyond the edge of nonwoven mat.

4.3.6. Ultraviolet-visible (UV-vis) analysis

Jasco V-550 UV-vis spectrophotometer was used to analyze the synthesized nanoparticles. The analysis was conducted by collecting the spectrum over a wavelength range of 300-750 nm with a resolution of 0.5 nm. Silver nanoparticle treated PP nonwoven sheets were characterized by carrying UV-vis analysis between the wavelength range of 320-750 nm. Prior to the measurements, nanoparticle solutions were diluted 40-fold with deionized water.

4.3.7. Transmission electron microscopy (TEM)

Silver nanoparticles prepared at different concentrations of silver nitrate were characterized using JEOL 2010F-FasTEM at an operating voltage of 200 kV. The samples were prepared by placing a drop of the nanoparticle colloid solution on a carbon-coated copper grid. The drop was allowed to dry overnight in air. Shape and size distribution of the nanoparticles were characterized by TEM. The histograms prepared by measuring the sizes of nanoparticles from TEM images reveal information about the size distribution of nanoparticles. Statistical analysis was performed by one-way ANOVA to determine the differences in nanoparticle sizes prepared at different concentrations of silver nitrate. P values less than 0.05 were considered to be statistically significant.
4.3.8. Zeta potential analysis

The zeta potential of silver nanoparticle suspensions was measured using Zetasizer Nano-ZS instrument. Nanoparticle suspensions are considered to be stable, if the zeta potentials are greater than + 30 mV or less than -30 mV.

4.3.9. Scanning electron microscopy (SEM)

The presence of nanoparticles and coverage of the nanoparticles after silver nanoparticle deposition on PP nonwoven surfaces was examined under Hitachi S-4700 operating at 20 kV in high vacuum mode after sputter-coating with a thin layer of gold-palladium metal.

4.4. Results and discussion

4.4.1. Preparation of silver nanoparticles at different molar concentrations

Silver nanoparticles were synthesized by chemical reduction of AgNO₃ with NaBH₄ in the presence of TSC as capping agent. The presence of TSC is to stabilize the nanoparticles by imparting anionic character to the nanoparticles providing electrostatic repulsion and prevents aggregation of the nanoparticle.²³-²⁵ UV-visible spectra of silver nanoparticles prepared at different concentrations as detailed in material and methods section are shown in Figure 4.1. We hypothesize that as the concentration of AgNO₃ in solution increases, the concentration of silver nanoparticles per unit volume increases accordingly. Figure 4.2 documents that the silver colloids are yellow in color at lower concentrations and show a variation in visible color to deep yellow color with the increasing molar concentrations of the pre-cursor. Particle size and size distribution of AgNPs in the colloids affect the position, shape and symmetry of the surface plasmon absorption band of silver particles. The absorption peak observed at ≈390 nm in Figure 4.1, corresponds to the typical surface plasmon resonance absorption of spherical silver nanoparticles with sizes ranging between 3-20 nm.²⁵,²⁶,²⁷ An increase in the UV absorption peak intensity was observed with increasing concentration of AgNO₃, which might be attributed to the occurrence of two phenomena including a significant change in the size of nanoparticle due to aggregation or increasing the
number density of nanoparticles in solution. However, no obvious shift in the absorption maximum towards the larger wavelengths is evident from the UV-visible spectra with increasing molar concentrations of silver pre-cursor suggesting that the increase in intensity might be the result of the latter phenomenon. Dong et. al. prepared silver nanoparticles from AgNO$_3$ using different concentrations of TSC and NaBH$_4$. The authors demonstrated that the decrease in the intensity of surface plasmon resonance is attributed to the decreased amount of AgNPs in solution. TEM images of silver colloids prepared at different molar concentrations are shown in Figure 4.3. The TEM images clearly reveal that nanoparticles are of nearly spherical in shape. AgNPs prepared at different concentrations of AgNO$_3$ exhibit similar shapes and sizes. The average size and size distribution of the AgNPs were determined by analyzing at least 150 nanoparticles except for a AgNO$_3$ concentration of 0.25 mM; size distribution histograms are shown in Figure 4.4. The number density of AgNPs observed on the TEM grid with $x_{Ag} = 0.25$ mM is smaller than other concentrations. The average particle size for silver nanoparticles is $\approx 5\pm 4$ nm; there is no noticeable change in mean size with increasing molar concentrations of the reagents. The statistical analysis using on-way analysis of variance reveals that there are no significant differences between the average sizes of nanoparticles prepared at all concentrations with confidence level of 95%. Owing to its strong reducing power, NaBH$_4$ reacts fast with silver nitrate and forms small nanoparticles. The total concentration of silver ions reduced to silver atoms was determined using silver ion-selective electrode (see Appendix C). Using the percent conversion of silver ions to silver atoms, the molar concentration of AgNPs was calculated by assuming that the nanoparticles have a spherical shape with a mean diameter of 5 nm (see Appendix C). The corresponding results are summarized in Table 4.1. From the results, it is evident that the molar concentrations of nanoparticles obtained with $x_{Ag} = 2$ mM is twelve times greater than that of $x_{Ag} = 0.25$ mM. This supports our presumption that increasing the concentration of AgNO$_3$ increases preferentially the number density of nanoparticles in the solution. Turkevich prepared gold nanoparticle solutions at different concentrations and demonstrated that when the initial concentration of solute is high, the formation of a large number of nuclei at the nucleation step is favored resulting in generating more nanoparticles.
with smaller sizes and narrow-down the size distribution. Similar results are obtained in our work, where the concentration of nanoparticles in the solution increases with increasing initial concentrations of the precursor. In addition, though the mean size of nanoparticle is ≈5 nm at all the concentrations, a large number of nanoparticles with sizes smaller than 5 nm is present for the case of $x_{Ag} = 1.0$ mM and $x_{Ag} = 2.0$ mM as compared to $x_{Ag} = 0.5$ mM (cf. Figure 4.4). The surface zeta potential for the nanoparticles synthesized with $x_{Ag} = 0.25$ mM was negative and $\approx -29.3 \pm 5$ mV. Cumberland et al.$^{31}$ reported similar zeta potential values for the nanoparticles synthesized from AgNO$_3$. This confirms that the TSC and excess NaNH$_4$ present in the colloidal solutions are adsorbed on the surface of the AgNPs ensuring a stable and well dispersed suspension resulting in high stability of nanoparticles. No significant differences in the zeta potentials of were observed for nanoparticles obtained with increasing molar concentrations of precursor indicating that all the suspensions are stable. AgNPs obtained with high molar concentrations (i.e., $x_{Ag} = 2$ mM) were used in this work to decorate on PP nonwoven substrates as discussed in the next sections.

