Generation of Functional Coatings on Hydrophobic Surfaces through Deposition of Denatured Proteins Followed by Grafting from Polymerization

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Supporting Information

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 nonspecific 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.

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 biofouling.1,2 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.3,4 Unfortunately, the major drawback of PE and PP is their low surface energy, which results in inherent hydrophobicity and poor biocompatibility.3,5,6 For example, materials designed for surgical implants must be biocompatible and resist adsorption of biospecies to avoid blood agglutination.7 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.8,9 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.\textsuperscript{10} However, the lack of functional
groups in polyolefins and their high chemical resistance make
grafting a difficult task without employing harsh and aggressive
pretreatments, which may adversely affect their bulk properties.
\textsuperscript{7,9,11}

To date, several methodologies have been reported that provide routes for pretreatment of hydrophobic surfaces.\textsuperscript{7,11} These approaches typically involve surface modification, including plasma treatment,\textsuperscript{2,12,13} irradiation with UV light,\textsuperscript{11,14,15} electron beam,\textsuperscript{46} ozone,\textsuperscript{17,18} or \(\gamma\)-rays,\textsuperscript{19} or adsorption of surface active amphiphiles (i.e., copolymers or surfactants).\textsuperscript{20} Alternatively, surface modification can be achieved during the manufacturing process by pre-treatment (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.\textsuperscript{21} Relevant work includes that of Wang et al.\textsuperscript{8} who reported on grafting of poly(N-vinyl-2-pyrrolidone) onto ozone-pretreated PP nonwovens to enhance the hydrophilicity and antifouling properties of PP nonwovens. Lavanant et al.\textsuperscript{22} grafted poly(poly(ethylene
glycol) methacrylate) brushes onto polyolefin surfaces premodified by photobromination. The work by Desai et al.\textsuperscript{23} demonstrated that alkyl bromide groups generated on polypropylene through photobromination 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 toward modifying hydrophobic substrates is inspired by the known affinity of proteins to solid surfaces (both hydrophobic and hydrophilic) as a result of nonspecific 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 absorb 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, that is, 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.\textsuperscript{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 nonspecific interactions between the protein and the substrate is maximized.\textsuperscript{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).\textsuperscript{28,30,32,35} Depending on the solution pH, protein molecules may contain either an effective positive or a 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.\textsuperscript{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.\textsuperscript{34,36,37}

Denatured proteins attach to hydrophobic substrates via relatively weak physical van der Waals and other nonspecific interactions.\textsuperscript{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).\textsuperscript{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.\textsuperscript{40,41}

This paper focuses on the formation of hydrophilic functional coating layers on model hydrophobic \(\alpha\)-octadecyltrichlorosilane (ODTS) surfaces by adsorbing proteins such as \(\alpha\)-lactalbumin (LALBA), lysozyme (LYS), fibrinogen (FIB), and soy globulins (glycinin and \(\beta\)-conglycinin). Prior to adsorption on the ODTS surfaces, proteins were denatured using urea or
by heating to elevated temperatures. After adsorption, the protein layer was immobilized by cross-linking 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 that 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.

**MATERIALS AND METHODS**

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 Gelset (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, Mₜ = 14.3 kDa, pI=11.3), fibrinogen (from human plasma, Mₜ = 340 kDa, pI=5.5), Cuc (99.99%), CuCl₂ (99.99%), 2,2′-bipyridine (bpy, 99%), 2-bromopropionyl-bromide (2-BPB), 2-hydroxyethyl methacrylate (HEMA, 98%), glutaraldehyde (GA), sodium borohydride (NaBH₄), and poly(allylamine-hydrochloride) (PAH, Mₜ = 56 kDa) were obtained from Sigma-Aldrich and were used as received. Poly(styrene sulfonate) (PSS, Mₜ = 75 kDa) was obtained from Polysciences, Inc. α-Lactalbumin (from bovine milk, Mₜ = 14.2 kDa, pI = 4.3) was received from Davisco Foods International Inc. Soy proteins were supplied by Archer Daniels Midland Company and glycinin (115) and β-conglycinin (7S) were obtained by standard fractionation methods.42 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 used. Poly (styrene sulfonate) (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 added to the substrates by sonication in 0.1 mg/mL nonionic Triton X-100 surfactant for 2 min.

