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USING CROSS-SECTIONED MULTILAYER POLYMER FILM AND SURFACE MODIFICATION TO FORM CHEMICALLY PATTERNED SUBSTRATES

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USING CROSS-SECTIONED MULTILAYER POLYMER FILM AND SURFACE MODIFICATION TO FORM CHEMICALLY PATTERNED SUBSTRATES

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Chemical Engineering

by
Chun Zhang
May 2007

Accepted by:
Dr. Douglas Hirt, Committee Chair
Dr. Scott Husson
Dr. Michael Kilbey
Dr. Igor Luzinov
ABSTRACT

Highly layered structures are important to micro- and nanofabrication technologies for understanding and controlling surface structures through manipulation of chemical and physical interactions. The objective of this work was to develop a new approach to create micro- and nanopatterned surfaces using multilayer polymer films of commercially available and inexpensive polymers instead of inorganic substrates. As an example, linear low density polyethylene (LLDPE) and ethylene-co-acrylic acid copolymer (EAA) were used as alternating inert and reactive polymers, respectively. Thin cross-sections of the multilayer molded sheets were prepared by ultra-microtoming and the highly layered microstructure was verified by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and polarized optical microscopy. The length scale of the micro- and nanolayer thickness varied from 7 µm to around 500 nm depending on the extent of layer multiplication with compression molding.

As a precursor to the multilayer work, surface modification of EAA was conducted to carefully control the chemical functionality on the surface by a variety of methods. Dansyl cadaverine and polyethylene glycol (PEG) derivatives were grafted on the surface of EAA film and in its subsurface region through formation of amides and esters, respectively. A two-step reaction was conducted. First, EAA film was activated with PCl₅ and then the acid chloride was reacted with dansyl cadaverine or a PEG derivative. It was found that dichloromethane yielded the highest grafting efficiency, with the dansyl cadaverine penetrating throughout the ATR-FTIR analysis region (~ 400 nm) in two
minutes. Moreover, two other reaction schemes were developed to covalently graft PEG chains on EAA surfaces. The schemes involved surface grafting of linker molecules l-lysine or polypropyleneamine dendrimer (AM64), with subsequent covalent bonding of PEG chains to the linker molecules. NHS and EDC were used to activate the carboxylic acid groups of the EAA in the outermost region of the film, estimated to be 20 nm by ATR-FTIR spectroscopy. Combining the data from ATR-FTIR, XPS, and contact angle goniometry, it was found that the PEG chains were grafted on the surface of the EAA film and larger surface coverage was achieved when the dendrimer was used as intermediate layer. This surface also had the lowest water contact angle.

Research was then conducted on the EAA-LLDPE multilayer cross-sectioned templates. Regionally confined chemical functionality was confirmed by grafting an amine-terminated biotin to the alternating layers of EAA. Subsequently, fluorescently labeled streptavidin selectively adsorbed on the biotin-modified EAA layers. As a further development, polyelectrolyte multilayers (PEM) were adsorbed on the nanopatterned surfaces to significant increase the areal density of reactive groups. Using PAH and PAA as the polyelectrolytes, the EAA nano-stripes were successfully modified by PEM films, forming a nanopatterned template with alternating hydrophilic and hydrophobic regions. This kind of nano-striped surface could serve as a template for many applications, including biomedical, separation, and electronics.
ACKNOWLEDGEMENTS

It is with immense pleasure that I dedicate this dissertation to my girlfriend and my family. I am grateful to Yijun for her love, inspiration, and support over the course of this work. My family’s unselfish support and unquestionable belief in me and my dreams have also been a constant source of encouragement which has helped me achieve my goals. I would like to thank them all and many others who have given me help and guidance.

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CHAPTER 1
INTRODUCTION

Polymer surfaces are generally defined as the phase boundaries between the polymer and the surrounding environment. In many applications, the surface properties play a critical role in the performance of polymeric materials. For example, most commercially important polymers are hydrophobic such that their unmodified surfaces are disadvantageous in terms of adhesion, printing, coating, and biocompatibility.

Surface modification of polymers is necessary to improve their chemical, physical, and biointerfacial properties. Methods such as flame treatment, corona discharge, and plasma exposure have been used widely in polymer surface modification; however, most of these techniques do not provide permanent modification. The surface composition tends to reorganize after exposure to the surrounding environment [1]. Additionally, these methods do not accurately control the chemical nature (surface functional groups) of polymer surfaces. In recent years, other surface modification techniques [2-9] were developed to precisely control the surface functionalities and properties. Among them, surface grafting has advantages over other methods in several respects, including covalent bonding of chains to the surface at controlled density to form tailored, durable surface layers.

In addition, it is also desirable to control the spatial distribution of functionalities and generate patterned structures with different physico-chemical properties through polymer surface modification and structural patterning. For example, if the surface is designed and
developed with hydrophobic and hydrophilic regions, it can be used as a template with high adsorption selectivity. Fuchs and coworkers [10] have demonstrated several types of patterned surfaces by selectively adsorbing gold clusters, dye molecules, and FeCl₃ molecules to mica surfaces. Well-defined patterned surfaces have received increasing interest over the past decades because of their potential applications in areas such as microelectronic devices [11, 12], optics [13], microarray sensors [14, 15], and microfluidics [16]. At the same time, the ability to pattern biomolecules (such as biotin, DNA, peptides, and proteins) on solid substrates has become increasingly important for the development of molecular and cellular biosensors [17, 18], biomaterials for tissue engineering [19, 20], fundamental studies of cell biology [21, 22], high throughput drug screening [23], and proteomic arrays [24, 25]. The ability to fabricate microscopic structures with well-defined micrometer-scale topography and tailored surface chemical functionalities became a key element for creating functional nanoscale devices and novel materials. In this chapter, I will first discuss a few examples of how researchers have used the patterned surfaces in various applications, especially in biotechnology. I will then briefly review currently available techniques for the nanofabrication of patterned surfaces. Subsequently, the objectives of this research work will be discussed in detail.

**Applications of Patterned Surfaces in Micro- and Nanoscale**

Driven by the technology platform of microfabrication in the semiconductor industry, surface patterning at the micro- and nanoscales has attracted significant attention in many emerging fields. Patterned surfaces have been used widely in fabricating functional devices, for instance, in cases where the integration of micro- and nanoparticles plays a
critical role in device performance. As an example, Whitesides and coworkers [26] patterned gold surfaces with regions of hydrophobic (CH$_3$-terminated) and hydrophilic (COOH-terminated) self-assembled monolayers (SAMs) of alkanethiolates using microcontact printing, followed by immersing the patterned surface in an aqueous solution of organic or inorganic salt and then removing it from the solution. In this way, the solution was distributed into a regular array of drops with controlled volume. After water evaporation, the patterned array of nanoparticles was formed as shown in Figure 1.1, and was proposed for applications such as diffraction gratings, masks in reactive ion etching, or arrays of functional materials [27, 28].

![Figure 1.1 SEM images of CuSO$_4$ particles and KNO$_3$ crystals formed on patterned surfaces with SAMs (Reprinted with permission from reference [26]).](image)

Instead of direct deposition of nanoparticles on a patterned substrate, Hammond and coworkers [29] utilized polyelectrolyte multilayer (PEM) films as intermediate layers, first depositing PEM films on a chemically patterned silicon substrate, then selectively adsorbing bare SiO$_2$ microspheres or functionalized polystyrene latex particles. Figure 1.2
shows the patterned surface structure. They also demonstrated that the modulation of surface charge density led to a range of deposition selectivities. The PEM platform proved to be amenable to the introduction of many other functionalities [30-33]. This kind of template could be used in a variety of applications, such as functional templates and catalysts for chemical and biological processes, sensor arrays, optical materials, and photonic crystal devices.

Figure 1.2 Optical micrograph of patterned (PDAC/SPS)₅ PDAC multilayers followed by immersion in a polystyrene latex sphere suspension (Reprinted with permission from reference [29]). PDAC denotes poly(diallyldimethylammonium chloride) and SPS denotes sulfonated polystyrene.

For the development of biologically integrated devices, it is crucial to organize multiple biomolecules on the surfaces at the micro- and nanoscales. Protein patterning has been investigated in order to integrate protein into miniature biological-electronic devices. There are three basic methods to immobilize proteins on surfaces in a controlled
fashion, and they are physical adsorption [34], covalent linking [35], and the use of high-affinity ligands [36]. The ability to generate protein patterns on surfaces is important for biosensor technology [37, 38], tissue engineering [39-41], and fundamental studies of cell biology [42-45]. For example, Langer and coworkers [36] used reactive coating material and microcontact printing to prepare patterned surfaces. The reactive coating was first deposited on the substrates by chemical vapor deposition polymerization of functionalized 2, 2’-paracyclophanes. Patterned surfaces were then generated on the reactive coating by microcontact printing of amino-terminated biotin ligand. After binding with streptavidin, a protein pattern was formed. They used another affinity site of streptavidin to bind biotin-tethered antibody and subsequently deposited mammalian cells through specific interactions between the antibody and endothelial cells. The fluorescent image in Figure 1.3 demonstrated the patterned surface after antibody binding.

![Fluorescence micrograph of a gold substrate with a reactive coating and patterned with a fluorescein-labeled secondary antibody (Reprinted with permission from reference [36]).](image)

**Figure 1.3** Fluorescence micrograph of a gold substrate with a reactive coating and patterned with a fluorescein-labeled secondary antibody (Reprinted with permission from reference [36]).
Using microphase-separated PS-\textit{b}-PMMA diblock copolymers, Hahm and coworkers [34] successfully patterned proteins through physical adsorption as shown in Figure 1.4. The model proteins they used were bovine IgG, protein G, and FITC anti-bovine IgG. They demonstrated that the protein was selectively adsorbed on PS microdomains due to the preferred interactions with polystyrene (PS) over poly(methyl methacrylate) (PMMA).

![AFM phase images of 4 µg/mL IgG molecules deposited selectively on PS domains in the PS-\textit{b}-PMMA ultrathin films spun on silicon oxide substrates (Reprinted with permission from reference [34]). The image size is 2 × 2 µm.](image)

**Figure 1.4** AFM phase images of 4 µg/mL IgG molecules deposited selectively on PS domains in the PS-\textit{b}-PMMA ultrathin films spun on silicon oxide substrates (Reprinted with permission from reference [34]). The image size is 2 × 2 µm.

In recent years, more attention has been paid to the development of methods for cell and tissue culturing. Through cell patterning, visualization of the effect of surface properties on cell functions and spatial control of cellular micro-organization could be achieved. It is well known that the performance of artificial biomaterials depends strongly on surface chemical functionalities and surface topography at the micro- and nanoscales. Controlled protein adsorption is a critical factor to optimize cell-surface
interactions, because cells could recognize only the protein layer but not the underlying surface. Cellular patterning has been employed successfully to understand the fundamental cellular activities, including cell-surface interactions, cell guidance, and morphological effects on cell function, migration, and proliferation. Potentially, spatial organization of animal cells could be beneficial for medical and dental implants, neuron regeneration, bioartificial organs, bioelectronics, and cell-separation techniques [21, 46]. In addition, cell-based biosensors [18, 47] have been developed for the functional characterization and detection of drugs, pathogens, toxicants, and odorants. Potentially, they could be used in high throughput drug discovery and clinical diagnostics.

Ho and coworkers [48] prepared cell-patterned surfaces on poly(lactic acid) (PLA) and poly (lactide-co-glycolide) (PLGA) substrates using microcontact printing and cell resistant polymeric materials. The cell resistant materials they used were poly(oligoethyleneglycol methacrylate) or poly(oligoethyleneglycol methacrylate-co-methacrylic acid). Through protein adsorption and subsequent NIH 3T3 fibroblast culturing for up to 2 weeks, fibroblasts were successfully confined within line-shaped patterns (Figure 1.5). Because PLA and PLGA have excellent biocompatibility, biodegradability, and low toxicity after degradation, the cell-patterned substrates on these materials could have significant applications in tissue engineering.
Figure 1.5 Micropatterned NIH 3T3 fibroblasts on PLGA films (Reprinted with permission from reference [48]). Cells were cultured on 30 µm-wide lines for (A) 3 days, (B) 7 days, and (C) 14 days. Scale bar is 50 µm.

Conventional and Unconventional Patterning Techniques

After highlighting several applications of micro- and nanopatterned surfaces, I will now briefly review methods that have been used to generate the patterned surfaces. The patterning methods commonly are characterized as “top-down” (e.g., photolithography, dip-pen nanolithography, electron beam lithography, nanoimprint lithography) and “bottom-up” (e.g., self-assembly, phase separation). Many techniques have been developed for fabricating patterned nanostructures by utilizing at least one of the following principles:

- interaction of materials with photons, energetic particles and scanning probes
- replication of the surface structures against masters
- self-assembly of molecules and nanoscale objects

Photolithography

Photolithography is the most widely and successfully used “conventional” technique to generate patterned surfaces on solid substrates [49-54]. In general, the solid surface is
coated with a thin layer of photosensitive polymer, then exposed to UV light through a 
photomask so that the pattern is transferred onto the photoresist film. Finally, this pattern 
can be further transferred into the underlying substrate by selective etching or lift-off as 
shown in Figure 1.6. Photolithography has been applied to a variety of substrates, but it 
requires the surface to be flat enough so that a thin film of photoresist material can be 
deposited uniformly.

![Figure 1.6 Schematic illustration of photolithography of positive and negative 
photoresists (Reprinted with permission from reference [55]).](image)

This technique has been extended to generate protein and cell patterns. For example, 
Toner and coworkers [56] developed a new process based on photolithography to pattern 
PEG hydrogel wells on glass. They used the PEG hydrogel structures to study protein
adsorption and cell adhesion. It was proven that the structure is very effective in guiding and confining adhesion of transformed 3T3 fibroblasts.

The major limitation of this technique is the large cost for generation of the photomask. Another problem is low throughput rates for nanometer sized features. In addition, it is hard to apply photolithography to non-planar surfaces and it provides almost no control over the chemistry of the patterned surfaces. Well-established techniques such as photolithography are limited by the fundamental barriers of diffraction and the harsh conditions required, which limits their use in biomolecular patterning.

**Soft lithography**

Microcontact printing (µCP) is used widely to transfer chemical molecules to a surface using a patterned elastomeric mold or stamp, which is typically fabricated from poly(dimethylsiloxane) (PDMS). Figure 1.7 shows a schematic representation. Developed in 1993 [57], this technique has been used to form patterns on various substrates, including patterning alkanethiols on gold [58, 59], alkylsiloxanes on silicon [60], and alkylphosphoric acids on aluminum oxide [61]. Proteins could then be adsorbed selectively on the patterned SAMs. For example, Lopez et al. [62] patterned gold surfaces into alternating regions of oligo(ethylene glycol) groups and methyl groups with microcontact printing, then immersed the patterned surface into a protein solution of fibronectin and other proteins. They found that the protein was adsorbed only on the methyl-terminated regions. Proteins have also been patterned on many polymer surfaces, for example, Chilkoti and colleagues [63] patterned biotin onto the surfaces of various polymers by microcontact printing. They first functionalized the polymer surface
chemically with reactive pentafluorophenyl ester groups. After reaction with biotin, the patterned surface selectively adsorbed streptavidin on the modified region.

![Microcontact printing technique](image)

**Figure 1.7** Microcontact printing technique. A PDMS mold is coated with the transfer molecules, such as protein shown here. Then the mold contacts the substrate and transfers the protein pattern on the surface (Reprinted with permission from reference [64]).

Microcontact printing is attractive because of its ease and low cost. However, the pattern resolution depends strongly on the resulting distortion of the stamp when a large patterned surface is required. In addition, µCP is limited to the micron scale when it is used to pattern proteins. In addition, when printing with water-based biological solutions, the poor wettability of the hydrophobic PDMS stamps typically does not provide good uniformity. Therefore, PDMS is normally pretreated to provide a more hydrophilic surface.

Many other soft lithography techniques have been developed, including replica molding (REM) [65], micro-transfer molding (µTM) [66], micromolding in capillaries (MIMIC) [67], and solvent-assisted micro-molding (SAMIM) [68]. REM uses a PDMS
polymer to duplicate structures of a master into multiple copies. For µTM, the stamp is filled with a pre-polymer, placed on a substrate and cured, then removed to get a patterned structure. In MIMIC, a PDMS mold in contact with a substrate surface forms a network of empty channels that are filled with a thermal or photo-curable liquid. SAMIM is a combination technique of replica molding and embossing. The surface of the stamp is wetted with a solvent and pressed against the polymer film at ambient conditions. The solvent dissolves a thin layer of the polymer, and the resulting gel-like fluid conforms to the elastomeric mold.

More recently, Delamarche and coworkers [69] extended MIMIC into microfluidic networks (µFNs) to pattern biological molecules such as immunoglobulins. By allowing protein solution to pass through microfluidic channels as shown in Figure 1.8, protein patterns were prepared on various substrates including gold, glass, and polystyrene. This approach enables simultaneous and highly localized immunoassays for the detection of different IgGs.

**Figure 1.8** µFN uses a patterned elastomer in contact with a substrate allowing the local delivery of a solution of biomolecules to the substrate (Reprinted with permission from reference [69]).
In general, it is unreliable to have production of defect-free molds using soft lithography. Because the molds are patterned through several steps, many variations are introduced so that the pattern fidelity, resolution, and replication will be affected. In addition, release agents used to assist with separation from the cured polymer have limited lifetimes. For biomolecule patterning, the main limitation of μCP, especially on polymers, is the low surface density of the biomolecules immobilized on the surface, because usually only a small amount of reactant is adsorbed on the stamp and the reaction time is limited. Therefore, its use has largely been restricted to self-assembled monolayers on gold or silicon, which are not suitable in many applications such as fabrication of biomaterials.

