Biomedical Applications of Nanostructured Polymer Films

by

Jonathan Brian Gilbert

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ABSTRACT

Functional polymeric thin films are often stratified with nanometer level structure and distinct purposes for each layer. These nanostructured polymeric materials are useful in a wide variety of applications including drug delivery, tissue engineering, controlling condensation and polymeric batteries; all of which will be discussed in this work.

The first area of my thesis will detail the use of C\textsubscript{60} cluster-ion depth profiling X-ray Photoelectron Spectroscopy (XPS) to fundamentally understand how thin film structure and function relate. This method has the unique capability to determine the atomic composition and chemical state of polymeric thin films with \textless 10nm nanometer depth resolution without any chemical labeling or modification. Using this technique, I probed the nanostructure of functional thin films to quantify the interlayer diffusion of the biopolymer chitosan as well as demonstrate methods to stop this diffusion. I also explored the role of interlayer diffusion in the design of hydrophobic yet antifogging ‘zwitter-wettable’ surfaces. Additionally, I probed the lithium triflate salt distribution in solid block copolymer battery electrolytes (PS-b-POEM) to understand the lithium-ion distribution within the POEM block.

In the second area of my thesis, I show how the nanostructure of materials control the function of polymeric particles \textit{in vitro} and \textit{in vivo}. One example is a ‘Cellular Backpack’ which is a flat, anisotropic, stratified polymeric particle that is hundreds of nanometers thick and microns wide. In partnership with the Mitragotri group at UCSB, we show that cellular backpacks are phagocytosis resistant, and when attached to a cell, the cell maintains native functions. These capabilities uniquely position backpacks for cell-mediated therapeutic delivery and we show \textit{in vivo} that immune cells attached to backpacks maintain their ability to home to sites of inflammation. In addition, we have designed polymeric microtubes that can control their orientation on the surface of living cells. Inspired by chemically non-uniform Janus particles, we designed tube-shaped, chemically non-uniform microparticles with cell-adhesive ligands on the ends of the tubes and a cell-resistant surface on the sides. Our results show that by altering the surface chemistry on the end versus the side, we can control the orientation of tubes on living cells. This advance opens the capability to control phagocytosis and design cellular materials from the bottom up.

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3. INTRODUCTION AND BACKGROUND

3.1 Introduction

My thesis is focused around the versatile potential of structured polyelectrolyte multilayer thin films. These easy-to-fabricate polymeric thin films can have an exceptionally wide range of applications depending on the materials and the chosen nanostructure. The Rubner and Cohen groups alone have researched functional films that are antibacterial(1), anti-fogging(2), optically active(3), drug delivery systems(4), stimuli-responsive(5) and many other properties.

My thesis focuses on two areas. First I study the nanostructure of stratified polyelectrolyte multilayer films primarily through the use of depth profiling X-ray Photoelectron Spectroscopy (XPS) to fundamentally understand how thin film structure and function relate. This method has the unique capability to determine the atomic and chemical state of a thin film with depth with nanometer resolution without any chemical labeling or modification. Exploring the application of this technique I probed the nanostructure of functional thin films for applications in antifogging surfaces and solid block copolymer batteries. In the second area of my thesis I apply insights derived from this powerful technique to effectively create functional biomedical materials including flat anisotropic polymeric particles we call ‘cellular backpacks’ and also orientation controlled microtubes. I show the how the film polymeric film nanostructure controls the function in vitro and in vivo.

3.2 Nanostructured Thin Films

One method to fabricate nanostructured films is through the use of polyelectrolyte multilayers (PEMs). These films are formed by dipping a surface of any shape into a solution of
species that adsorbs and forms a conformal layer. The advantages of this method include that it is simple to perform, aqueous based, and can contain a diversity of materials. Previous work has used proteins(6), small molecules(7), nanoparticles(8), natural and synthetic polymers(9, 10), DNA(11), inorganic platelets(12) and many other materials as material to build functional thin films.

The film growth can be driven by a variety of interactions including hydrogen bonding, biological recognition, hydrophobicity and covalent interactions, but the most common method is electrostatic interactions as first described by Decher in the 1990s. The figure below shows the process of using electrostatic interactions to create a bilayer of polymers on a surface (13).

![Figure 3-1: Procedure to create electrostatic Polyelectrolyte Multilayers (PEMs) A) The surface is alternately dipped in solutions of oppositely charged polymers and rinsed in between. B) The polymers are adsorbed and sequentially build a film. – Adapted from (13)](image_url)

Depending on the choice of materials, PEMs can have a wide range of applications. For applications in biology, the PEM technique is especially relevant since the active materials can be deposited under physiological conditions, allowing for the incorporation of biomolecules. As a result, PEMs are important to biomedical problems such as drug delivery, tissue engineering and biomedical materials (14). Another application of PEMs explored is the control of water
condensation for applications in antifogging surfaces. Enabled by the capability to easily deposit films using the layer-by-layer assembly method, we studied the antifogging properties of a variety of materials ranging from hydrophilic biopolymers and hydrophobic fluorine containing polymers.

Furthermore, by altering the materials included, the films can become stimuli responsive. One type of stimuli responsive PEM of particular interest are hydrogen bonding films. If a film of hydrogen bonding polymers is raised above a critical pH value, then the ionization of the polymers will result in the film dissolving. This can be used to create free floating films which have a variety of biomedical applications (15, 16). The nomenclature for PEM films for the rest of this thesis follows the convention ("poly1"X/"poly2"Y)Z, where X and Y represent the pH of the polymer solutions used during assembly, and Z is the total number of bilayers deposited.

3.2.1 Fundamental Studies of Nanostructured Films

Functional thin films often require stratified structures with a distinct purpose for each layer and nanometer level control over the structure (17). However, due to dynamic diffusion of the species between layers, the composition and function of these layers may not be as desired. Analysis of the diffusion of species between layers has proven difficult with other methods (FRET, neutron reflectivity, confocal microscopy, FTIR, SIMS), but high-resolution depth profiling X-ray Photoelectron Spectroscopy (XPS) using polymer-friendly cluster-ion C60 sputtering can detect new details. This technique has never before been applied to functional nanostructured films, and allows for the direct determination of the composition and chemical bonding properties of polymeric films.
Applying this technique to a model nanostructured film in Chapter 4, we explored common issues in the field such as the dynamic competition between hydrogen bonding and electrostatic interactions during fabrication, blocking interlayer diffusion, the exchange of film components with a surrounding solution, and the kinetics of interlayer diffusion. Using high resolution depth profiling XPS we analyzed the diffusion of chitosan, a commonly used biopolymer, within the PEM. Furthermore, we quantified the effect of this diffusion on the film structure and function and finally showed a method to stop this interlayer diffusion. We propose that our results address some common issues of interlayer diffusion and could be applied to multiple fields utilizing organic thin films.

I further applied this technique to two very distinct problems: one in surface science and one in polymer batteries. In Chapter 5, in collaboration with Hyomin Lee of the Rubner/Cohen Group, we analyzed the molecular profile and nanostructure of zwitter-wettable surfaces to understand the role of polymer interlayer diffusion on antifogging and antifrosting surfaces. Zwitter-wettable surfaces are unique surfaces with the capability to absorb molecular water directly into the film, yet still present a hydrophobic character to macroscopic water droplets. This uncommon combination provides the ability to overcome the limitations of current hydrophilic anti-fogging surfaces.

In Chapter 6, I demonstrate the resolution limits of the depth profiling technique to resolve the structure of lamellar block copolymer systems. This work is in collaboration with Ming Luo of the Epps Group at the University of Delaware. We analyze aligned lamellar films of polystyrene-b-poly(oxyethylene methacrylate) that have been swollen with lithium salts for applications in solid state lithium-ion batteries. This block copolymer architecture is an exciting type of solvent-free polymer electrolyte, since the PEO based region can solubilize the lithium
ions while the polystyrene can provide the mechanical rigidity needed to maintain the separation of the anode and cathode(18). For this application, we are interested in whether XPS depth profiling can resolve the distribution of lithium within the nanostructured PEO region. Previous research has seen significant increases in conductivity with larger (higher molecular weight) domains and it has been hypothesized that the lithium ions are concentrated in the center of the POEM. However, the experimental proof is limited(19). Using depth profiling XPS, we believe that better analysis capabilities may enable a thorough understanding of the phenomenon and potentially allow for more effective polymer electrolyte design.

3.2.2 Biomedical Applications of Nanostructured Films

The second area of my thesis is focused on the design of nanostructured materials for biological applications. In particular, I have chosen to focus on drug delivery since effective material delivery to desired regions in the body has the potential to greatly improve human health. Drug delivery has been enabled by designing the surface chemistry and, more recently, the shape of particles for therapeutic targeting (20). In particular, recent research from the Mitragotri group at UCSB clarified the effect of shape on cellular response and applied these insights for enhanced drug delivery(21). Furthermore, recent studies have started using cell-mediated drug delivery which uses the cell’s natural function, such as homing to inflammation or disease sites, to deliver the desired therapeutic materials(22). This synergistic combination has great potential to increase the efficiency of drug delivery in the future.

In Chapter 7, I discuss the development of a uniquely shaped polymeric particle termed a cellular backpack that conjugates strongly to the surface of immune cells through specific interactions and has many applications in cell-mediated delivery. The technology was first developed by Albert Swiston in the Rubner/Cohen group in 2008 and he initiated our work with
immune cells and UCSB. In general, backpacks are anisotropic, stratified polymeric particles that are hundreds of nanometers thick and microns wide(23). Since a backpack leaves most of the cell surface unaltered, the biological functions and characteristics of cells have not shown to be greatly changed upon its conjugation with the cell surface. Additionally, Swiston noticed that the backpacks were not being phagocytosed, and in partnership with the Mitragotri group at UCSB, we further explored this potential. Our continuing collaboration with the Mitragotri group has shown that the backpack is uniquely positioned for cell-mediated therapeutic delivery in areas such as inflammation or cancer.

Chapter 8 discusses cellular microtubes as another application of nanostructured materials for biomedical applications. The cellular microtube is built on the idea that shape, surface chemistry and size can be designed to increase the function of a material. In particular, it has been found that the local shape of an anisotropic particle in contact with the cell determines the internalization rate of the particle(24). This insight drives the desire to design anisotropic particles that orient themselves on the surface of living cells to either promote or resist internalization depending on the desired purpose. For example, one can imagine using the controlled orientation interactions of anisotropic particles to form stable cell-biomaterial hybrids for cell-mediated drug delivery. Inspired by the use of chemically non-uniform Janus or patchy particles to control the local orientation of synthetic particles in colloid systems(25), we designed a tube-shaped, chemically non-uniform microparticle with the capability to control its orientation on cell surfaces. This advancement opens the possibility to design new cell-biomaterial hybrids for a variety of biomedical applications.
4. DEPTH PROFILING XPS ANALYSIS OF INTERLAYER DIFFUSION IN POLYELECTROLYTE MULTILAYERS


4.1 Introduction

Layer-by-Layer assembly of polyelectrolyte multilayers (PEMs) allows for the precise deposition of ultrathin organic films that can conformally coat features of any shape and size. These films can incorporate a variety of species, leading to a wide range of applications including antifogging(26), anti-reflection(27), drug delivery(14, 23, 28-30), fuel cells(12) and responsive materials(31). Since the multilayer films are assembled through a sequential self-limiting adsorption process onto a substrate(13), a major advantage of the technique is the ability to constrain the location of certain materials within the film at the nanoscale by simply controlling the order of material deposition. Such nanoscale spatial control has allowed the creation of complex periodic heterostructures not easily realized by other deposition techniques(32). In some cases however, diffusion of the constituent macromolecular species in and out of the film may occur, changing the desired film stratification, composition, growth profile and properties(17). This type of diffusion, known as interlayer diffusion, is often detrimental to the desired properties but also could be an opportunity to impart new functions. Yet, interlayer diffusion has proven difficult to fully characterize and control and a more thorough understanding is needed.
Interlayer diffusion in PEM films can be detrimental if the desired stratified heterostructure is lost during the assembly process, a post-assembly treatment or in use. For example, in the cases of the sequential release of therapeutics\(^{(30)}\), structural color\(^{(33)}\), organic LED devices\(^{(34)}\), solar cells\(^{(35)}\) and on-demand release of PEM films\(^{(23, 36)}\), the loss of stratification due to interlayer diffusion results in the loss of the desired function. However in other cases, such as surface planarization for the creation of higher efficiency dye-sensitized solar cells\(^{(35)}\) or ordering of the internal or surface arrangement of PEM films\(^{(37, 38)}\), interlayer diffusion can be harnessed to provide functional benefits. Interlayer diffusion can also be used to tune material properties such as the critical dissolution pH in hydrogen bonding systems through the addition of small amounts of electrostatic cross-links\(^{(36)}\) or viscoelasticity through diffusion of stiffer polymer components\(^{(39)}\). In all of these cases, interlayer diffusion must be understood and controlled.

Interlayer diffusion also affects the mechanism of PEM film growth. Some polymer systems show linear growth where the bilayer thickness is invariant with deposition cycles, while other systems show exponential growth with progressively increasing bilayer thicknesses\(^{(40)}\). It is widely believed that exponential growth arises from rapid interlayer diffusion of polymers throughout the film during the fabrication steps\(^{(40, 41)}\), however some disagree with this conclusion\(^{(42)}\). Improved analysis techniques that provide spatial information about the location of specific molecules within a multilayer thin film are therefore clearly needed.

Due to the importance of understanding interlayer diffusion, a variety of techniques have been employed to analyze it with varying degrees of success. These techniques include confocal microscopy\(^{(43-45)}\), fluorescence resonance energy transfer (FRET)\(^{(46, 47)}\), FTIR\(^{(48)}\), neutron reflectivity\(^{(49-51)}\) and X-ray reflectometry\(^{(52)}\). Confocal microscopy is limited in spatial
sensitivity since films much thicker than the typical PEM thickness (500 nanometers or less) are required due to a relatively low z-resolution (43-45). FRET is more sensitive but relies on fluorescent modification of polymers for indirect measurements of diffusion (46, 47). FTIR can provide valuable information on the exchange of polymers in solution with PEM film components, but commonly probes the full thickness of the film, limiting the ability to spatially resolve the effects of diffusion (48). Neutron reflectivity and x-ray reflectometry (49-52), require nuclear contrast and electron density contrast respectively in the film, and commonly require the use of special deuterated polymers. In comparison, x-ray photoelectron spectroscopy (XPS) is a highly sensitive surface analysis method that probes the top 10 nm of a film. When combined with sputtering or etching sources to slowly remove material between analysis cycles without damaging underlying material, depth profiling XPS enables high resolution chemical analysis of polymer films. The information provided by this technique could expand the understanding of how to control PEM structure, what compositional/structural changes occur with interlayer diffusion and when polymers in the film exchange with deposition/post-assembly solutions.