4.4.2. Deposition of silver nanoparticles on protein-coated PP nonwoven surfaces

In our previous work, we reported on the formation of stable denatured protein layers on hydrophobic surfaces. Figure 4.5 shows a schematic of method leading to adsorption of heat-denatured proteins on PP nonwoven surfaces. Specifically, heat-denatured LYS was adsorbed on flat surfaces coated with hydrophobic $n$-octadecyltrichlorosilane (ODTS) and PP nonwoven surfaces through non-specific interactions. The amount of protein adsorbed (protein coverage) on hydrophobic ODTS surfaces was varied by changing conditions implemented during protein adsorption including concentration of protein in the solution, pH of the solution and adsorption time. The following conditions provided LYS-modified ODTS substrates with variation in fractional protein coverages of 0.44 (PP-lys(0.44)), 0.75 (PP-lys(0.75)) and 1.1 (PP-lys(1.1)) are a) 0.01 mg/mL LYS adsorbed at pH 7.4 and 85°C for 15 min, b) 0.01 mg/mL LYS adsorbed at pH 10 and 85°C for 15 min and c) 1 mg/mL LYS adsorbed at pH 10 and 85°C for 15 min.$^{22}$ Substrates with different amounts of
adsorbed protein were used for the deposition of silver nanoparticles. The presence of protein on PP nonwoven surfaces was confirmed by IR spectroscopy shown in Figure 4.6. The characteristic amide I (C=O stretching) and amide II (C-N stretching and N-H bending) bands observed at wave numbers of 1700-1550 cm\(^{-1}\) and 3420-3250 cm\(^{-1}\) respectively, verify the protein immobilization on PP surface.\(^{32,33}\) The isoelectric point of LYS is \(\approx 11.3.\)\(^{22}\) Thus, depending upon the pH of the solution, LYS carries net positive or negative charges, below or above its isoelectric point, respectively. Hence, LYS-adsorbed PP nonwovens exhibit cationic character at neutral pH. By taking advantage of these positive charges, negatively-charged AgNPs were deposited through electrostatic interactions. A schematic of silver nanoparticle deposition on protein-coated PP nonwoven surfaces is shown in Figure 4.7. Particularly, dipping PP nonwovens with varying fractional protein coverages in silver nanoparticle sols at pH 7.4 resulted in the deposition of silver nanoparticles. The nanoparticle adsorption was evident by a prominent color appearing over the fiber surfaces as shown in Figure 4.8. Protein precoated surfaces provided numerous accessible sites on the periphery of the protein layer that facilitated easy fixation of silver nanoparticles through electrostatic interactions. To some extent, the inherent affinity of cysteine groups of protein also promoted the uptake of silver nanoparticles. As shown in Figure 4.9, the appearance of a characteristic surface plasmon resonance peak at \(\approx 404\) nm after the deposition of nanoparticles on PP nonwoven substrates clearly indicates the presence of AgNPs. A shift in the surface plasmon peak towards larger wavelengths (404 nm) was noticed as compared to surface plasmon peak (390 nm) of silver nanoparticles in solutions. This red shift might be an outcome of AgNPs that are either in close proximity to each other on the surface of PP nonwoven after deposition or due to the agglomeration of nanoparticles that might have occurred during the deposition step.\(^{34}\) As demonstrated in Figure 4.8, when the amount of protein adsorbed on PP substrate is increased, the intensity of yellow color increased indicating deposition of increased number density of AgNPs. This finding is supported by UV-visible spectra of nonwoven surfaces as shown in Figure 4.9, which clearly show an increase in intensity of the absorption peak at 404 nm with increasing protein coverage. The increased amounts of protein lead to the generation of a large fraction of amino-acid
functional and charged groups on the surface that anchor increased amounts of AgNPs through electrostatic interactions.

SEM images of unmodified PP and LYS-modified PP nonwoven surfaces after the deposition of silver nanoparticles is displayed in Figure 4.10. Attachment of AgNPs was not observed on the surface of unmodified PP nonwoven; in contrast, AgNPs are clearly visible on the surface of LYS-modified PP surfaces. This results support the conclusion that the electrostatic interactions between the positive charges present at the top surface of protein layer and the negatively charged AgNPs promote particle deposition. In addition, SEM images also demonstrated that the attached silver nanoparticles on fiber surfaces are sufficiently dispersed and not aggregated.

The ability of the silver nanoparticle coatings on PP nonwoven mats to provide effective antibacterial characteristics was tested by using method (a) as detailed in the Materials and methods section. Figure 4.11 shows bacterial colony forming units (CFU) grown on culture plates for control PP, PP-LYS, and PP-LYS-AgNP as a function of concentration of proteins on its surface. The results obtained from antibacterial tests of the nonwoven surfaces are summarized in Table 4.2. The control plate was covered with a higher number density of bacterial colonies, whereas 46% bacterial reduction is observed for nonwovens treated with LYS protein. The work by Ibrahim and co-workers\textsuperscript{35} reported that heat denatured LYS exhibited enhanced antibacterial activity though its enzymatic activity is lost as compared to its native structure. The action of LYS against bacteria is due to its interaction with the bacterial membrane and its subsequent disruption of the cell membrane. The protein-coated surfaces treated with AgNPs exhibited enhanced biocidal function. The mode of action of AgNPs on microorganisms is not well known. Kittler \textit{et al}.\textsuperscript{36} reported that released silver ions from the citrate capped silver nanoparticles inhibit the bacterial growth. Specifically, an approximate 2 log reduction (99% reduction) in bacteria was observed for PP-LYS(0.44)-AgNP nonwoven in comparison to the control PP fabric. On the other hand, approximately 7 log reduction (100% reduction) in bacterium was observed in PP-LYS(0.75)-AgNP and PP-LYS(1.1)-AgNP nonwoven surfaces. The results clearly demonstrate that the biocidal
activity increased due to the increased concentration of nanoparticles on the surface with increased protein coverage.

Antibacterial testing of PP nonwoven specimens was carried also out using method (b) as described in the Materials and methods section. **Figure 4.12** shows the LB agar plates with PP nonwovens before and after modification with silver nanoparticles to which *E. coli* was applied. No clear zone of inhibition was observed around the control PP and LYS-PP nonwoven mats. The presence of nanoparticles on the nonwoven surfaces inhibited the bacterial growth and exhibited a clear zone of inhibition. The sizes of the zone of the inhibition increased with an increase in the amount of silver nanoparticles on the nonwoven surfaces. The corresponding optical images at the bottom each Petri dish clearly showed that no bacterial colonies were observed at the edge of surface. The number of cells inhibited in the zone of inhibition was calculated for AgNPs-treated surfaces including PP-LYS(0.44)-AgNP, PP-LYS(0.75)-AgNP and PP-LYS(1.1)-AgNP assuming a homogeneous distribution of cell throughout agar slurry (the numbers in the parentheses represent corresponding fractional coverages of protein). The results are summarized in **Figure 4.13** assuming 100 percent bacterial efficiency for PP-LYS(1.1)-AgNP. The results indicate that the efficacy of the coating in imparting bacterial growth increases with increasing the amount of protein on the surface increases, which ultimately increase the density of the AgNPs. Sambhy *et al.*\(^1\) demonstrated that the diffusion of the silver ions from nanoparticles into the surrounding aqueous medium inhibited the bacterial growth. Parameswari *et al.*\(^37\) reported an increase in the size of zone of inhibition with increased silver nanoparticle concentrations. Therefore, increasing the nanoparticle concentration on fiber surface results in higher degree of active silver ions that have leached into the surrounding aqueous medium thus providing higher zones of bacterial inhibition. Hence, silver nanoparticles deposited on nonwoven surfaces served as efficient antibacterial coatings.
4.5. Conclusions

A simple and novel method was developed to form functional coatings on PP that imparts antibacterial characteristics via the deposition of silver nanoparticles. Silver nanoparticles were prepared at different molar concentrations followed by their characterization with UV-vis spectroscopy and TEM. Using positively-charged functional groups of protein primers on deposited on PP surfaces at neutral pHs, negatively charged citrate capped silver nanoparticles were attached through electrostatic interactions. The concentration of AgNPs increased with increasing the amount of protein primer on the PP surface. The deposition of AgNPs on the surface was confirmed by UV-vis spectroscopy and SEM. Denatured LYS-coated surfaces exhibited killed *E.coli* to some extent due to its inherent antibacterial characteristics. The antibacterial activity of the surfaces was improved to significant extent with the deposition of silver nanoparticles on PP materials.
4.6. References


(13) Sondi, I.; Salopek-Sondi, B. *J. Colloid Interface Sci.* **2004**, 275, 177-182.


(22) Goli, K. K.; Rojas, O. J.; Ozçam, A. E.; Genzer, J. Biomacromolecules 2012.
Table 4.1. Molar concentrations of silver nanoparticles with different initial concentrations of silver nitrate.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Conversion of silver ions to silver atoms in nanoparticle suspensions</th>
<th>Molar concentration of nanoparticles (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a ($x_{Ag} = 0.25$ mM)</td>
<td>62.3</td>
<td>$4.06 \times 10^{-8}$</td>
</tr>
<tr>
<td>b ($x_{Ag} = 0.5$ mM)</td>
<td>89.1</td>
<td>$1.16 \times 10^{-7}$</td>
</tr>
<tr>
<td>c ($x_{Ag} = 1.0$ mM)</td>
<td>97.1</td>
<td>$2.53 \times 10^{-7}$</td>
</tr>
<tr>
<td>d ($x_{Ag} = 2.0$ mM)</td>
<td>98.4</td>
<td>$5.22 \times 10^{-7}$</td>
</tr>
</tbody>
</table>
Table 4.2. Antibacterial activity of the PP nonwoven surfaces before and after modification with proteins and silver nanoparticle coating.