**Block-copolymer lithography**

Block copolymers (BCP) consist of chemically distinct polymer chains linked covalently to form a single molecule. Dissimilar blocks tend to segregate into different domains due to their mutual repulsion. Self-organized periodic microstructures result from the competing effects between random coiled molecules and stretched blocks. At the same time, the microstructure of BCPs could be controlled by choice of molecular weight, composition, and chain architecture. Figure 1.9 shows various orientations (parallel, perpendicular, and close packed) of lamellar, cylindrical, and spherical microdomains of BCPs along with possible surface characteristics (non-patterned, chemically and/or topographically patterned) [70]. As mentioned earlier, Hahm and coworkers [34] used this technique to fabricate protein patterns by selectively adsorbing proteins on one of the microdomains. However, spontaneous assembly of components has limited its application for nanofabrication due to many issues such as pattern defects,
small-range order of the patterned structures, and control of domain orientation [71]. To obtain a preferred and controlled orientation, other external biases have to be used, such as a mechanical flow field [72], temperature gradient [73], electric field [74], and patterned substrate [75, 76].

![Figure 1.9](image)

**Figure 1.9** Schematic of various orientations of lamellar, cylindrical, and spherical microdomains of BCPs (Reprinted with permission from reference [70]).

**Dip-pen nanolithography**

Another patterning technique is dip-pen nanolithography (DPN) [77-81]. It uses an atomic force microscope (AFM) tip “inked” with a solution of the material of interest. The adsorbed materials are transferred to the surface in a controlled fashion as shown in Figure 1.10. SAMs have been patterned on gold surfaces using this technique, followed by binding with oligonucleotides, proteins, and viruses [82, 83]. For example, Chilkoti and coworkers [84] used DPN to fabricate chemically reactive nanoscale features by
patterning a SAM of COOH-terminated alkanethiol on a gold surface. Biotin was then covalently immobilized on the nano-patterned SAM, and streptavidin was subsequently adsorbed on the biotin-modified regions.

**Figure 1.10** Schematic representation of DPN (Reprinted with permission from reference [77]). A water meniscus forms between the AFM tip coated with 1-octadecanethiol and the Au substrate.

The mechanism of material transfer by DPN is not yet clear, but a possible explanation is that a pool of water develops between the tip and surface. DPN can precisely deposit molecules on a surface. But this technique is relatively slow and requires expensive instrumentation such that it is not cost effective for many applications. In addition, there are many factors that affect the patterned structure, such as humidity, temperature, and surface roughness of the substrate.
Other patterning techniques

Researchers have continued to pursue other approaches to generate micro- and nanoscale patterned surfaces in a more cost effective and efficient way. For example, in nanoimprint lithography [85-87], a thermoplastic polymer film (such as PMMA) is heated above its glass transition temperature and a rigid mold is pushed into the film. The surface is textured and separated from the mold after the polymer is cooled. This method, also known as hot embossing or thermal imprinting, requires high processing temperature and pressure to enable the polymer to flow.

Lithographically induced self-assembly (LISA) [88, 89] was also developed and utilized to produce patterned surfaces. In this method, a mask with protruded patterns was placed a certain distance above a flat polymer melt (PMMA), and the polymer was then attracted toward the mask protrusions. Electrostatic force is assumed to be the driving force for this technique.

Ink-jet printers have been used to prepare patterned surfaces [90]. This technique has the advantages of high speed and high throughput. It could also be used to deposit proteins and cells with control [91, 92]. However, it is difficult to achieve nanoscale dimensions and the substrates could not be covered by the “inks” uniformly.

Research Objectives

Micropatterning and nanopatterning are becoming increasingly important for the development of biomedical devices such as biosensors as well as genomic and proteomic arrays. But for those applications, silicon may not be an optimal choice because of its incompatibility with biomolecules and high cost. Therefore, it is highly desirable to
fabricate a patterned surface with high throughput and repeatability. From a cost standpoint, it would also be advantageous to fabricate patterned surfaces using commercial polymers.

The objective of the present work is to generate reactive, patterned surfaces using multilayer polymer films. The cross-section of a highly multilayered film is used as a template to form functionalized, patterned surfaces. Figure 1.11 shows a schematic representation. The advantages of this approach, compared with other techniques described in this chapter (summarized in Table 1.1), are the use of inexpensive polymers (one of which could be reactive) to create flexible, nanostriped patterns. As an example (Figure 1.11), I used poly(ethylene-co-acrylic acid) (EAA) copolymer as a reactive material and linear low-density polyethylene (LLDPE) as an inert material. The combination of multilayer polymer films and subsequent surface modification provides us the opportunity to tailor the functionality of the patterned surfaces.

Figure 1.11 Schematic representation of multilayer polymer film approach to generate reactive, nanopatterned surface.
Table 1.1 Comparison of advantages and disadvantages of various patterning techniques described in this chapter.

<table>
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<th>Technique</th>
<th>Description</th>
<th>Advantages</th>
<th>Disadvantages</th>
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| Photo-
  lithography                | Using ultraviolet (UV) and rigid photomask to create patterned structures    | Fast, reproducible, high pattern fidelity | Expensive, not applicable for non-planar surfaces, limited control over the surface chemistry |
| Microcontact printing (µCP)  | Printing inks such as biomolecules onto surfaces with an elastomeric stamp   | Low-cost, fast, applicable for many kinds of “inks” | Stamp distortion due to the deformation, low resolution                      |
| Block copolymer lithography  | Fabrication of patterned surfaces using the self-assembly nature of block copolymer | Fast, spontaneous                      | Pattern defects, difficult to form large-range order of the patterned structures |
| Dip-pen Nano-lithography     | Directly writing an “inked” AFM tip on a substrate                          | High-resolution, applicable for various “inks” | Slow, limited to inorganic substrates such as gold and silicon               |
| Our approach                 | Direct using cross-sectioned multilayered film as a stripe-patterned surface | Low-cost, fast, unlimited in one dimension, possible to tailor the surface chemistry | Stripe-patterned surfaces only                                             |

Based on the overall objective of this research, it is critical to first study the surface modification of EAA copolymer. This part of the research work is described in Chapters 3 and 4. In Chapters 5 and 6, nano-patterned surfaces prepared using multilayer polymer films, protein patterning, and polyelectrolyte multilayer (PEM) patterning are described. The specific objectives of the work are as follows:
1. To study the penetration behavior and grafting depth of biomolecules during the surface modification of EAA films.

2. To immobilize poly(ethylene glycol) (PEG) on EAA surfaces with controlled grafting depth to improve biocompatibility and to minimize cell adhesion.

3. To develop a novel approach for protein patterning by carefully functionalizing the carboxylic acids on alternating EAA layers of the patterned surface with amine-terminated biotin and subsequently adsorb streptavidin.

4. To form a nano-striped surface with alternating wettability, hydrophilic and hydrophobic stripes by deposition of PEM films and subsequent crosslinking.
References


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CHAPTER 2
ANALYTICAL TECHNIQUES

Attenuated Total Reflectance (ATR) FTIR Spectroscopy

A Nicolet Avatar 360 FTIR spectrometer (Nicolet Analytical Instruments, Madison, WI) equipped with a nitrogen-purged chamber was used to investigate the chemical changes after each reaction step and to provide some evidence concerning the penetration of reactants into the EAA film. ATR-FTIR spectroscopy was conducted with a horizontal multibounce attachment using a germanium crystal and a 45° angle of incidence. All spectra were taken at 4 cm\(^{-1}\) resolution and reported as an average of 256 scans. The penetration depth \(d_p\) of the evanescent wave from the reflected light was calculated knowing the refractive indices of the crystal \((n_1 = 4.0)\) and film specimen \((n_2 = 1.5)\), the angle of incidence \((45^\circ)\), and the wavelength of interest \((\lambda)\) [1]. The calculations determined that the average penetration depth of the evanescent wave for the dansyl cadaverine grafted EAA film at \(\lambda = 1645\) cm\(^{-1}\) was approximately 403 nm, while that for the carboxylic acid grafted EAA film at \(\lambda = 1705\) cm\(^{-1}\) was approximately 400 nm and that for PEG modified EAA film at \(\lambda = 1743\) cm\(^{-1}\) was approximately 381 nm. For many other samples, adsorption peaks of their FTIR spectra were identified according to a reference book [2].

The basis for quantitative analysis of absorption spectrometry is Beer’s law. For a single compound in a homogeneous medium, the absorbance at any frequency is expressed as:
where \( A \) is the measured sample absorbance at the given frequency, \( a \) is the molecular absorptivity at the frequency, \( b \) is the path length of source beam in the sample, and \( c \) is the concentration of the sample. This law basically states that the intensities of absorption bands are linearly proportional to the molecular absorptivity of each component in a homogeneous mixture or solution for a given concentration. For our reaction system, the absorptivities of the carboxyl functionality for \(-\text{COOH}, -\text{COCl}, \text{and } -\text{COOR}\) are 32, 100, and 57, respectively [3]. Therefore, the peak area at the same concentration can be rank ordered as \( A_{\text{COCl}} > A_{\text{COOR}} > A_{\text{COOH}} \).

**X-ray Photoelectron Spectroscopy (XPS)**

Chemical analysis of polymer film was performed using a Kratos Axis Ultra photoelectron spectrometer with Al K\( \alpha \) radiation (15 kV, 225 W). All spectra were collected at an electron take-off angle of 90° to the sample surface. Before testing, each sample was transferred from the ambient into the untrahigh vacuum chamber through a high vacuum cell and dried overnight with N\(_2\). Low-resolution survey and high-resolution spectra were obtained for each sample. Survey scans were recorded over the 0-1200 eV binding energy range, using a pass energy of 80 eV. High-resolution spectra of the C 1s, O 1s, and N 1s core levels were also recorded. Spectral analysis was done using casaXPS software and all binding energies were referenced to the C 1s binding energy of 285 eV. Peak shifts for the carbon and nitrogen functionalities were taken from the literature [4, 5].
Contact Angle Goniometry

Static contact angle measurements were conducted using a Krüss G10 instrument. HPLC water was used and the measurement was taken immediately after the drop formed on the surfaces. The average static contact angle and 95% confidence interval were calculated using at least eight droplets on each specimen.

Dynamic contact angle measurements were made with the same instrument. HPLC water and diiodomethane were chosen as probe liquids and sessile drop measurements were performed at room temperature. Through increasing or decreasing the drop size by 0.5 µL until the three phase boundary moved, the advancing ($\theta_a$) and receding ($\theta_r$) contact angles were obtained. Five measurements for each specimen were performed, yielding the mean dynamic contact angle and standard deviations.

Fluorometry

Fluorometry measurements were performed using a GENios Multi-Detection Reader (Phenix Research Products). The excitation and emission wavelengths for the dansyl group were 340 nm and 510 nm, respectively. After reaction, these EAA-g-Dansyl films were extracted with dichloromethane in a Soxhlet apparatus for 24 h and then dried at 60 °C for 2 h before measurement. For each polymer surface, 16 readings were performed at different locations and averaged to report the fluorescence intensity.

Differential Scanning Calorimetry (DSC)

DSC experiments were performed using TA Instruments MDSC 2920. Specimens of EAA films weighing approximately 5 ~ 10 mg were cut from each of the materials. The
specimens were sealed in aluminum pans with covers and then placed in the DSC. The samples were first held at -50 °C for 30 seconds and then heated at a constant rate of 10 °C/min until they reached 120 °C followed by cooling at a constant rate of -10 °C/min until they reached 25 °C. Two scans were carried out for each specimen. Heating was carried out in a nitrogen environment. The heat flow recorded in the second scan was used to construct a DSC plot of heat flow (mW) versus temperature (°C). Calculation of the crystallization percentage was based on the heat of fusion for a perfect crystal of polyethylene [6].

**Optical Microscopy**

An Olympus BX60 microscope with crossed polarizer and analyzer was used to characterize the microstructure of thin sections and 200x magnification was applied. Image analysis was performed with Image-Pro 4.1 software. Images of streptavidin-modified patterns were recorded by fluorescence optical microscopy (Nikon, HFX-DX). FITC filter was used so that the modified region for the patterned surface appeared green. Images were captured with a CCD camera and processed with the camera software.

**Confocal Fluorescence Microscope**

Confocal fluorescence images were obtained using a Zeiss LSM510 microscope. Samples were mounted on glass slides and then fixed onto the stage with cover slip down. An Ar-ion laser (488 nm) for excitation and a 510 ~ 525 nm bandpass filter was used for fluorescein (FITC) detection. Image analysis was performed with the LSM 510 software from the original images.
**Atomic Force Microscopy**

Atomic force microscopy (AFM) images (both phase and morphology) were obtained using a Digital Instruments Nanoscope IIIa in tapping mode with a rectangular Si$_3$N$_4$-coated cantilever (325 kHz, 40 N/m). For all the images, frequency was set as 0.5 Hz and scanning number as 256 per image. Root-mean-square (RMS) roughness of each specimen was evaluated from the images using the NanoScope Software Version 4.23.

**Scanning Electron Microscopy (SEM)**

Low voltage (5 kV) scanning electron microscopy (LVSEM, Hitachi, Model S4700) was used to study the more highly layered structures at 5000x magnification. Samples were pressed against carbon tapes and then mounted on aluminum stubs. Fractured surfaces and ultrathin sections were coated with about 100 Å of platinum before scanning.

**Transmission Electron Microscope (TEM)**

Transmission electron microscope (TEM) images were obtained (Hitachi, Model H7600T) operating at 100 kV at 15000x magnification. The ultrathin sections of 4032-layer films were stained with osmium tetroxide vapor at room temperature for several hours in order to selectively stain the less dense EAA layers of the patterned structure.
References


CHAPTER 3

PENETRATION BEHAVIOR AND SUBSURFACE GRAFTING OF DANSYL CADAVERINE AND POLYETHYLENE GLYCOL (PEG) DERIVATIVES IN POLY (ETHYLENE-CO-ACRYLIC ACID) (EAA) FILM

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**Introduction**

Poly(ethylene-co-acrylic acid) (EAA), a commercially available copolymer, has excellent physical and mechanical properties. It is used commonly as a protective coating because its carboxylic acid groups promote adhesion to metal [1]. EAA has also been investigated to improve the miscibility with polystyrene (PS) as a polymeric emulsifier [2]. However, the low surface energy of EAA limits its biotechnological applications due to its non specific adsorption and poor wettability. Therefore, research is being conducted to obtain improved surface properties through controlled surface modification. For example, Chong et al. [3] explored the use of EAA as a surface coating in biosensor applications, where the shielding EAA layer protected the silver coated quartz-crystal-microbalance (QCM) and was tethered covalently with DNA oligonucleotides.

Because EAA contains reactive carboxylic acid groups, it can undergo chemical coupling after activation with phosphorus pentachloride [4, 5], thionyl chloride [6], or
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) [3, 7]. In addition, functional groups exist not only on the outer surface of EAA but also throughout the bulk, and surface rearrangement may result in carboxylic acid groups migrating into the subsurface region of the film [8]. Therefore, it is possible to graft long chain molecular or macromolecular species onto the surface and in the subsurface of EAA.

Currently, much attention is focused on attaching PEG onto surfaces to reduce adsorption of protein, bacteria, and cells. For example, Zhu et al. [9] studied the configuration of PEG molecules on silicon, finding that PEG with low molecular weight exhibits brushlike configurations, while PEG with higher molecular weight resembles a coil configuration. Some theories suggest that the brushlike configuration is more protein resistant because it provides maximum entropic repulsion between the proteins and surfaces [10]. Tajouri et al. [11] compared the behavior of PEG chains grafted on silica both in the presence of and without a solvent, finding that PEG can flatten on the surface or be swollen and spread out depending on the interactions among the chains, the solvent, and the solid surface in a heterogeneous system. In an effort to use polymers as biomaterials and biosensors, grafting techniques have also been employed to immobilize PEG on various polymer surfaces [12-19], including polypropylene, polyethylene, poly(ethylene terephthalate), polyaniline, polyurethane, polysulfone, poly(vinylidene fluoride), and poly(acrylonitrile-co-maleic acid).

The issue of reactant penetration into the polymer and subsequent reaction in the subsurface or bulk, which can be prevalent in a solution reaction system, is not often
addressed. If the reaction occurs deeply within a film, the reaction would result in a bulk modification in addition to a surface modification such that the bulk properties may be affected [20], even though better surface wettability was achieved. Therefore, it is important to know the extent to which reactants penetrate into a polymer film and subsequently react with the reactive groups. ATR-FTIR spectroscopy is a good technique for studying the penetration reaction behavior within depths of tens and hundreds of nanometers [21, 22]. The aim of this work was to study the subsurface grafting behavior and penetration reaction of a relatively small biomolecule (dansyl cadaverine) and PEG derivatives (Nonoxynol-9 (N9)) [23]. Dansyl cadaverine normally is used as a fluorescent label to quantitatively determine the concentration of carboxyl, hydroxyl, and amino acids on polymer surfaces [24, 25]. In this research, it served as a fluorescent molecular label anchored onto the activated EAA surface and subsurface. We also used a series of N9s denoted as PEG4, PEG11, PEG39 and PEG99, where the number corresponds to the number of ethylene glycol repeat units. They were chosen for this reaction for several reasons. First, they are commercially available with nearly monodisperse molecular weights. Second, different chain lengths of the PEG derivatives aid in characterizing the penetration behavior of a series of oligomeric and polymeric chains for comparison with dansyl cadaverine. Third, they serve as well defined materials for PEG grafting on EAA surfaces, which is important for future protein resistance studies.
**Experimental**

**Materials**

Poly(ethylene-co-acrylic acid) (EAA, PRIMACOR 1410 from Dow Chemical Co., 9.5% (w/w) acrylic acid) films (thickness ~ 50 μm) were used as received from the Cryovac Division of Sealed Air Corp. (Duncan, SC). Phosphorous pentachloride (PCl₅, 95% by weight), dichloromethane, acetone, methanol, pH 7 phosphate buffered saline (PBS) solution, potassium hydroxide solution (KOH in water, 85% ACS reagent), dansyl cadaverine, and Nonoxynol-9 (N9) (Igepal CO-990, 890, 720, 520) were purchased from Aldrich and used as received. Figure 3.1 shows the chemical structures of dansyl cadaverine and the PEG derivatives.

**Conversion of carboxylic acid groups into acid chlorides**

The EAA film was first washed with acetone and then dried at room temperature. To transform the carboxylic acids into acid chlorides, a film specimen was immersed in 20 ml of a 3% (w/w) PCl₅ solution in dichloromethane for 9 h to ensure high conversion. The film was then washed sequentially with dichloromethane and acetone. This film was denoted as EAA-Cl and used for the next reaction after being dried at room temperature.