Development of less destructive sputtering or etching sources has been the enabling step in advancing polymer depth profiling capabilities. Many depth profiling techniques use single-atom sputtering sources like argon, applicable to inorganic materials but severely damaging to polymers (53-55). Only recently have cluster ion sources like $\text{C}_{60}^+$ been used in conjunction with XPS for analysis of polymer films with depth (53, 56, 57). Cluster ion $\text{C}_{60}^+$ sputtering is much less damaging since the energy transfer from the ion to the material occurs primarily at the surface, minimizing the chemical damage deep into the film (58). Therefore most of the damaged material is removed from the surface, minimizing its interference with the proper analysis of the exposed surface (59).
One strategy in the fabrication of functional PEM films is the use of blocking layers to minimize interlayer diffusion. Earlier studies(30, 44, 60-62) have shown that the properties of a successful blocking layer depend on the diffusing species under consideration and the conditions of diffusion. Some have found that covalent cross-linking is the only way to stop interlayer diffusion of polymers(30, 63) while others have noted that electrostatic interactions can be utilized to stop interlayer diffusion (44, 64, 65). Of interest to this study is how blocking layers enable the controlled production of free-floating PEM films by maintaining the desired dissolution properties of a sacrificial region that anchors a pH stable PEM film to a substrate surface. Once released, these free-floating assemblies have been used for tissue engineering(66) and drug delivery(23, 29).

In this paper we designed a model system that enables the study of common attributes of interlayer diffusion found in many PEM systems including blocking layer effectiveness. The sacrificial component of this model PEM system is a hydrogen bonded region (poly(acrylic acid)/poly(ethylene oxide)) that is insoluble at low pH but becomes soluble at a critical higher pH. A pH stable PEM system based on chitosan and hyaluronic acid (HA) is assembled on top of this sacrificial region with the goal of creating an on-demand pH triggered release of the chitosan/HA multilayer films. A related approach has previously been used by us to create cellular backpacks that attach to immune system cells via specific interactions between HA and CD-44 receptors on the cell surface(23). From these earlier studies, it became apparent that the assembly of chitosan/HA onto a hydrogen bonded region rendered the entire multilayer system insoluble under pH conditions that should dissolve the sacrificial region. Thus, this work seeks to determine if interlayer diffusion of chitosan/HA causes the changes in solubility and how suitable blocking layers can prevent these changes. From a fundamental perspective, this model
system allows the exploration of elements such as the competition between hydrogen bonding and electrostatic interactions in multilayers, the design of an effective blocking layer, the exchange of film components with a surrounding solution and the extent and kinetics of interlayer diffusion. Utilizing XPS depth profiling data acquired with $\text{C}_{60}^+$ cluster ion sputtering, we find that chitosan diffuses effectively into the hydrogen bonded region of the multilayer film and displaces the hydrogen bonded component poly(ethylene oxide). In addition, we show that this interlayer diffusion process can be blocked completely with only a single adsorbed layer of a polycation.

4.2 Experimental Set-up

The model PEM films examined in this work were assembled on glass slides coated with an 80nm poly(diallyldimethylammonium chloride) (PDAC) and poly(styrene sulfonate) (SPS) adhesion promoting multilayer. Above this adhesion layer is a hydrogen bonded film comprised of poly(acrylic acid) (PAA) and poly(ethylene oxide) (PEO). The solubility of this hydrogen bonded PEM system is pH sensitive and above a pH of 3.6, it will dissolve in water(67). Figure 4-1 shows a cartoon representation of the multilayer heterostructures examined in this work along with the experimentally determined thicknesses of the various regions of the multilayer. In the top case (Figure 4-1A), the experiments involved immersing a hydrogen bonded multilayer film in a chitosan solution for varying amounts of time. In the bottom case (Figure 4-1B), blocking layers containing varying numbers of poly(allylamine hydrochloride) (PAH) and SPS layers were deposited on top of the hydrogen bonded region followed by the assembly of a hyaluronic acid (HA) and chitosan (CHI) multilayer.
Figure 4-1: Schematic of systems used to test (A) chitosan diffusion into the hydrogen bonded region and (B) electrostatic blocking layer effectiveness. The number after the polymer abbreviation is the deposition solution pH.

These stratified films were dried and then analyzed using depth profiling XPS paired with C$_{60}^+$ sputtering to collect C1s, O1s, N1s and Si2p high resolution spectra. It is important to note that prolonged X-ray exposure and C$_{60}^+$ sputtering can alter the chemical composition of PEMs and decrease the interface resolution (56, 68). As described in Fig. S1, the choice of XPS data acquisition parameters and sputtering conditions is very important since long periods of X-ray exposure reduced the O to C ratio and in particular the signal of the carboxyl peak at ~289eV(69). As a result we chose acquisition parameters and C$_{60}^+$ sputtering conditions to minimize the total X-ray exposure time while still obtaining an acceptable resolution and signal to noise ratio at each point in the depth profile.

4.3 Diffusion of Chitosan in Hydrogen Bonded Multilayers

To explore the question of whether the adsorbed chitosan diffuses into the swollen hydrogen bonded region and by how much, the nitrogen signal from the amine on chitosan was analyzed as a function of depth for hydrogen bonded multilayers exposed to a 0.1% (w/v) chitosan solution at pH 3 for a specified amount of time. The chitosan solution acted as an infinite supply for diffusion of chitosan into the hydrogen bonded region. All samples were
rinsed with water for four minutes and dried with nitrogen gas prior to analysis. The compiled spectra for chitosan exposure times of 1, 3, 10 and 60 minutes (CHI1, CHI3, CHI10 and CHI60) are plotted in Figure 4-2A-D. Color was added to highlight the approximate locations of the distinct regions of the PEM film, using the same color scheme shown in Figure 4-1A. The depth of the (red) chitosan region was determined by analyzing the intensity of the N1s signal with depth. When the N1s signal dropped to background levels the spectrum was colored yellow to denote the hydrogen bonded region. Finally, the (black) adhesion layer starts when the N1s signal increases at the base of the film due to the presence of nitrogen-containing PDAC.

Figure 4-2: Diffusion of chitosan into hydrogen bonded films. Spectra of hydrogen bonded (PAA3/PEO3) films exposed to chitosan solution for different amounts of time. (A) 1 min exposure (B) 3 min exposure (C) 10 min exposure (D) 60 min exposure to chitosan. The color scheme is the same as that of Figure 1A. Red spectra represent chitosan infused areas, yellow spectra represent the hydrogen bonded (PAA3/PEO3) area and black spectra represent the (PDAC4/SPS4) adhesion layer. (E) Quantification of A-D to determine the atomic concentration of nitrogen with depth in the film. Data points are individual dots and line shown is the result of a Savitzky-Golay five point quadratic algorithm.

The spectra from Figure 4-2 were analyzed to determine the atomic percentage of nitrogen with depth as seen in Figure 4-2E. A film not exposed to chitosan is shown in Figure 4-7. The concentration of nitrogen in the multilayer film increased systematically with time of exposure to the chitosan solution. Also, the maximum depth at which an appreciable nitrogen
signal was observed increased with time. Separate experiments showed that the PDAC from the adhesion layers does not enter the hydrogen bonded region during the assembly process even after many hours at pH 3 (Figure 4-7). Thus, the only source of nitrogen in the film, above the 80nm adhesion layer, is from the chitosan that diffused from the top of the hydrogen bonded region. For the samples CHI1, CHI3, and CHI10 the location of the diffusion front, (where N1s concentration is 50% of the maximum value), advanced 181, 238 and 299 nm respectively as measured in dry films. Since the final dry thickness remained relatively constant, independent of chitosan diffusion depth, the diffusion of chitosan does not greatly expand or collapse the film. As a result, the dry diffusion distance of chitosan directly correlates with the thickness of the portion of the (PAA3/PEO3) film that was altered by chitosan diffusion. Therefore, to estimate the diffusion coefficient, the chitosan penetration distances were multiplied by a factor of 2.5 to account for the 250% swelling of the (PAA3/PEO3) film in pH 3 water (Table 4-1 and Table 4-4). After 60 minutes in the chitosan solution (CHI60), the chitosan has diffused through the entire hydrogen bonded region as seen by the uniformly high nitrogen content throughout the film in Figure 4-2E. These results clearly show that the adsorbed chitosan diffuses into the hydrogen bonded region. Chitosan is known to be highly diffusive due to a lower charge density than typical polyamines like PAH and the presence of multiple hydrogen bonding acceptors(45, 46).
Table 4-1: Diffusion of Chitosan in a Swollen Hydrogen Bonded Film

<table>
<thead>
<tr>
<th>Sample</th>
<th>t (sec)</th>
<th>Dry film distance (nm)</th>
<th>Swollen film distance (nm)</th>
<th>D (cm²/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHI 1 min</td>
<td>360</td>
<td>181</td>
<td>452</td>
<td>1.42E-12</td>
</tr>
<tr>
<td>CHI 3 min</td>
<td>720</td>
<td>238</td>
<td>594</td>
<td>1.23E-12</td>
</tr>
<tr>
<td>CHI 10 min</td>
<td>900</td>
<td>299</td>
<td>748</td>
<td>1.56E-12</td>
</tr>
</tbody>
</table>

Dry distance multiplied by 2.5 to account for 250% film swelling at pH 3

To estimate the diffusion coefficient of chitosan into the hydrogen bonded region we used the data in Table 4-1 and the characteristic diffusion length, \( L = \sqrt{4Dt} \) (70). As seen in Table 4-1 the calculated diffusion coefficient is consistent for the three time points sampled and is \(~1.4\times10^{-12}\) cm²/sec. Recent reports on interlayer diffusion coefficients in polyelectrolyte multilayers range from \(10^{-20}\) cm²/sec for SPS in linearly growing (PAH/SPS) films(49) to \(10^{-7}\) cm²/sec for poly(L-lysine) in exponentially growing poly(L-lysine)/hyaluronic acid films(40). This wide range of reported interlayer diffusion coefficients is due to a fundamental difference in the film growth mechanism between linearly and exponentially growing films. In linear growth conditions, the deposited polymers generally interact only with the top surface and thus generally have interlayer diffusion coefficients below \(10^{-17}\) cm²/sec(49-51). In comparison, exponentially growing systems require some amount of interlayer diffusion to occur during the dipping cycle(40) and as a result have higher reported interlayer diffusion coefficients in the range of \(10^{-16}\) cm²/sec to \(10^{-7}\) cm²/sec depending on the conditions and polyelectrolytes used(40, 46). A recent paper by Lundin et al. (46) used fluorescence resonance energy transfer (FRET) and showed that the interlayer diffusion of chitosan in exponentially growing films made of chitosan and heparin was \(~10^{-15}\) cm²/sec for 150kDa chitosan. Our reported interlayer diffusion coefficient of about \(10^{-12}\) cm²/sec for chitosan of roughly the same molecular weight is larger,
but well within the range of other exponentially growing polymer systems previously studied. In addition, the diffusion coefficient we report would be higher than the diffusion coefficient of chitosan in a pure film of chitosan/heparin since Xu et al. showed that weaker matrix interactions enable a higher diffusion coefficient(50). Given that in our study chitosan is diffusing in a hydrogen bonded (PAA3/PEO3) matrix with a low interaction strength as measured by dissolution pH and is without internal structure as measured by neutron reflectivity(71) it would thus allow for a higher diffusion coefficient than the more strongly interacting electrostatic matrix of chitosan and heparin.

4.4 Displacement of PEO from Film by Chitosan

One possible effect of interlayer diffusion in PEMs is the displacement of materials from the film into solution(48, 60, 72). In our model system, interlayer diffusion of chitosan into the hydrogen bonded region changes the dominant interaction from hydrogen bonding between PAA and PEO to electrostatic interactions between PAA and chitosan. FTIR confirmed these new electrostatic interactions. In films with large amounts of chitosan diffusion, the PAA ionization level increased due to the titration of carboxylic acid groups to carboxylate groups by cationic chitosan (Figure 4-8)(9). Therefore upon chitosan diffusion, electrostatic interactions between chitosan and PAA displace the weaker hydrogen bonding interactions between PEO and PAA. As a result, the hydrogen bonding acceptor PEO is no longer associated with the film and can diffuse out.

Enabled by the high sensitivity of XPS, the displacement of PEO can be directly explored. Figure 4-3 shows the high resolution C1s data from the CHI1 and CHI3 samples using the color scheme from Figure 4-1A (CHI10 and CHI60 data in Figure 4-9). The red region is infiltrated with chitosan and its location in this region was determined by analyzing the intensity
of the N1s signal with depth as in Figure 4-2. In the remaining depth of the film, the N1s signal is very low (<0.5% atomic conc. N), signifying that in this yellow region, the film contains little to no chitosan. To analyze the displacement of PEO from the film, the C1s spectra from the (red) chitosan infiltrated regions were compared to the spectra in the (yellow) hydrogen bonded regions of the film. In particular we focus on the change in C1s signal intensity at 286.5eV. Both PEO and chitosan have a signal at this point, but since the extent of chitosan diffusion can be independently determined by the nitrogen signal, the changes in C1s spectra can be used to analyze the displacement of PEO from the film. The C1s spectra of pure PEO, chitosan and PAA can be seen in Figure 4-10.

Figure 4-3: High resolution C1s XPS depth profiling of a hydrogen bonded film exposed to chitosan solution. (A) 1 min (B) 3 min exposure to chitosan. The color scheme is the same as that of Figure 4-1A. Red spectra represent chitosan infused areas, yellow spectra represent the hydrogen bonded (PAA3/PEO3) areas and black spectra represent the (PDAC4/SPS4) adhesion layer. Comparing the red chitosan diffused areas to the yellow hydrogen bonded areas the chitosan diffused areas have a lower signal at 286.5eV since PEO has diffused out. (C) Comparison of C1s spectra with different chitosan exposure times to the initial yellow
hydrogen bonded area. The longer exposure to chitosan solution, the more the PEO signal at 286.5eV decreases. All chitosan exposed spectra were from ~450 nm above the glass surface.