<table>
<thead>
<tr>
<th>Substrate a)</th>
<th>% Killing efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control PP</td>
<td>-</td>
</tr>
<tr>
<td>PP-LYS</td>
<td>46.5</td>
</tr>
<tr>
<td>PP-LYS(0.44)- AgNPs</td>
<td>99</td>
</tr>
<tr>
<td>PP-LYS(0.75)-AgNPs</td>
<td>100</td>
</tr>
<tr>
<td>PP-LYS(1.1)-AgNPs</td>
<td>100</td>
</tr>
</tbody>
</table>

a) PP-LYS(xx) represents the fractional coverage of *.* of lysozyme on PP.
Figure 4.1. UV-visible spectra of silver nanoparticles prepared in aqueous solutions at four different molar concentrations of AgNO₃; (a) $x_{Ag} = 0.25$ mM, (b) $x_{Ag} = 0.5$ mM, (c) $x_{Ag} = 1.0$ mM and (d) $x_{Ag} = 2.0$ mM.
Figure 4.2. Photographs of solutions containing AgNPs synthesized with different molar concentrations of AgNO₃ represented by (a) $x_{\text{Ag}} = 0.25$ mM, (b) $x_{\text{Ag}} = 0.5$ mM, (c) $x_{\text{Ag}} = 1.0$ mM and (d) $x_{\text{Ag}} = 2.0$ mM. A variation in visible color can be seen with increasing molar concentrations of (a)-(d).
Figure 4.3. TEM images of silver nanoparticles prepared from molar concentrations of AgNO$_3$; (a) $x_{\text{Ag}} = 0.25$ mM, (b) $x_{\text{Ag}} = 0.5$ mM, (c) $x_{\text{Ag}} = 1.0$ mM and (d) $x_{\text{Ag}} = 2.0$ mM.
Figure 4.4. Size distribution histograms of AgNPs prepared from molar concentrations of AgNO₃: (a) $x_{\text{Ag}} = 0.25$ mM, (b) $x_{\text{Ag}} = 0.5$ mM, (c) $x_{\text{Ag}} = 1.0$ mM and (d) $x_{\text{Ag}} = 2.0$ mM.
Figure 4.5. Schematic illustration of the adsorption of denatured protein layers on PP nonwoven surfaces. Pictures were not drawn on proportional scales (in reality, size of lysozyme is much smaller than diameter of the nonwoven fiber).
**Figure 4.6.** IR spectra of untreated PP and PP modified with LYS.
Figure 4.7. Schematic illustration representing the deposition of silver nanoparticles on protein-coated PP nonwoven surfaces. Pictures were not drawn on proportional scales (in reality, size of lysozyme and silver nanoparticles are much smaller than diameter of the nonwoven fiber).
Figure 4.8. Protein modified PP nonwovens coated with silver nanoparticles. The sample dimensions are $\approx 3 \times 3 \text{ cm}^2$. 
Figure 4.9. UV-visible absorbance spectra of PP-LYS (1.1) nonwoven (black), silver nanoparticle coated PP-LYS with varying amounts of proteins; PP-LYS (black), PP-LYS(0.44)-AgNPs (blue), PP-LYS(0.75)-AgNPs (magenta) and PP-LYS(1.1)-AgNPs (olive green).
Figure 4.10. SEM images of the control PP and protein-coated PP nonwovens after treatment with silver nanoparticles.
Figure 4.11. LB plates corresponding to the *E.coli* suspension recovered from control and treated PP nonwovens. The colonies on the plates were counted and the percent killing efficiency is reported in Table 4.2.
Figure 4.12. The antibacterial activity of control PP and silver nanoparticle treated PP nonwoven mats. Zone of inhibition (ZOI) can be observed around the silver nanoparticle treated mats and are shown at the top right corner of the plates. The sample dimensions are ≈2 x 2 cm². The images at the bottom of petri dish show the corresponding enlarged optical microscopy images taken at the edge of nonwoven mat.
Figure 4.13. Antibacterial efficiency of silver nanoparticle deposited PP surfaces calculated from test method (b) with increasing amount of protein on the surface.
5.1. Summary

The main objective of this Ph.D. thesis is to devise novel and alternative technologies for the modification of hydrophobic surfaces. In particular, surface properties of hydrophobic $n$-octadecyltrichlorosilane (ODTS) and PP were altered to impart functional properties through the adsorption of proteins. The functional groups of the protein molecules were utilized to create new functional polymer coatings that possess special properties, including anti-fouling characteristics by surface initiated polymerization of monomers, followed by their subsequent modification. In addition, anti-bacterial performance was incorporated by attaching silver nanoparticles to protein-coated hydrophobic PP substrates.

Hydrophobic ODTS and polymeric PP were modified by physisorption of protein molecules through non-specific interactions. Protein molecules adsorbed strongly onto ODTS and PP surfaces. Specifically, the adsorption of urea and heat-denatured protein molecules including $\alpha$-lactalbumin (LALBA), lysozyme (LYS), fibrinogen (FIB), and two soy globulin proteins (glycinin and $\beta$-conglycinin) endowed hydrophobic surfaces with enhanced wettability due to surface enrichment with amino-acid functionalities. The wettabilities of modified surfaces were dependent on the coverage of proteins on the surface as well as their chemical composition; soy proteins exhibited slightly better wettabilities than LALBA, LYS and FIB. Characterization of the modified surface with circular dichroism, ellipsometry, contact angle and Fourier transfer infrared (FTIR) spectroscopy in the attenuated total reflection mode confirmed the attachment of proteins on the hydrophobic surfaces. The stability of protein coating in broad range of selected solvents was improved by cross-linking the adsorbed protein molecules by forming covalent bonds between the amine and hydroxyl groups using glutaraldehyde in the presence of sodium borohydride.
Hydrophobic substrates immobilized with cross-linked protein layers were used as primers for surface-initiated polymerization using several functional monomers including 2-hydroxyethyl methacrylate and \( N \)-isopropylacrylamide. In particular, these protein coatings provided sufficient number of amine- and hydroxyl- groups on the surface, which allowed the attachment of atom transfer radical polymerization (ATRP) initiator via amide and ester linkages. Anchoring of the polymerization initiator, 2-bromopropionyl-bromide (2-BPB) was confirmed with X-ray photoelectron spectroscopy (XPS). In addition, single or multilayered polyelectrolytes were formed on the adsorbed proteins through electrostatic interactions to generate uniform and homogeneous distribution of functional groups throughout the substrate. Characterization with ellipsometry, contact angle and FTIR confirmed the deposition of polyelectrolytes and growth of polymer brushes. Incorporated polymer brushes enhanced the wettability of ODTS surface and a considerable increase in wicking was noticed on PP nonwoven sheets due to the enrichment with polar hydroxyl groups on the surface. The grafted poly(2-hydroxyethyl methacrylate) (PHEMA) brushes were further functionalized by post-polymerization modification with selected fluorinated species. Successful modification of PHEMA brushes with fluorinated agents (Fx) resulting in amphiphilic polymer brushes was confirmed by ellipsometry, angle-resolved XPS (AR-XPS), and wettability measurements. Modified substrates exhibited altered surface properties based upon the type of fluorinated agent grafted to PHEMA chains due to varying degrees of self-organization of the fluorinated agents at the surface. Antifouling characteristics of PHEMA-Fx modified PP nonwoven sheets were tested by exposing them to fluorescein isothiocyanate-labeled bovine serum albumin as a model fouling protein. The results showed that the presence of amphiphilic polymer grafts on PP surfaces hindered the protein adsorption to a significant extent.