**Hydrophobic ODTS Layers Assembled on Silicon Wafers.** ODTS layers were prepared on silicon wafers as described in our previous work.43 The ellipsometric thickness of the obtained ODTS films was ±2.5 ± 2 Å, which is consistent with the theoretical thickness of a covalently bound ODTS molecule of 26.2 Å.44,45 In addition, the ellipsometric thickness of the obtained ODTS layers were approximately a monolayer.46 Wettability of ODTS layers was characterized by water contact angle measurements to determine the thickness and wettability, respectively, of the substrates with adsorbed protein coatings.

**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 bandwidth 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 by 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

**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) sonication in DI water for 5 min, (3) incubating in THF solution for 12 h at room temperature, or (4) sonication in 0.1 mg/mL nonionic Triton X-100 surfactant for 2 min.

**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, PAH was deposited as the first layer followed by the subsequent deposition of the PAH layer. The built-up of polyelectrolyte layers was carried out according to the procedure as described by Balachandra and co-workers.50

**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 of dry THF. Both solutions were cooled to 0 °C prior to the addition of initiator solution. Initiator solution was prepared 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 preheated to 85 °C for 3 min before immerging 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 to determine the thickness and wettability, respectively, of the substrates with adsorbed protein coatings.
added dropwise 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. A similar procedure was used to immobilize initiator molecules over PP nonwoven surfaces.

**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) of methanol, 1.82 mL (101 mM) of 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.

**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 for ODTS and protein coatings was 1.5 for PHEMA brushes. 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.

**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.

**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.

**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 the 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.

### RESULTS AND DISCUSSION

**Urea and Thermal Denaturation of Proteins.** Protein denaturation (thermal or chemical) and adsorption is illustrated schematically in Figure 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).

Figure 2a,b shows 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

![Image](image-url)
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. The CD spectra of native protein obtained are in a good agreement with previous reports. In general, molar ellipticity measurements at 222 nm are considered to be the index of protein denaturation and at this wavelength, α-helix has maximal optical activity. To put these data into context, ellipticity measurements at 222 nm for a range of urea concentrations are replotted in Figure 2c. The monotonous increase of negative ellipticities toward zero with increasing urea concentrations indicates that both proteins under investigation exhibited a reduction in the α-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 et al. for BSA protein denatured with 8 M urea. Fernandez-Sousa et al. also observed similar alterations in ellipticity values for LYS (from insect eggs) proteins, denatured with 4% sodium dodecylsulfate. 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 α-helix contents present in LYS and FIB reported in the literature are 42 and 30−35%, respectively.

We also unfolded protein structures using thermal denaturation, which involves heating protein solutions. Thermal denaturation leads often to permanent conformational unfolding of proteins, in contrast to denaturation using urea (because, in the latter case, protein can fold back partially with time). As can be observed from Figure 3, the negative ellipticities of FIB and LYS protein solutions decrease with increasing temperature thus implying unfolding of proteins by breaking the α-helices. Analogous reductions in ellipticity values were observed for FIB protein solutions heated from 40 to 110 °C by Chen et al. The results clearly suggest that protein denaturation increases with increasing protein solution temperature.

**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, submonolayers, or multilayers depending on the protein type and experimental conditions. The dry thickness was calculated and expressed as a “fractional surface coverage” and is summarized in Figure 4 (top; details on the fractional surface coverage calculation are provided in Supporting Information). 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. 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 Å³), LYS (30 × 30 × 45 Å³), and FIB (60 × 60 × 450 Å³) 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 (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) and “hard” (LYS and FIB) 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 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 Wösthen 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. Because 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°, Wösthen and co-workers mention that high wettability 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.

**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 incubation time, or the degree of denaturation (cf., Figure 5, left ordinate). This behavior is due to charges present over the protein molecules that minimize adsorption as well as aggregation. Lu et al.29 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 protein 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 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.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 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 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).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 ≈77.1 °C and ≈93.3 °C, respectively, as reported in the literature.69 7S and 11S solutions were heated to temperatures of ≈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 ≈ 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 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 ≈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 depends 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.

Cross-Linking and Stability of Protein Coatings. Adsorbed protein molecules were cross-linked to improve the stability of the adsorbed layers (see details about protein cross-linking in the Supporting Information). 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 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.
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 un-cross-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.