As shown in Figure 4-3 A and B, the red regions where chitosan has diffused into the film, have a markedly lower signal at 286.5eV than the yellow hydrogen bonded region. The change in the signal intensity at 286.5eV is highlighted in Figure 4-3 C which compares the chitosan infiltrated regions from Figure 4-3 A and B to the (PAA3/PEO3) hydrogen bonded region. The spectra of all chitosan exposed samples were obtained from ~450nm above the glass surface to minimize differences due to X-ray exposure time or C60+ sputtering time. The decrease in signal intensity at 286.5eV is a result of chitosan diffusion displacing PEO and allowing it to diffuse out of the film. Since chitosan also has a peak at 286.5eV, if PEO was not diffusing out of the film, this signal would increase. Figure 4-3 C also shows that the decrease in the signal at 286.5eV correlates with the holding time in chitosan solution, which is consistent with the diffusion of PEO out of the film.

4.5 Electrostatic Blocking Layer Stops Chitosan Diffusion

In many cases it is desirable to stop interlayer diffusion to maintain distinct functional regions of a multilayer heterostructure. The effect of electrostatic blocking layers on the diffusion of chitosan into the hydrogen bonded region was investigated using the film architecture shown in Figure 4-1 B. Unlike the previous study, above the hydrogen bonded region there is an electrostatic blocking layer that varies from a single layer of PAH (<1nm) to 9.5 bilayers of (PAH3/SPS3) (10nm). On top of the blocking region, the final region is a 20nm (HA3/CHI3) multilayer film. To determine the location of the distinct regions of the PEM film and apply the color scheme shown in Fig. 1B, information from the C1s and N1s spectra was combined. For example, in Figure 4-4A the transition from the red HA/CHI region at the surface to the green (PAH3/SPS3) blocking region was determined by the change in shape of the C1s
spectrum. The shape change is a result of more carbon-carbon bonds at 285eV and fewer carbon-oxygen bonds at 286.5eV in the green region compared to the red region. Since XPS analyzes approximately the top 10nm and the blocking layers are less than 10nm, the green spectrum representing the blocking layers likely contains signal from an adjacent region as well. Yet the C1s spectra of the blocking layers remain distinct from the red and yellow C1s spectra. The end of the green region was determined by the drop in N1s signal and thus the yellow hydrogen bonded region begins. Finally, the black adhesion layer starts when the N1s signal increases at the base of the film due to the presence of nitrogen-containing PDAC. These data reveal that each of the various regions of the multilayer heterostructure illustrated in Figure 4-1B can be identified in XPS depth profile spectra.

Figure 4-4: Effect of a blocking layer on interlayer diffusion of chitosan (A) C1s and N1s regions from depth profiling XPS of a hydrogen bonded sample with a (PAH3/SPS3)3.5 blocking layer topped with (HA3/CH13)3.5. The color scheme is the same as that of Figure 4-1B. Red spectra represent (HA3/CH13), green spectra represent the (PAH3/SPS3)
electrostatic blocking layer, yellow spectra represent the (PAA3/PEO3) hydrogen bonded region and black spectra represent the (PDAC4/SPS4) adhesion layer. (B) Quantification of the nitrogen signal for different blocking layer systems tested. Data points are individual dots and the line is the result of a Savitzky-Golay five point smoothing algorithm. All films had (HA3/CHI3)$_{3.5}$ deposited on top of the blocking layer.

The N1s spectra from depth profiling samples with blocking layers were analyzed to determine the atomic percentage of nitrogen with depth as seen in Figure 4-4B (N1s spectra from all samples with blocking layers seen in Figure 4-11). From Figure 4-4B it is clear that all three electrostatic blocking layers tested (PAH3/SPS3)$_z$ ($z=0.5, 3.5, 9.5$) with approximate thicknesses of <1nm, 4nm and 10nm respectively, effectively stop the diffusion of chitosan into the hydrogen bonded region as seen by the absence of any detectable nitrogen signal in the bulk of the film. Remarkably, even though the films spent over 30 minutes in chitosan solution during the (HA/CHI) film fabrication, even a single adsorbed layer of PAH was sufficient to block its diffusion into the hydrogen bonded region. At pH 3, PAH is a fully charged polycation (pKa ~8)(9) so it has a strong electrostatic interaction with the ionizable polyanion PAA found at the top of the hydrogen bonded region(73). Due to the strong electrostatic interaction, the PAH blocking layer is kinetically trapped at the top of the film (48) and effectively stops the diffusion of chitosan into the hydrogen bonded region under the conditions employed in this study. Recent literature (30, 44, 60, 63, 64) has shown that different blocking layers work well for different polymer systems and annealing conditions. In some cases covalently cross-linked blocking layers are needed(30, 63), but in other cases electrostatic blocking layers can stop interlayer diffusion as well(44, 64). In the conditions tested in this work, a single electrostatic blocking layer is all that was needed to block the interlayer diffusion of chitosan.

In the absence of a blocking layer, chitosan from the (HA3/CHI3) multilayer film diffuses into the entire hydrogen bonded film during fabrication, producing a high nitrogen signal
throughout as seen in Figure 4-4B. Even though the film has fewer total layers deposited than the multilayer films with blocking layers, the interlayer diffusion of chitosan allows for a large increase in the final film thickness as shown by the leftmost data point in Figure 4-4B. This large increase in thickness is characteristic of exponential growth which is caused by the diffusion of polymers and polymer pairs in and out of the film during deposition(40). As a result we expect that chitosan from not only the first bilayer deposition, but from the subsequent depositions to be present throughout the film. Similar chitosan diffusion into the hydrogen bonded region is also observed when the order of polymer deposition is switched from hyaluronic acid first (HA3/CHI3) to chitosan first (CHI3/HA3) as revealed in Figure 4-12. As a result of macromolecules like chitosan diffusing throughout a film, hydrogen bonded films could be used as scaffolds for easily loading of drugs or other macromolecules of interest(14, 74).

An interesting question to ask concerning the use of depth profiling XPS with $C_{60}^+$ sputtering is what level of vertical resolution is possible and is it sufficient to probe PEM heterostructure interfaces? The interface used to analyze this resolution was the sharp boundary between the (PAH3/SPS3) blocking layers and the hydrogen bonded region seen in Figure 4-4B. The precipitous drop of nitrogen signal from the blocking layer to the hydrogen bonded region occurs between two data points or approximately 15nm. Reducing the sputtered thickness between successive XPS spectra would allow for the interface resolution to increase to approximately 10nm since this is commonly the depth of analysis during an XPS cycle. However, near this limit of resolution, extended sputtering time may cause radiation induced diffusion and surface roughening which need to be considered during experimental design(68).
4.6 pH Sensitivity of the Hydrogen Bonded Region

The purpose of a blocking layer is to maintain the distinct properties of each region of a multilayer film. In our model system the as-assembled (PAA3/PEO3) hydrogen bonded region dissolves above pH 3.6(67). If the film is altered by sufficient chitosan interlayer diffusion, the multilayer becomes insoluble at neutral pH. Therefore to test whether the desired properties of the hydrogen bonded region can be maintained through the use of blocking layers, all films characterized previously were exposed to a buffered salt solution of PBS pH 7.4 for 30 minutes. After 30 minutes the residual dry film thickness was compared to the initial dry film thickness to determine if the hydrogen bonded film dissolved. As see in Table 4-2, all multilayer films with a blocking layer dissolved in PBS. Therefore even a single layer of PAH is able to effectively block chitosan diffusion and maintain the pH sensitive solubility of the hydrogen bonded region.

Table 4-2: pH Sensitivity of the Hydrogen Bonded Region

<table>
<thead>
<tr>
<th>Top layer</th>
<th>Blocking Layer</th>
<th>Dissolve in PBS pH 7.4?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 4-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(HA3/CHI3)₃.₅</td>
<td>(PAH3)</td>
<td>Yes</td>
</tr>
<tr>
<td>(HA3/CHI3)₃.₅</td>
<td>(PAH3/SPS3)₁.₅</td>
<td>Yes</td>
</tr>
<tr>
<td>(HA3/CHI3)₃.₅</td>
<td>(PAH3/SPS3)₉.₅</td>
<td>Yes</td>
</tr>
<tr>
<td>(HA3/CHI3)₃.₅</td>
<td>none</td>
<td>No</td>
</tr>
<tr>
<td>Figure 4-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHI 60</td>
<td>none</td>
<td>No</td>
</tr>
<tr>
<td>CHI 10</td>
<td>none</td>
<td>No</td>
</tr>
<tr>
<td>CHI 3</td>
<td>none</td>
<td>No</td>
</tr>
<tr>
<td>CHI 1</td>
<td>none</td>
<td>No</td>
</tr>
</tbody>
</table>

After exposure to pH 7.4 PBS for 30 min, the remaining thickness was compared to the initial thickness to determine if the hydrogen bonded region dissolved

In the absence of a blocking layer, the multilayer films no longer dissolved due to pH stable electrostatic crosslinks formed between the diffused chitosan and PAA. In some cases like CHI1, CHI3 and CHI10 this result was unexpected since chitosan had not diffused all the way through the film (Figure 4-2E) before PBS exposure. However, depth profiling of CHI10 after
PBS exposure shows that chitosan has diffused throughout the film and stabilized it to pH changes (Figure 4-13). Therefore, in the absence of a blocking layer, chitosan interlayer diffusion was stopped by drying for analysis but continues after exposure to PBS solutions. Since hydrogen bonded PEMs require a minute or two to dissolve(75), this brief time allows for further chitosan diffusion, rendering the film insoluble in PBS.

4.7 Conclusions

XPS with C$_{60}^+$ cluster ion sputtering is a powerful technique for analyzing the atomic composition and chemical state of organic nanostructured films. With the correct choice of conditions to minimize sample damage it can directly determine to within 15 nm the location of polymers through the thickness of a film, allowing for analysis of interlayer diffusion; in addition to testing the efficacy of various blocking layers. Using our model system we have shown that chitosan is highly diffusive with an interlayer diffusion coefficient around $1.4\times10^{-12}$ cm$^2$/sec in hydrated hydrogen bonded (PAA3/PEO3) films. Also, the high resolution capabilities of XPS show the displacement of hydrogen bonded PEO in favor of electrostatic interactions between chitosan and PAA. Finally, various thicknesses of PAH-containing blocking layers were explored, including a single layer of PAH that can stop the diffusion of chitosan into the hydrogen bonded region.

We believe that the ability to control and measure the interlayer diffusion in PEMs will have applications in a variety of areas. For example, exchanging hydrogen bonding interactions for electrostatic may be important for loading of biological molecules through post assembly modification of films. Furthermore, the design of films capable of blocking macromolecule diffusion is relevant to structured films, sequential drug release and production of free-floating PEM films.
4.8 Materials and Methods

**Materials:** poly(acrylic acid) (PAA, Aldrich, M=450kDa), poly(allylamine hydrochloride) (PAH, Aldrich, M=15kDa), poly(ethylene glycol) (PEO, PolySciences, M=20kDa), poly(diallyldimethylammonium chloride) (PDAC, Aldrich, M=200-350kDa in 20% aqueous solution), poly(styrene sulfonate) (SPS, Aldrich, M=70kDa), hyaluronic acid (HA, from Streptococcus equi, Fluka, M~1580kDa), acetic acid (Sigma) and low MW chitosan (CHI, deacetylation 0.9, Sigma, M= 50-190kDa) were used as received. The nomenclature for PEMs follows (poly1X/poly2X)z, where X is the pH of the polymer solutions and z is the number of bilayers deposited (1 bilayer = poly1+poly2). A non-integer value of z indicates the assembly was terminated with poly1.

![Chemical structures of polymers](image)

**Figure 4-5: Chemical structure of the polymers in this study**

**Multilayer Film Deposition:** Polymers solutions were made from Milli-Q 18.2MΩ water. Solutions of PAA, PDAC and SPS were 0.01M and solutions of PEO, HA and CHI were 0.1% (w/v). CHI solutions included 0.1M of acetic acid to aid dissolution. PDAC and SPS solutions for the adhesion layer had 0.1M NaCl at pH 4.0. All other solution pHs were adjusted to pH 3.0 with 1M HCl and no added salt. Glass substrates were sequentially dipped in the polymer solutions using an automated Zeiss programmable slide stainer or nanoStrata dipping unit.
Substrates were held in polymer solutions for 10 minutes and then rinsed for a total of 3 minutes in water with mild agitation. The time in chitosan solution was altered for diffusion studies, but the rinse cycle employed the same time profiles. Dry film thickness was measured with a P-16 profiler (KLA Tencor Corp.). Layer-by-Layer assembly was executed using the Zeiss programmable slide stainer apparatus for all of the regions except for the HA/CHI regions, which were built in the nanoStrata unit. The dipping time for polymers in the Zeiss apparatus was 10 minutes, followed by two pH 3 Milli-Q water rinses for 2 and 1 minutes with mild agitation. In the nanoStrata unit, polymer depositions were done for 10 minutes, followed by three pH3 Milli-Q water rinses: one for 2 minutes and two for 1 minute, while the substrate was rotating at about 100rpm. To minimize the changes that could arise from drying we dried all samples in Figure 4-2 and Figure 4-3 with nitrogen immediately after the aqueous assembly was complete. All samples in Figure 4-4 were dried passively at ambient conditions. Time from Table 4-1 started when the sample entered solution and ended when the sample was dried with N₂.

**X-ray Photoelectron Spectroscopy (XPS):** Chemical composition of the surface was characterized using a PHI Versaprobe II X-ray photoelectron spectrometer with a scanning monochromated Al source (1486.6 eV, 50 W, spot size 200 μm). The takeoff angle between the sample surface and analyzer was 45° and the X-ray beam collected C1s, N1s, O1s and Si2p elemental information while rastering over a 200 μm x 700 μm area. Detailed XPS acquisition parameters are found in Table 4-3. Depth profiling was accomplished using the instrument's C₆₀⁺ ion source operated at 10 kV, 10 nA and rastered over a 3x3 mm area at an angle 70° to the surface normal. Sputtering occurred in 1 minute intervals while the sample was moved using concentric Zalar rotation at 1 rpm. Atomic composition was determined based on photoelectron peak areas and the relative sensitivity factors provided in PHI's Multipak processing software.
All data were background subtracted, smoothed using a five point quadratic Savitzky-Golay (S-G) algorithm and charge-corrected so that the carbon-carbon bond has a binding energy of 285.0 eV. The surface of the glass was defined as the point at which the atomic concentration of silicon reached 5% in the depth profiling data. The thickness as measured by profilometry was compared to the number of sputter cycles that occurred before reaching the surface of the glass. Data was plotted using Matlab.