Another application of these protein-coated surfaces is directed towards the introduction of antibacterial properties. Specifically, PP nonwovens were pre-coated with LYS proteins, followed by their subsequent cross-linking with glutaraldehyde. Adsorbed protein layers provided large number of accessible sites on the periphery including metal binding residues.
and positively charged groups by modulating the solution pH. These groups facilitated “easy fix” of silver nanoparticles. The presence of nanoparticles on protein-coated PP surfaces was characterized by scanning electron microscopy (SEM) and UV-VIS spectroscopy. The antibacterial properties of PP nonwovens decorated with silver nanoparticles were tested against gram-negative bacteria, *E. coli*. It was demonstrated that the resultant silver nanoparticle attached PP fibers exhibited antibacterial activity by inhibiting the growth of *E. coli*.

5.2. Outlook

The following section gives detailed description of future experiments, potential approaches, suggestions and insights towards the advancement of this simple and versatile technology for the modification of surfaces.

5.2.1. Effect of adsorption time on protein coverage

As mentioned in Chapter 2, urea-denatured proteins were adsorbed on hydrophobic surfaces using a dip-coating technique for 12 h. In order to employ the deposition process routinely it is important to optimize and minimize adsorption times required for the formation of the best possible protein coverage layer. Hence, it is critical to explore the kinetics of protein adsorption by monitoring the protein layer thickness at varying adsorption times on hydrophobic surfaces including ODTS and PP spin-coated substrates. For kinetic studies, selected protein concentrations must be as low as possible so that rapid saturation of the substrate by proteins from the solution can be prevented or reduced, which might be encountered at higher protein concentrations.

In our preliminary experiments, we adsorbed urea-denatured LALBA and FIB at concentrations of 0.1 mg/mL and at the respective isoelectric point during different incubation times (0.5, 1, 3, 6, 12 and 24 h) onto flat PET substrates (PET films spun-coated on top of flat silicon wafers). The coverage of proteins was monitored using ellipsometry and the data was summarized in Figure 5.1. As observed in our adsorption studies in
Chapter 2. surface coverage of LALBA was greater than that of FIB at all the adsorption times. The effect of adsorption times is insignificant on protein adsorption for these specific conditions. As mentioned earlier, in-depth understanding of protein adsorption kinetics can be obtained by performing adsorption studies at varying protein concentrations and wider range of adsorption times.

Our studies described in Chapter 2 confirmed that the adsorption of heat-denatured proteins is more effective than urea-denatured proteins. We demonstrated that LYS coverage depends on the pH of adsorbing solution, protein concentration in solution, and protein adsorption times at 85 °C. It is important to optimize all the parameters to obtain a protein layer with a monolayer coverage with minimum input of energy and the amount of material used. For example, protein adsorption studies can be carried out at a pH closer to the isoelectric point while decreasing the adsorption times to less than 15 min at 85°C.

To complete this research, it is necessary to perform protein adsorption studies as a function of surface hydrophobicity. The use of gradient surfaces can reveal the effects of surface coverage of proteins with gradual variation of hydrophobicity/surface chemistry and adsorption time. A linear gradient in hydrophobicity can be generated on flat silica surfaces with $n$-octyltrichlorosilane (OTS) molecules by using the vapor diffusion methodology developed by Chaudhury and Whitesides.\textsuperscript{1} Another gradient in varying adsorption times of protein can be generated perpendicular to the linear gradient of hydrophobicity resulting in orthogonal gradients as shown in Figure 5.2.\textsuperscript{2} Hence, gradient surfaces may permit the exploration of broad range of parameters with improved efficiency and lower cost in a single substrate.\textsuperscript{3}

5.2.2. Protein denaturation using extreme solution conditions and different chemical denaturants

Urea-based denaturation of proteins demands using a large amount of urea and causes changes in properties of aqueous solutions including viscosity and absorption properties in
the far-UV region to some extent.\textsuperscript{4} In general, extreme pH conditions in solution result in partial denaturation of proteins. At pH far from the isoelectric point of the respective proteins, proteins will acquire either positive or negative charges. In ranges of large charge density, the like charges will induce unfavorable repulsive electrostatic interactions resulting in unfolding of protein molecules.\textsuperscript{5} We presume that performing adsorption of heat-denatured proteins at extreme pH might assist in reducing the denaturation temperatures of proteins. In addition, one must also consider using different types of denaturants such as sodium dodecyl sulfate (SDS) (10 mM) and 2-mercapto-ethanol (10 mM) which can be added at lesser quantities.\textsuperscript{6,7} In contrast to urea and guanidinium chloride, 2-mercapto-ethanol denatures protein molecules irreversibly by reducing disulfide linkages.\textsuperscript{6} Hence, exploring the effects of different denaturants may help in obtaining effective protein unfolding.

5.2.3. Spray-assisted protein adsorption

Modification of hydrophobic surfaces through protein adsorption using a dip-coating technology is likely to be a batch process, which may be time-consuming for many applications. Applications that require a variety of functions or different functionalities at specific points or sections of the surface of a film necessitate the deposition of multilayer coatings of proteins using the layer-by-layer (LbL) assembly. In addition to the deposition of multiple protein layers, the combination of protein and nanoparticle or enzyme sequential layers is a pre-requisite for specific biological activities. Numerous deposition steps involved under such conditions increase the complexity of the entire system and limit the feasibility of using dip-coating methods. However, spray-coating technologies reported in the literature for polycations may increase the rate and convenience of deposition.\textsuperscript{8,9} Additionally, spray coating can potentially offer the technology to treat the material in a continuous fashion rapidly.\textsuperscript{8,9} One of the main problems in this technology, as compared to dip-coating, is the reduced control over deposition resulting in inconsistent and non-uniform coatings.\textsuperscript{8,9} One must thus optimize the spray-assisted LbL process to obtain conformal coatings by adjusting the spray flow rates and spray direction. We propose that one should
concentrate on developing protein-based hydrophilic coatings based on this spray-technology, which would represent a major advancement in the field of surface modification.

5.2.4. Protein adsorption from organic solvents and co-solvents

Throughout this Ph.D. Thesis protein adsorption studies were carried out in aqueous solutions. One can reduce the energy costs associated with aqueous based wet-processing technology by using high vapor pressure organic solvents. Solubility of LYS in various solvents and co-solvents were reported in the literature.\textsuperscript{10} For instance, the solubility of LYS in a pure organic solvent such as methanol (boiling point of 64.7 °C) was reported to be > 10 mg/mL.\textsuperscript{10} In general, alcohols destabilize the tertiary structure of proteins while increasing their $\alpha$-helical content (secondary structure).\textsuperscript{11} Lai et al.\textsuperscript{12} reported that the denaturation temperature of LYS in an aqueous solution at the concentration of 3.75 mg/mL is 74.3 °C. The same authors demonstrated that the addition of co-solvent, \textit{i.e.}, 15 % trifluoroethanol (TFE, boiling point 73.5 °C) to aqueous solution reduced the denaturation temperature to 45.7 °C. Hence, carrying out protein adsorption in either organic solvents or co-solvents might assist in reducing the denaturation temperature of proteins and thereby lowering the operating costs. However, one must characterize the changes associated with heat-induced denaturation of proteins in solvents and co-solvents in a detailed manner using circular dichroism (CD) spectroscopy prior to protein adsorption.