**Grafting of dansyl cadaverine to the EAA-Cl film**

Dansyl cadaverine has a primary amine that can be linked covalently to an acid chloride. The EAA-Cl film was immersed in a 0.1 mg/ml solution of dansyl cadaverine in dichloromethane for 9 h at room temperature. Two other solvents were also used to study
he effect of solvent in the grafting reaction, namely, 4% (w/w) aqueous KOH solution (pH 12), and a pH 7 PBS solution. Modified films were dried under vacuum at 60 °C for 2 h before characterization. The EAA-Cl film was also immersed in a 1 mg/ml solution of dansyl cadaverine in dichloromethane to obtain higher reaction rates, and the reaction kinetics were studied over a time period of 5 sec to 2 min. These EAA-g-Dansyl films were extracted with dichloromethane in a Soxhlet apparatus for 24 h and then dried at 60 °C for 2 h before characterization.

**Differential scanning calorimetry (DSC)**

DSC experiments were performed using TA Instruments MDSC 2920. Five to 10 mg of film was heated from -50 to 120 °C with heating and cooling rates at 10 °C/min. Crystallinity of the polymers was calculated according to the equation

\[ \chi(\%) = \left( \frac{\Delta H}{\Delta H^0} \right) \times 100 \]  

(1)

where \( \Delta H \) is the heat of fusion of the film samples and \( \Delta H^0 \) is the heat of fusion of perfectly crystalline polyethylene (PE).
Figure 3.1 Reaction scheme for grafting dansyl cadaverine and PEG derivatives on an EAA surface and into the subsurface region.

Estimation of the surface densities of the functional groups

Assuming the outermost surface to be 5 Å in depth and the carboxylic acid groups distributed uniformly throughout the EAA film, the surface density of the carboxylic acid
groups, $\sum$ (number/nm$^2$), was obtained by [26]

$$\sum = D \times \rho \times \eta \times \frac{N_A}{M}$$  \hspace{1cm} (2)

where $D$ is 5 Å, $\rho = 0.938$ g/cm$^3$ is the density of the polymer, $\eta = 0.095$ is the acrylic acid fraction (w/w), $N_A$ is Avogadro’s number, and $M = 72$ g/mol is the molecular weight of acrylic acid.

The distance between functional groups F was estimated by

$$F = \left(\frac{4}{\pi \sum}\right)^{1/2}$$  \hspace{1cm} (3)

**Results and Discussion**

The carboxylic acid groups in EAA films can be used as reactive sites for surface grafting. In this work, studies focused on grafting dansyl cadaverine and PEG derivatives on the EAA surface and in its subsurface region. The reactions were performed in the two steps shown in Figure 3.1. First, EAA film was reacted with PCl$_5$ for 9 h at room temperature to convert the carboxylic acid groups to acid chlorides, which could occur throughout the film depending on reaction conditions. Second, the acid chlorides were reacted with dansyl cadaverine or PEG derivatives to form a modified film. The penetration rate and reactivity of dansyl cadaverine in EAA film was studied initially as a
function of solution concentration and immersion time.

A 0.1 mg/ml solution of dansyl cadaverine was prepared in three different solvents, namely, dichloromethane, 4% (w/w) KOH aqueous solution (pH 12), and pH 7 buffer solution. When dansyl cadaverine reacts with an acid chloride in the EAA-Cl film, an amide bond should form as evidenced by an IR absorption peak at 1645 cm⁻¹. Figure 3.2 shows the ATR-FTIR spectra for neat EAA (spectrum 1), EAA-Cl (spectrum 2), and EAA-Cl immersed in the various dansyl cadaverine solutions for 9 h (spectra 3-5). The neat EAA showed a peak at 1705 cm⁻¹, which is characteristic of the carbonyl stretching of the acid portion of EAA. For the EAA-Cl, the 1705 cm⁻¹ peak was not observed as the carboxylic acids were converted to acid chlorides, indicated by the peak at 1793 cm⁻¹. Spectrum 3, corresponding to the reaction in the KOH solution, showed evidence of amide formation with a peak around 1645 cm⁻¹; the KOH solution was the only case where this was observed. Reactions did not appear to occur in the aqueous pH 7 or CH₂Cl₂ dansyl cadaverine solutions. Although it is generally considered that the acid chlorides will hydrolyze rapidly in aqueous solution, it was interesting to note that the ATR-FTIR spectra did not show any hydrolysis. But there was still a large amount of acid chlorides in the film for both of the aqueous solutions, even after a 9-h reaction time. A control experiment was conducted in which a piece of EAA-Cl film was immersed in water for 2 days, but no acid peak was observed in the ATR-FTIR spectrum, indicating that a large amount of acid chlorides in the subsurface were not hydrolyzed. By neutralizing hydrochlorides produced in the reaction, KOH solution promoted the
anchoring reaction of dansyl cadaverine at the surface, but the aqueous solution still could not reach the acid chlorides in the subsurface region.

The static water contact angles for the unmodified and modified EAA films were measured and it was found that the majority of the contact angles were approximately 98-101°, not only because of the low coverage of carboxylic acids on the surface but also because of the hydrophobic nature of the two-member rings on dansyl cadaverine. In fact, the high contact angle values provided some hint of the surface density of carboxylic acids. As estimated by Holmes-Farley [24], the contact angles for polyethylene (PE), PE-COOH (treated by chromic acid), and acid-terminated monolayers are 103°, 55°, and 0°, respectively. For our EAA film, the contact angle was approximately 99°, indicating that the acrylic acid surface coverage on EAA film was low. This is reasonable since the weight percentage of acrylic acid in this EAA was 9.5%, which means that the polymer consisted of approximately 25 ethylene units for every acrylic acid. Therefore, the acid content is only 4% on a molar basis, and the Cassie equation [26] would then predict only a small deviation from the contact angle of neat polyethylene. The bulk density of the EAA film (Primacor 1410) is 0.938 g/cm³, and the weight percentage of acrylic acid is 9.5%. Assuming that the “surface layer” is 5 Å thick and the carboxylic acid groups are distributed uniformly within the layer, then the site density of the carboxylic acid groups is estimated to be 0.37 nm⁻² from equation (2). Therefore, the distance between grafting sites is approximately 1.8 nm from equation (3). In addition, the surface heterogeneity of EAA forms ethylene-rich and acrylic-acid-rich regions, so it is reasonable to conclude
that grafting small molecules on the EAA surface would not modify its wettability significantly, particularly with only 9.5% acrylic acid content.

We were curious as to why there was no reaction using dichloromethane as the solvent, so we conducted another series of experiments by immersing EAA and EAA-Cl film specimens in a more concentrated (1 mg/ml) dansyl cadaverine dichloromethane solution for a shorter time (10 s). Fluorescence intensities for those samples are shown in Figure 3.3 and the height of each bar denotes the fluorescence intensity for each specimen. It was observed that there was no significant difference between neat EAA film, EAA-Cl film, and EAA and EAA-Cl films attempted to be modified in dilute solution of dansyl cadaverine, but a dramatic increase occurred for EAA-Cl film reacted in the more concentrated solution. The control experiment in which unmodified EAA film was immersed in the concentrated solution showed a fluorescence intensity equivalent to that of neat EAA, as expected. The dramatic increase of fluorescence intensity could be explained by the higher penetration rate of dansyl cadaverine from concentrated solution than from dilute solution, so that a large amount of dansyl cadaverine was rapidly absorbed into the film and grafted over a period of 10 s. In the concentrated-solution case, the increased intensity was due to dansyl cadaverine grafted to the film since the fluorescence measurement was conducted after 24 h of Soxhlet extraction in dichloromethane.
Figure 3.2 ATR-FTIR spectra for EAA activation and dansyl cadaverine grafting: (1) neat EAA; (2) EAA-Cl; (3) EAA-Cl reacted in dansyl cadaverine/KOH solution; (4) EAA-Cl reacted in dansyl cadaverine/pH 7 solution; (5) EAA-Cl reacted in dansyl cadaverine/CH₂Cl₂ solution. Solution concentrations were 0.1 mg/ml and reaction time was 9 h.
**Figure 3.3** Fluorescence intensities: (1) neat EAA and EAA-Cl; (2) EAA and EAA-Cl films after immersion in 0.1 mg/ml dansyl cadaverine/CH\(_2\)Cl\(_2\) solution at room temperature for 9 h; (3) EAA and EAA-Cl films after immersion in 1 mg/ml dansyl cadaverine/CH\(_2\)Cl\(_2\) solution at room temperature for 10 s. Soxhlet extraction was then performed for 1 day in dichloromethane to remove unreacted material from the film.

To estimate the speed of the dansyl-cadaverine-attaching reaction in a 1 mg/ml dansyl cadaverine solution with dichloromethane, a series of reactions was conducted for different immersion times from 1 s to 2 min. Figure 3.4 summarizes the ATR-FTIR results. The amide peak at 1645 cm\(^{-1}\) increased while the acid chloride peak at 1793 cm\(^{-1}\) decreased as the reaction proceeded. The intensity of the amide peak was used to study the time-dependence of the amide formation within the analysis depth of the ATR-FTIR.
The grafting depth can be estimated as

$$d = \frac{A}{A_\infty} \times d_p$$

(4)

where $A$ is the peak area at 1645 cm$^{-1}$ at any given time, $A_\infty$ represents the peak area at 24 hrs when all of the acid chloride groups were converted into amides ($A_\infty \sim 0.5$), and $d_p$ is the analysis penetration depth of ATR-FTIR ($d_p = 403$ nm at $\lambda = 1645$ cm$^{-1}$ [22]). Results obtained using the ATR-FTIR data and equation (4) are shown in Figure 3.5. Also shown are the fluorescence intensities at $\lambda_{\text{emission}} = 510$ nm measured directly using $\lambda_{\text{excitation}} = 340$ nm for all samples. As expected, the rate of amide formation within the subsurface region of the film indicated a gradual decrease with time, faster at the beginning and then slowing down. The fluorescence data indicated the same trend. The ATR-FTIR and fluorescence results in Figure 3.5 could be superimposed if the y-axis of the fluorescence data were chosen properly. However, the intent is not to superimpose the data, but to demonstrate that the two sets of data can be correlated.
Figure 3.4 ATR-FTIR spectra as a function of grafting time in 1 mg/ml dansyl cadaverine solution in dichloromethane. From bottom to top, the spectra correspond to immersion times of the film after 1, 5, 10, 20, 40, 60 and 120 s at room temperature.

Figure 3.5 Relationship of reaction depth (obtained from the peak area from ATR-FTIR data) and fluorescence intensity with immersion time for 1 mg/ml dansyl cadaverine solution in dichloromethane.
It was evident that the relatively small dansyl cadaverine penetrated into the EAA film. To assess the depth of penetration as a function of molecular size, a subsequent grafting study was performed using a series of PEGs with various molecular weights. The hypothesis was that the grafting would occur first on the surface and then polymer chains would have to overcome a potential barrier to penetrate into the film. Initially, a control experiment was conducted in which a reaction was attempted between PEG99 and EAA-Cl film in dichloromethane at room temperature. Comparing ATR-FTIR spectra for EAA-Cl film and EAA-Cl film reacted with PEG99, no grafting was evident over a 1-day reaction period. However, at 60 °C in dichloromethane, the acid chloride peak decreased while an ester peak at 1743 cm\(^{-1}\) and a 1110 cm\(^{-1}\) peak (corresponding to the ether groups in the grafted PEG) appeared. The contact angle also showed a large decrease of approximately 40° for EAA-g-PEG99. However, as reaction time increased, hydrolysis occurred with the appearance of a peak at 1705 cm\(^{-1}\), and there was still a large amount of acid chloride groups left in the film, suggesting that after the outermost PEG layer formed, it was more difficult for other PEG chains of that size to penetrate and react. As the grafting rate is affected by temperature, 60 °C was used for all subsequent PEG grafting. A higher temperature was avoided so that any crystallinity changes in EAA, a semi-crystalline polymer with a melting point of 95 °C, would be minimized. To demonstrate that the 60 °C was not altering the EAA crystallinity during grafting, DSC experiments were conducted. DSC data of neat EAA, EAA-Cl, and PEG39 grafted films showed crystallinities of 18%, 17%, and 20%, respectively, based on the measured
enthalpies of crystallization and a value of $\Delta H^o = 286 \text{ J/g}$ [27], the heat of fusion of perfectly crystalline polyethylene.

![Figure 3.6](image)

Figure 3.6 ATR-FTIR data showing the penetration reaction process of PEG39 in EAA-Cl film at 60 °C in 0.01 mol/l dichloromethane solution. From bottom to top, the spectra represent immersion times of 2, 4, 6, 12, 24, 72 and 120 h.

The extent of the grafting reaction as a function of immersion time in dichloromethane was first studied. Figure 3.6 presents the FTIR spectra of the film grafted with PEG39. It can be observed that the acid chloride peak at 1793 cm$^{-1}$ decreased with time, while the ester peak at 1743 cm$^{-1}$ increased. However, hydrolysis occurred at later times, as evidenced by the increase in the 1705 cm$^{-1}$ peak, also consuming acid chloride groups within the analysis depth. When the reaction time was
extended to 120 h, it was found that the acid chloride peak at 1793 cm\(^{-1}\) disappeared with the appearance of a large carboxylic acid peak at 1705 cm\(^{-1}\), suggesting that all of the acid chlorides not grafted with PEG were hydrolyzed to acids.

**Figure 3.7** Simplified schematic of the penetration reaction process inside of EAA-Cl film.

When a control experiment was conducted using neat EAA films immersed in the same reaction solution at the same temperature, no peaks appeared at 1743 cm\(^{-1}\) and 1110 cm\(^{-1}\), and the contact angles of those samples were similar to neat EAA and EAA-Cl within ±5°. Therefore, no PEG grafting occurred on neat EAA without activation. It also suggested that covalent bonding played an important role in the penetration reaction process. A depiction of the penetration reaction process is shown in Figure 3.7.
from top to bottom, the figure has three regions within 400 nm, the grafted, hydrolyzed, and activated regions. Hydrolysis occurred beneath the grafted region, extending deep inside the film over time, because a small amount of water was taken up into the film by the grafted hydrophilic PEG chains. Based on the acid peaks, the hydrolysis increased with PEG size at a given time, since, on a molar basis, more water was associated with larger PEG chains.

The accessibility and reactivity of a PEG’s hydroxyl group to the acid chloride should limit the esterification reaction rates. Therefore, PEGs with different molecular weight should have different subsurface grafting behavior because larger molecules can not penetrate as deeply into the film, resulting in a relatively low grafting efficiency but potentially improved surface hydrophilicity. The grafting process was performed under the same conditions as the earlier experiments, the only difference being the use of four PEGs. Equation (2) was used to estimate the grafting depths for the PEGs, where the peak area of interest was the ester peak at 1743 cm$^{-1}$. To determine $A_\infty$ for this case, methanol was used as a small molecule that would penetrate into the film and convert all of the acid chlorides to esters (analogous to hydroxyl-terminated PEGs reacting with acid chlorides). All of the acid chlorides were converted to esters within 24 h of immersion in methanol to yield $A_\infty \sim 0.18$. The grafting depth as a function of reaction time for different PEG derivatives is shown in Figure 3.8. The grafting depth for PEG4 and PEG11 increased continuously but the rate of increase slowed beyond 6 h. The larger PEG39 and PEG99 chains achieved less grafting depth to confine the grafting nearer to
the surface. Therefore, the grafting depth can be controlled either by grafting time or molecular size.

![Graph showing grafting depth versus reaction time for PEG4, PEG11, PEG39 and PEG99](image)

**Figure 3.8** Grafting depth versus reaction time for PEG4, PEG11, PEG39 and PEG99 (0.01 mol/l in dichloromethane at 60°C). The figure does not show the grafting depth for PEG4 and PEG11 at 72 h, because they had already reached the analysis penetration depth of ATR-FTIR at 24 h.

Static water contact angles can be used to assess the wettability of the outermost few Å of the modified films. Figure 3.9 showed that the static water contact angle of neat EAA was approximately 99°. After grafting with different PEG derivatives, the contact angle decreased continuously to approximately 60° for EAA-g-PEG99. This decrease is reasonable because larger PEG molecules will cover more surface area with hydrophilic ethylene glycol repeat units.
Summary

Dansyl cadaverine and PEG derivatives were grafted successfully on EAA surfaces and in its subsurface region. For dansyl cadaverine grafting, ATR-FTIR spectra and fluorescence intensities indicated that the acid chloride groups of EAA-Cl were not hydrolyzed in aqueous solution because the moisture could not reach the acid chlorides in the subsurface regions, thus protecting those reactive functional groups. Concentrated dansyl cadaverine solution in dichloromethane promoted subsurface grafting with a higher penetration rate than in dilute solution. In a few minutes, the dansyl cadaverine penetrated throughout the analysis region (~400 nm) afforded by ATR-FTIR spectroscopy. In a subsequent study, four PEGs, PEG99, 39, 11, and 4, were grafted on the EAA surface.
and in the subsurface region. Three regions were proposed to explain the penetration behavior of PEG chains, including the grafted, hydrolyzed, and activated regions. It was found that the two larger PEGs achieved less grafting depth to confine the grafting nearer to the surface. For PEG99, the grafting depth was about 180 nm after 24 h of reaction time. However, after grafting with different PEG derivatives, the static water contact angle decreased to approximately 60° for EAA-g-PEG99, almost 40° lower than that of neat EAA.
References


CHAPTER 4
SURFACE GRAFTING OF POLYETHYLENE GLYCOL (PEG) ONTO POLY(ETHYLENE-CO-ACRYLIC ACID) FILMS

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**Introduction**

Currently, several techniques are being used to attach small molecules and thin polymer films to solid substrates, including physisorption [1, 2], chemical coupling [3-6], electrostatic adsorption [7-9], and graft polymerization [10-12]. Chemical coupling and graft polymerization allow for a more permanent surface modification through covalent bonds between grafted molecules and the substrate. The substrate of interest in this work is poly(ethylene-co-acrylic acid) (EAA), which contains carboxylic acid groups not only on the outermost surface but also throughout the bulk. Because EAA contains reactive carboxylic acid groups, it can undergo chemical coupling after activation with phosphorus pentachloride [13], thionyl chloride [14], DCC [15] or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) [16]. In previous studies of surface grafting on EAA, phosphorus pentachloride was used to activate the carboxylic acid groups, however, it was found that phosphorus pentachloride in dichloromethane penetrated into the EAA films, easily converting all of the carboxylic acid groups to acid chlorides [17].
Likewise for graft polymerization (e.g., photografting), monomers may also penetrate into the polymer film, leading to polymerization within the film bulk instead of being confined to the surface [10]. Generally, the issue of reactant penetration into the polymer and subsequent reaction in the subsurface or bulk can be prevalent in a solution reaction system. Better control of a surface modification at the nanometer scale provides versatile surface functionality without changing the bulk properties. In this work, two strategies were investigated in order to achieve covalent grafting of poly(ethylene glycol) (PEG) on EAA film with minimal penetration and grafting in the subsurface region. In recent years, the immobilization of PEG on material surfaces to improve biocompatibility and to minimize cell adhesion has been well studied and various techniques have been used [18-25]. Relatively little has been published on EAA film related to PEG grafting, but it is a substrate that is amenable to surface chemistry and impervious to many solvents, making it attractive for potential applications in biotechnology [16]. In addition, EAA has been used extensively as an adhesive layer on metals, glass, cellulosics, and plastics. Thus, it could be coated as a thin layer on many biomedical implants and devices to provide a wide range of functionality.