Table 4-3: Detailed XPS Acquisition Parameters

<table>
<thead>
<tr>
<th>Element</th>
<th>Binding Energy Range (eV)</th>
<th>Pass Energy (eV)</th>
<th>Data spacing (eV/step)</th>
<th>Sweeps</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1s</td>
<td>280-290</td>
<td>11.75</td>
<td>0.200</td>
<td>3</td>
</tr>
<tr>
<td>N1s</td>
<td>393-404</td>
<td>117.4</td>
<td>0.125</td>
<td>6</td>
</tr>
<tr>
<td>O1s</td>
<td>526-537</td>
<td>117.4</td>
<td>0.500</td>
<td>1</td>
</tr>
<tr>
<td>Si2p</td>
<td>95-107</td>
<td>117.4</td>
<td>1.000</td>
<td>1</td>
</tr>
</tbody>
</table>

pH Sensitivity of Hydrogen Bonded Region: Films were tested by a 30 minute immersion in PBS (pH 7.4) with 100rpm agitation. After drying with N₂, the thickness was measured using profilometry and was compared to the initial thickness to determine if the hydrogen bonded region dissolved.

Film details listed in order of fabrication (substrate to exposed surface).

Fig. 1A Chitosan Diffusion test: (PDAC4/SPS4)_{15.5} (PAA3/PEO3)_{30.5} (CHI3 exposed for designated time)

Fig. 1B Electrostatic Blocking Layers test: (PDAC4/SPS4)_{15.5} (PAA3/PEO3)_{30.5} (PAH3/SPS3)_{z} (z=0,0.5,3.5,9.5) (HA3/CHI3)_{3.5}

As others have found, the thickness/bilayer of (PAA3/PEO3) region can vary between batches (67, 76). As expected in the hydrogen bonded regions, the molar ratio of PAA to PEO in
(PAA3/PEO3) based on the qualitative peak intensity is approximately 1:1 matching literature (75)

4.9 Supplemental Information

**Table 4-4: Swelling of (PAA3/PEO3) in pH3 water as measured by wet ellipsometry**

<table>
<thead>
<tr>
<th>Film</th>
<th>(PAA3.0/PEO3.0)$_{16.5}$</th>
<th>(PAA3.0/PEO3.0)$_{20.5}$ exposed to CHI 60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry Thickness (nm)</td>
<td>123.48 ± 3.5</td>
<td>224.07 ± 0.95</td>
</tr>
<tr>
<td>Wet Thickness (nm)</td>
<td>302.56 ± 9.63</td>
<td>580.00 ± 4.43</td>
</tr>
<tr>
<td>Percentage Swelling</td>
<td>245.03 ± 4.26%</td>
<td>258.85 ± 0.87%</td>
</tr>
</tbody>
</table>

\[
Swelling(\%) = \frac{\text{wet thickness}}{\text{dry thickness}} \times 100
\]

For swelling experiments in pH3 water, a custom-built quartz cell was used to measure film thickness using variable-angle spectroscopic ellipsometry as described in a previous publication (4). All ellipsometry measurements were made using a Woolham Co. ellipsometer operating at a 70° angle of incidence. Measurements were made from 300 to 1000 nm, and all data analysis was done using the WVASE32 software. The refractive index of the pH 3 water was assumed to be the same as water (1.333).
Figure 4-6: Increasing X-ray Exposure Time Decreases Carboxyl Atomic Concentration:
To study the effect of X-ray exposure and sputtering conditions identical (PAA3/PEO3)\textsubscript{30.5} (PAH3/SPS3)\textsubscript{9.5} samples were exposed to a variety of X-ray exposure levels while depth profiling. Data was analyzed with Multipak software (Physical Electronics, USA) and then the carboxyl peak at 289eV was fitted using CasaXPS software. Different XPS data acquisition parameters were tested along with different C\textsubscript{60}\textsuperscript{+} cluster ion sputtering conditions to determine conditions with minimal decrease in carboxyl signal, high signal to noise ratio and many data points while depth profiling. The carboxyl signal was chosen to determine X-ray damage since it is highly sensitive to degradation. It was determined that the condition 5 shown above provided a good balance of these demands and the final acquisition parameters are found in Table 4-3. The degradation of the film due to extended X-ray exposure or sputtering is a limitation that should be carefully considered by others before using this technique. C\textsubscript{60} deposition was not observed since the C-C bond at 285eV did not systematically increase.
Figure 4-7: XPS Depth profile in absence of chitosan. (A) N1s signal from a (PAA3/PEO3) film before exposure to chitosan. The only N1s signal is from the (PDAC4/SPS4) adhesion layer. (B) PDAC from (PDAC4/SPS4) adhesion layer does not diffuse into the hydrogen bonded region (PAA3/PEO3) on top of it even after 15 hours in pH 3 water as shown by the absence of nitrogen signal above the adhesion layer.

Figure 4-8: GATR-FTIR results showing ionization of PAA upon chitosan diffusion. Two distinct peaks from the carboxylic acid functional group of PAA are shown: one at ~1550 cm\(^{-1}\) is associated with the asymmetric stretching of the ionized carboxylate (COO\(^{-}\)) and the other at ~1710 cm\(^{-1}\) which is associated with the C=O stretching of the carboxylic acid (COOH) groups. In comparison to the pure hydrogen bonded sample (PAA3/PEO3), the sample exposed to chitosan for 10 minutes has a higher amount of ionized carboxylate groups.
Figure 4-9: High resolution C1s XPS depth profiling of a hydrogen bonded film exposed to chitosan solution. (A) 10 min and (B) 60 min exposure to chitosan. The color scheme is the same as that of Figure 4-1A. Red spectra represent chitosan infused areas, yellow spectra represent the hydrogen bonded (PAA3/PEO3) areas and black spectra represent the (PDAC4/SPS4) adhesion layer.

Figure 4-10: Pure component C1s XPS spectra. (A) Poly(ethylene oxide) (B) Chitosan (C) Poly(acrylic acid)
Figure 4-11: N1s spectra from depth profiling XPS of hydrogen bonded films with different blocking layers. All samples were topped with (HA3/CHI3)$_{3.5}$. (A) No blocking layer (B) (PAH3/SPS3)$_{9.5}$ layer (C) (PAH3/SPS3)$_{3.5}$ layer (D) Single (PAH3) layer
Figure 4-12: (CHI3/HA3)_{3.5} on top of a hydrogen bonded region. Independent of whether CHI or HA is deposited first onto the hydrogen bonded region, the chitosan diffuses fully through the film as seen by the nitrogen signal throughout the film.

Figure 4-13: Comparison of CHI10 nitrogen signal before and after PBS exposure. CHI10 sample did not dissolve after a 30 minute PBS soak. It was stabilized due to the diffusion and electrostatic cross-linking of chitosan as seen by high levels of nitrogen throughout the film.
5. DESIGNING ZWITTER-WETTABLE SURFACES

Given the capabilities of depth profiling XPS we could potentially explore a range of nanostructured polymeric films. One application of interest to the Rubner/Cohen group was developing a full understanding of the important characteristics in the design of ‘zwitter-wettable’ films. Developed by fellow labmate Hyomin Lee, these films use a unique nanostructured system with a thin hydrophobic capping layer and a thick hydrophilic reservoir. This system presents a hydrophobic surface to macroscopic water droplets, yet allows molecular water to diffuse through to the reservoir and thus creates an anti-fogging surface. This chapter represents a joint effort with equal contributions by Hyomin Lee and me to probe the molecular structure and source of the ‘zwitter-wettable’ phenomenon. My experimental addition to the project focused on the depth profiling analysis and creation of patterned films for controlling condensation. The following paper is currently in preparation for publication.

Authors: Hyomin Lee, a, ‡ Jonathan B. Gilbert, a, ‡ Francesco E. Angile, b Rong Yang, a Daeyeon Lee, b Michael F. Rubner c, * and Robert E. Cohen a, *

a Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge 02139

b Department of Chemical and Biomolecular Engineering, University of Pennsylvania, Philadelphia,

c Department of Materials Science and Engineering, Massachusetts Institute of Technology, Cambridge
5.1 Introduction

Control of water condensation from vapor on a surface is critical for a broad range of applications including heat-transfer,(77, 78) fog-harvesting,(79) and high-performance optical devices.(2, 80, 81) As a result, significant attention has been devoted to study the effect of surface chemistry, and roughness on water condensation over the past few decades. Many interesting new phenomenon such as jumping-droplets during coalescence,(82) and low-volatility liquid impregnated surfaces with enhanced heat transfer(78) has been discovered and expanded potential applications by engineering surfaces with optimal surface roughness and surface wetting characteristics.

For example, in the area of controlling water condensation for optically clear antifogging coatings, smooth hydrophilic(2) or nano-textured superhydrophilic coatings(8, 83, 84) were found to significantly reduce light scattering by facilitating film-like condensation.(2, 85) While many of these coatings perform satisfactorily in specifically defined condensing environments, these hydrophilic surfaces (i) may exhibit frost formation or excess and non-uniform water condensation under aggressive fogging conditions, which results in poor optical performance of the coating,(80) and (ii) may have reduced durability due to fouling by contaminants.(86)

To overcome the limitations of the currently accepted hydrophilic antifogging method new insights into the design of antifogging materials are needed. Recent developments by us(80) and others(87, 88) have presented possible solutions by reporting surfaces that can be both antifogging and hydrophobic. These counter-intuitive surfaces consist of polymeric networks of hydrophilic as well as hydrophobic moieties that can imbibe a considerable amount of water molecules from the vapor phase directly into the hydrogel-like film while the surface maintains a hydrophobic appearance. We recently defined these surfaces as zwitter-wettable surfaces,(80)
due to the capability to simultaneously absorb molecular water from the environment while exhibiting hydrophobic character to water droplets. It was also shown that incorporating hydrophilic poly(ethylene glycol) (PEG) segments resulted in antifogging coatings with frost-resisting capabilities. The presence of PEG increased the nonfreezing water capacity of the thin film due to its strong hydrogen bonding capability.\(^{(80)}\) While the major focus of these studies was to achieve antifogging coatings that perform well in very harsh conditions (below the freezing point of water), the detailed explanation correlating the abnormally high water contact angles (60-70°\(^{(87)}\), or even as high as 110°\(^{(80)}\)) and the antifogging performance was rather limited. Furthermore, the experimental complexity of these individual systems restricted their thorough characterization which limited our understanding of the underlying mechanism.

In our previous work, we have shown that smooth hydrophilic antifogging surfaces result in a sheet of water on the surface and thus additional droplets form an effective water contact angle of 0°. Yet with zwitter-wettable surfaces, a water droplet on top of a film containing condensed water still exhibited a water contact angle above 90, indicating that the water molecules in the vapor phase were directly imbibing into the film. Therefore, we hypothesized that in addition to roughness and surface chemistry, a third factor, the capability of the film to transport molecular water to the underlying reservoir must be considered in the design of antifogging coatings. Thus, it was anticipated that a hydrophilic reservoir capped with a thin hydrophobic layer with high molecular water permeability could limit the nucleation and growth of water droplets on the surface during condensation and thus produce a hydrophobic and antifogging surface.
5.2 Results and Discussion

Figure 5-1: (a) Structures of the polymers described in this paper. Polycations and polyanions are labeled with parenthesis (+), (-), respectively. (b) Water advancing contact angle of the samples tested. (c) Photographs taken immediately after transfer to ambient lab conditions (22 ± 1 °C, 40 ± 10% RH) from a -1°C refrigerator (1 hr). Only the hydrophilic surface and zwitter-wettable surface resisted fog formation. (d) Schematic
representation of hydrophilic surface with sessile drop and during condensation. (e) Schematic representation of zwitter-wettable surface with sessile drop and during condensation.

To test this hypothesis, the layer-by-layer (LbL) self-assembly technique was chosen to assemble various coatings with heterostructured architecture.(89) For the base platform, hydrophilic polysaccharides (chitosan (CHI) as the polycation (+) and carboxymethyl cellulose (CMC) as the polyanion (-)) as shown in Figure 5-1a were LbL assembled electrostatically to create a hydrophilic antifogging coating as reported earlier.(2) As shown in Figure 5-1b, 30-bilayers of CHI/CMC on a glass substrate has a water advancing contact angle of ~20°. Then the surface wetting characteristics of this 30-bilayer hydrophilic antifogging surface was altered to become hydrophobic with either 3-bilayers of (CHI/Nafion) by LbL technique or chemical vapor deposited (CVD) with poly(perfluorodecyl acrylate) (PPFDA). 3-bilayers of CHI/Nafion increased the water advancing contact angle up to ~110° and the PPFDA coating enhanced the water advancing contact angle to ~125. The number of 3-bilayers was chosen because fewer bilayers would exhibit transient contact angle behavior due to surface rearrangement(90) which complicates the analysis. All samples chosen for our analysis do not exhibit significant contact angle changes with time (Supporting Information Figure 5-5).

As shown in Figure 5-1c, photographs were taken immediately after transfer to ambient lab conditions (22 ± 1 °C, 40 ± 10% RH) from a -1°C refrigerator (1 hr) and only the hydrophilic surface and the CHI/Nafion coated hydrophilic surface resisted fog formation. It has been reported earlier(2) that the hydrophilic CHI/CMC coating remains optically clear during condensation due to their superior water-absorbing characteristics that form a uniform, non-light-scattering film of water on the surface as shown in Figure 5-1d. However, when this same
surface was subsequently coated with hydrophobic CHI/Nafion, this coating exhibited a zwitter-wettable behavior where water molecules in the vapor phase directly imbibed into the film, while the surface remains hydrophobic to water droplets as shown in Figure 5-1e. Yet, when the same hydrophilic surface was coated with PPFDA, the film exhibited fog formation. As revealed in Table 5-1, film thickness change after subsequent assembly are similar in its extent (69 nm and 62 nm, respectively) and the water advancing contact angles are both well above 90°. Two control samples where (CHI/Nafion)_30 and PPFDA are solely coated on a glass substrate also exhibited fog formation even though the surface wetting characteristics resemble the zwitter-wettable (CHI/Nafion)_3 coated hydrophilic surface. These results clearly indicate that the surface wetting characteristic does not solely determine whether a surface will fog and that the presence of the underlying hydrophilic “reservoir” is indeed critical.

Table 5-1: Thickness of the multilayer films used in this work.