5.2.5. Grafting of polymers using free-radical polymerization

Hydrophobic ODTS and PP surfaces were grafted with PHEMA brushes using living radical polymerization, as described in Chapters 2 and 3 of this Ph.D. Thesis. A cross-linked protein coating provides a stable and robust layer on hydrophobic surfaces that is rich in amino- and hydroxyl- functionalities, which can be utilized to conjugate a free radical initiator molecules such as 4,4’-azobis(4-cyanopentanoic acid) (ABCA).\textsuperscript{13} The initiator immobilized on the surface can be utilized to graft numerous polymers by free radical polymerization including poly(methyl methacrylate), polystyrene and many others.
5.2.6. Formation of amphiphilic polymers on poly(ethylene terephthalate) (PET) surfaces

In Chapter 3 of this thesis, we presented a simple method to endow PP fibers with amphiphilic coatings using protein layer as a primer. Alternatively, we propose to impart flexible transparent PET films with amphiphilic coatings by chemically attaching 3-aminopropyltrimethoxysilane (APTES) as a primer. PET is among the most popular polymers in the plastics industry and is known for its superior mechanical strength and solvent resistance. The major limitation of this polymer is its hydrophobicity and limited number of chemically-active species. Different methods have been reported to pre-activate the surfaces, including, saponification, plasma treatment and aminolysis. However, these treatments change the surface topography by increasing surface roughness leading to PET degradation. Numerous studies reported that 3-aminopropyltrimethoxysilane (APTES) modification endows PET surfaces with silanol groups without affecting the bulk PET properties. Specifically, APTES reacts with PET by amidation reaction resulting in ethoxysilane groups, which upon subsequent hydrolysis yields silanol groups that permit surface attachment of initiator molecules. “Grafting from” atom transfer radical polymerization from such modified PET surfaces were employed to form PHEMA brushes. In addition, these PHEMA brushes can be post-modified with selected fluorinated species of different chain lengths to generate amphiphilic brushes to endow surfaces with anti-fouling activity. One can also create flexible and transparent conductive films from PET by incorporating or attaching gold nanoparticles to the synthesized polymer brushes including poly(N-isopropylacrylamide) (PNIPAAm). Bhat and Genzer reported that nanoparticles tend to attach to PNIPAAm through the hydrogen bonding interactions between citrate groups present on the surface and amide groups of PNIPAAm.

We propose to generate surface-grafted polymer assemblies with gradients in grafting density on APTES modified PET by creating density gradients of silanol groups first which serve as anchoring sites for attaching initiator molecules. This can be accomplished by placing the n-octyltrichlorosilane (OTS) open reservoir at an edge of APTES modified PET films. As OTS molecules evaporate, they diffuse in the vapor phase and react with silanol
groups of APTES-modified substrates generating a concentration gradient of hydroxyl groups along the substrate as evident in generating surface initiator (1-trichlorosilyl-2-(m/p-chloromethylphenyl) ethane) gradients on silicon wafers and other gradients as described in previous papers.\textsuperscript{3,17,18} A molecular gradient in polymerization initiator on the above substrate can be formed by reacting available hydroxyl groups of APTES molecules with (11-(2-bromo-2-methyl)propionyloxy)-undecyl trichlorosilane (BMPUS). The resultant substrates can be subjected to surface-initiated atom transfer polymerization to generate various polymer brushes of interest, including PHEMA brushes.

5.2.7. Molecular weight determination of surface grafted polymer brushes

Determining the molecular weight of the surface grafted polymer is the ideal way to characterize a brush. To date, there are no reports on the determination of the molecular weight of the surface grafted polymer brushes. It is important to cleave off the bond that connects grafted polymer to the surface in order to understand the polymerization mechanism in a detailed manner such as grafting density and molecular weight determination.\textsuperscript{19,20} This can be facilitated by carrying out the degrafting reaction in a good solvent which would allow easy accessibility of the groups to be cleaved.\textsuperscript{19} Most degrafting studies were carried out using nonporous silica gel particles rather than silicon wafers to recover higher amounts of polymer after cleavage due to higher surface areas of particles as compared to flat silicon surfaces.\textsuperscript{21} Hence, it is important to use planar silicon wafers of large dimensions to graft polymer brushes so that sufficient amounts of polymer can be degrafted for subsequent analysis. The degrafted polymers can be characterized by using GPC. Hydrofluoric acid was used to remove the polymer attached to a silica gel through dissolution process.\textsuperscript{19} However, this treatment did not yield successful results due to the possible shielding of the silica core from the harsh solvent by the hydrophobic polymer layer.\textsuperscript{19} Prucker et. al.\textsuperscript{19} used acid-catalyzed transesterification with methanol to cleave the polystyrene brushes from silica gel. They demonstrated that the ester group that connects the polystyrene polymer to the surface was cleaved completely by the transesterification. In some instances, the substrate might be inherently made up of ester linkages, for example in case of PET, employing such harsh
treatments might lead to the degradation and depolymerization of the substrate-grafted polymer. In addition, if the polymer graft contains ester linkages, acids would attack these bonds leading to graft polymer damage. Hence, there is a need to develop a system where specifically the bond that connects to the substrate surface would be broken.

In Chapters 2 and 3 of this dissertation, we have prepared polymer brushes by surface-initiated polymerization pre-functionalized with protein adsorption. Proteins can be digested by using different types of enzymes, which are specific for different types of amino-acid residues. Abubakar et al. reported on digestion of whey protein using seven kinds of protease enzymes, specifically, pepsin, trypsin, chymotrypsin, proteinase K, actinase E, thermolysin and papain. The optimal conditions for digestion using seven proteases are reported in Table 5.1. The enzymes can be inactivated during the digestion process by heating them at 98 °C for 10 min. Canfield et al. demonstrated the digestion of egg-white LYS with trypsin. Specifically, trypsin cleaves the peptide bond next to arginine and lysine residues through hydrolysis reaction. Trypsin can be a promising agent for digesting LYS since it is composed of high contents of arginine and lysine. Wu et al. reported that proteinase K is an extremely robust enzyme that chops out proteins into small peptides by attacking the peptide bonds after the carboxyl group of N-substituted hydrophobic aliphatic and aromatic amino acids. However, one can reduce the digestive activity of proteinase K by increasing the pH of the solution. We propose that adding different amounts of proteases to LYS protein solutions followed by their characterization using SDS-PAGE. SDS-PAGE provides information about the molecular weights of amino-acid units resulted from digestion of protein molecules. This might facilitate the optimization conditions for protein digestion. In the next step, polymer grafts formed on protein primer substrates of known “dry thickness” can be exposed to protease solutions for different time periods. It is believed that an enzyme digests the protein layer as shown in Figure 5.3. The digestion of protein layer can release the polymer brushes from the substrate, which can be characterized by using SEM. SEM gives information about the molecular weight distribution of polymers.
grown from protein primers. In addition, the polymer grafted substrates can be characterized by measuring the “dry thickness” of the substrates before and after the protein digestion.

5.2.8. Patterning of surface grafted polymer brushes

Patterning of polymer brushes is an important and rapidly developing field in integrated circuits, information storage devices, light emitting displays, fabrication of semiconductor microelectronics, microelectromechanical systems (MEMS), miniaturized sensors, micro- or nanofluidic devices, biochips and photonic crystals. Traditionally, many methods were developed to produce patterned polymer microstructures. Here we propose a patterning and fabrication strategy using two approaches based on surface topography and chemistry.

In the first approach, ODTS patterns can be created on silica surface by using either self-assembled microsphere monolayers developed by Chen et al. or by using microcontact printing stamps as demonstrated by Husemann and co-authors. Once the ODTS patterned SAMs are generated on silica surfaces, the unreacted or unoccupied hydroxyl groups of silica can be used to attach polyethylene glycol polymer in solution or in the vapor phase as performed by Desai and co-authors. Another method was developed by Cai et al. to produce OTS-PEG patterned surfaces. The formation of PEG polymers must be carefully optimized to prevent the adsorption of protein layers. In the next step, proteins can be adsorbed on to these patterned surfaces; it is predicted that protein adsorbs on to the hydrophobic surfaces while the protein adsorption is prevented on PEG patches as shown in Figure 5.4. Protein-modified patterned surfaces can be used to grow PHEMA polymer brush or other grafted polymers as carried out in Chapters 2 and 3 of this Thesis.