Our previous work showed that the activation step of converting EAA acid groups to acid chlorides was associated with significant penetration of the PCl₅ into the film bulk. However, water did not significantly penetrate the film. Therefore, an aqueous system
offers another strategy for activation of carboxylic acids for subsequent surface modification. In this work, N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were used to activate EAA surfaces in aqueous solution, and the activated EAA films were subsequently grafted with \textit{l}-lysine or dendrimer to serve as intermediate layers. Our objectives were to minimize the grafting depth and to study the effect of an increase in surface functional groups on subsequent PEG grafting. The number of functional groups was increased using a dendrimer, which was an amine-terminated, commercially available polypropyleneamine, Astramol-Am-64 (AM64). To be consistent with the amine-terminated dendrimer, we used \textit{l}-lysine as an intermediate layer to provide amine-grafting sites, albeit at a lower surface density. Also, the \textit{l}-lysine did not penetrate significantly into the activated EAA films, so it could contribute to the objective of minimizing the grafting depth. The terminal amine groups from \textit{l}-lysine or AM64 were reacted with PEG chains as shown in Figure 4.1. Each step of the surface modification was followed by ATR-FTIR spectroscopy, XPS, and contact angle goniometry.
Experimental

Materials

Poly(ethylene-co-acrylic acid) (EAA, PRIMACOR 1410 from Dow Chemical Co., 9.5% w/w acrylic acid) films (thickness ~ 50 micron) were used as received from the Cryovac Division of Sealed Air Corp. (Duncan, SC). Hydroxyl-terminated methyl poly(ethylene glycol) ether (PEG, $M_W = 750$) was purchased from Aldrich and used without further purification. Sodium chloride (NaCl) and hydrochloric acid (HCl) were obtained from VWR. Polypropyleneamine dendrimer, Astramol-Am-64 (AM64), L-lysine, glycine, 1,1’-carbonyldiimidazole (CDI), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and N,N’-dicyclohexylcarbodiimide (DCC) were purchased from Aldrich. Phosphate buffered saline (PBS) tablets were obtained from EMD Biosciences Inc.

Synthesis of carboxyl-terminated PEG (mPEG)

Hydroxyl-terminated methyl PEG ether ($M_W = 750$) was reacted with equal moles of CDI in dichloromethane, and then stirred with an excess amount of aqueous glycine solution ($pH = 7.0$) at room temperature. After 20 min, NaCl was added to the solution to saturate it and HCl was added to adjust the pH to 2.0. The organic phase was washed with acidic brine (HCl + NaCl) ($pH = 2.0$) three times and then neutral brine ($pH = 7.0$) three times. Dichloromethane was removed by vacuum drying to yield a wax-like product,
denoted mPEG (yield ~ 90%). FTIR peaks (cm⁻¹) for PEG: 3474 (OH), 1110 (C-O-C); for mPEG: 1710 (C=O acid), and 1110 (C-O-C).

**Activation of EAA surfaces (EAA-NHS)**

EAA carboxylic acid groups were activated by immersing EAA film in NHS/EDC (200 mM/100 mM) phosphate buffered saline (PBS, pH = 7.4) solution for 1 h with sonication. The film was then rinsed with PBS buffer three times for a period of 5 min each with fresh solution each time (film denoted as EAA-NHS). In addition, another EAA film was sonicated for 1 h and immersed in the same solution for 2 days to study the effect of time on the activation depth of reaction. Activation experiments were also done using only EDC (without NHS) to study the extent of penetration in the near surface region of the film.

**Coupling of mPEG on activated EAA films using l-lysine (EAA-Lysine-PEG)**

The activated EAA-NHS specimens were washed in PBS buffer solution and then used for a l-lysine reaction. First, EAA-NHS film was immersed in a 3% (w/w) l-lysine pH 10 buffer solution and shaken gently for 24 h at room temperature using platform shaker. The film was then thoroughly rinsed with HPLC water three times to remove unreacted l-lysine and treated in pH 12 buffer solution for 2 h to deprotonate the remaining primary amine of l-lysine, introducing another free amine group for subsequent PEG grafting. The specimen was dried in a vacuum oven for 6 h at room
temperature for further use and denoted as EAA-Lysine. The PEG was grafted on EAA-Lysine surface in dichloromethane. First mPEG and DCC (1 mol/0.8 mol) were added to 20 ml dichloromethane, and the solution was left for 6 h with gentle shaking using platform shaker. EAA-Lysine film was immersed in the reaction solution for two days at room temperature. The film was then rinsed with dichloromethane and acetone consecutively three times and dried at reduced pressure. The film was denoted as EAA-Lysine-PEG.

**Coupling of mPEG on activated EAA films using a dendrimer (EAA-AM64-PEG)**

As above, AM64 was grafted on the EAA-NHS surface by immersion in aqueous AM64 solution (5 mM) for 1 day at room temperature. After rinsing with copious amounts of HPLC water three times with sonication, the film was dried at reduced pressure overnight. The specimen (EAA-AM64) was used for PEG grafting using the same method described above. The grafted specimen was denoted as EAA-AM64-PEG.

**ATR-FTIR spectroscopy**

A Nicolet Avatar 360 FTIR spectrometer was used to investigate the chemical changes after each reaction step and to provide some evidence concerning the penetration of reactants into the EAA film. The penetration depth $d_p$ of the evanescent wave from the reflected light was calculated according to the method in reference [26]. The calculation determined that the average penetration depth of the evanescent wave for the carboxylic
acid grafted EAA film at $\lambda = 1705$ cm$^{-1}$ was approximately 400 nm. Please refer to Chapter 2 for details.

**X-ray photoelectron spectroscopy (XPS)**

XPS data were obtained with a Kratos Axis Ultra photoelectron spectrometer with Al K$\alpha$ radiation (15 kV, 225 W). Peak shifts for the carbon and nitrogen functionalities were taken from the literature [27, 28]. Please refer to Chapter 2 for details.

**Contact angle measurement**

Dynamic contact angle measurements were made with a Krüss G10 instrument. Please refer to Chapter 2 for more information. Five measurements for each specimen were performed, yielding the mean dynamic contact angle and standard deviations. Contact angle hysteresis, $H$, was obtained by the following equation [29, 30]:

$$H = \theta_a - \theta_r$$

(1)

Surface energies were calculated from the dynamic contact angle data using the harmonic mean approximation of Young’s equation [29]:

$$(1 + \cos \theta_i)(\gamma_i^d + \gamma_i^p) = 4 \left( \frac{\gamma_i^d \gamma_s^d}{\gamma_i^d + \gamma_s^d} + \frac{\gamma_i^p \gamma_s^p}{\gamma_i^p + \gamma_s^p} \right)$$

(2)

where $\theta_i$ is the advancing contact angle of liquid $i$ on the polymer surface, $\gamma_i^d$ and $\gamma_i^p$ are the dispersive and polar surface energy components of liquid $i$, and $\gamma_s^d$ and $\gamma_s^p$ are the dispersive and polar surface energy components of the polymer.
Results and Discussion

Poly(ethylene-co-acrylic acid) (EAA) contains carboxylic acid groups on the outermost surface and throughout the bulk, which can be used as reactive sites for surface grafting. In this work, studies focused on covalently grafting PEG chains on EAA film surfaces with an aim of minimizing grafting depth. Two reaction systems were studied as shown in Figure 4.1. l-Lysine or dendrimer AM64 were grafted onto the EAA surface in aqueous solution to introduce primary amines for further PEG grafting. Table 4.1 summarizes the various specimens and their notations.

Table 4.1 Experimental conditions and sample notations for surface grafting of PEG on EAA films.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Pretreatment</th>
<th>Intermediate layer</th>
<th>PEG grafting</th>
<th>Notation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>EAA</td>
</tr>
<tr>
<td>2</td>
<td>HPLC water</td>
<td></td>
<td></td>
<td>EAA-D-water</td>
</tr>
<tr>
<td>3</td>
<td>dichloromethane</td>
<td></td>
<td></td>
<td>EAA-D-dichloromethane</td>
</tr>
<tr>
<td>4</td>
<td>EDC/NHS</td>
<td>l-Lysine</td>
<td></td>
<td>EAA-NHS</td>
</tr>
<tr>
<td>5</td>
<td>EDC/NHS</td>
<td>l-Lysine</td>
<td>DCC, mPEG</td>
<td>EAA-Lysine-PEG</td>
</tr>
<tr>
<td>6</td>
<td>EDC/NHS</td>
<td>Dendrimer</td>
<td>DCC, mPEG</td>
<td>EAA-AM64</td>
</tr>
<tr>
<td>7</td>
<td>EDC/NHS</td>
<td>Dendrimer</td>
<td>DCC, mPEG</td>
<td>EAA-AM64-PEG</td>
</tr>
</tbody>
</table>
Figure 4.1 Strategies for surface modification with PEG on EAA surfaces.
Activation of carboxylic acids

NHS and EDC were first used to activate the carboxylic acid groups of EAA. Successful formation of an ester intermediate with controlled depth was dependent on the penetration of EDC and NHS into the film from the PBS buffer solution. In Figure 4.2, the bottom spectrum for EAA showed a peak at 1705 cm\(^{-1}\), characteristic of the acid groups. When only EDC was used to activate the acids, the acid peak (Figure 4.2) decreased noticeably, and two new peaks appeared at 1820 and 1745 cm\(^{-1}\) that represent the C=O stretching vibrations of anhydride [15, 31]. When NHS and EDC were used together to activate the carboxylic acids, ATR-FTIR spectra showed that a small shoulder appeared at 1734 cm\(^{-1}\) due to the formation of NHS ester, but the acid peak did not decrease as significantly as when only EDC was used because ATR-FTIR was still detecting a large number of acids within its 400 nm probe depth. As discussed in Chapter 2 on Beer’s law, the peak area of –COOR should be about two times larger than that of –COOH at a given concentration. When an even smaller shoulder at 1734 cm\(^{-1}\) appeared, it indicated that only the carboxylic acids on the outermost surface were activated, forming NHS ester. The grafting reaction occurred on the outermost surface and no significant penetration occurred even after 2 days’ reaction period. Therefore, the activated depth was lower than that when only EDC was used, because NHS replaced the EDC after activation and was stable on the surface as shown in Figure 4.3. The NHS existing on the
EAA surface apparently prevented EDC from penetrating significantly and inhibited the formation of anhydrides deeper into the film. We believe that the vast majority of the EDC and NHS molecules that penetrated into the film react with acid groups. This is not to say that there is absolutely no free EDC or NHS remaining in the film, but the amount is so low that we cannot detect them accurately. No distinct peaks are observed that can be attributed to free EDC or NHS. In addition, if there was a significant amount of unreacted EDC or NHS, XPS would have shown a much higher than expected nitrogen content, which was not observed (see the XPS discussion below).

Figure 4.2 ATR-FTIR spectra of NHS and EDC activation (bottom to top, EAA, EAA-EDC with 1 h sonication, EAA-NHS with 1 h sonication, and EAA-NHS for 2 days).
Figure 4.3 Activation of carboxylic acid groups with NHS and EDC.

ATR-FTIR is not truly surface-sensitive, but it can provide information about chemistry occurring within depths of several hundred nanometers or a few microns depending on the configuration of the system. For our ATR-FTIR configuration, the average penetration depth of the evanescent wave for the carboxylic acid formation at $\lambda = 1705 \text{ cm}^{-1}$ was approximately 400 nm. To estimate the activation depth of NHS anchored in the EAA subsurface, area ratios of the peaks $1705 \text{ cm}^{-1}$ and $2850 \text{ cm}^{-1}$ were used. The peak at $1705 \text{ cm}^{-1}$ represents the carboxyl stretch of the acid groups while that at $2850 \text{ cm}^{-1}$ represents the out-of-phase stretching of the hydrogen atoms in the CH$_2$ groups [27]. On the basis of ATR-FTIR data, the activation depth was estimated from
\[ d = (1 - \frac{(A_{1705} / A_{2850})_1}{(A_{1705} / A_{2850})_0}) \times d_p \]  

(3)

where \((A_{1705} / A_{2850})_1\) is the peak area ratio for NHS anchored film, \((A_{1705} / A_{2850})_0\) is that for neat EAA, and \(d_p\) is the analysis penetration depth due to ATR-FTIR. Based on ATR-FTIR spectra from five different specimens, we estimated that the NHS activation depth was limited to 20 nm with a 0.5 nm standard deviation, much less than one would observe using PCl₃ as an activating agent [17].

Further analysis was done using XPS, including survey scans and high resolution C 1s spectra (Figure 4.4). Table 4.2 summarizes the elemental compositions. For neat EAA, the oxygen content was 4.1% and corresponds well with the theoretical oxygen content of this EAA film of 4.9%. Carboxylic acids of EAA film were activated by EDC and NHS with the formation of NHS ester, introducing nitrogen and oxygen in the spectra. From the survey scans of EAA and EAA-NHS, a nitrogen peak appeared at 401 eV and the oxygen content increased at 530 eV, as expected. Assuming all the carboxylic acid groups were derivatized through ester formation in the NHS reaction, the calculated atomic ratio of O/C should be around two times the O/C ratio of EAA, which was 0.04 from Table 4.2. Experimental survey XPS data showed that the O/C ratio of EAA-NHS was around 0.09, indicating the conversion of the activation step was complete in the region probed by XPS (around 10 nm).
Figure 4.4 XPS survey scans (inset) and high resolution spectra for: (A) EAA and (B) EAA-NHS.
Table 4.2 Summary of XPS elemental composition for derivatization of modified EAA samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>C</th>
<th>N</th>
<th>O</th>
<th>N/C</th>
<th>O/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAA</td>
<td>95.9</td>
<td>0</td>
<td>4.1</td>
<td>0.00</td>
<td>0.04</td>
</tr>
<tr>
<td>EAA-NHS</td>
<td>89.6</td>
<td>2.5</td>
<td>7.9</td>
<td>0.03</td>
<td>0.09</td>
</tr>
<tr>
<td>EAA-Lysine</td>
<td>88.0</td>
<td>4.1</td>
<td>7.9</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>EAA-Lysine-PEG</td>
<td>85.7</td>
<td>4.2</td>
<td>10.1</td>
<td>0.05</td>
<td>0.12</td>
</tr>
<tr>
<td>EAA-AM64</td>
<td>86.6</td>
<td>6.1</td>
<td>7.3</td>
<td>0.07</td>
<td>0.08</td>
</tr>
<tr>
<td>EAA-AM64-PEG</td>
<td>81.0</td>
<td>5.9</td>
<td>13.1</td>
<td>0.07</td>
<td>0.16</td>
</tr>
</tbody>
</table>

High resolution C 1s spectra provided more detail in Figure 4.4 and Table 4.3. Unmodified EAA exhibited three peaks, 289.5, 285.5 and 285.0 eV, which are characteristic of COOH, C-COOH, and C-C species, respectively. The peak at 289.5 eV confirmed the presence of carboxylic acid groups existing on the surface of the film, and the amount of carboxylic acid groups was 1.5% (Table 4.3). However, through theoretical calculation by acrylic acid content in EAA film, this fraction was expected to be 1.9%, slightly higher than observed experimentally. Thus the acid content at the surface was less than that in the bulk for EAA films. This phenomena was also observed by Yasuda et al. [32]. After reaction with NHS, newly formed ester groups were detected at 289.2 eV, and new peaks appeared at 288.2 and 286.3 eV, corresponding to the carbon atoms of -C=O (N<) in the ring and the carbon atoms next to it.
Table 4.3 XPS high resolution C (1s) of NHS/EDC activation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Binding Energy (eV)</th>
<th>Corresponding Species</th>
<th>Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAA</td>
<td>285.0</td>
<td>C-C</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>285.5</td>
<td>C-COOH</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>289.5</td>
<td>COOH</td>
<td>1.5</td>
</tr>
<tr>
<td>EAA-NHS</td>
<td>285.0</td>
<td>C-C</td>
<td>84.9</td>
</tr>
<tr>
<td></td>
<td>285.5</td>
<td>C-COOH,C-COOR</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>286.3</td>
<td>C-CON&lt; or C-EDC</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>288.2</td>
<td>CON&lt;</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>289.2</td>
<td>COOH or COOR</td>
<td>2.7</td>
</tr>
</tbody>
</table>