<table>
<thead>
<tr>
<th>(CHI/CMC)_{30}</th>
<th>(CHI/CMC)_{30}</th>
<th>(CHI/CMC)_{30}</th>
<th>(CHI/Nafion)_{30}</th>
<th>PPFDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>(CHI/Nafion)_{3}</td>
<td>PPFDA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>615 ± 16 nm</td>
<td>684 ± 5 nm</td>
<td>677 ± 1 nm</td>
<td>18 ± 3 nm</td>
<td>72 ± 2 nm</td>
</tr>
<tr>
<td>(+ 69 nm)^a</td>
<td>(+ 62 nm)^a</td>
<td></td>
<td></td>
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</tbody>
</table>

^afilm thickness change after assembling a capping layer.
Figure 5-2: (a) Schematic diagram of the polymer patch fabrication process. (b) Scanning electron microscopy (SEM) image of the polymer patch. Scale bar is 50 μm. Outlet image shows the enlarged SEM image and the average carbon and fluorine signal obtained from energy dispersive X-ray spectrometry (EDS). (c) Optical microscopy image of the polymer patch after exposure to humid air. (d) ESEM images of the condensation of water vapor on a polymer patch. Water droplets start to form on the off-patch areas and at the edges of the patches.

Previously, Varanasi et al., demonstrated that spatial control in the condensation of water molecules from the vapor phase can be achieved by patterning a surface with significant contrast in local intrinsic wettability to preferentially guide water molecules to nucleate and grow on the
hydrophilic regions. To demonstrate the unique capability of our zwitter-wettable films to spatially control condensation of water molecules from the vapor phase, patterned films with zwitter-wettable characteristics were fabricated (Figure 5-2a). First, patches of the hydrophilic (CHI/CMC) reservoir (10-bilayers) were fabricated using lift-off photolithography. 10-bilayers were chosen because higher numbers of bilayers would result in incomplete patterning during the fabrication process. Then, the hydrophobic thin film (CHI/Nafion) (3-bilayers) was deposited, resulting in a surface with uniform (CHI/Nafion) over both the small patches of (CHI/CMC) as well as the off-patch areas. The uniform fluorine EDS signal (Figure 5-2b) confirms the consistent coverage of the Nafion, while the non-uniform carbon signal shows the presence of the underlying hydrophilic reservoir ((CHI/CMC)) in the patch area. When the patterned film is exposed to progressively higher water vapor pressure (Figure 5-2c and Figure 5-2d) the water condensation is spatially controlled. Initially the water condenses in the hydrophilic reservoir of the patch, changing the refractive index and thus the color of the patch, however minimal droplets are seen on the surface (Figure 5-2c). As the water vapor pressure increases, progressively larger droplets start to form on the interstitial fogging areas, while no droplets are seen on the patch areas with zwitter-wettable characteristics. The capability to spatially control the condensation of water through patterning of the hydrophilic reservoir, while still presenting a uniform hydrophobic surface is indeed unique to zwitter-wettable surfaces.

In addition, contact angle measurements were performed on the (CHI/Nafion) 3 coated hydrophilic reservoir using both high and low surface tension nonpolar liquids, diiodomethane ($\gamma_{LV} = 50.8$ mN/m) and hexadecane ($\gamma_{LV} = 27.5$ mN/m) to further investigate the uniqueness of this zwitter-wettable surface. These results are shown in Supporting Information Figure 5-6. Both liquids exhibited reasonably high advancing contact angles with low contact angle
hysteresis indicating that surface exhibits a Teflon-like behavior to nonpolar liquids. Unlike typical hydrophilic surfaces where low surface tension liquids would completely spread, organic contaminants could be removed relatively easily due to the weak retention force offered by the hydrophobic capping layer.

Figure 5-3: (a) Schematic of depth-profiling x-ray photoelectron spectroscopy (XPS) used to acquire atomic concentration profile and molar ratio profile of various samples tested. Here, atomic concentration of (CHI/Nafion)\textsubscript{3} coated hydrophilic surface is shown as an example. (b) Molar ratio of hydrophilic surface ((CHI/CMC)\textsubscript{30}) with depth in the film. (c) Molar ratio of (CHI/Nafion)\textsubscript{3} coated hydrophilic surface with depth in the film. (d) Molar ratio of PPFDA coated hydrophilic surface with depth in the film.

To investigate the origin of the (CHI/Nafion)\textsubscript{3} coated hydrophilic reservoirs favoring direct imbibing of water molecules into the film, the axial chemical composition analysis was performed. C\textsubscript{60} cluster-ion depth profiling X-ray Photoelectron Spectroscopy (XPS) was used to
study the chemical 1 CHI:CMC molar ratio in the hydrophilic reservoir and that this ratio is
effectively maintained composition with depth on various coatings with heterostructured
architecture. Previously our group showed that this technique can provide atomic concentration
information as well as the chemical state of polymer thin films with a resolution around
15nm(92). This capability was only recently enabled by the development of cluster-ion $C_{60}$
etching which is much less damaging to polymers than the single-ion etching methods
commonly used.(93) As shown in Figure 5-3a the depth profiling process uses iterative $C_{60}^+$
etching and XPS acquisition to acquire an atomic concentration profile with depth. This atomic
concentration with depth can be further analyzed using a mass balance to determine the molar
repeat ratio of the various polymers with depth, allowing for the analysis of polymer interlayer
diffusion. From Figure 5-3b it is clear that there is a 2:1 when the reservoir is coated with either
the $(CHI/Nafion)_3$ (Figure 5-3c) or the PPFDA film (Figure 5-3d). Due to the lack of interlayer
diffusion, it is clear that the different antifogging performance can be attributed to the
characteristics of the thin capping layer alone.
Figure 5-4: (a) Diffusivity (D) of water vapor in hydrophilic reservoir and (CHI/Nafion)_3 coated hydrophilic reservoir (b) Solubility of water vapor in hydrophilic reservoir and (CHI/Nafion)_3 coated hydrophilic reservoir. (c) Schematic representation of the effect of the ability of thin hydrophobic capping layer to transport water molecules: For low diffusivity capping layer, water molecules nucleate and grow on the surface while for high diffusivity capping layer, water molecules in the vapor phase prefer to directly imbibe into the underlying hydrophilic reservoir.

With the insight gained from the depth-profiling XPS study, the ability of thin hydrophobic capping layer to transport water molecules were investigated by permeability measurements using quartz crystal microbalance with dissipation monitoring (QCM-D). It has been previously demonstrated that QCM-D enables real-time measurements of water vapor uptake by a thin film with high precision and provides quantitative information on water diffusivity (D), and solubility (S).(94) Briefly, quartz crystals coated with various films are introduced into a chamber where the changes in the frequency and dissipation are monitored upon exposure to a steam of humidified nitrogen and fitted using the Voigt viscoelastic model to predict the increase in thickness under humidified nitrogen. Then, the diffusivity (D) of water vapor in the film is determined by using Fickian diffusion model fitting and the solubility (S) by calculating the change in mass between dry nitrogen and wet nitrogen. Detailed method of measurement and assumptions used for one-dimensional diffusion of water molecules can be found in the Supporting Information. As shown in Figure 5-4a and Figure 5-4b, the diffusivity (D), solubility (S) were measured for the following samples: (1) (CHI/CMC)_{30} hydrophilic
reservoir and (2) (CHI/Nafion)_3 coated hydrophilic reservoir. Comparing the hydrophilic reservoir alone to the hydrophilic reservoir coated with (CHI/Nafion)_3, both the diffusivity (D) and the solubility (S) were similar in their values. However, a hydrophobic capping layer that reduces the capability of the film to transport water molecules would thus favor nucleation on the surface which is indeed the case for PPDFA coated hydrophilic reservoir. This phenomenon is schematically shown in the top of Figure 5-4c. Since the diffusivity and solubility of the hydrophilic reservoir did not change when coated with the (CHI/Nafion) capping layer this indicates that a (CHI/Nafion) capping layer does not hinder water transport to the underlying hydrophilic reservoir. However, the (CHI/Nafion) layer without a reservoir does not have sufficient capacity to absorb water and eventually fogs after being saturated with water during condensation, agreeing with the antifogging test results in Figure 5-1c.

Previously, it has been reported(91) that a hydrophilic surface (θ ~25° ) nucleates tens of orders of magnitude faster than a hydrophobic surface (θ ~110° ) at a typical saturation ratio of p/p_{\text{in}}=1.7. This allows water nucleation and subsequent growth to preferentially occur on more hydrophilic regions. In our zwitter-wettable surface, on the other hand, the surface is hydrophobic while highly diffusive to water molecules. Thus, the antifogging capability of the surface is further enabled since surface hydrophobicity retards nucleation and growth, while the CHI/Nafion capping layer allows facile diffusion of water molecules to the hydrophilic reservoir. This unique combination allows a zwitter-wettable surface to be hydrophobic and also antifogging.

Simple but careful design of this model system allowed the previous complex network of hydrophilic and hydrophobic moieties to be decoupled into a heterostructured film consisting of
hydrophilic reservoir and a hydrophobic capping layer. While surface chemistry and roughness were the main factors for controlling water condensation until now, a third factor, the capability of the film to transport water molecules needs to be considered. Furthermore, no surface rearrangement in our model zwitter-wettable surface allowed the film to exhibit truly hydrophobic behavior to water droplets unlike the previously studied zwitter-wettable system (80, 95) where the film exhibited a hydrophobic to hydrophilic transition when contacted with water.

Enabled by the experimental techniques to determine diffusivity (D) and solubility (S) via QCM-D, the design considerations discussed in this work can be further expanded to fabricate optimal hydrophilic reservoirs and hydrophobic capping layers depending on the application requirements. Furthermore earlier observations such as a critical film thickness requirement for hydrophilic antifogging coatings (2) can be clarified in detail with respect to diffusivity (D) and solubility (S). In addition, optimal material selection for a specific condensation environment, governed by initial substrate temperature, and exposing environment (relative humidity, and temperature), can be further fine-tuned allowing additional physicochemical properties such as enhanced mechanical durability or frost-resisting capabilities to be taken into account while designing future zwitter-wettable surfaces.

In summary, we showed that when a thin hydrophobic layer with sufficient permeability is assembled on top of a hydrophilic reservoir, antifogging coatings with zwitter-wettable characteristics can be prepared. Enabled by depth profiling XPS and QCM-D, the required film properties were detailed for the design of future zwitter-wettable surfaces. Future work incorporating new surface chemistry and roughness may further extend zwitter-wettable surfaces to enable the fabrication superhydrophobic antifogging surfaces. Additionally we believe further
applications of directed condensation of molecules from the vapor phase include water separation and purification.

5.3 Methods
Layer-by-Layer assembly of the polymer thin films

Sequential adsorption of polymer layers were performed using a StratoSequence VI spin dipper (nanoStrata Inc.), controlled by StratoSmart v6.2 software, at 80 rpm. The concentrations of chitosan (CHI, low molecular weight), carboxymethylcellulose (CMC, Mw = 250 000 g/mole) and Nafion dispersion (Alfa Aesar, 5% (w/w) in water and 1-propanol) in the dipping solutions were 1 mg/mL, 1 mg/mL and 0.25 % (w/w) respectively. For chitosan, 0.3 % (v/v) acetic acid was added prior to dissolving the polymer and was filtered with 200 μm pore filter (VWR) after stirring overnight. Distilled water (>18 MΩ•m, Millipore Milli-QTM) water (DI water) was used in formulating the solution and in all rinsing procedures. The dipping time in the CHI, CMC, and Nafion solutions were each 10 min followed by three sequential rinse steps (of 2, 1, and 1 min). All solutions and their respective rinse solutions were adjusted to pH 4.0 with either NaOH or HCl respectively. Glass substrates were first degreased by sonication in a 4% (v/v) solution of Micro-90 cleaner (International Products Co.) for 15 min, subsequently sonicated twice in DI water for 15 min. The substrates were blow-dried with dry air and treated for 2 min with oxygen plasma (PDC-32G, Harrick Scientific Products, Inc.) at 150 mTorr before the LbL assembly.

Contact angle measurements

Transient contact angle measurements as well as advancing and receding contact angles of probe liquids on various samples were performed using a Rame-Hart model 590 goniometer, by dispensing liquid droplets of volume V ~ 10 μL.
Preparation of poly(perfluorodecyl diacrylate) (PPFDA) coating using initiated chemical vapor deposition (iCVD)

iCVD polymerization of perfluorodecyl diacrylate (PFDA) (97%, Aldrich) was conducted similar as previously described.(77) Briefly, the peroxide initiator, TBPO (98%, Aldrich), was delivered into the reactor through a mass flow controller (MKS Instruments) at a constant flow rate of 3.2 sccm. PFDA was vaporized in a glass jar that was heated to 80 °C. The flow rates were controlled using needle valves and kept constant at 0.2 and 0.6 sccm. The filaments were resistively heated to 230 °C using a DC power supply (Sorensen), and the temperature was measured by a K-type thermocouple (Omega Engineering). The sample stage was backcooled at 30 °C using a recirculating chiller/heater (Neslab RTE-7). The working pressure was maintained at 200 mTorr using a throttle valve (MKS Instruments). The reactor was covered with a quartz top (2.5 cm) that allows in-situ thickness monitoring by interferometry with a 633 nm HeNe laser source (JDS Uniphase). Final thickness of the polymer deposited on the hydrophilic surface corresponds to ~70 nm.

Permeability measurements using QCM-D

Gold-coated QCM-sensors (Q-Sense Inc.) are cleaned with UV/ozone treatment for 10 min, then immersed in 2% (v/v) Sodium Deodecyl Sulfate (SDS) for 30 min in room temperature, rinsed with DI water, and finally blown dry with compressed air. Also, another UV/ozone treatment was done for 10 min before the baseline measurement. The baseline frequency and dissipation are first recorded with a blank crystal under dry nitrogen and calibrated to zero for the sorption measurements. All the multilayer films are then formed on the crystals through the similar procedure used for glass substrate. Here, gold crystals were immobilized on a plastic microscope
slide (P11011P) in between two grooves made with a razor blade. The film on the back side of crystal is carefully removed using 1.0 M NaOH with a cotton swab and then rinsed thoroughly with DI water. After the multilayer coated sensors are loaded in the QCM chamber (E4 QCM-D unit (Q-Sense Inc.)), the frequency and dissipation shifts are monitored at varying humidity conditions.

The measured frequency and dissipation are fitted using the Voigt viscoelastic model incorporated in Q-Sense analysis software (QTools) to predict the increase in film thickness under wet nitrogen (~80% RH) due to the absorption and diffusion of water in the film similar to previously described procedure.(94) Average density of chitosan, carboxymethyl cellulose and Nafion was used to determine the film thickness from QCM-D measurements.