Deposition of Polyelectrolyte Layers and Surface-Initiated ATRP of Polymer Brushes. Figure 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., PAH₁) or multilayers (i.e., PAH₁/PSS/PAH₂) of polyelectrolytes were formed through electrostatic interactions on top of which the 2-BPB initiator was attached, as shown in methods (ii) and (iii), respectively. The reason for depositing polyelectrolyte layers was to create a more uniform and homogeneous 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 mechanical stability. 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 (pKₐ = 8.5), and PSS is negatively charged (pKₐ = 1). The data in Figure 9 (top row) show thickness increases 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 PAH₁ (9 Å) on top of FIB layer was less than the PAH₂ (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.²¹ 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 polyelectrolytes 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 PAH₁ 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 ≈70°, while PSS-terminated layers are more hydrophilic (θ ≈ 53°). While it is possible to grow or add more polyelectrolyte layers onto ODTS surfaces modified with preadsorbed 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/PAH₁). The resultant thickness and contact angle measurements are summarized in Figure 9 (bottom row). Specifically, the adsorbed amount of PSS was 7 Å on top of the 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 the PSS layer) was noted after the adsorption of the PSS layer above the 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 Supporting Information). 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 in 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 \( \approx 49 \pm 2^\circ \). 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 \( \approx 99^\circ \) 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 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.

**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 10 we present AFM images of surfaces coated with ODTS only, LYS layers deposited onto ODTS precoated 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 the 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, that is, the variation between the extremes in height is \( \approx 3 \) 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. To understand the detected roughness effects, one has to monitor the overall modification process in situ, which can give some insight into 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 on these phenomena.

**Modification of Hydrophobic Fibers.** 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 11 we plot IR
spectra of (a) PP nonwoven surfaces, (b) FIB-modified PP fibers, and (c) PP nonwoven surfaces with PHEMA brushes. The appearance of new peaks located between 1700 and 1550 cm\(^{-1}\) corresponds to the presence of amide I (C\(\equiv\)O stretching) and amide II bands (C\(\equiv\)N stretching and N\(\cdots\)H bending). In addition, the emergence of amide A (N\(\cdots\)H stretching) and O\(\cdots\)H stretching between 3420 and 3250 cm\(^{-1}\) confirms the attachment of protein molecules to PP supports.

Figure 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/NaBH\(_4\) (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/NaBH\(_4\). 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 has been reversed relative to that of the thickness.

Figure 10. AFM images, \(3 \times 3\) \(\mu\text{m}\), of control ODTS (left), LYS-coated ODTS (middle), and PHEMA-coated ODTS (right) surfaces.
Because protein molecules contain several amino acids, the bands appeared to be featureless and broadened in nature.\textsuperscript{72,73} HEMA growth is evident from the appearance of a strong carbonyl peak at 1720 cm\textsuperscript{-1}. The peaks at 1250 and 1080 cm\textsuperscript{-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 a significant increase in its intensity as well as broadness between 3500 and 3100 cm\textsuperscript{-1} also confirms the growth of PHEMA brushes.\textsuperscript{3,51,74} In addition, the wetting experiments presented as videos in the Supporting Information 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, a considerable increase in wicking was noticed after the growth of PHEMA brushes due to the presence of polar hydroxyl groups on the surface.

CONCLUSIONS

Denatured α-lactalbumin (LALBA), lysozyme (LYS), and fibrinogen (FIB) were used to alter the physicochemical properties including surface chemistry (functional groups) and surface wettability of hydrophobic ODTS and PP nonwoven surfaces through 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 β-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 on PP 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 functionalized materials might be explored include capture of metals or other contaminants from waters, prevention of protein adsorption, attachment of metallic nanoparticles, and many others.

ASSOCIATED CONTENT

Supporting Information

Information on XPS analysis of initiator immobilized surfaces. In addition, the results from the SDS-PAGE analysis are presented that confirm cross-linking of proteins in solutions after the addition of GA. The growth of PNIPAAm brushes on FIB- and LYS-coated substrates is also reported. The video shows wettability of water on parent PP fiber mats before and after modification with protein and after attachment of PHEMA brushes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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