**Covalent grafting with intermediate layers and attachment of activated PEG**

After activation of carboxylic acids with NHS, l-lysine or AM64 were grafted as intermediate layers. l-lysine has α- and ω-amine groups, with the ω-amine exhibiting stronger basicity (pKₐ = 10.53) than the α-amine (pKₐ = 8.95) [13]. Therefore, in a pH 10 buffer solution, the α-amines were more extensively deprotonated, which were replaced covalently by NHS groups through an amidation reaction. Then ω-amine was treated by pH 12 buffer solution before it reacted as linker group to attach PEG onto the EAA surface. The same amidation reaction also occurred between dendrimer AM64 and the EAA-NHS surface, with the dendrimer providing more primary amine groups for further grafting. After immersion in l-lysine solution or dendrimer solution for 1 day, ATR-FTIR
spectra did not show obvious peaks at 1645 cm$^{-1}$ (amide I) and 1545 cm$^{-1}$ (amide II), attributed to the covalent bond formation between primary amines and NHS ester groups. These peaks were not observed because the ATR-FTIR spectra were overwhelmed by the chemical changes in subsurface region (400 nm deep), while the reactions were occurring on the surface of EAA film. It was reasonable to conclude that the dendrimer did not penetrate into the film because of its large molecular size (around 2.3 nm) [33]. However, whether the l-lysine penetrated into the activated EAA film was still not known. A control experiment was done with l-lysine grafted onto EAA-Cl, which had reactive acid chloride groups throughout the film (preparation of EAA-Cl was described elsewhere [17], and the control experiment was conducted using the same steps as above). The activated EAA-Cl film was used because l-lysine will not graft to the carboxylic acids in aqueous solution without activation and we wanted activated acids deep into the bulk of the film. As shown in the ATR-FTIR spectra (Figure 4.5), peaks appeared at 1645 and 1420 cm$^{-1}$, which were attributed to amide formation from the reaction of the l-lysine with acid chlorides. However, the acid chloride peak at 1790 cm$^{-1}$ did not decrease substantially (even with sonication), so the l-lysine did not exhibit significant penetration into the film. Therefore, either large molecules or charged species could be used to control the surface modification precisely on the outermost surface of an EAA film.
For these two modified surfaces, EAA-Lysine and EAA-AM64, there were three well resolved carbon peaks in the C 1s region (Table 4.4): 285.0, 288.4, and 286.0 eV, which correspond to C-C, CO-NH, and carbons next to amides and primary amines, respectively. Based on XPS survey scan data (Table 4.2), the N/C ratio of EAA-Lysine was almost two times that of EAA-NHS, while the O/C ratio was the same, as expected from the reaction scheme since l-lysine has two nitrogen atoms and NHS has only one. The successful grafting of dendrimer was also clearly shown from the substantial increase in nitrogen content and higher N/C ratio. Additionally, a high resolution C 1s scan of EAA-AM64
(Table 4.4) showed a lower percentage of carbon in –CONH- group but a higher percentage of carbon in –C-NHCO- and –C-NH2-, indicating a relatively small amount of amine reacted with NHS on the surface, so a high concentration of free amine groups were still available for further PEG grafting.

**Table 4.4** XPS high resolution C (1s) results for PEG-modified samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>285.0 eV</th>
<th>286.0 eV</th>
<th>288.4 eV</th>
<th>286.6 eV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C-C</td>
<td>C-NH-CO, C-NH2</td>
<td>CONH</td>
<td>C-O-C</td>
</tr>
<tr>
<td>EAA-Lysine</td>
<td>78.6</td>
<td>16</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>EAA-Lysine-PEG</td>
<td>75.6</td>
<td>7</td>
<td>7.3</td>
<td>10.1</td>
</tr>
<tr>
<td>EAA-AM64</td>
<td>75.5</td>
<td>20.8</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>EAA-AM64-PEG</td>
<td>66.8</td>
<td>1.8</td>
<td>3.4</td>
<td>28.1</td>
</tr>
</tbody>
</table>

Upon PEG-grafting, a small amount of PEG was observed by XPS on the EAA-Lysine-PEG surface, which showed an increase in O/C ratio from 0.09 to 0.12 (Table 4.4). For the EAA-AM64-PEG surface, the O/C ratio increased from 0.08 to 0.16. High resolution C 1s XPS showed four well-resolved carbon peaks (Figure 4.6), with the one at 286.6 eV (corresponding to C-O-C ether of grafted PEG) clearly showing a greater concentration of PEG chains on EAA-AM64-PEG surfaces than on EAA-Lysine-PEG.
Figure 4.6 XPS high resolution C 1s spectra for: EAA-Lysine-PEG (left) and EAA-AM64-PEG (right).

Surface refinement and wettability contrast

Water and diiodomethane (DIM) were used to investigate the surface wettability. Table 4.5 shows the dynamic contact angles and surface roughness (determined by AFM). Before activation, the advancing water contact angle of neat EAA was 101° and the receding water contact angle was 71°, yielding a hysteresis of 30°. After the EAA was activated in EDC/NHS aqueous solution, the advancing contact angle decreased to 84° and the receding contact angle decreased to 47°. However, a control experiment was done
where a neat EAA film was immersed in the same PBS buffer solution without EDC and NHS and it was found that the advancing contact angle also decreased to 88°. The EAA surface, therefore, reconstructed and became more hydrophilic during immersion in the aqueous buffer solution [34].

Table 4.5 Dynamic contact angles and surface free energies for each sample (H = hysteresis and RMSR = root mean square roughness).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Water</th>
<th>Diodomethane</th>
<th>Surface Energies (mJ/m²)</th>
<th>RMSR (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>θₐ</td>
<td>θᵣ</td>
<td>H</td>
<td>θₐ</td>
</tr>
<tr>
<td>EAA</td>
<td>101</td>
<td>71</td>
<td>30</td>
<td>52</td>
</tr>
<tr>
<td>EAA-D-water</td>
<td>88</td>
<td>39</td>
<td>49</td>
<td>46</td>
</tr>
<tr>
<td>EAA-D-dichloroform</td>
<td>94</td>
<td>73</td>
<td>21</td>
<td>44</td>
</tr>
<tr>
<td>EAA-NHS</td>
<td>84</td>
<td>47</td>
<td>37</td>
<td>51</td>
</tr>
<tr>
<td>EAA-Lysine</td>
<td>80</td>
<td>38</td>
<td>42</td>
<td>48</td>
</tr>
<tr>
<td>EAA-Lysine-PEG</td>
<td>84</td>
<td>34</td>
<td>50</td>
<td>42</td>
</tr>
<tr>
<td>EAA-AM64</td>
<td>70</td>
<td>5</td>
<td>65</td>
<td>43</td>
</tr>
<tr>
<td>EAA-AM64-PEG</td>
<td>53</td>
<td>0</td>
<td>53</td>
<td>49</td>
</tr>
</tbody>
</table>

As compared to unmodified EAA, EAA-Lysine did not increase the surface wettability as shown by the advancing and receding contact angles for both water and DIM. However, for EAA-AM64, the advancing water contact angle decreased to 70°, and the receding water contact angle was 5°. For measurements with water, the hysteresis of samples increased from 30° for neat EAA to around 65° after grafting with dendrimer
AM64, but for diiodomethane the hysteresis was less than 20°. Typically, hysteresis is attributed to surface roughness, surface chemical heterogeneity, swelling with liquid penetration [35], as well as the reorientation of polar groups on the surface in contact with a polar liquid, such as water [36]. One possible explanation for the high hysteresis is that the polar interaction became stronger and led to increased hysteresis as more polar groups were introduced on the surface after attachment of l-lysine or dendrimer AM64. Further supporting evidence was provided when apolar DIM was used. In these cases, hysteresis values were lower than 20°, which meant that there was no significant polar interaction occurring on the surfaces of modified film specimens. Another possible explanation for the higher hysteresis for the dendrimer-grafted surfaces is the correspondingly higher RMS roughnesses shown in Table 4.5 (the advancing contact angle may increase with roughness while the receding contact angle remains the same [37]). However, comparing the EAA and EAA-AM64-PEG surfaces for example, the roughness changed by less than a factor of 2, and all of the surfaces were relatively smooth with order-of-magnitude RMS roughnesses ~1 nm. Additionally, θ_a did not increase with roughness as might be expected. The decrease in θ_a from EAA to EAA-AM64 to EAA-AM64-PEG was another indicator of the high concentration of PEG chains grafted to the surface.
Comparing the two methods to attach PEG chains on the EAA surface via L-lysine or AM64, advancing water contact angle for EAA-AM64-PEG decreased most until 53° and was attributed to more fractional coverage of PEG on EAA. The receding water contact angle for EAA-AM64-PEG was zero, indicating enhancement of attraction between receding water molecules and grafted PEG chains. Although not all of the primary amine groups were anchored with activated PEG, it still showed better wettability than EAA-Lysine-PEG.

The polar and dispersive components of the surface free energy for each surface were calculated using advancing contact angle data [29]. Surface free energies increased step by step. EAA-AM64-PEG had the highest surface energies, indicating higher PEG coverage. In contrast, the other PEG modified surface (EAA-Lysine-PEG) did not show high surface free energies because of high advancing water contact angle. It was also noted that there was no more than 14% change of the dispersive component after each step, compared with that of neat EAA, but the polar component increased dramatically and was as high as 22.5 mJ/m² for EAA-AM64-PEG. This increase was attributed to the increase in polar groups on the surface, such as primary amines and PEG repeat units.

**Surface morphology and phase**

Figure 4.7 shows 10 × 10 μm AFM phase images of modified films after each reaction step. Generally, a phase image indicates the surface composition difference
based on different mechanical or adhesive properties of surfaces. The $10 \times 10 \mu m$ phase images showed that small isolated domains formed after $l$-lysine was grafted and they increased further when PEG reacted with the $\omega$-amine of $l$-lysine. Using dendrimer AM64 as an intermediate layer, the clusters of dendrimer covered the surfaces more uniformly. The PEG-grafted surface appeared flat and uniform with higher coverage, consistent with the lower water contact angles and higher surface energy.

But it still can be recognized that the surface became more heterogeneous after EDC/NHS modification (Figure 4.8). This may because that the acrylic acid region of EAA was repelled by ethylene region in the solvent and surface grafting increased the difference of mechanical properties between these two regions. Continuously with the dendrimer and PEG grafted, surface heterogeneity of surfaces changed again. The individual round features which are apparent in this figure were attributed to clusters of dendrimer. The domain of PEG crystalline was also apparent in the last images. Step by step, rms roughness of film samples on $1 \mu m$ scale increased from 3.5 nm for neat EAA surface to 6.6 nm for EAA-AM64-PEG, based on the topographical analysis.
Figure 4.7 AFM phase images (10 × 10 µm) for (A) EAA, (B) EAA-NHS, (C) EAA-Lysine, (D) EAA-Lysine-PEG, (E) EAA-AM64, and (F) EAA-AM64-PEG.
Figure 4.8 AFM topography (left) and phase (right) images (1 × 1 µm) for (A) EAA, (B) EAA-NHS, (C) EAA-AM64, and (D) EAA-AM64-PEG.
Summary

Two reaction schemes were studied to covalently graft PEG chains on EAA surfaces. In aqueous solution, NHS and EDC were used to activate the carboxylic acid groups of EAA film. From ATR-FTIR spectra, it was found that the activation depth (estimated as 20 nm) was lower when NHS and EDC were used together to activate the carboxylic acids than that when only EDC was used. XPS data showed that the conversion of this activation step was nearly complete in the detected region of XPS. After activation of carboxylic acids, L-lysine and dendrimer AM64 were grafted onto the EAA surface with minimized grafting depth. They also introduced single or multiple primary amines for PEG grafting. Combining the data from ATR-FTIR and XPS, it was found that PEG chains were grafted on the surface of EAA films and a larger surface coverage was achieved when dendrimer was used as the intermediate layer. Using L-lysine as a linker molecular to attach PEG did not increase the surface wettability although L-lysine was anchored to the surface with high conversion. When dendrimer was used as a linker layer, the advancing water contact angle decreased to around 50° and the receding water contact angle was 0°. Therefore, using dendrimer AM64 as the intermediate layer provided more functionality and larger fraction of PEG coverage than L-lysine. Calculations suggest that the dispersive component of the surface free energy did not change significantly with surface modification, but the polar component changed appreciably with replacement of
carboxylic acids with dendrimer and PEG chains. AFM images revealed that small isolated domains formed without large scale bumps after l-lysine was grafted and they increased further in size when PEG reacted with the ω-amine in l-lysine.
References


CHAPTER 5

PATTERNING PROTEINS ON SURFACES OF CROSS-SECTIONED MULTILAYER POLYMER FILMS

[As published in Macromolecular Rapid Communication 2006, 27, 1173-1179]

Introduction

Surface modification of polymers is often necessary to improve their chemical, physical, and biological properties. Several techniques have been used to modify polymer surfaces, including physisorption [1], chemical coupling [2, 3], electrostatic adsorption [4, 5], and graft polymerization [6, 7]. Sometimes pretreatments, like flame treatment, corona discharge, and plasma exposure, are used to introduce surface functional groups, but they do not provide permanent modifications. However, for many biomedical devices, a permanent modification is required. Moreover, a patterned surface may be desirable, especially patterned surfaces of biological ligands, proteins, or cells. Various techniques have been developed recently to generate biomolecular patterns on solid surfaces, including microcontact printing (µCP) [8, 9], ink-jet printing [10, 11], dip-pen nanolithography [12], imprint lithography [13], microfluidic channel networks [14], and phase separation of polymer blends and block copolymers [15, 16]. Each has its advantages and inherent limitations, but multiple steps are normally needed to generate functionalized patterned surfaces. Therefore, it is highly desirable to fabricate a patterned surface with high throughput and repeatability. From a cost standpoint, it would also be advantageous to form the patterned substrates from commercial polymers.
The main motivation of this study was to generate a polymeric patterned substrate with two types of surface regions, one inert polymer and the other containing reactive carboxylic acid groups. Subsequent surface chemistries would occur only on the reactive regions. Multilayer films with two or more polymers are widely used for flexible packaging, in which barrier, mechanical, and thermal properties are optimized based on layer structure and thickness. Highly layered films have also been studied for photonic applications [17], utilizing the pronounced optical interference characteristics when two transparent polymers have different refractive indices. Here, we use the cross-section of a highly multilayered film as a template to form functionalized, patterned surfaces.

As an example, films were produced with alternating layers of ethylene-co-acrylic acid copolymer (EAA) and LLDPE. The acid-containing EAA layers served as the reactive regions [18-22], while the LLDPE layers served as the inert regions. There is also good adhesion between these two polymers so that the multilayer structure stays intact upon microtoming to reveal the cross-section. As a proof-of-principle, we immobilized amine-terminated biotin on the alternating EAA layers, and the biotin-modified EAA layers subsequently adsorbed fluorescent streptavidin.

**Experimental**

**Materials**

Seven-layer film of EAA (A) and LLDPE (B) was provided by Cryovac Division of Sealed Air Corp. (Duncan, SC). The structure of the film was A/B/A/B/A/B/A, with each layer approximately 7 µm thick. N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and bovine serum albumin
(BSA) were purchased from Aldrich. Phosphate buffered saline (PBS) tablets were obtained from EMD Biosciences Inc. (+)-Biotinyl-3,6,9-trioxaundecanediamine and Alexa488 labeled streptavidin were purchased from Pierce. All chemicals were used as received.

**Generation of patterned polymer platforms with highly layered structure**

To prepare samples with highly layered structures, a prescribed number of 7-layer films (1 × 1 cm square) were stacked together in a Teflon mold (3 × 3 × 0.15 cm), which was sandwiched between two Teflon sheets. Samples were pressed at 130 °C in a compression press (Carver model 30-12-2T). All samples were pressed at ~ 117 kPa for 25 s initially to remove air pockets, and then pressed at ~ 70 MPa for 1 min. The samples pressed between Teflon sheets were quenched rapidly to room temperature with cold water. After this step, the samples were removed from the mold and dried in a vacuum oven. First, 64 pieces of 7-layer film were pressed into a 448-layer film. Then the sample was cut into four equal pieces, with one used for analysis. The other three pieces were stacked together in the same mold. After pressing at the same conditions as above, the polymer films were quenched and dried in a vacuum oven. That film was cut into four pieces and the process was repeated. In this way, we obtained 1344- and 4032-layer films, but only the 7-, 448-, and 4032-layer films were analyzed in this study.

Thermal properties were studied with a TA Instruments MDSC 2920 DSC. A few pieces of 7-layer film were stacked together in the DSC pan to prepare a sample with a weight of around 10 mg. The sample was first held at 0 °C for 1 min and then heated at a constant rate of 20 °C/min until it reached 200 °C; thereafter, it was cooled at constant rate of 20 °C/min until it reached 0 °C. Two scans were performed and the results were
recorded with the Perkin-Elmer Analysis Program. The sample was also held at 200 °C for 6 h to study its thermal stability after both scans. After it was removed from the DSC pan, it was embedded directly in the wax. Thin sections of about 10 µm thick were obtained by rotary microtome (Leica RM2155) with a steel blade at room temperature. Polarized optical microscopy with 200x magnification was used to observe the polymer phases and the interfaces between EAA and LLDPE before and after heat treatment.

Specimens of 7-, 448-, and 4032-layer films were each embedded in wax and microtomed (Leica RM2155) at a 45° angle at room temperature. Thin sections were examined by polarized optical microscopy and AFM. 7-layer film was also immersed in liquid nitrogen for 5 min, and fractured immediately afterward. The layer structure was examined by SEM. Ultrathin sections (~ 100 nm thick) of 448- and 4032-layer films were prepared using a RMC Powertome X with a cryo system equipped with a diamond knife at -100 °C. Specimens were then imaged by SEM and TEM to obtain better contrast and higher resolution. Ultrathin sections were also examined by AFM.

**Surface grafting with biotin and streptavidin adsorption on alternating layers**

Before grafting on the EAA layers, the 448-layer thin section was rinsed with hexane to remove any residual wax from microtoming and dried in a vacuum oven. The thin section was then immersed in a NHS/EDC (200 mM/100 mM) phosphate buffered saline (PBS, pH = 7.4) solution for 1 h to activate carboxylic acid groups on the surface of EAA layers (Figure 5.1), followed by rinsing with PBS buffer solution. After activation, the sample was immersed in the amine-terminated biotin ligand solution (10 mM in ethanol) for 2 h and rinsed with ethanol and PBS buffer solution. The sample was then immersed in a solution of fluorescence-conjugated streptavidin (0.1 mg/ml) in PBS buffer solution
with 0.1% (w/v) BSA for another 2 h. After rinsing several times with PBS buffer solution, the sample was dried in a vacuum oven and examined by fluorescence microscopy as described in Chapter 2.