The diffusivity (D) of water vapor in the film is determined from the plot of the thickness change versus time under wet nitrogen (~80% RH) using a numerical model for one-dimensional diffusion of water into a thin film as shown previously.(94)

\[
\frac{m_t - m_0}{m_\infty - m_0} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n + 1)^2 \pi^2} \exp \left[ -\frac{D(2n + 1)^2 \pi^2 t}{l^2} \right]
\]

where \( m_\infty, m_t, \) and \( m_0 \) are the mass of the thin film and water at time \( \infty, t, \) and 0, respectively. \( t \) and \( l \) are the time and thickness of the film, respectively. Since the film mass is linearly proportional to the thickness, above equation can be simplified to,

\[
-\frac{1}{\pi^2} \ln \left[ \frac{(m_\infty - m_t)\pi^2}{8(m_\infty - m_0)} \right] = \frac{D}{l^2} t
\]

where the slope can be used to determine the diffusivity (D). The solubility (S) of water vapor in
the film is simply determined by calculating the change in the mass of the film between under dry nitrogen and wet nitrogen, \( m_e - m_o \). The water permeability (\( P \)) can be subsequently be determined by the following relationship: \( P = D \times S \), the effect of water adsorption on blank QCM crystals have been corrected as reported earlier.(94)

For this measurement, a humidified stream is introduced at 10 cm\(^3\) (STP) min\(^{-1}\), with the corresponding mass transfer analog Biot number to be in the order of \( 10^4 \), indicating that the water-vapor transport process is diffusion-limited. It is known that the lateral diffusion is much faster than the normal diffusion through the film(96) and thus our measurement will be dominated by one-dimensional diffusion of water normal to the film and the film swelling occurs dominantly in one dimension.

*Scanning electron microscopy (SEM) measurements*

Scanning electron microscopy micrographs were obtained using a JEOL 6010LA in back scattered electron shadow image mode (BES) operated at an accelerating voltage of 10 kV. Energy dispersive X-ray spectrometry (EDS) was performed as well to perform elemental analysis of the Zwitter-wettable patch sample. Environmental scanning electron microscope (ESEM, Zeiss Evo 55) was used to investigate the nucleation and condensation phenomena *in situ.*

*Depth Profiling X-ray Photoelectron Spectroscopy*

Chemical composition of the surface was characterized using a PHI Versaprobe II X-ray photoelectron spectrometer with a scanning monochromated Al source (1486.6 eV, 50W, spot size 200 um). Depth profiling was accomplished using the instrument's C\(_{60}\)\(^+\) ion source. The
takeoff angle between the sample surface and analyzer was 45° and the X-ray beam collected C1s, O1s, F1s, N1s and Si2p elemental information while rastering over a 200 μm x 350 μm area. Detailed XPS acquisition parameters are in the table below. Sputtering occurred in 1 minute intervals while the sample was moved using concentric Zalar rotation at 1 rpm. The C₆₀⁺ source was operated at 10 kV and 10 nA and rastered over a 3x3 mm area at an angle 70° to the surface normal. Atomic composition was determined based on photoelectron peak areas and the relative sensitivity factors provided in PHI's Multipak processing software. All data were background subtracted, smoothed using a five point quadratic Savitzky-Golay (S-G) algorithm and charge-corrected so that the carbon-carbon bond has a binding energy of 285.0 eV. The surface of the glass substrate was defined as the point at which the atomic concentration of silicon reached 5% in the depth profiling data. The molar ratio profile was calculated via an oxygen mass balance. To minimize experimental bias, pure films of chitosan, CMC, Nafion and PPFDA were analyzed via Depth Profiling XPS as the basis for the effective molecular repeats.

Table S1. Detailed XPS Depth Profiling Conditions

<table>
<thead>
<tr>
<th>Element</th>
<th>Binding Energy (eV)</th>
<th>PassEnergy (eV)</th>
<th>Data Spacing (eV)</th>
<th>Sweeps</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1s</td>
<td>280-298</td>
<td>117.4</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>O1s</td>
<td>526-538</td>
<td>117.4</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>Si2p</td>
<td>95-107</td>
<td>117.4</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>F1s</td>
<td>682-695</td>
<td>117.4</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>N1s</td>
<td>393-405</td>
<td>117.4</td>
<td>0.5</td>
<td>2</td>
</tr>
</tbody>
</table>

Zwitter-wettable Patch Fabrication

Patch fabrication was based on a photolithographic lift off procedure. Briefly, the slides were cleaned with 2% (v/v) solution of Micro-90 cleaner (International Products Co.), 1M NaOH and
then rinsed with water. Next, the photoresist was spin coated onto the surface and developed as previously described. After deposition of the uniform hydrophilic layer via layer by layer processing, the film was patterned by dissolving the remaining photoresist in sonicating acetone for 10 seconds, leaving behind patches of hydrophilic (CHI/CMC). Finally the hydrophobic (CHI/Nafion) film was deposited uniformly over the surface via layer by layer processing.

5.4 Supporting Information

Figure 5-5: (a) Water contact angle evolution over time for (CHI/CMC)$_{30}$ subsequently coated with 1-, 2-, and 3- bilayers of (CHI/Nafion) by LbL technique. (b) Water contact angle evolution over time for various samples tested in this work.

The linear decrease in water contact angle can be attributed to evaporation of water from the static drop over the elapsed time of the experiment. 

The linear decrease in water contact angle can be attributed to evaporation of water from the static drop over the elapsed time of the experiment.
Figure 5-6: Advancing and receding contact angle of water, diiodomethane, and hexadecane on (CHI/Nafion)$_3$ coated hydrophilic reservoir.
6. ION DISTRIBUTION IN BLOCK COPOLYMER ELECTROLYTE THIN FILMS

An additional application of depth profiling XPS is the analysis of the nanostructure in block copolymer films. In collaboration with Ming Luo of the Epps Group at the University of Delaware, I have studied the distribution of lithium-ions in aligned lamellar films. A better understanding of the ion distribution in block copolymer films is important for the design of solid polymer battery systems. My work is focused on the depth profiling characterization and analysis while Ming focused on film fabrication and other characterization methods. This chapter represents the joint effort and equal contributions of Ming Luo and me. The following paper is currently in preparation for publication.

Authors Jonathan B. Gilbert,† Ming Luo,‡ Wei-Fan Kuan,† Cameron Shelton,‡ Thomas H. Epps, III,*† Michael F. Rubner,*‖ Robert E. Cohen*†

†Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States

‡Department of Chemical & Biomolecular Engineering, University of Delaware, Newark, Delaware 19716, United States

*‖Department of Materials Science and Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States
6.1 Introduction

Over the past several decades, battery technology has been primarily driven by the need for longer operating time, longer cycle life, smaller size, lighter weight, and improved safety and stability. Lithium-ion batteries, a commonly used rechargeable system, have several desirable features including a high energy density, a long lifespan, and minimum memory effects. However, the electrolytes in most commercialized lithium-ion batteries are organic solvents which are vulnerable to fires under mechanical deformation or overvoltage conditions. Significant efforts have focused on designing solid-state lithium-ion batteries with a solvent-free electrolyte. Amorphous poly(ethylene oxide) (PEO)-based materials doped with alkali-metal salts are favored candidates and have sufficient ionic conductivities for practical use ($10^{-4}$ S/cm). These organic solvent free electrolyte systems enable the construction of lightweight batteries and are adaptable to continuous roll-to-roll processing which potentially lowers the cost of manufacture. Yet, poor resistance to mechanical stress hampers their large-scale production and commercialization.

Block copolymers (BCP) with microphase separated domain structures are an attractive avenue to achieve the mechanical strength required for commercial use while maintaining the high ionic conductivities needed. The BCP systems accomplish this by using one domain to conduct ions while the other provides rigidity. Since conductivity and mechanical strength are decoupled it becomes possible to design battery systems with appealing mechanical properties and ion conductivities. One of the most extensively studied systems are Poly(styrene-block-ethylene oxide) (PS-PEO) copolymers and several groups have achieved the desired mechanical properties (shear modulus on the order of $10^8$ Pa at 90 °C) as well as ionic conductivity levels similar to the corresponding homopolymer electrolytes. However, low
conductivity at room temperature due to PEO crystallization has limited the application of these PS-PEO electrolytes. Comb-branched polymers with short PEO side chains such as poly(oligo(oxyethylene) methacrylate) (POEM) have been demonstrated to effectively eliminate PEO crystallization and exhibit high ionic conductivity at room temperature.(105, 106) Furthermore given the new design parameter of the PEO side chain length, the conductivity and crystallization characteristics of the block copolymer could be further improved.

To optimize the solid BCP electrolyte, the effect of molecular weight on ionic conductivity and mechanical properties needs to be clearly understood. It is known that the stiffness of polymers increases with increasing molecular weight. However, the effect of molecular weight on the ionic conductivity is more complex. Yuan et al. demonstrated that the ionic conductivity of symmetric PS-PEO blended with lithium bis(trifluoromethanesulfone)imide (LiTFSI) slightly decreased with increasing molecular weight of the PEO block at low molecular weights ($M_{\text{PEO}}<$10 kg/mol).(107) Additionally, Panday et al. showed an order of magnitude increase in conductivity as the molecular weight of the PEO block ($M_{\text{PEO}}$) increased from 7 to 98 kg/mol.(104) They ascribed the initial decrease in conductivity to hindered PEO segmental motion near the interfacial zone between PS and PEO blocks. This caused the ionic conductivity of the PEO to be affected by the glass transition temperature of PS block. As the molecular weight of the PEO block increased above 10 kg/mol, the width of the conducting PEO channel increases and the fraction of ions affected by the interfacial zone became negligible. This effect was further corroborated by Gomez et al. who presented the first direct imaging of ions in a BCP bulk using energy-filter transmission electron microscopy (EFTEM).(19) They showed that with increasing molecular weight the lithium salt is progressively more localized near the middle of
the PEO lamellae. They also developed a theory that the increasing ionic conductivity can be attributed to increased chain end and ion mobility in the middle of the PEO channels.

Experimentally quantifying the ion distribution is important to elucidate the relationship between ionic conductivity and BCP domain structures. EFTEM is powerful; however challenges remain due to the relatively small scattering cross section of lithium ions, tedious sample preparation and cautious image analysis, especially for thin film systems. A new potential method to analyze the ion distribution in BCP films is via cluster-ion depth profiling X-ray Photoelectron Spectroscopy (XPS). This method recently showed its effectiveness in resolving both chemical and atomic information with depth in nanostructured polymeric films. The development of cluster-ion etching sources that induce much less damage than single-ion sources has been essential to the accurate depth profiling of polymeric materials. Single-ion sources like argon-ion cause severe damage in the film and change the chemical composition. In comparison, cluster-ion sources like C_{60}^{+} result in much less damage since the energy transfer from the ion to the material occurs primarily at the surface, minimizing the damage deep into the film.

In this work we studied a lamellar forming PS-POEM thin film system with lithium trifluoromethanesulfonate (CF_{3}SO_{3}Li). Though the proven room temperature conductivity of these PEO grafted BCP is very exciting, the lithium-ion distribution in the POEM domain has not been explored and it is unclear whether a similar lithium concentration effect would be seen. To accomplish this we analyzed the structure of the neat film and the lithium salt doped films with 6:1 and 12:1 PEO monomer to lithium ions using atomic force microscopy (AFM) and X-ray reflectivity (XRR). Furthermore we found that a new technique to analyze nanostructured polymer films, XPS depth profiling with C_{60}^{+} sputtering, can resolve the chemical and atomic
structure of nanometer level domains found in block copolymers. Through this analysis we confirmed the presence of the lithium salt in the POEM region and found that the lithium distribution is directly correlated to the POEM concentration. Furthermore we find that depth profiling XPS with $C_{60}^+$ sputtering is readily applicable to analyze the nanostructure of block copolymers. However when applying this technique one must account for the large area being analyzed and roughness induced etching that may alter the results.

6.2 Results and Discussion

The PS-POEM BCP used in this study had an overall molecular weight of 36 kg/mol ($PS_{20k}$-$POEM_{16k}$, determined by size exclusion chromatography using polystyrene as standards), a polydispersity index of 1.09, and a bulk domain spacing ($L_0$) of 26.1 nm (determined from primary peak in small angle X-ray scattering). As seen in Figure 6-1, blends were made in THF solution in an Ar glovebox and methanol was added to the mixture to help dissolve the polymer-salt complex. The number of lithium ions to PEO monomer was varied from 0, 1:12 to 1:6. Next gradient thickness films (80-130 nm) were cast on toluene-rinsed, ultraviolet-ozone (UVO)-treated silicon wafers using the flow coating technique.(108) The samples were then vacuum oven annealed at 135 °C for 6 h to promote parallel oriented lamellar structure which is essential for the characterization of the thin films.
Figure 6-1: Fabrication of lamellar PS-POEM lithium containing films.

To characterize the domain structure of the sample we analyzed it using optical images, atomic force microscopy (AFM), and X-ray reflectivity (XRR). From the optical images, we saw the gradient thickness films exhibited cyclic changes of islands/holes structures and uniform free surfaces (Figure 6-2A). As the free surface morphology is primarily determined by the commensurability conditions (ratio of film thickness to BCP domain spacing, $L_0$)(109), we first acquired the domain spacing from the height of the island/hole structures through AFM. We further found the results are consistent with small angle X-ray scattering (SAXS) measurements of the bulk system (Figure 6-2B). The growth in the domain spacing of the block copolymer electrolyte with salt loading (from 26.8 nm to 36 nm to 41.3 nm) is expected as the salt is known to preferentially swell the PEO domain. From commensurability calculations, the PS-POEM films showed island/hole structures at film thickness of $nL_0$, and uniform surfaces at $(n+0.5) L_0$, which indicated asymmetric wetting conditions. We also note that the bulk sample with 6:1 salt developed a mixed lamellar and cylindrical structure (Figure 6-6); however, the thin film sample clearly exhibited lamellar structure due to strong surface interactions.
Figure 6-2: (A) Optical images of gradient thickness PS-POEM films (neat, 12:1 salt and 6:1 salt) annealed at 135 °C for 6 h; (B) AFM height images and corresponding sections show that the difference between the high and low regions is \( L_0 \), which is consistent with SAXS measurement of the bulk system.