The second approach is based on surface chemistry. We can generate polymer patterns as shown in Figure 5.5. First, patterns of ODTS can be generated by using the techniques discussed earlier. Hydrophobin tends to self-assemble at hydrophilic and hydrophobic interfaces altering the chemical nature of the surfaces. Wösten et al. reported that adsorption of class I hydrophobin protein on Teflon produces hydrophilic surfaces in nature with water contact angles of \( \approx 22^\circ \). Alternatively, adsorption on hydrophilic surfaces
generates hydrophobic surfaces in nature with contact angles of \(\approx 110^\circ\). We propose to adsorb this special kind of class I hydrophobin protein onto patterned ODT/SiO\(_2\) substrates. Based on the surface chemistry of the adsorbing substrate, the majority of the hydrophilic amino-acids should reside in the outer environment on OTS patches while the hydrophobic amino acids of protein reside in the exterior environments on SiO\(_2\) patches. In the next step, polymerization initiator molecules attach preferentially to hydrophilic amino-acids of proteins while a negligible deposition is noticed on hydrophobic amino-acids generating surface patches rich in initiators. Subsequent polymerization from the surfaces generates polymer brush patterns as shown in Figure 5.5. Potential applications include patterned polymer brushes including poly(heptadecafluorodecyl acrylate) (PHFA) brushes to form superhydrophobic surfaces.\(^{33}\)
5.3. References


Table 5.1. Optimal conditions for digestion of LYS from enzymes.\textsuperscript{22}

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Buffer</th>
<th>pH</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepsin</td>
<td>0.05 M HCl</td>
<td>2.0</td>
<td>25</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0.02 M Tris-HCl</td>
<td>8.0</td>
<td>37</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>0.02 M CH\textsubscript{3}COONH\textsubscript{4}</td>
<td>8.0</td>
<td>25</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>0.02 M Tris-HCl</td>
<td>7.5</td>
<td>37</td>
</tr>
<tr>
<td>Actinase E</td>
<td>0.02 M Tris-HCl</td>
<td>8.0</td>
<td>37</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>0.02 M Tris-HCl</td>
<td>8.0</td>
<td>37</td>
</tr>
<tr>
<td>Papain</td>
<td>0.02 Sodium phosphate</td>
<td>7</td>
<td>37</td>
</tr>
</tbody>
</table>
Figure 5.1. Ellipsometry profiles of urea-denatured protein coatings on PET spin-coated flat surfaces.
Figure 5.2. Schematic illustrating the generation of orthogonal gradients from the combination of two linear gradients whose properties are perpendicular to each other. (adapted from reference 2)
Figure 5.3. Schematic illustrating the digestion of protein layer in the presence of an enzyme to release the surface-immobilized polymer brushes.
**Figure 5.4.** Schematic depicting the technological steps for the formation of polymer patterns based on surface topography.
Figure 5.5. Schematic depicting the technological steps for the formation of polymer patterns based on surface chemistry.
APPENDIX A - SUPPORTING INFORMATION FOR
CHAPTER 2

A.1. Fractional surface coverage of proteins
The amount of protein adsorbed on ODTS quantified as a “fractional surface coverage”. The coverage is expressed here as the measured dry thickness (H) relative to the thickness expected for an ideal monolayer adsorption (assuming protein cube-shape packing) (H₀ = \[M_o/(\rho N_A)\]^{1/3}), i.e., H/ H₀, where, M₀ is the molecular mass or molecular weight of the protein, ρ is the density of the protein (assumed 1 g/cm³), and N_A is Avogadro’s number.

A.2. Protein cross-linking and SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) of uncross-linked and cross-linked proteins
Protein cross-linking was employed to improve the stability of the coating layer. In solution, the aldehyde groups of GA react with alcohol and amine groups of proteins to form either hemiacetals or Schiff bases at low and high pH, respectively. Migneault et al.\(^1\) reported that the Schiff base formed during the cross-linking reaction is not stable in aqueous solutions and is thus liable to hydrolysis. Cross-linking in the presence of a reducing agent such as sodium borohydride (NaBH₄) can reduce the double bonds of the Schiff bases to stable secondary amine groups thus endowing coatings with higher stability.\(^2\)

SDS-PAGE is a method that can be employed to separate proteins based upon their size. SDS-PAGE experiments were carried out with LYS solutions with and without GA/NaBH₄; they were prepared at pH of 7, 9, 11 and 12 using bromophenol blue as a marker. Lysozyme solutions at concentrations of 0.5 mg/mL were prepared in 10 mL PBS buffer. In addition, 100 µL of 50% GA & 100 µL of 1 % NaBH₄ were added to these protein solutions. Specifically, to a small centrifuge tube, 15 µL of resultant protein solution and 5 µL of
bromophenol blue (marker) were added and mixed gently. The protein molecules were negatively charged with SDS (sodium dodecyl sulfate). The gel was made up of pores of different sizes; hence, when the resultant protein solutions were loaded in a SDS-PAGE gel under electric field, protein molecules tended to move towards the positive pole resulting in separation based upon size. SDS-PAGE was run for 50 min at a 200 V and 120 mA. The resultant gels were destained and photographed.

As shown in Figure A.1, only a single band appears in the SDS-PAGE gel of LYS corresponding to 14 kDa, which is expected for this protein. In case of LYS solution containing GA/NaBH₄ (represented as X-LYS), different bands present along the SDS-PAGE gel imply that GA cross-links LYS proteins producing dimers, trimers, and tetramers, etc. The results indicate that LYS undergoes cross-linking with GA at all pH values. The marker present at the extreme left part of the SDS-PAGE photograph represents a standard, which indicates various sizes of molecules eluting along the gel.

A.3. XPS analysis of immobilized ATRP Initiator (2-BPB)

The surface chemical compositions of ODTS, FIB-coated ODTS and FIB-coated ODTS substrates with ATRP initiator coupling (both uncross-linked and cross-linked) were determined by x-ray photoelectron spectroscopy (XPS). A Kratos AXIS ULTRA DLD X-ray photoelectron spectrometer employing monochromated Al Kα radiation with charge neutralization was utilized to study the surface chemical composition of the aforementioned samples. Survey and high-resolution spectra were collected with pass energies of 80 and 20 eV, respectively, by using both electrostatic and magnetic lenses for single angle spectra collection. The XPS measurements were obtained at a take-off angle of 90° (with respect to the sample surface). The resultant data were analyzed using the CasaXPS software. The elemental composition of protein-coated and initiator-immobilized substrates obtained from the XPS analyses are summarized in Table A.1.

Figure A.2 depicts a schematic representation of immobilization of 2-BPB (ATRP initiator) to protein-modified ODTS surfaces. ODTS substrates decorated with FIB protein
coatings evidenced the appearance of a strong N1s peak binding energies (BEs) of \( \approx 400 \text{ eV} \) (cf. Figure A.3),\(^3\) which corresponds to peptide bonds and amino acids, confirming the deposition of protein molecules with a surface chemical composition of 9.9 atomic % of N. The N1s was present even after the immobilization 2-BPB on the top of protein layer, which confirms that protein was not delaminated from the surface during immobilization step. As expected, the atomic % Br in the native FIB layer is zero.

The immobilization of 2-BPB to uncross-linked and cross-linked protein layer was confirmed by the appearance of peaks at BEs of \( \approx 70 \text{ eV} \), corresponding to Br3d (cf. Figure A.3).\(^3\) The amount of Br present in uncross-linked and cross-linked protein layers was estimated by using the Br/C atomic ratio. The Br/C ratio of uncross-linked protein layer is \( \approx 0.03 \), which is higher than that for the cross-linked protein layer (\( \approx 0.02 \)). This decrease in bromine content is attributed to the fact that some of the available surface hydroxyl and amine groups of cross-linked protein layer were involved in cross-linking reaction with GA. The amount of Br content present in cross-linked protein layer was reduced by 33% in comparison to uncross-linked protein layers, which is attributed to the proportional reduction of accessible functional groups. Overall, the XPS data confirm that a considerable number of -OH and -NH\(_2\) groups is still available after the cross-linking reaction; these provide attachment points for the polymerization initiator.

A.4. Deposition of polyelectrolyte layer and surface initiated ATRP of polymer (PNIPAAm) brushes.