**Figure 5.1** Scheme of the patterned surface generation and subsequent modification on EAA layers.
Surface characterization

An Olympus BX60 microscope with crossed polarizer and analyzer was used to characterize the microstructure of thin sections. Low voltage scanning electron microscopy (LVSEM, Hitachi, Model S4700) was used to study the more highly layered structures. Fractured surfaces and ultrathin sections were coated with about 100 Å of platinum before scanning. For 4032-layer films, the ultrathin sections were stained with osmium tetroxide vapor at room temperature and transmission electron microscope (TEM) images were obtained (Hitachi, Model H7600T) operating at 100 kV. Atomic force microscopy (AFM) images were obtained using a Digital Instruments Nanoscope IIIa in tapping mode. Images of streptavidin-modified patterns were recorded by fluorescence microscopy (Nikon, HFX-DX). Please refer to Chapter 2 for more information.

Results and Discussion

In Figure 5.2, images are presented for the 7-, 448-, and 4032-layer films. Various microscopy techniques were used to obtain the images depending on the resolution and contrast. The initial 7-layer film had EAA as the skin (outer) layers. When 7-layer films were pressed together, thicker EAA layers were formed as shown clearly in Figure 5.2B. Uniform multilayer structures were obtained and the layer thickness was measured from the images and also calculated knowing the total film thickness and number of layers. The results (Figure 5.2D) demonstrated good agreement between the two methods. It was also demonstrated that submicron-thick layers can be achieved, even with compression molding. The maximum total film thickness among the specimens in Figure 5.2D was approximately 2 mm (4032 layers x 0.5 microns/layer), but for many devices a thicker
film or sheet may be desirable. With further stacking and compression molding, we produced a specimen that was about 1 cm thick that is easier to handle and provides more surface area for adsorption. While compression molding does not lend itself well to a continuous process, more efficient methods are available to produce highly multilayered films [23, 24]. These methods will be explored in the near future.

Because the multilayer structures were generated by compression molding of hundreds of 7-layer films, it is important to know whether the two polymers, EAA and LLDPE, are miscible or not at the working temperature. If they are completely miscible or partially miscible, when the microlayers become thinner, inter-diffusion will potentially blur the layer structures at the interface between each microlayer. Even if they are immiscible, there exist strong enough interaction forces between EAA and LLDPE layers to provide sufficient adhesion and keep the layers intact.

To estimate the miscibility of this polymer pair, the interaction parameter between the two polymers was calculated according to Hildebrand [25] from the solubility parameters of the components in a polymer blend as follows:

\[
\chi_{AB} = \frac{V_r (\delta_A - \delta_B)^2}{RT}
\]

(1)

where \(\chi_{AB}\) is the interaction parameter between polymers A and B, \(\delta_i\) are the solubility parameters of the two polymers, \(R\) is the gas constant, and \(T\) is the absolute temperature. \(V_r\) is a reference volume, set at 100 cm\(^3\)/mol. By comparing this interaction parameter with the critical interaction parameter \(\chi_{cr}\), which was calculated according to the method of Krause [26], it is possible to evaluate the miscibility of the two polymers. If \(\chi_{AB} > \chi_{cr}\), then the two polymers will be immiscible.
Figure 5.2 Microstructure of the multilayer films: (A) optical and (A’) SEM images of 7-layer film, (B) optical and (B’) SEM images of 448-layer film, (C) optical and (C’) TEM images of 4032-layer film. (D) Relationship between the layer thickness and the number of layers.
where the $DP_i$ are the degrees of polymerization of the polymers in terms of the reference molar volume $V_r$. As estimated by Krause, the critical interaction parameters are 0.002 for two polymers with molecular weights of 100,000 Da, 0.004 for 50,000 Da, and 0.02 for 10,000 Da. The solubility parameters in this study were obtained from the literature [27, 28]. For EAA random copolymer, the solubility parameter $\delta_c$ was calculated as follows:

$$\delta_c = (1 - \phi)\delta_{PE} + \phi\delta_{PAA}$$

where $\delta_{PE}$ and $\delta_{PAA}$ are the solubility parameters of the homopolymers and $\phi$ is the volume fraction of acrylic acid in the copolymer.

Therefore, a graph (Figure 5.3) was made according to the calculation method above. This phase diagram can be used to predict the amount of acrylic acid needed in the EAA copolymer for full miscibility with LLDPE at a certain temperature. Because the EAA used in the study contains 9.5 wt% of acrylic acid, and both polymers had molecular weights much larger than 10,000 Da, complete immiscibility was expected for this polymer pair at the processing temperature of 200 °C.
Figure 5.3 Miscibility of EAA and LLDPE calculated using Flory-Huggins theory.

To confirm the prediction about the immiscibility of EAA and LLDPE, DSC traces of the seven-layer films were obtained from 0 to 200 ºC at the heating rate of 20 ºC/min, since the key indicator for miscibility and co-crystallization of polyethylenes with the broad molecular weight distribution and branching heterogeneity typical comes from a single melting peak. Figure 5.4 showed the first run, the second run after cooling from 200 ºC at 20 ºC/min, and the third run after annealing for 6 h at 200 ºC and cooling back from 200 ºC at 20 ºC/min. It was observed that there are two well-separated melting peaks, one at around 90 ºC for EAA and the other peak for LLDPE at around 120 ºC, showing apparent bimodal melting behavior. This result suggested that the EAA and LLDPE crystallized as separate phases, even after long time (24 h) annealing at 200 ºC.
Therefore, upon compression molding, the EAA melt first and the skin EAA layers adhered together to form well-defined multilayered structures. DSC trace after annealing for 6 h at 200 °C did not show significant change for LLDPE melting peaks. The unchanged melting temperature for LLDPE meant that the crystallization of LLDPE in the system was not affected by EAA. However, the melting temperature of EAA shifted a little bit to lower temperature, indicating small amount of co-crystallization of EAA and LLDPE chains at the boundaries of multilayers. During the compression molding, intimate contact in the melt state allowed the inter-diffusion and subsequent co-crystallization of EAA and LLDPE. The image of the annealed sample showed large isolated phases in the cross section. This is because when the temperature increased above the melting temperature of EAA and LLDPE, they became fluid like so that the EAA layers started contracting, formed holes and then enlarged.

Although there was some co-crystallization at the interfaces of layers, this transcry stalline region must be very thin, because there was no new crystallization peak observed in the cooling DSC traces. Normally, the co-crystallization regions will form on the copolymer side at the boundaries and appear either tightly packed or relatively diffuse, depending on the density of the nuclei at the interface [26]. But with the current results from DSC traces and polarizing optical microscope observation, it was hard to determine how the co-crystallization and inter-diffusion occurred. At least, the presence of this thin transcry stalline region at the interface did improve the adhesion between EAA and LLDPE, which is very important for the formation and stability of the patterned surfaces. The layers were intact without any fracture after immersing into liquid nitrogen, different from most of other polymer pairs. It is proposed that the hydrogen bonds, which play a
cross-linking role, restrain the movement of PE segments of EAA and prevent them from inter-diffusion and forming large transcrystalline with LLDPE. However, the PE chains with lower molecular weight in LLDPE are still able to diffuse into EAA when it is completely molten, and subsequently co-crystalline with PE segments of EAA, while carboxyl groups that form hydrogen bonding systems remain in the amorphous region of EAA.

**Figure 5.4** DSC curves of EAA/LLDPE 7-layer films: (A) melting and (B) crystallization. (C) optical image of microstructure after 6 h annealing at 200 °C.
The multilayer films of EAA and LLDPE contain hundreds of alternative layers. Subsequently, these films were microtomed to generate the patterned surfaces. Upon microtoming the multilayer films, the cross-section revealed patterned (striped) surfaces of alternating reactive EAA layers. The generated thin sections have highly layered structure while carboxylic acids of EAA are available for reaction with various functional groups, including epoxy, amino, and hydroxyl. Microtome is used routinely to prepare thin sections for structure analysis using AFM, SEM, or TEM. This is the first time, in our knowledge, that it was used for generation of patterned templates for surface modifications utilizing the highly layered structures of multilayer films.

When microtomed with a steel blade at room temperature, it was observed that the steel blade left substantial scratch marks on the thin section and cut surfaces. Meanwhile, the LLDPE layers bumped to about 200 nm higher than EAA layers as shown in Figure 5.5. This volume relaxation was resulted from the residual stresses stored in the individual layers when they were extruded and compressed, because the LLDPE crystallized before EAA. The surface topography was stable and no significant change was observed (Figure 5.5) by AFM after the sample was kept at 60 ºC for 24 h.
Figure 5.5  AFM 3D topography images and section profiles (100 × 100 µm) of 448-layer section by (A) RT microtome and (B) after 60 °C treatment.

The 448-layer films were also cryo-ultramicrotomed at approximately -100 °C. The polymers at this temperature were glassy. Figure 5.6 shows the AFM topography image and section profile. It was apparent that the surface roughness decreased while the LLDPE bumped only about 100 nm above the EAA layers. Similar result was obtained for the cut surface, in which the scale of section profile was even less than 50 nm. Thus the volume relaxation occurred much less in cut surfaces than in thin sections. Note that
the section-analysis plots in Figure 5.6 may be misleading because the x and y axes are not on the same scale. If the x axis were plotted with the same nanometer scale as the y axis, the section would appear much smoother. Relatively flat surfaces were obtained for 4032-layer films. When the sample was cryo-ultramicrotomed at -100 °C, layered topography was not as clear as that for 448-layer specimens (Figure 5.7).

Figure 5.6 AFM 3D topography images and section profiles (100 × 100 µm) of (A) 448-layer section by cryo-ultramicrotome and (B) the bulk surface.
To achieve the covalent attachment of biomolecules onto the EAA layers of patterned surfaces, we used the common method of EDC and NHS ester as reactive intermediates to activate the carboxylic acids of EAA [2]. Subsequently, amine-linked biotin was covalently “grafted to” the confined EAA layers by formation of an amide bond between

**Figure 5.7** AFM (A) topography and (B) phase images and (C) section profiles of 4032-layer section (50 × 50 μm and 5 × 5 μm zoom in).
the primary amine of biotin and the NHS groups of activated EAA. Biotin was chosen because the amination is well-known chemistry that results in high conversion [29-32]. The modified, patterned surface was incubated in a solution of fluorescence (Alexa488) conjugated streptavidin to bind with the biotin-grafted layers. Figure 5.8 shows the images taken by fluorescence microscopy. Figures 5.8B and 5.8C showed the streptavidin was bound mainly on the biotin modified EAA layers. The specimen in Figure 5.8A served as a control in which a neat (non-activated) patterned surface was immersed in protein solution for a prescribed time, but no significant fluorescence was observed after rinsing, indicating a low level of non-specific adsorption. This is not to say that there was no non-specific binding, which would occur particularly on the hydrophobic LLDPE layers. To minimize non-specific binding, another polymer, such as poly(PEG methacrylate), could be used as the non-reactive layer. As seen in Figures 5.8B and 5.8C, the surfaces are not perfect stripes as there is evidence of defects in the fluorescence images. Those defects do not come from irregularities in the multilayer structure but primarily from the microtoming process. The microtoming conditions need to be optimized further to create a defect-free section, but this proof-of-concept study has demonstrated the potential of forming functionalized nanostripes from multilayer polymer films.

This approach has favorable characteristics compared with micro-contact printing (µCP), ink-jet printing, and other patterning techniques: (1) highly layered pattern surfaces are generated with high repeatability and high throughput; (2) the EAA layers have reactive carboxylic acids in alternating regions, which are available for reaction with various functional groups (e.g., epoxy, amino, and hydroxyl); (3) the reaction can be
conducted in solution to high conversion. Additionally, the polymer film substrate is more flexible than rigid, inorganic substrates. The multilayer films potentially can be useful for patterning (in various scales) small biological ligands, proteins, and cells onto prestructured polymer surfaces for biomaterial and biotechnology applications. The advantage of grafting biotin with subsequent streptavidin adsorption is that streptavidin has two pairs of binding sites for biotin on opposite faces. Therefore, one pair could be used to attach to the solid surfaces and the other could be used for further high affinity binding with biomolecules with biotin end groups [33].

**Figure 5.8** Fluorescence images for: (A) neat patterned surface and (B, C) Alexa488-streptavidin patterned surface. The same brightness and contrast were used to obtain all images.
Summary

Multilayer films have been used to fabricate patterned surfaces with highly layered structures, combining the techniques of co-extrusion and compression molding. The layer thickness varied from several microns to a few hundred nanometers. Thermal analysis showed that there was a very thin region of transcrystalline at the interfaces of microlayers and it improved the adhesion between EAA and LLDPE, which is very important for the formation and stability of the patterned surfaces. Using AFM, it was found that the LLDPE layers bumped up to about 200 nm higher than EAA layers for thin sections, and these patterned surfaces were stable after annealing at 60 ºC. By grafting amine-terminated biotin and subsequently adsorbing streptavidin to the alternating layers of EAA, we demonstrated the possibility to carefully functionalize the carboxylic acids on EAA layers. Fluorescent images showed that streptavidin adsorption was confined to the modified EAA stripes. This approach is a simple but effective technique developed for fabricating highly layered micropatterns with various pattern sizes. It allows confining the reactive carboxylic acid groups instead of confining the chemistry occurring on the surface, allowing more complicated modification in solution for biomaterial and biotechnology applications.
References


CHAPTER 6

LAYER-BY-LAYER SELF-ASSEMBLY OF POLYELECTROLYTE MULTILAYERS ON CROSS-SECTION SURFACES OF MULTILAYER POLYMER FILMS AS A NANO-PATTERNING TEMPLATE

Introduction

Poly(ethylene-co-acrylic acid) (EAA) is a commercially available copolymer with outstanding thermoplastic and adhesion properties. It has been used widely as an adhesive layer on metals, glass, cellulosics, and plastics. It has also been used to protect metal surfaces from attack of moisture, acids, and oily materials when used as a thin-film coating. Recently, the applications of EAA in biotechnology have been increasing rapidly in areas such as biosensors [1], biofouling reduction [2], and surface patterning [3, 4] where the carboxylic acids provide convenient reactive groups. However, there are many situations where a high surface concentration of acid groups is desirable. This increase can be achieved by using an EAA with higher acrylic acid content, but the rheological properties of that material are greatly altered, which can significantly influence processing behavior.

Researchers have used a variety of methods to conduct surface modification of EAA to introduce desired chemical functionality [2, 5-10]. The modification procedures have typically included activation of the carboxylic acids and chemical coupling with other species. In some cases, even after it was successfully modified with small molecules, the EAA surface tended to reconstruct to maintain its hydrophobic nature [2, 10]. Others have used polymers like poly(glycidyl methacrylate) (PGMA) or polypropyleneamine dendrimer as anchoring layers to introduce more functionality on EAA-film surfaces [2,
However, the acrylic acid content formed tiny domains in the EAA copolymer because of hydrogen bonding between the carboxylic acids, leading to incomplete coverage even when anchoring layers were used to modify the surfaces. Since the carboxylic acids on an EAA surface can be deprotonated easily to yield negative charges (-COO\(^-\)), we have performed layer-by-layer self-assembly of polyelectrolyte multilayers (PEM) to introduce more surface functional groups. Besides electrostatic interactions, it is also possible to produce PEM films by hydrogen bonding [12, 13] or hydrophobic-hydrophobic interactions [14, 15]. Therefore, this approach has the potential to provide more extensive functional-group coverage on EAA film. Acrylic acid domains will be covered by PEM films through electrostatic interactions, while ethylene domains will be covered through hydrophobic-hydrophobic interaction.

First introduced by Decher [16], electrostatic PEM self assembly is a well established method to construct vertically structured multilayer films of polyelectrolytes. This technique is a simple, fast, and economic approach. It involves alternating immersions of a charged substrate in polycation- and polyanion-containing solutions with rinsing between each adsorption step, and has been used for polymer surface modification [17-20]. The great advantage of the PEM approach is that it is possible to incorporate a variety of other materials into the films, such as functional polymers [21], nanoparticles [22, 23], proteins, and drugs [24]. In recent years, weak polyelectrolytes have received a great deal of attention, because they provide opportunities to control the average charge on the polymer chain so that the layer-layer interactions can be controlled. The charge of weak polyelectrolytes, such as poly(acrylic acid) (PAA), poly(allylamine hydrochloride) (PAH), and poly(ethylene imine) (PEI), can vary depending on the pH of the medium [25,
26]. In this work, PAH/PAA pairs were used as the weak polyelectrolytes because carboxyl and amine groups of PEM films could crosslink to improve their stability [27, 28] and also provide anchoring sites for functionalization [29].

Hammond and coworkers [14, 30] have demonstrated that the PEM films could be directed on patterned structures with surface chemistry. They used chemically patterned substrates with two regions of different chemical functionality, one promoted adsorption and another resisted adsorption of polyions onto the surface. Patterned PEM films potentially can be used in various applications such as biosensors [31], tissue engineering [32, 33], and electronic and photonic devices [34, 35]. In our previous study [3], a new approach was introduced to create stripe patterned surfaces at the sub-micron scale using multilayer polymer films that contained alternating layers of two different polymers (EAA/LLDPE). EAA acted as a reactive layer for protein patterning, while LLDPE was used as inert polymer. This method used cross-sectioned surfaces of multilayer polymer films as the substrate, which had greater flexibility than rigid, inorganic substrates that typically are fabricated with other patterning methods [36-38]. In addition, the patterned surfaces using multilayer films could be generated with high repeatability and throughput.