We further conducted XRR experiments on the thin film samples (12:1, 6:1 salt) to confirm the layered structures. Figure 6-3 shows the X-ray reflectivity profiles for PS-POEM thin films with 12:1 and 6:1 salt that are both \( 5L_0/2 \) thick. The “o” symbols represent the reflectivity profile calculated using the scattering length density profile shown in the inset. The profile is characterized by a capping layer of PS at the air surface followed with POEM, PS, POEM, PS and a wetting layer of POEM at the substrate. The lamellar unit cell thickness
(consisting of a POEM layer and a PS layer) is 35.8 nm (or 34.9 nm) for 12:1 salt sample and 40.0 nm (or 39.8 nm) for 6:1 salt sample, respectively. We compared these values with the domain spacing measured from AFM and SAXS, and found they are in reasonable agreement. We further note that the roughness of the interfaces for the domains is on the order of ~1nm which is expected since the PS-POEM with lithium salt system is strongly segregating. It should be emphasized, though, that the models used to calculate the reflectivity profiles may not be unique. However, we believe the models presented herein should be very close to an accurate description of the morphology in the films.
Figure 6-3: X-ray reflectivity profile for PS-POEM films with salt ratio 12:1 and 6:1. The solid line denotes measured profile and the "o" symbol denotes fitted profile.

For detailed chemical analysis of the BCP electrolyte we turned to depth profiling XPS. Enabled by the iterative etching and analysis of depth profiling XPS we are able to visualize the discrete nanostructured lamellar regions (Figure 6-4A). To maximize the distinction between the regions we chose film thicknesses that are free of island/hole structures (neat film thickness = 117 nm, 6:1 salt film thickness = 100 nm). Figure 6-4B shows the alternating C1s and O1s signal of the neat BCP lamellar thin film. Near the silicon substrate we see significant signal from the Si2p peak as well as an increase in the O1s signal due to SiO₂. Figure 6-4C shows the alternating intensity of the O1s spectra due to the large amounts of oxygen in the red spectra representing...
the POEM region. The C1s signal in Figure 6-4D displays a similar alternating structure and two C1s peaks (285 eV and 286.5 eV) can clearly be seen. In the blue spectra representing the PS rich regions, the C1s peak at 285 eV corresponds to carbon-carbon bonds and is dominant. In the red spectra representing the POEM rich regions, the C1s peak at 286.5 eV corresponds to the ether bonds in the PEO side chain. There is also a carbon-carbon bonding peak in the red spectra at 285 eV due to the carbon-carbon backbone and some signal from the adjacent PS region. The atomic profile also supports that the film is asymmetrically wetting, with PS at the air interface and POEM in contact with the substrate.
Figure 6-4: Depth Profiling XPS of 36 kDa PS-POEM without lithium salt. (A) Schematic of depth profiling XPS analysis. (B) Atomic concentration versus thickness above the substrate. (C) and (D) O1s and C1s photoelectron spectra respectively, showing the alternating intensity of a lamellar block copolymer film. The red and blue spectra are spectra primarily in the POEM and PS region respectively.

To analyze the distribution of ions within BCP films, 6:1 salt was added to maximize the lithium signal from the lamellar system. As seen in Figure 6-2, the addition of 6:1 salt significantly increases the domain spacing of the film from ~26 nm to ~41 nm. Figure 6-5A
shows the repeating structure of the lamellar film and it is clear that in the O1s rich POEM regions there is significant F1s and Li1s signal supporting the segregation of the lithium and counter-ion into the POEM region. To further analyze the lithium-ion distribution within the POEM region, Figure 6-5B shows only low concentrations. To directly compare the distribution of the salt anion with the lithium cation, we can multiply the F1s signal by the molar ratio of Li1s to F1s (1:3). The resultant atomic profile (Li(F)) overlays very closely with the Li1s signal, showing that the lithium cation and fluorine containing anion concentrations are similar in distribution(19).

The lithium-ion distribution can be determined by further analyzing the atomic and chemical composition of the film as seen in Figure 6-5C. In the system tested there are only two sources of oxygen, first from the POEM region and second from the anion of the lithium salt; by separating the two sources we can better analyze the material distribution. By subtracting the lithium salt derived oxygen signal from the total oxygen signal we can determine the location of the POEM block within the film (Figure 6-5C). This was accomplished by simply subtracting the F1s signal from the O1s signal since it is known that for every fluorine in the salt anion there is one oxygen. Another independent method to analyze the distribution of ions in the POEM block is through component analysis of the C1s region. As seen in Figure 6-5D the C1s region contains a variety of peaks, each corresponding to different chemical bonding states present within the BCP. For example the alternating photoelectron peaks found at 285eV and 286.5eV relate to carbon-carbon bonds in the PS region and ether bonds in the POEM region respectively. Using the known binding energy of the polymer components the multiple C1s peaks can be deconvoluted and attributed to the lithium salt, PS region, or POEM region. An example of fitting the spectra with their corresponding peaks in both a PS rich region and a POEM rich
region is shown in Figure 6-7. Comparing the results of the O1s subtraction method and the C1s fitting method we see that the independent methods found very similar POEM distributions. We suspect that the lower POEM signal in the domain closer to the substrate is due to cumulative X-ray damage induced from the high energy settings needed to detect the lithium.

We can next compare the distribution of the POEM signal compared to the Li1s signal by multiplying by the molar ratio of F1s to POEM (4.25:10.5, in 6:1 salt films Table 6-1). F1s was used in place of the Li1s signal since the F1s signal has much less noise and the previous analysis showed that the F1s and Li1s concentration are tightly correlated (Figure 6-5B). Since the F1s signal follows the POEM amount closely, we can determine that the lithium salt concentration corresponds with the POEM concentration. If the lithium concentration was non-uniformly distributed, we would not expect to see the coupling of the signals as seen in Figure 6-5C. It is also important to note that the unique capability to analyze the chemical composition as well as the atomic concentration is not possible in other analysis techniques such as secondary ion mass spectrometry (SIMS) or Energy-filtered TEM, displaying the unique potential of depth profiling XPS to analyze BCP thin films.
Figure 6-5: XPS depth profiling of 36kDa PS-b-POEM with 6:1 Li salt added. (A) Atomic concentration profile with depth. (B) Zoom-in of low concentration and overlay of fluorine atomic ratio comparison. (C) Distribution of PEO containing side block within the film analyzed via O1s signal minus O1s signal from the salt (dashed green), fitting the C1s peak (dashed blue) or F1s ratio to POEM (dashed red) (D), (E), (F) and (G) 3D spectra of the C1s, Li1s, F1s and O1s regions respectively. The Li1s figure is rotated for clarity.
By analyzing other individual spectra we can garner further details on the film structure and the limitations of depth profiling XPS to analyze BCP films. Figure 6-5E shows the Li1s spectra with depth and due to the low scattering factor of lithium, the signal intensity is relatively low. Increasing the lithium concentration was not possible since higher concentrations of lithium (4:1 salt) changed the morphology to a mixed cylinders and lamellae system (Figure 6-6) which is not easily analyzed via depth profiling XPS. Figure 6-5F also shows the strong alternating signal from F1s and also shows a small peak around 685eV. This minor peak at 685eV is potentially due to X-ray damage from the high X-ray power needed to detect lithium. Figure 6-5F also shows the strong alternating pattern of the O1s signal.

As opposed to the sharp interface expected, the sinusoidal nature of the profile in Figure 6-5A can potentially be attributed to the material characteristics sources such as material defects, interfacial roughness and interface wiggling. The profile could also be caused by experimental effects like etching induced roughness and the inelastic mean free path (IMFP) of the photoelectron being measured. To determine the effect of the IMFP of the photoelectron on the atomic profile, the phenomenon was modeled (Figure 6-8 and Table 6-2) and shows this effect contributes, but does not alone cause the sinusoidal profile see in Figure 6-5A.

The sinusoidal atomic concentration profile as opposed to the sharp interface expected shows the limits of depth profiling XPS to analyze nanostructured films. However, one advantage unique to depth profiling XPS is that the chemical state of the film can be analyzed enabling new analysis never done before. Even though the technique requires a large area for analysis (near 1mm$^2$) to minimize X-ray damage and has the potential for etching induced roughness causing interface roughening, we believe the unique advantages of C$_{60}^+$ depth profiling XPS will enable it to be a powerful tool for the analysis of BCP thin films.
6.3 Supporting Information

Figure 6-6: Bulk TEM image of salt-doped PS-POEM with EO:Li = 6:1. Dark domains correspond to RuO$_4$-stained POEM; B. AFM height image of salt-doped PS-POEM thin film with EO:Li = 4:1, indicating a mixed cylinder and lamella structure.

Figure 6-7: Peak fitting to the different regions of the C1s spectra. A) POEM region displaying peaks from the POEM backbone (C-C) PEO side chain (PEO), ester linkage (Ester Carbon) and lithium Salt (CF$_3$). B) PS region displaying Carbon bonds (C-C) a very small peak from PEO side chain (PEO) and C-C bond shake up peak due to pi stacking.
Figure 6-8: Effect of Inelastic Mean Free Path (IMFP) on Depth Profiling Interface Analysis. Comparing experimental data ‘o’ to the modeled system we see that though the IMFP would affect the shape of the profile, but not to the extent seen in our data.

Table 6-1: Atomic Composition Ratios

<table>
<thead>
<tr>
<th>Component</th>
<th>C</th>
<th>F</th>
<th>Li</th>
<th>O</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS domain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>%</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Lithium salt (6:1, PEO:salt)</td>
<td>8.5/6</td>
<td>8.5/6*3</td>
<td>8.5/6</td>
<td>8.5/6*3</td>
</tr>
<tr>
<td>POEM domain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POEM</td>
<td>5+2*8.5</td>
<td>0</td>
<td>0</td>
<td>2+8.5</td>
</tr>
<tr>
<td>% (total = 43.833)</td>
<td>23.4167/43.833 = 53.4%</td>
<td>4.25/43.833 = 9.7%</td>
<td>1.42/43.833 = 3.2%</td>
<td>14.75/43.833 = 33.6%</td>
</tr>
</tbody>
</table>
Table 6-2: IMFP information used for modeling from Tanuma et al. (110). Also shown is the relationship between the Intensity (I) of each electron from different depths (d) within the film depending on its IMFP (\(\lambda\)).

<table>
<thead>
<tr>
<th>Photo-electron</th>
<th>Binding Energy [eV]</th>
<th>IMFP ((\lambda)) [nm]</th>
<th>% Signal From Top 3.5nm</th>
<th>(I(d) = I_o e^{-d/\lambda(E)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li(_{1s})</td>
<td>55eV</td>
<td>0.69</td>
<td>99.9</td>
<td></td>
</tr>
<tr>
<td>C(_{1s})</td>
<td>285eV</td>
<td>1.26</td>
<td>98.0</td>
<td></td>
</tr>
<tr>
<td>O(_{1s})</td>
<td>533eV</td>
<td>1.96</td>
<td>92.0</td>
<td></td>
</tr>
<tr>
<td>F(_{1s})</td>
<td>688eV</td>
<td>2.37</td>
<td>88.4</td>
<td></td>
</tr>
</tbody>
</table>

6.4 Materials and Methods

The synthesis of PS-POEM block copolymers was achieved via atom transfer radical polymerization (ATRP). The PS block was synthesized in a mixture of copper (I) bromide (CuBr, amount), \(N, N', N'', N'''\)-pentamethyldiethylenetriamine (PMDETA, amount), and anisole (amount) using propargyl 2-bromoisobutyrate (PgBiB, amount) as initiator at 100 °C. The reaction was allowed to proceed for 12 h and terminated by immediate cooling to room temperature and exposing to air. The polymer was purified by passage through a neutral alumina column and precipitated from methanol. The PS-Br was reinitiated as a macroinitiator for the semibatch growth of POEM block. The final PS-POEM block copolymer was purified by passage through a neutral alumina column and precipitated from cold isopropanol. (By Wei-fan Kuan)

The PS-POEM solution with lithium salt was prepared as described in the main manuscript. The PS-POEM thin film was cast using flow coating technique (cite Stafford’s paper). Film thickness was measured using a reflectance spectrometer (Filmetrics F20-UV). Optical microscopy images were collected on a Nikon microscope equipped with a 5 MP CCD camera (Nikon Eclipse LV100). The topologies of polymer films were assessed by atomic force
microscopy (Veeco Dimension 3100). Silicon probes (Tap 150G, BudgetSensors) were used in tapping mode. A typical set point ratio was 0.9.

X-ray reflectivity (XRR) X-ray reflectivity (XRR) was performed for the polymer thin films on a Ultima IV unit (Rigaku). A thin, parallel beam of Cu-Kα radiation, \( \lambda = 0.154 \text{ nm} \), was incident on the samples. The beam was sized so as to best capture the critical edge of the samples for best results and fit accuracy. XRR profiles were collected by scanning a small incident angle (\( \theta \)) of X-rays from the source and a detection angle (2\( \theta \)) of reflected X-rays (\( 0^\circ < 2\theta < 3^\circ \)). The electron density profiles across the film thickness were obtained by using Motofit software.

X-ray Photoelectron Spectroscopy (XPS): Chemical composition of the surface was characterized using a PHI Versaprobe II X-ray photoelectron spectrometer with a scanning monochromated Al source (1486.6 eV, 100W, spot size 200 μm). Depth profiling was accomplished using the instrument's \( \text{C}_{60}^+ \) ion source. The takeoff angle between the sample surface and analyzer was 45° and the X-ray beam collected C1s, O1s, F1s, Li1s and Si2p elemental information while rastering over a 200 μm x 1400 μm area. Detailed XPS acquisition parameters are in Table 6-3 below. Sputtering occurred in 1 minute intervals while the sample was moved using concentric Zalar rotation at 1 rpm. The \( \text{C}_{60}^+ \) source was operated at 10 kV and 10 nA and rastered over a 4x4 mm area at an angle 70° to the surface normal. Atomic composition was determined based on photoelectron peak areas and the relative sensitivity factors provided in PHI's Multipak processing software. All data were background subtracted, smoothed using a five point quadratic Savitzky-Golay (S-G) algorithm and charge-corrected so that the carbon-carbon bond has a binding energy of 285.0 eV. The surface of the silicon substrate was defined as the point at which the atomic concentration of silicon reached 5% in the
depth profiling data. Spectra peaks were fit in CasaXPS and data was plotted and analyzed using Matlab.