In addition to PHEMA brushes (described in the Chapter 2), poly(N-isopropylacrylamide) PNIPAAm brushes were grown on ODTS surfaces functionalized with proteins and polyelectrolytes using ATRP. ATRP of PNIPAAm was carried out in methanol and water as co-solvents according to the procedure as described by Bhat and co-workers.\(^4\) NIPAAm (6.30 g, 55.67 mM) was dissolved in a mixture of methanol (4.86 g, 151.57 mM) and water (6.3 g, 350 mM) in an Ar-purged Schlenk flask. The system was degassed by three freeze-thaw cycles. To this degassed flask, N,N,N',N''-pentamethyldiethylene-
triamine (PMDETA) (0.56 g, 3.23 mM) and CuBr (0.16 g, 1.11 mM) were added followed by an additional freeze-thaw cycle. The molar ratios of the various reactants were [NIPAAm]:[PMDETA]:[CuBr] = 50 : 2.9 : 1. Bare and the protein-modified ODTS substrates were subjected to polymerization using the above solution for 9 h. The thickness and contact angle data of the resultant PNIPAAm brushes are summarized in Figure A.4. The thickness of PNIPAAm brushes produced from FIB and LYS films as well as their respective polyelectrolyte layers is \( \approx 740 \pm 30 \ \text{Å} \); the water contact angles at room temperature are \( \approx 46 \pm 2^\circ \).

While polymer layers comprising only two specific chemistries (i.e., PHEMA and PNIPAAm) have been studied, it is reasonable to assume that our method of functionalizing hydrophobic surfaces with denatured protein layers and decorating them with macromolecular grafts prepared by “grafting from” polymerization is applicable to other chemical chemistries as well.
A.5. References


Table A.1. Chemical composition (atom % C, O, N and Br) on different surfaces.

<table>
<thead>
<tr>
<th>Sample</th>
<th>C atom %</th>
<th>O atom %</th>
<th>N atom %</th>
<th>Br atom %</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODTS</td>
<td>66.19</td>
<td>33.81</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>ODTS/FIB</td>
<td>65.14</td>
<td>24.88</td>
<td>9.98</td>
<td>0.00</td>
</tr>
<tr>
<td>ODTS/FIB/2-BPB</td>
<td>64.57</td>
<td>23.98</td>
<td>9.53</td>
<td>1.92</td>
</tr>
<tr>
<td>ODTS/cross-linked FIB/2-BPB</td>
<td>65.15</td>
<td>24.59</td>
<td>9.06</td>
<td>1.20</td>
</tr>
</tbody>
</table>
Figure A.1. SDS-PAGE of LYS and LYS proteins cross-linked in the presence of GA with NaBH4 at different pH conditions.
Figure A.2. Immobilization of ATRP initiator to cross-linked protein-coated ODTS surfaces.
Figure A.3. XPS spectra of (from bottom to top) ODTS (black), ODTS surface coated with denatured fibrinogen (FIB) layer (green), ODTS-FIB surface immobilized with 2-BPB (dark purple), and ODTS/cross-linked-FIB surface immobilized with 2-BPB (magenta). The inset shows magnification of the spectra at approximately 70 eV.
Figure A.4. (top row) Dry thickness (left) and DIW contact angle (right) from PNIPAAm brushes grown from functional layers deposited on top of flat ODTS/FIB-coated silica substrates. The layer comprising denatured FIB was cross-linked with GA/NaBH₄. (bottom row) Dry thickness (left) and DIW contact angle (right) from PNIPAAm brushes grown from functional layers deposited on top of flat ODTS/LYS-coated silica substrates. The layer comprising denatured LYS was cross-linked with GA/NaBH₄. Data corresponding to fabrication methods (i)-(iii) denoted pictorially in Figure 2.8 in the Chapter 2 are shown in the top row. Note that in the method (iii) we only use a bilayer PSS/PAH for the ODTS/LYS substrate. The data corresponding to LYS protein primer are shown in the bottom row. For clarity the order in the DIW data have been reversed relative to that of the thickness.
B.1. Relating ellipsometry thickness with chemical composition of P(HEMA-co-fHEMA)

A simple model was developed by Arifuzzaman et al.\textsuperscript{1} to relate the ellipsometry thickness to chemical composition of P(HEMA-co-fHEMA). The grafting density of a PHEMA brush is given by:

\[
\sigma_{\text{PHEMA}} = \frac{h_{\text{PHEMA}} \rho_{\text{PHEMA}} N_A}{M_{\text{PHEMA}}}, \tag{B.1}
\]

where \(h_{\text{PHEMA}}\) is the ellipsometric dry thickness, \(\rho_{\text{PHEMA}}\) is the density of PHEMA, \(M_{\text{PHEMA}}\) is PHEMA molecular weight, and \(N_A\) is the Avogadro’s number.

Similarly, the grafting density of the amphiphilic random copolymer, P(HEMA-co-fHEMA) is given as:

\[
\sigma_{P(\text{HEMA-co-fHEMA})} = \frac{h_{P(\text{HEMA-co-fHEMA})} \rho_{P(\text{HEMA-co-fHEMA})} N_A}{M_{P(\text{HEMA-co-fHEMA})}}. \tag{B.2}
\]

Assuming there is no change in the grafting density of the polymer after coupling reaction, \textit{i.e.}, no chain cleavage occurs during the fluorination reaction; \(\sigma_{P(\text{HEMA-co-fHEMA})} = \sigma_{\text{PHEMA}}\):

\[
\frac{h_{P(\text{HEMA-co-fHEMA})}}{h_{\text{PHEMA}}} = \frac{\rho_{\text{PHEMA}}}{\rho_{P(\text{HEMA-co-fHEMA})}} \frac{M_{P(\text{HEMA-co-fHEMA})}}{M_{\text{PHEMA}}}. \tag{B.3}
\]

Considering that the degree of polymerization of the PHEMA brush does not change after fluorination/coupling reaction, one can write:

\[
\frac{h_{P(\text{HEMA-co-fHEMA})}}{h_{\text{PHEMA}}} = \frac{\rho_{\text{PHEMA}}}{\rho_{P(\text{HEMA-co-fHEMA})}} \frac{M_{0,\text{HEMA-co-fHEMA}}}{M_{0,\text{HEMA}}}, \tag{B.4}
\]
where \( M_{o,\text{HEMA-co-fHEMA}} \) and \( M_{o,\text{HEMA}} \) are the effective molecular weights of fluorinated co-polymer and HEMA units, respectively.

By using mass balance one can calculate the density and molecular weight of P(HEMA-co-fHEMA) using the following equations, respectively:

\[
\frac{1}{\rho_{P(\text{HEMA-co-fHEMA})}} = \frac{w_{\text{HEMA}}}{\rho_{\text{HEMA}}} + \frac{1-w_{\text{HEMA}}}{\rho_{\text{fHEMA}}},
\]

\[
\frac{1}{M_{0,\text{HEMA-co-fHEMA}}} = \frac{w_{\text{HEMA}}}{M_{0,\text{HEMA}}} + \frac{1-w_{\text{HEMA}}}{M_{0,\text{HEMA}}},
\]

where \( \rho_{\text{HEMA}} \) and \( M_{o,\text{HEMA}} \) are the density and effective molecular weights of fHEMA, respectively, \( \rho_{\text{fHEMA}} \) is the density of HEMA (assumed to be equal to that of PHEMA), and \( w_{\text{HEMA}} \) represents the weight fraction of fHEMA in P(HEMA-co-fHEMA).