In this study, we have extended that work by depositing PAH/PAA films onto the patterned surfaces to introduce more functional groups on alternating EAA layers. After chemical crosslinking, the nano-striped surface would have stable hydrophilic regions adjacent to hydrophobic regions. This kind of structured surface with alternating wettability could be used as a template in many applications of selective adsorption [39]. In addition, the hydrophilic PEM films provide moieties that can be used as a functional part of a device, thus offering a means to creat inexpensive bio-devices.
We have three main objectives in this study. First, we investigated the deposition of PAH/PAA films on both EAA and LLDPE films. Second, we crosslinked the PEM films in aqueous solution and studied their stability in solvent and acid or base solutions. Third, we deposited the PEM films on the nano-striped surfaces generated from multilayer polymer films. It was hypothesized that the PEM films on the alternating EAA regions would form hydrophilic thin films, but those on LLDPE regions would be removed due to the lack of linkage on those chemically inert stripes.

**Experimental**

**Materials**

Poly(ethylene-co-acrylic acid) (EAA, PRIMACOR 1410 from Dow Chemical Co., 9.5% w/w acrylic acid) and linear low-density polypropylene (LLDPE, Dowlex 2517) films were used as received from the Cryovac Division of Sealed Air Corp. (Duncan, SC). To fabricate multilayer polymer films, seven-layer film of EAA (A) and LLDPE (B) was used and the structure of the film was A/B/A/B/A/B/A, with each layer approximately 7 µm thick. Poly(allylamine hydrochloride) (PAH, Mw = 10,000), sodium chloride (NaCl), hydrochloric acid (HCl), and HPLC water were obtained from VWR. N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), poly(acrylic acid) (PAA, Mw = 5,100) was purchased from Aldrich. Fluorescein cadaverine (FITC-NH₂) was obtained from Fisher Scientific and used without purification.
Surface modification of polymer films

Covalent attachment of PEM films on EAA films involved three steps: deprotonation of the carboxylic acids on EAA, layer-by-layer self assembly of PAH/PAA films, and chemical cross-linking in aqueous EDC/NHS solution. EAA film was deprotonated in a 1 M aqueous NaOH solution, followed by rinsing with HPLC water and drying with N₂. Kinetics of deprotonation were studied over a time period from 15 s to 30 min. The resulting film was denoted as EAA-COO⁻.

To deposit PEM films, EAA-COO⁻ films were immersed in a 0.02 M PAH aqueous solution for 15 min at pH 7.5 and then rinsed with HPLC water for 2 min. After drying with N₂, the film was immersed in a 0.02 M PAA aqueous solution for another 15 min at pH 3.5 and then rinsed with HPLC water for 2 min. Consecutive cycles of alternating adsorption of PAH/PAA bilayers was performed for a prescribed number of times. The deposition solutions also contained 0.5 M NaCl and were adjusted to the pH value using 1 M NaOH or 1 M HCl solutions.

Crosslinking of PAH/PAA films was performed by immersing the PEM-modified EAA film in a solution of NHS/EDC (100 mM/200 mM), which was freshly prepared with a 0.2 M NaCl solution. The ionic linkages between –COO⁻ and –NH₃⁺ were converted into covalent bonds through a carbodiimide coupling reaction to yield a cross-linked structure. The film was then rinsed and sonicated with HPLC water three times (5 min for each) and dried with N₂. To test the stability of crosslinked PEM, the film was immersed in different solvents for 3 days, including methanol, 1 M HCl, and 1 M NaOH solutions.
To check the possibility of grafting other species on the PEM films, the modified EAA film was immersed in FITC-NH$_2$ (1 mg/ml) solution for 4 h. The solution was prepared by dissolving FITC-NH$_2$ in NHS/EDC (100 mM/200 mM) solution, so the crosslinking reaction and attachment of FITC occurred at the same time. The film was then rinsed and sonicated three times (5 min each) and dried with N$_2$.

**Preparation of nano-striped surfaces and deposition of PAH/PAA films**

The nano-striped surfaces were prepared using a multilayer polymer film [3]. A prescribed number of seven-layer films (A/B/A/B/A/B/A where A = EAA and B = LLDPE) were stacked and pressed in a compression molding machine at 130 °C. In this way, a 4032-layer film was prepared and the thickness of the individual layers was about 500 nm. To obtain smooth, nano-patterned surfaces, the 4032-layer film was microtomed at –100 °C using a RMC Powertome X with a cryo system equipped with a diamond knife. The smooth surfaces generated by cryo-microtoming were used as the nano-striped template for PEM deposition. After formation of cross-linked PEMs, the modified nano-striped template was sonicated in HPLC water and methanol, respectively, for three times each to remove the PEM films from the LLDPE layers.

**Characterization**

Attenuated total reflection FTIR (ATR-FTIR) spectroscopy measurements were conducted on the modified polymer films using a Thermo Nicolet Megna 550 single bounce FTIR spectrometer equipped with a Thermo-Spectra-Tech Foundation Series Diamond ATR with Deuterated Triglycine Sulfate (DTGS) detector. XPS data were obtained with a Kratos Axis Ultra photoelectron spectrometer with Al K$_\alpha$ radiation (15kV, 225 W). Other instrumental details were described in Chapter 2.
Results and Discussion

Figure 6.1 depicts the procedure of the PEM patterning, including nano-striped pattern formation, PEM deposition, and chemical cross-linking. Using this procedure, the EAA-confined PEM films should form a stable hydrophilic coating, providing multiple functionalities, while PEM films on the LLDPE regions should not bond to the substrate and be removed easily. Since it is difficult to detect the chemical changes on the cross-section of these multilayer films, surface characterization of the layer-by-layer (LbL) procedure was first studied on the major surfaces of neat EAA and LLDPE films.

Figure 6.1 (A) Illustration of the PAH/PAA multilayer patterning on cross-section surfaces of polymer multilayer films; (B) Reaction scheme of the crosslinking step.
Layer-by-layer PEM deposition on EAA-COO\textsuperscript{-} and LLDPE

The carboxylic acids of EAA film were first deprotonated in a base solution (NaOH 1M). The deprotonation process was monitored by ATR-FTIR using the peaks at 1550 and 1400 cm\textsuperscript{-1}, which are attributed to the asymmetric and symmetric stretches of -COO\textsuperscript{-} groups (Figure 6.2). It was found that the deprotonation process occurred in the first 15 min and then plateaued, while the peak area of carboxylic acid at 1705 cm\textsuperscript{-1} kept diminishing. After being deprotonated by the base solution, the carboxylic acids were converted to carboxylate ions and the surface became negatively charged. Therefore, PAH could be attached on the EAA-COO\textsuperscript{-} through electrostatic interaction since the PAH was highly charged at the selected pH of 7.5 (pK\textsubscript{a} for PAH is 8.5). PEM films of PAH/PAA were deposited on the film by alternatively immersing EAA-COO\textsuperscript{-} films in dilute aqueous solutions of PAH at pH 7.5 and PAA at pH 3.5 (pK\textsubscript{a} for PAA is 4 ~ 5.5). Rinsing three times with HPLC water followed each deposition to remove weakly bound polyelectrolytes. The formation of PEM films was confirmed by ATR-FTIR and contact angle measurements. From Figure 6.3, it was observed that the peak at 1705 cm\textsuperscript{-1} increased with the deposition of alternating PAH/PAA layers due to the carbonyl stretch of uncharged acids in the PAA layers. Another peak appeared at 1650 cm\textsuperscript{-1} when more polyelectrolyte layers were deposited on the EAA film, indicating that a small amount of amide was formed between –NH\textsubscript{3}\textsuperscript{+} and –COO\textsuperscript{-}. A broad peak around 3000 cm\textsuperscript{-1} was attributed to the C-OH bonds.
**Figure 6.2** FTIR spectra of EAA and deprotonated films in NaOH solution for 5, 20 sec, 1, 5, 15, 30, 60, 360 min.

**Figure 6.3** FTIR spectra of EAA-COO\(^-\) and PAH/PAA multilayer deposited films up to 10 layers.
XPS was used to provide more detailed information about the chemical compositions of EAA surfaces after deposition of PEM films. Figure 6.4 shows the C 1s high resolution spectra for neat EAA, EAA-PEM2, EAA-PEM6, and EAA-PEM10, representing neat EAA film and EAA films with one, three, and five pairs of PAH/PAA bilayers. For neat EAA, there were two well-resolved peaks at 289.5 and 285.0 ev, the first corresponding to carboxylic acids existing within the outmost 10 nm of the film and the second corresponding to C-C. When alternating layers of PAH/PAA were adsorbed on the deprotonated EAA surfaces, the C 1s high resolution peaks could be resolved into three peaks at 285.0, 286.0 and 288.6 ev, corresponding to C-C, C-NH$_3^+$, and COO'NH$_3^+$, respectively. As shown in the figure, some carboxyl groups were introduced on the surface of EAA-PEM2, and even more on the surface of EAA-PEM6. But comparing the spectra for EAA-PEM6 and EAA-PEM10, the contents of carboxyl groups were 21% and 18%, respectively. This small difference was likely due to the thickness of these deposited PEMs being greater than the 10 nm probe depth of XPS. As measured on glass slides, the thickness of a dried PAH/PAA bilayer was about 10 nm [40]. Based on our XPS data, there is a significant difference between the carboxyl contents of EAA-PEM2 and EAA-PEM6, but the difference diminished upon further deposition.
Figure 6.4 High-resolution XPS C 1s spectra for PEM deposited EAA films.
Water contact angle measurements were performed after each deposition step. As shown in Figure 6.5, treatment with base solution to yield COO⁻ improved the hydrophilicity of EAA film slightly. After adsorption of PAH at pH 7.5, the water contact angle continued decreasing to about 75°. Subsequent adsorption of PAA at pH 3.5 led to a significant decrease of water contact angle. The value is close to 30°, which is so far the lowest value observed in surface modification studies of EAA film. However, when more PAH/PAA layers were deposited, the surface topped with PAH had a higher water contact angle ranging from 80 to 90°, which was probably because of an increased charge density of the polyelectrolytes leading to a more hydrophobic surface. Surface roughness was also obtained from AFM topography images, and it was found that the roughness increased slowly at the beginning, and then significantly after six depositions of PAH/PAA. As can be seen from AFM images (Figure 6.6), nanoporous films were obtained on the EAA surface when more bilayers were deposited. These nanoporous films have also been observed on silicon substrates [41, 42]. It is believed that pH-induced cleavage of ionic bonds leads to phase separation and the formation of nanoporous PEM films. However, polyelectrolyte films on EAA were quite uniform for the first few layers of PAH/PAA, as reflected by the relatively stable RMS roughness values.

To determine whether the driving force for polyelectrolyte adsorption on EAA film is electrostatic or secondary interactions, control experiments were conducted on LLDPE film, which does not contain charged groups. However, PAH contains a hydrophobic backbone that will have stronger hydrophobic-hydrophobic interactions with LLDPE than will PAA. Thus, PAH could also adsorb on the LLDPE films after immersion in the
dilute PAH solution at pH 7.5. The contact angle decreased to values comparable to those on EAA with further adsorption of PAH/PAA, but AFM images showed large micropores formed in the PEM films (Figure 6.6). Further analysis showed that clusters formed upon the first layer of PAH adsorption. We believe that the ions on the EAA surface played an important role in spreading out the PAH chains on the surfaces and provided more coverage than on inert LLDPE. Since the hydrophobic-hydrophobic interactions were weaker than electrostatic interactions, significant desorption of adsorbed chains occurred during the rinsing steps.

Figure 6.5 Water contact angle of PEM deposited EAA film as a function of number of layers and the relationship with surface RMS roughness.
Figure 6.6 AFM topography (A and C) and phase (B and D) images of LbL PEM deposition on EAA-COO⁻ and LLDPE films. All images 10 × 10 µm.
Chemical cross-linking of the PEM films

There are several methods to crosslink the PEM films, such as thermal crosslinking [27, 43] and photo-crosslinking [44, 45]. Thermal crosslinking generally requires temperatures above 100 °C, which is above the melting temperature (90 °C) of EAA, so it was not suitable for EAA films. Photo-crosslinking needs a photoinitiator, which limits its application in many polyelectrolyte pairs. An EDC/NHS coupling method was then selected for this phase of the study. After deposition on EAA film, the PEM films were crosslinked in aqueous EDC/NHS solution by a carbodiimide coupling reaction between the PAA and PAH chains.

The cross-linking reaction was monitored by ATR-FTIR (compare the lower two spectra in Figure 6.7). The amides formed during cross-linking showed peaks at both 1650 (amide carbonyl stretch) and 1550 cm\(^{-1}\) (N-H amide bend and COO\(^{-}\) asymmetric stretches). It was observed from the spectra that the peak at 1650 cm\(^{-1}\) increased, while the intensity of the peak at 1550 cm\(^{-1}\) decreased slightly. This result suggested that only small amounts of the acid ions were converted into covalent amide bonds through amidation reaction, saying that the PEMs on EAA film were not highly cross-linked. But the linkage with the EAA film made the PEMs stable. The films were stable even when treated with methanol, HCl, and NaOH solutions for 3 days. As shown in Figure 6.7, the peaks associated with the amide bonds remained intact after the various treatments. The peak at about 1705 cm\(^{-1}\) indicated that there were still a significant number of carboxylic acids existing in the PEM films. A control experiment was conducted for uncrosslinked PEM films and showed that they disassociated in only a few minutes of immersion in the solutions.
After treatment with 1 M HCl solution, most of the –COO⁻ was protonated back to –COOH, making further linkage with other important molecules possible. As an example, amine-terminated FITC was used to react with the crosslinked PEM on EAA. Fluorescence images were obtained and shown in Figure 6.8. All of the settings were kept the same for the various samples. The fluorescence images indicated that the number of carboxylic acid groups increased significantly after deposition and crosslinking of PEM6 vs PEM2.

**Figure 6.7** FTIR spectra of modified EAA films (bottom to top: EAA-PEM10, after crosslinking, stability test after immersed in methanol, HCl, and NaOH solutions for 3 days).
One of the goals of this work was to fabricate PEM patterns on a nanostriped surface revealed after microtoming a multilayer polymer film. The nanostructure of cross-sectioned surfaces of a 4032 layer EAA/LLDPE film has been described elsewhere [3].

To generate a hydrophilic coating on EAA regions, the PEM deposition procedure

**Figure 6.8** Fluorescence images of neat EAA film, FITC grafted film, crosslinked and FITC grafted PEM2 and PEM6 on EAA film.

**PEM patterning on cross-section surfaces of multilayer films**

One of the goals of this work was to fabricate PEM patterns on a nanostriped surface revealed after microtoming a multilayer polymer film. The nanostructure of cross-sectioned surfaces of a 4032 layer EAA/LLDPE film has been described elsewhere [3]. To generate a hydrophilic coating on EAA regions, the PEM deposition procedure
discussed earlier was conducted on this cross-sectioned, nano-striped surface. Three steps were involved: (i) LbL self-assembly of PAA/PAH, (ii) crosslinking in an aqueous EDC/NHS solution, and (iii) sonicking three times in both HPLC water and methanol.

We conducted the cross-linking reaction in the presence of amine-terminated FITC, so that the patterned structure could be detected by confocal fluorescence microscopy if a fluorescent pattern was formed. Figure 6.9 shows the results from several different experiments conducted to see if it was possible to form the hydrophilic PEM films on EAA stripes only. By removing the PEM from LLDPE stripes through sonication in HPLC and methanol, we expected to obtain nano-striped PEM patterns. As shown in Figure 6.9A, the entire surface showed strong fluorescence without sonication, indicating that the PEM covered both the EAA and LLDPE stripes. However, after sonication, the nano-striped structure was clearly observed by confocal fluorescence microscopy (Figure 6.9B), where the green layers represent FITC grafted to the PEM on EAA layers and the dark layers represent inert LLDPE layers. Control experiments of FITC grafting without prior PEM deposition were performed on the original microtomed surface, but no fluorescence and contrast were observed. Therefore, the PEM layers were deposited and stabilized only on the EAA stripes, and provided many more functional groups for further linkage with amine-terminated FITC. In addition, the 500 nm PEM stripes deposited on EAA were clearly observed by SEM, as shown in Figure 6.9C. The light stripes represent EAA regions covered with PEM. This striped structure was not detectable via SEM before PEM deposition because of the compositional similarity between LLDPE and EAA copolymer (9.5 % AA by weight).
Figure 6.9 Fluorescence images (20 × 20 µm) of crosslinked PEM films (3 sets of bilayers) on nano-striped surfaces: (A) before washing and (B) after washing off the crosslinked complex from LLDPE layers; (C) SEM image of the surface after washing, noting that the nano-stripes were not seen by SEM before washing.
It is interesting to note that the PEM was removed from the LLDPE stripes, even after crosslinking. Although the carbodiimide coupling reaction occurred between the PAA-PAH layers, we speculate that the cross-link density was relatively low such that the PEM not strongly bound to LLDPE through secondary interaction could be removed. A similar phenomena was observed by Mallwitz et al. [27] when they prepared freestanding PEMs (PAH/PAA) in the meshes of supports with large pores. They successfully formed freestanding PEMs if there were no rinsing steps between each deposition of polyelectrolyte. The freestanding film was then thermally crosslinked to increase its stability. However, if there were rinsing steps, as there were in this study, no freestanding film formed, indicating that the PEMs were not highly cross-linked films that spanned the pore openings. Meanwhile, the covalent binding of PEM films to EAA was strong enough to hold the film through linkages between the carboxylic acids of EAA and the initial PAH layer, thus forming stable hydrophilic stripes.

Finally, with flexible multilayer polymer films (Figure 6.10A and B), which could contain hundreds or thousands of nano-layers, it is possible to bend the material into a variety of shapes. To demonstrate the flexibility of the surface-modified template, I formed our multilayer EAA-LLDPE film into a spiral and embedded it in wax (Figure 6.10C). The fluorescence image of this spiral was clearly observed using fluorescence microscopy (Figure 6.10), where a thick fluorescent region is made up of 4032 layers (each thin layer ~ 500 nm thick). Therefore, using this approach, flexible patterned surfaces can be formed, with crosslinked PEMs linked to reactive layers to serve as functional moieties to adsorb or bond with other molecules.
Figure 6.10 (A, B) photograph of the flexible multilayer polymer film (EAA/LLDPE); (C) multilayer polymer film after embedding in wax, forming a spiral sharp; (D) fluorescence image after hydrophilic PEM coating and FITC grafting, with the single ring having 4032 nanolayers.

Summary

We successfully demonstrated that the surface negative charges on EAA films affected the morphology of deposited PEM films, and microporous thin films were formed without the electrostatic interaction between the PEM and the underlying substrates. When the PEM films were crosslinked by a carbodiimide coupling reaction in aqueous EDC/NHS solution, its stability was improved significantly. We also extended the PEM assembly technique to nano-striped surfaces, which were generated using the
cross-section of multilayer polymer films made from EAA and LLDPE. The PEM adsorbed on both the EAA and LLDPE regions, but was washed from the LLDPE with sonication due to the lack of linkage between PEM and LLDPE. Moreover, the carboxylic acids introduced by the PEM reacted with amine-terminated FITC to yield a stripe-patterned surface detected clearly by fluorescence microscopy.
References


CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

Conclusions

This research focused on developing an innovative nano-patterning technique coupled with tailored surface chemistry. Highly layered multilayer films with better uniformity were prepared using multilayer co-extrusion technique and followed by compression molding hundreds of multilayer films together. As an example, ethylene-co-acrylic acid copolymer (EAA) and linear low-density polyethylene (LLDPE) were used as the two components in the multilayer films, where EAA serve as the reactive material and LLDPE serve as inert material. Thermal analysis showed that there was a very thin transcrystalline region at the interfaces of microlayers and improved the adhesion between EAA and LLDPE, which is very important for the formation and stability of the patterned surfaces. Using SEM, TEM, and AFM, it was found that uniform multilayer structures were generated by microtoming and the LLDPE layers bumped up to about 200 nm higher than EAA layers for the thin sections. These patterned surfaces were stable after annealing at 60 °C for a long period of time. By grafting amine-terminated biotin and subsequently adsorbing streptavidin to the alternating layers of EAA, we demonstrated the feasibility of carefully functionalizing the carboxylic acids on EAA layers with ligands. Fluorescent images showed that streptavidin adsorption was confined to the modified EAA stripes. This approach is a simple but effective technique developed for fabricating highly layered micropatterns with various pattern sizes.
To improve the surface properties of EAA, including hydrophilicity and biocompatibility, other approaches were developed in this work. Dansyl cadaverine was successfully grafted on EAA surfaces and in its subsurface region. ATR-FTIR spectra and fluorescence intensities indicated that the acid chloride groups of EAA-Cl were not hydrolyzed in aqueous solution because the moisture could not reach the acid chlorides in the subsurface regions, thus protecting those reactive functional groups. Concentrated dansyl cadaverine solution in dichloromethane promoted subsurface grafting with a higher penetration rate than in dilute solution.

To minimize the grafting depth of surface modification for EAA films, NHS and EDC in aqueous solution were used to activate the carboxylic acid groups of EAA film. From ATR-FTIR spectra, it was found that the activation depth (estimated as 20 nm) was lower when NHS and EDC were used together to activate the carboxylic acids than that when only EDC was used. After activation of carboxylic acids, L-lysine and dendrimer AM64 were grafted onto the EAA surface with minimized grafting depth. They also introduced single or multiple primary amines for PEG grafting. Combining the data from ATR-FTIR and XPS, it was found that PEG chains were grafted on the surface of EAA films and a larger surface coverage was achieved when dendrimer was used as the intermediate layer. At the same time, the advancing water contact angle decreased to around 50° and the receding water contact angle was 0°.

I also successfully demonstrated that the surface negative charges on EAA films could be used to deposit PEM films (PAH/PAA), forming a hydrophilic coating and improving its stability by chemical cross-linking. I extended the PEM self-assembly technique to nano-striped surfaces, generated using multilayer polymer films. Importantly,
the carboxylic acids introduced by PEM films could react with amine terminated molecules for further functionality.

**Recommendations for Future Work**

In this research, I developed an innovative approach to fabricate reactive, nano-patterned surfaces using multilayer polymer films for a variety of applications. A few ideas derived from this research are recommended here for further investigation:

- Weak polyelectrolytes, polyallylammonium hydrochloride (PAH) and polyacrylic acid (PAA), were used to generate PEM patterns with alternating hydrophilic and hydrophobic regions. It was found that the PEM films were first adsorbed on both EAA and LLDPE regions through electrostatic and hydrophobic interactions respectively, but were removed from the LLDPE regions after crosslinking and sonication. Therefore, it would be interesting to study the adsorption of strong polyelectrolytes such as poly(diallyl-dimethylammonium chloride) (PDAC) and sulfonated polystyrene (SPS) on the patterned surfaces because electrostatic interactions will dominate so that the PEM films will be selectively deposited on EAA regions only. These strong PEM deposited, nano-patterned surfaces could be used as templates to direct charged particles or many other functional materials.

- Hydrophilic-hydrophobic patterned surfaces were fabricated by covering the EAA layers of the patterned surfaces with hydrophilic PEM films. The hydrophilic stripes could serve as templates to generate nanowires through deposition of conductive thiophene polyelectrolyte. Additionally, deposition of copper [1] is another possible approach, where electroless metallization of EAA layers is conducted in three steps:
Sn\textsuperscript{2+} ions are first coordinated to the oxygen atoms of the surface, then Pd is deposited as a catalyst through chemisorption, finally copper is deposited from a Cu\textsuperscript{2+} solution with formaldehyde.

- The EAA layers contain reactive carboxylic acids which are available for further functionalization. Instead of using LLDPE as the inert material, it will be worthwhile to use another reactive polymer, such as Nylon [2], providing reactive amine groups after pretreatment. Therefore, EAA regions could be modified to have one kind of affinity for a protein, and Nylon regions could be modified to have another kind of affinity. Protein patterns can be prepared with two alternating regions covered with different proteins. One region can also be modified to provide a high resistivity to protein adsorption, yielding a protein patterned surface with alternative protein adsorption and resistant regions.

- Extend the patterning technique to tissue engineering applications, including protein and cell patterning, using biodegradable polymers as the layer materials. For example, we can use poly(lactic acid) (PLA) as the inert polymer because it has excellent biocompatibility and biodegradability, and thermoplastic poly(vinyl alcohol) (PVOH) as the reactive polymeric material so that it could be processed with extrusion techniques and also provide reactive hydroxyl groups. The hydroxyl groups in alternating region can be used as initiators for ring-opening polymerization [3], introducing different functionalities for a variety of applications.

- The EAA surface was modified by grafting various PEGs to provide protein resistance. When propylenamine dendrimer was used as a linker layer, the EAA
surface was covered by PEG to a larger extent compared to other linkers, like l-lysine. To obtain an even larger surface coverage of PEG chains and make the process more straightforward, poly(l-lysine)-g-poly(ethylene glycol) (PLL-g-PEG) [4, 5] can be used directly without using a linker layer. Since the carboxylic acids of EAA can be charged in base solution, the polycationic PLL backbone of PLL-g-PEG would be attracted to the negatively charged EAA surface. Covalent bonds between –COO⁻ of EAA and –NH₃⁺ of PLL can be formed through a carbodiimide coupling reaction in aqueous EDC/NHS solution.
References


APPENDICES
Appendix A

Surface Modification of Ethylene-Acrylic Acid Copolymer (EAA) Films Using Poly(glycidyl methacrylate) (PGMA) as Linker Layer

Introduction

Ethylene acrylic acid copolymer (EAA) contains carboxylic acid groups not only on the outermost surface but also throughout the bulk. It provides exceptional adhesion to a wide range of substrates, including metal, polymers, and woods. However, the low surface energy of EAA limits its biotechnological applications due to its non specific adsorption and poor wettability. As a result, there are continuous interests in development of new strategies to modify the surface properties. In this work, another strategy was investigated in order to achieve covalent grafting of poly(ethylene glycol) (PEG) on EAA film with minimal penetration and subsurface modification.

Figure A.1 shows the approach, using poly(glycidyl methacrylate) (PGMA) as an efficient intermediate layer for the attachment of PEG chains. Some of the epoxide groups of PGMA were reacted to the EAA surface and the remaining epoxides were used for subsequent grafting with PEG.

![Diagram of the surface modification process](image)

**Figure A.1** Strategy for surface modification of EAA surfaces with PEG.
Experimental

Poly(ethylene-co-acrylic acid) (EAA, PRIMACOR 1410 from Dow Chemical Co., 9.5% w/w acrylic acid) films (thickness ~ 50 micron) were used as received from the Cryovac Division of Sealed Air Corp. (Duncan, SC). Carboxyl-terminated PEG (denoted as mPEG) was synthesized according to the method described in Chapter 4. Poly(glycidyl methacrylate) (PGMA) was provided by Dr. Igor Luzinov’s group (School of Materials Science and Engineering, Clemson University); its molecular weight (Mn) was about 140,000 g/mol as measured by GPC. The synthesis steps of PGMA were described in detail elsewhere [1-3].

EAA film was first dip coated (Mayer Feintechnik, model D-3400) into 0.2% (w/v) PGMA solution in acetone and epoxide groups were allowed to react with acid groups of EAA at 60 °C for 2 days. The modified EAA film was then washed with acetone to remove unreacted PGMA and dried at room temperature (film denoted as EAA-PGMA). The EAA-PGMA specimens were then smeared uniformly with mPEG and placed in a vacuum oven for 2 days at 60 °C to graft the PEG chains. The specimens were washed with sonication for 5 min in acetone (denoted as EAA-PGMA-PEG). ATR-FTIR and AFM measurements were done as described in Chapter 2.

Results and Discussion

Ethylene acrylic acid copolymer (EAA) contains carboxylic acid groups not only on the outermost surface and throughout the bulk. These can be used as reactive sites for surface grafting. In this work, studies were focused on covalently grafting PEG chains on EAA film surfaces with an aim of minimizing modification depth. As shown in Figure
A.1, PGMA was grafted onto the EAA surface to introduce functional groups for further PEG grafting with minimal penetration.

The reaction was confined to the surface by conducting reaction from the “melt” state because of the relatively slow diffusion of polymer chains in the melt state. This was the rationale for first attempting the grafting with PGMA as an intermediate layer. Figure A.2 shows the ATR-FTIR spectra for unmodified EAA film (bottom spectrum), EAA grafted with PGMA (middle), and EAA-PGMA grafted with PEG chains (top). The carboxylic acid peak at 1705 cm\(^{-1}\) did not decrease significantly but there appeared a small shoulder at 1743 cm\(^{-1}\), which may correspond to the ester formation of the PMGA reacted to the EAA surface. After reaction with mPEG for 2 days, a new peak was observed at 1110 cm\(^{-1}\), attributed to the ether groups in the grafted PEG.

AFM images revealed that the first step of dip coating in PGMA solution and subsequent reaction (EAA-PGMA) led to large, isolated surface domains that were microns in scale. Although it was proven that PGMA and mPEG were grafted on the surface through covalent bonding, phase separation occurred on the surface in the first step, suggesting that the PGMA coalesced into droplets when solvent was evaporated at room temperature. Since a more uniform surface coverage was desired, modification in solution was studied subsequently (Chapter 4).
Figure A.2 PGMA grafting IR spectra (bottom to top: EAA, EAA-PGMA, EAA-PGMA-PEG).

Figure A.3 AFM phase images (10 × 10 µm) for (A) EAA-PGMA, (B) EAA-PGMA-PEG.
Summary

Surface modification in the “melt” state was studied to covalently graft PEG chains on EAA surfaces with minimal penetration. PGMA was successfully used as a macromolecular link layer to anchor PEG chains in melt, but AFM figures showed that only large isolated surface domains (microns in scale) were obtained because of phase separation during dip coating and drying of the PGMA layer.
References


Appendix B

Blend Morphology and Thermal Behavior of LLDPE-EAA Extruded Films

Produced by Continuous Chaotic Advection

Introduction

Originally used to gain an understanding of mixing, chaotic advection has also been used to develop a wide variety of polymer blend morphologies, including multilayer, interconnected layer, platelets, interpenetrating network, and droplet [1, 2]. In this research, a continuous chaotic advection blender (CCAB) was used to prepare the polymer blends of ethylene acrylic acid copolymer (EAA) and linear low-density polyethylene (LLDPE). Specified morphologies could be developed by controlling the motion of stir rods to agitate melts.

Using the cross-section surfaces of the extruded polymer blends as templates, we tried to create nanopatterned surfaces functionalized with a variety of chemical constituencies. Such nanopatterned surfaces will have different micro- and nanostructures depending on the internal blend morphology developed in the “smart” blender. Because we will introduce surface functionalities through further surface modification of EAA regions, it becomes critical for us to study the interaction between these two polymers, their miscibility, interface boundary, and thermal stability. It was hypothesized that the low molecular weight, amorphous chains of LLDPE migrate to the EAA/LLDPE interface, forming a boundary layer. Moreover, the adhesion between domains of two polymers should provide enough strength to keep the microtomed templates intact. In this appendix, the morphology development of EAA/LLDPE blends formed with the CCAB device and their thermal properties of the structured polymer blends are described.
Experimental

Ethylene acrylic acid copolymer (EAA) (Primacor 3004, Dow Chemical) was used as minor component, and linear low density polyethylene (LLDPE) (Dowlex 2517, Dow Chemical) was used as major component. Polymer blends with 10 and 30% (w/w) EAA were produced. Viscosities were measured with a cone and plate viscometer (Rheometric Scientific, ARES, Piscataway, NJ) in the low shear rate range at 190 °C. As shown in Figure B.1, the viscosity ratio (EAA/LLDPE) varies from 1.9 to 2.4 in the applicable shear rate range. Blends were extruded as 500 micron thick and 20 cm width films, and then solidified on a chill roll maintained at 20 °C. Details about the CCAB device and process control were described elsewhere [3]. Optical microscopy and DSC measurements were done as described in Chapter 2.

![Figure B.1 Viscosity and viscosity ratio of EAA/LLDPE blends at 190 °C.](image-url)
Results and Discussion

Using the continuous chaotic advection blender, two melt components of LLDPE and EAA moved toward an extrusion point, and recursive stretching and folding was occurring in response to the continuing rotation of the stir rods. For the blends with 30% EAA, when the pairs of rotation number was zero ($N = 0$), two layered extruded film was obtained as shown in Figure B.2. According to the percentage of EAA and LLDPE in extruded films, it was observed that the dark region represented EAA and light region represented LLDPE. When the rod started rotating, these two melt components were stretched, folded and driven along the barrel. Different morphologies were developed through sequential morphology transitions. Layer structure was the initial mother structure for any other derivative structures. At blending period of $N = 7$, layer structure was defined as the thickness of EAA layer was $1 \sim 5 \mu m$. Further chaotic advection, which corresponds to large pairs of rotation number ($N = 9$), made the thickness of some layers decreased below $1 \mu m$. With continuous stretching and folding, platelet morphology formed at $N = 11$ because of the enlarging holes in the very thin layers. However, it was observed that the thickness of platelets was a little larger than that obtained at $N = 9$, indicating that the interfacial interaction between LLDPE and EAA was trying to decrease the interfacial strength by increasing the interface area. With the break of those platelets, droplets formed at larger $N$ and the diameters of the dispersed droplets were $1 \sim 3 \mu m$. For 10% EAA extruded films, the same morphology development was observed, except that the droplet structure formed early at the blending period of $N = 7$, with less dispersed droplets and similar diameter distribution.
Figure B.2 Various blend morphologies controlled by pairs of rotation number (N) of stir rods for EAA-LLDPE blends with 30% (w/w) EAA.

Figure B.3 (A) showed the DSC traces of EAA, LLDPE, and their blends at the heating rate of 10 °C min⁻¹. It was observed that the melting temperatures for EAA and LLDPE are 98 and 108 °C respectively. There was bimodal melting behavior for pure LLDPE. For their blends, there was no significant shift observed for the melting peaks, indicating that these two polymers (EAA and LLDPE) are immiscible at the working temperature. This result matched well with the theoretical calculation using Flory-Huggins theory discussed in Chapter 5. For the crystallization behavior of LLDPE, as shown in Figure B.3 (B), the crystallization peak intensity decreases proportionally with the decrease of the LLDPE content. Similar as the melting behavior, no shift was found for the crystallization peaks. There were two single crystallization peaks for the
EAA/LLDPE blends, and the crystallization temperatures were the same as those for the individual components of the blends. Therefore, EAA and LLDPE crystallized into two phases and no thick transcrystalline regions were formed at the interface of the polymer blends, as observed in Figure B.4. However, the adhesion at an interface between the polymers was strong enough to keep the thin microtomed sections intact. Several reasons can be presented for the narrowness of LLDPE/EAA blends, but the chemical properties of EAA seem to be the most possible reason. The acid units are capable of inter- as well as intra-molecular hydrogen bonding. Additionally, the acid groups probably form dimers in the amorphous region of the copolymer, which may hinder the migration of polyethylene chains into the EAA copolymer and limit the formation of transcrystalline regions.

Figure B.3 Heating (A) and cooling (B) thermograms of, LLDPE, EAA/LLDPE blends with 10, and 30% (w/w) of EAA, and EAA as shown in order.
Figure B.3 Heating (A) and cooling (B) thermograms of, LLDPE, EAA/LLDPE blends with 10, and 30% (w/w) of EAA, and EAA as shown in order (Continued).

Figure B.4 Optical micrograph of the interface of EAA/LLDPE blends with 30% (w/w) EAA extruded by CCAB.
**Summary**

Extruded films of linear low density polyethylene (LLDPE) and ethylene-co-acrylic acid copolymer (EAA) were fabricated by a continuous chaotic advection blender. Various morphologies, including multilayer, platelets, and droplets, were investigated using SEM. It was observed that the thickness of EAA layers decreased to less than 1 µm in the multilayer structure. DSC showed two distinct melting temperatures \((T_m)\) characteristic of the component polymers for the extruded polymer blends. After melting the multilayer films at high temperature and subsequent cooling, it was observed that the \(T_m\)s did not change, indicating that the two polymers are immiscible and the interdiffusion at the interface is very slow. Two single crystallization peaks also showed that EAA and LLDPE crystallized in two separate phases, and no thick transcrystalline was formed, as observed by polarized optical microscopy.
References


Appendix C

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