The density and molecular weight of the fHEMA components can be obtained using:

\[
\frac{1}{\rho_{\text{HEMA}}} = \frac{w_{f}}{\rho_{f}} + \frac{1-w_{f}}{\rho_{\text{HEMA}}},
\]

\[
\frac{1}{M_{0,\text{HEMA}}} = \frac{w_{f}}{M_{0,f}} + \frac{1-w_{f}}{M_{0,\text{HEMA}}},
\]

where \( w_{f} \) is the weight fraction of the fluorinated modifier:

\[
w_{f} = \frac{M_{o,f}}{M_{o,f} + M_{o,\text{HEMA}}}
\]

where \( M_{o,f} \) correspond to the fluorinated modifier molecular weight in the bonded form rather than its native form. For example, for F7, we calculate the molecular weight of -(C\text{\textsubscript{7}}F\text{\textsubscript{15}}CO)- rather than C\text{\textsubscript{7}}F\text{\textsubscript{15}}COCl. In addition, we assume that the density of fluorinated modifier in the bonded form is similar to that of its native form. The results are summarized in Tables B.1 & B.2.

**B.2. FT-IR spectroscopy of LYS-modified PP nonwoven**

Figure B.1 summarizes the IR spectra for PP non-woven sheets following their modifications with LYS and amphiphilic polymer brushes. Hydrophilic PHEMA and
amphiphilic coatings were prepared on PP nonwoven surfaces using LYS protein as a primer by repeating the same technological steps as for the case of FIB primer presented in the Chapter 3. The characteristic IR peaks observed in Figure B.1 are similar to those of Figure 3.3; they clearly confirm that functional coatings were produced using LYS protein as primer. Hence, our method can be a generalized approach to develop functional coatings using any kind of protein as a primer.

B.3. FT-IR spectroscopy of FIB-modified ODTS flat surfaces

Fourier transform infrared (FTIR) spectroscopy spectra were recorded on a Nicolet 6700 infrared spectrometer in an attenuated total reflection (ATR) mode equipped with Ge crystal and purged with dry nitrogen before and during measurements for modified ODTS flat substrates. For each sample, the resolution was set at 4 cm$^{-1}$ and 1024 scans were recorded with simultaneous background and ATR corrections in order to enhance the signal-to-noise ratio. The data were analyzed using the OMNIC software.

Figure B.2 summarizes the IR spectra of FIB-coated ODTS surfaces modified with PHEMA and amphiphilic polymer brushes. The development of PHEMA brushes and the respective PPM reaction steps were repeated using the same experimental scheme as for the case of PP nonwoven surfaces represented in the Chapter 3. The characteristic IR peaks obtained from flat ODTS surfaces after their modifications are in analogous with our PP nonwoven IR spectra as shown in Figure 3.3 and previous works.$^1$
B.4. References

(1) Arifuzzaman, S.; Özçam, A. E.; Efimenko, K.; Fischer, D. A.; Genzer, J.

(2) Vickerman, J. C.; Gilmore, I. *Surface Analysis: The Principal Techniques*; 2nd ed.;
Table B.1. Chemical composition of fHEMA in P(HEMA-co-fHEMA) developed on LYS-coated ODTS surface.

<table>
<thead>
<tr>
<th>LYS</th>
<th>Individual components</th>
<th>fHEMA</th>
<th>P(HEMA-co-fHEMA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component</td>
<td>$M_o$ (g/mol)</td>
<td>$P$ (g/cm$^3$)</td>
<td>$w_f$</td>
</tr>
<tr>
<td>PHEMA</td>
<td>129</td>
<td>1.07</td>
<td>0</td>
</tr>
<tr>
<td>F1</td>
<td>210.03</td>
<td>1.51</td>
<td>0.43</td>
</tr>
<tr>
<td>F3</td>
<td>232.5</td>
<td>1.56</td>
<td>0.6</td>
</tr>
<tr>
<td>F7</td>
<td>432.5</td>
<td>1.56</td>
<td>0.75</td>
</tr>
</tbody>
</table>
Table B.2. Chemical composition of fHEMA in P(HEMA-co-fHEMA) developed on FIB-coated ODT5S surface.

<table>
<thead>
<tr>
<th>FIB Component</th>
<th>Individual components</th>
<th>fHEMA</th>
<th>P(HEMA-co-fHEMA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$M_o$ (g/mol)</td>
<td>$P$ (g/cm³)</td>
<td>$w_f$</td>
</tr>
<tr>
<td>PHEMA</td>
<td>129</td>
<td>1.07</td>
<td>0</td>
</tr>
<tr>
<td>F1</td>
<td>210.03</td>
<td>1.51</td>
<td>0.43</td>
</tr>
<tr>
<td>F3</td>
<td>232.5</td>
<td>1.56</td>
<td>0.6</td>
</tr>
<tr>
<td>F7</td>
<td>432.5</td>
<td>1.56</td>
<td>0.75</td>
</tr>
</tbody>
</table>
Figure B.1. FTIR spectra collected in transmission mode (from bottom to top) on PP nonwoven sheet (black), PP-sheet coated with denatured lysozyme (LYS) layer (dark magenta), PP-LYS fiber with PHEMA brushes (orange), PP-LYS-PHEMA modified with various fluorinated agents (green), \textit{i.e.}, F1, F3, or F7, similar to Figure 3.3.
Figure B.2. FTIR spectra collected in ATR mode for flat ODTS surfaces modified with FIB protein as primer. ODTS-FIB surface with PHEMA brushes (orange), ODTS-FIB-PHEMA modified with various fluorinated agents (green), \textit{i.e.}, F1, F3, or F7, similar to Figure 3.3.
C.1. Determination of the reduction of Ag\(^+\) to Ag\(^0\)

The degree of conversion of silver ions to silver after reduction process was determined by using ion-selective electrode; perfectION™ comb Ag/S2 combination electrode (Mettler Toledo). The electrode selectively measures the free silver ions present in the prepared nanoparticle suspensions. A calibration curve was plotted by measuring the electron voltage potential (mV reading) of known concentration of standard silver nitrate standard solutions. Then, the electron voltage potential of the silver nanoparticle suspension was measured to determine the concentration of free silver ions present in the solution. The % conversions of silver ions after the preparation of silver nanoparticles are tabulated in Table C.1. The data suggests that the reduction of silver ions during the synthesis of nanoparticles has increased as the concentration of silver nitrate is increased from 0.25 mM to 2 mM.

C.2. Determination of the molar concentration of silver nanoparticles (C) in solutions

Assuming that the nanoparticles obtained have a spherical shape and a uniform fcc crystalline structure, the average number of silver atoms per nanoparticle (N) can be calculated using the equation C.1.\(^1\)

\[
N = \frac{\pi \rho D^3}{6 M} N_A, \quad (C.1)
\]

where \(\rho\) is the density of silver (10.5 g/cm\(^3\))\(^2\), \(D\) is the mean diameter of nanoparticle determined from TEM images (\(\approx 5\) nm), and \(M\) is the atomic weight of silver (108 g/mol).
The ratio of total number of silver atoms added to the reaction solution as AgNO₃ ($N_r$) to the average number of gold atoms per nanoparticle ($N$) gives the molar concentration of nanoparticle in the solution ($C$) according to the equation C.2.¹

$$C = \frac{N_r}{NVN_A} q ,$$  \hspace{1cm} (C.2)

where $V$ is the reaction solution volume, $N_A$ is Avogadro’s constant, $q$ is the percent conversion of Ag⁺ to Ag⁰.
C.3. References


Table C.1. Determination of silver ion reduction to silver atom after nanoparticle synthesis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial amounts of Ag⁺ in AgNO₃ (ppm)</th>
<th>Amount of free Ag⁺ in nanoparticle suspensions (ppm)</th>
<th>% Conversion of Ag⁺ to Ag⁰</th>
</tr>
</thead>
<tbody>
<tr>
<td>a (xAg = 0.25 mM)</td>
<td>27</td>
<td>10.2</td>
<td>62.3</td>
</tr>
<tr>
<td>b (xAg = 0.5 mM)</td>
<td>54</td>
<td>5.9</td>
<td>89.1</td>
</tr>
<tr>
<td>c (xAg = 1.0 mM)</td>
<td>108</td>
<td>3.1</td>
<td>97.1</td>
</tr>
<tr>
<td>d (xAg = 2.0 mM)</td>
<td>216</td>
<td>3.4</td>
<td>98.4</td>
</tr>
</tbody>
</table>