**Table 6-3: Detailed XPS Acquisition Parameters for BCP Depth Profiling**

<table>
<thead>
<tr>
<th>Element</th>
<th>Binding Energy (eV)</th>
<th>Pass Energy (eV)</th>
<th>Data Spacing (eV)</th>
<th>Sweeps</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1s</td>
<td>278-296</td>
<td>11.75</td>
<td>0.2</td>
<td>3</td>
</tr>
<tr>
<td>O1s</td>
<td>528-537</td>
<td>11.75</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>Si2p</td>
<td>95-107</td>
<td>117.4</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>F1s</td>
<td>680-693</td>
<td>58.7</td>
<td>0.25</td>
<td>2</td>
</tr>
<tr>
<td>Li1s</td>
<td>48-66</td>
<td>187.4</td>
<td>0.20</td>
<td>8</td>
</tr>
</tbody>
</table>
7. CELLULAR BACKPACKS

7.1 Introduction and Background

The potential of synthetic material designed to interact with biological materials for therapeutic purposes has gained attention recently(111). One type of material explored in this thesis is a ‘cell backpack’. Cell backpacks are flat, micron-sized polymeric particles assembled through LbL and are nanostructured with each backpack containing distinct regions for release, payload and attachment. The backpack was originally developed by Albert Swiston, who found that it could be fabricated using a combination of PEMs and photolithography(23). In brief, a glass slide is patterned with 7μm holes where the photoresist is removed. The different regions of the backpack are then conformally deposited using layer by layer dipping. Next, the remaining photoresist is dissolved in sonicating acetone. During this step, the PEM above the photoresist is ripped and removed. This results in posts of PEMs on the surface as displayed in Figure 7-1. These posts are less than one micron thick and 5-7 microns in diameter.
The backpacks have three regions with distinct purposes. The top region is the cell adhesive layer. The composition of this region can be optimized for the particular cell of interest. In particular, we investigated three options. One option developed by Albert Swiston and used in the first generation of the backpack is the presentation of hyaluronic acid at the surface to interact with B-cells through the CD44 receptor (112) (Figure 7-2A). This layer has been thoroughly studied and optimized for its effectiveness at capturing CH-27 B-cells (10), and was also used in the orientation controlled cell-tube study(113). To generalize the attachment to a variety of cell types, two new attachment methods were developed. One method is a covalent chemistry attachment method using either maleimide or activated NHS esters to covalently react with the surface proteins (Figure 7-2B). However, due to the short half-life of the reactive groups and inability to target particular cell types to the necessary reactive groups, we considered other
attachment methods. An alternative method developed used the strong and specific biotin and streptavidin interaction so that any biotinylated antibody could be used. To accomplish this, poly(allylamine hydrochloride) was functionalized with biotin before incorporation into the film. After backpack fabrication, the film was incubated in streptavidin, rinsed and then exposed to the biotinylated antibody of choice (Figure 7-2C). For details of the attachment process see the methods in section 7.3.2. In the majority of the backpack related work, we used IgG-b. However, by simply changing the antibody, the backpack could apply to a wide range of cell types.

![Figure 7-2: Potential Methods of Cell Attachment](image)

A) Attachment of red labeled B-cells onto FITC labeled (hyaluronic acid/chitosan) patches (reproduced from Al Swiston). scale bar 10µm  B) Attachment of unlabeled Monocytes onto (PAA4/PAH4) patches activated with EDC-NHS. scale bar 10µm  C) Attachment of unlabeled Monocytes onto green labeled IgG presenting patches. scale bar 50µm.

The next region is the payload region. To design effective in vivo backpacks, the system must be loaded with a therapeutic agent. An advantage of the layer by layer system is that the payload region can be separately designed since it is independent from the release region and cell adhesive region. This flexibility allows the payload region of the backpack to be tuned to the desired application while the other regions maintain the same structure. For example, the payload region can contain magnetic nanoparticles which could be used for diagnostic tracking or to induce hyperthermia at disease sights. Significant work was focused to expand the potential loading of this region. However, the fabrication step where the backpack is sonicated in solvent
made this more difficult than expected. Small molecules were especially difficult to work with, but Roberta Polak has recently discovered a way to incorporate liposomes containing the small molecule doxorubicin into the backpack. Table 7-1 shows the materials successfully loaded into the backpack during my work.

**Table 7-1: Explored Payloads in a Cellular Backpack**

<table>
<thead>
<tr>
<th>Payload Type</th>
<th>Purpose</th>
<th>Specific Type</th>
<th>Figure Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnetic nanoparticles</td>
<td>Diagnostic Tracking Hyperthermia</td>
<td>Ferrotech 10nm particles</td>
<td>Figure 7-4</td>
</tr>
<tr>
<td>Quantum Dots</td>
<td>Fluorescent Tracking</td>
<td>Ocean Nanotech 665nm COOH functionalized</td>
<td>Figure 7-19</td>
</tr>
<tr>
<td>DNA/siRNA(114)</td>
<td>Gene delivery</td>
<td>Poly(I:C)</td>
<td>Figure 7-18</td>
</tr>
<tr>
<td>Proteins</td>
<td>Therapeutic</td>
<td>Enzymes, Anti-receptors</td>
<td>Figure 7-6</td>
</tr>
</tbody>
</table>

Finally, the release region is a dissolvable region that releases the patch upon exposure to stimuli (16). This region can be built of a variety of hydrogen bonding systems including poly(acrylic acid) and poly(ethylene glycol), or poly(methacrylic acid) and poly(vinylpyrrolidone). The poly(acrylic acid)/poly(ethylene glycol) hydrogen bonding film is useful for analyzing interlayer diffusion (see Chapter 0) since the components lack nitrogen, thus allowing the nitrogen signal to track the diffusion of components like poly(allylamine hydrochloride) or chitosan. The poly(methacrylic acid)/poly(vinylpyrrolidone) film is stable up to pH 6.5(16) and thus was used for the backpack studies to have the maximum flexibility in payload and attachment region functionality. Another more advanced hydrogen bonding system developed by Albert Swiston involves poly(N-isopropylacrylamide) and poly(methacrylic acid). Poly(N-isopropylacrylamide) is a temperature sensitive polymer and using this effect and hydrogen bonding, we have found that this system is only soluble above neutral pH and at low temperature (4°C)(23).
The backpacks can then be attached to the cell in a variety of ways. In Figure 7-3, the attachment method detailed allows for one to one cell to backpack ratios; however we have found that larger scale attachment is possible when the backpacks are freed from the surface by dissolving the release layer and mixed as a free floating solution with cells. Using this method and using IgG-b as the attachment antibody, we can attach ~50% of the incubated monocytes (Figure 7-12).

Figure 7-3: Attachment Scheme of Cell Backpacks a) PEM posts with three regions remains on the surface after the removal of the photoresist. b) The posts are incubated with cells to attach them to the posts c) The hydrogen bonding region is then dissolved, resulting in cells with backpacks attached. Reprinted with permission from Swiston, A.J., Cheng, C., Um, S.H., Irvine, D.J., Cohen, R.E., and Rubner, M.F. Nano Lett. 8, 4446-4453 (2008). Copyright 2008 American Chemical Society.

A further question that arises is the composition of the backpack facing the extracellular environment after attachment to a cell. Due to some interlayer diffusion it is expected that this composition might be a combination of the release layer and also the payload region. We have presented some preliminary work showing this effect in Appendix 11.
7.2 Phagocytosis Resistance of Cell Backpacks

Reproduced in part from: Doshi, N., Swiston, A.J., Gilbert, J.B., Alcaraz, M.L., Cohen, R.E., Rubner, M.F., and Mitragotri, S. Adv. Mater. 23, H105-H109 (2011). Copyright © 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. The work in this section was primarily completed by Albert Swiston and Nishit Doshi with my contribution focusing on the drug release study. I have included much of the original text and figures since the unique capabilities discussed in this paper have driven our continued collaboration with UCSB.

7.2.1 Introduction

Cellular backpacks anisotropic and their round disc-like shape imparts unique functionalities that are not see in most microparticles. Importantly, Champion et al.(24) showed that the local curvature of the particle near a macrophage greatly affects particles’ internalization and therefore providing a theory of the unique capability of the BP. In previous studies it was found that the cellular backpack can be immobilized on a cell without affecting cell functions. In particular the backpacks have shown not to affect the acute vitality or mobility if they are attached to B-cells or macrophages(23, 29). Further the backpack did not affect the distribution of CD44 receptors(23).

To study the phagocytosis resistance of backpacks we worked with macrophages. Macrophages are ubiquitous phagocytic cells in the human immune system and they play a key role in homeostatic, immunological and inflammatory processes(115, 116). Macrophages are widely distributed in various tissues and play a central role in clearing invading pathogens, dead cells and foreign entities through phagocytosis(117). Their wide presence in various organs and tissues makes them particularly suited to provide an immediate defense against invading threats. Moreover, macrophages are rapidly recruited to the diseased site by signaling molecules such as cytokines. Hence, macrophages are involved in a wide repertoire of pathological conditions.
including cancer, atherosclerosis, various inflammatory diseases such as vasculitis and asthma and many others.

Since macrophages play an indispensable role in most pathological conditions, they represent an ideal target for therapeutic applications. Several approaches seeking to use macrophages for targeted therapies involve feeding therapeutic nanoparticles to macrophages ex vivo, followed by re-injection of the macrophages to target the diseased site. This approach has shown promising results for treating HIV infections(118), brain disorders(119) and solid tumors(120). While this strategy is effective for certain conditions, its applications are limited by the fact that the drug carriers are sequestered within the phagosome of macrophages, which reduces the release rates, and in certain cases, degrades the drug. This limitation can be potentially addressed by designing particles that: (i) attach to the macrophage surface, (ii) avoid internalization, (iii) do not interfere with macrophage function and (iv) release the encapsulated drugs in a controlled manner. However, development of materials that simultaneously fulfill these requirements is a significant challenge. Herein, we report the ability of cellular backpacks to successfully encapsulate and controllably release drugs, and avoid phagocytic internalization while remaining on the macrophage’s surface. These characteristics point to new possibilities in creating cell-based bio-hybrid devices which leverage both the functions of the encapsulated cargo (drugs, nanoparticles, etc.) and the native functions of the cell.

7.2.2 Results
Cellular backpacks are fabricated using a standard photolithography lift-off technique of a layer-by-layer and spray deposited film. Briefly, a positive photoresist is patterned with regularly spaced 7 μm diameter holes that extend down to the substrate. Next, a layer-by-layer deposited film consisting of alternating hydrogen bond donor-acceptor pairs is deposited, and this layer
comprises the release region that tethers the rest of the backpack to the substrate. Two hydrogen-bonded regions were used, and details can be found elsewhere(23). Next, a PEM of either (FITC-PAH/MNP) or (PDAC/SPS) is deposited to provide sufficient mechanical rigidity for the backpack to survive the final acetone sonication step. In the protein-eluting backpack construction, a PLGA/FITC-BSA film is then sprayed onto the existing two layers, and forms the hydrolytic payload region of the backpack. An optional second (PDAC/SPS) or (PAH/SPS) film is deposited, and finally the cell-adhesive (HA/CHI) region is added to the top of the heterostructure. A schematic of the three final backpack films is found in Figure 7-4(i-iii). The last fabrication step is sonicating the film in acetone, which dissolves the photoresist and simultaneously lifts off the deposited heterostructured film in all areas except those adsorbed directly to the substrate. A SEM micrograph of an as-fabricated backpack may be found in Figure 7-4b.
Figure 7-4: Backpacks for cell-based drug delivery devices: (a) schematic of the different backpack layers, all of which deposited on top of a photoresist-patterned glass substrate and (b) scanning electron micrograph of a backpack with a PMAA/PVPON release region fabricated by the above strategy. The average diameter is ~ 6 μm and the backpacks have a flat disk shape. (c) Time lapse microscopy image sequence of macrophages interacting with HA/CHI coated backpacks. The arrow indicates an internalized backpack. All other backpacks (n=7) are attached to macrophage surfaces for the entire time lapse sequence of 50 minutes, exhibit strong resistance to macrophage phagocytosis. (d) Quantitative analysis of 6 μm diameter particles internalization by macrophages. Data is presented in terms of internalization percentage (percentage of particles observed that were internalized by phagocytosis). At least 100 particles were observed for each condition. Amine modified spheres act as a positive control and show a high internalization percentage. HA coated backpacks show significantly lower internalization percentage compared to both HA...
coated spheres and amine modified spheres. (e) Scanning electron micrographs showing the interaction of backpacks and spheres with macrophages after 3 hours of incubation in standard cell culture conditions: (i) HA coated backpack attached to the surface of a macrophage, and (ii) three 6 μm diameter HA coated spheres internalized within a macrophage.

**Backpack Interactions with Macrophages**

Attachment and phagocytosis of backpacks was studied using J774 mouse macrophages as a model cell, and 6 μm diameter HA-coated spheres were used as control particles. Around 95% of backpacks added to the cells attached to the macrophage surfaces (n=100 backpacks). Once attached, macrophages did not release backpacks from their surface, suggesting a strong attachment between the two. Time lapse video microscopy provided more insights into the cell-backpack interactions Figure 7-4c. Backpacks remained attached to macrophage surfaces without internalization even 50 minutes following introduction. In contrast, HA-coated spheres were phagocytosed within 30 minutes of attachment to the macrophage surface (see Supporting Information for time lapse microscopy video).

These qualitative observations were quantified in terms of percent internalization. Particles were observed for 3 hours following attachment to the macrophage surface. At least 100 particles were observed for each condition for statistical significance. Amine modified (positively charged at physiological pH) and HA-coated (negatively charged) spheres (diameter = 6 μm) were used as positive controls (121). As expected, amine-modified particles exhibited high internalization with almost 80% of attached particles being phagocytosed. HA-coated spheres were internalized to a lesser extent (35% of attached particles), however a large proportion was still internalized. The difference between HA-coated and amine-modified spheres likely originates from a difference in their surface charge. HA was used for coating backpacks since interactions between HA and macrophages are specific, mediated through the cell surface
receptor CD44(122). Backpacks exhibited almost 30-fold lower extent of internalization compared to positive controls and about 14-fold lower extent of internalization compared to HA-coated spheres (Figure 7-4d). It was found that backpacks typically attach to macrophages by their flat face (Figure 7-4e).

Effect of Backpacks on Macrophages

We assessed whether backpack attachment alters the cellular functions of macrophages. Two behaviors of macrophages with attached backpacks were assessed; (i) the ability to internalize spherical particles (i.e., easily phagocytosed targets) and (ii) motility. We measured the internalization capability of macrophages with attached backpacks. PS spheres (diameter = 3 μm) were chosen as the target since particles of this diameter have been shown to be preferentially phagocytosed by macrophages(123). Macrophages were first incubated with backpacks to allow sufficient attachment and then 3 μm PS spheres were added to the suspension. Time-lapse video microscopy clearly shows that backpack-laden cells were able to internalize 3 μm spheres while simultaneously remaining attached to a backpack (Figure 7-5a). Macrophage motility was measured by tracking their center of mass for 3 hours. No statistical difference was found between the mobility of macrophages with and without backpacks (Figure 7-5b).
Figure 7-5: Backpack attachment to macrophages does not affect cellular health and functions. (a) Time lapse video microscopy showing that a backpack-laden macrophage can still efficiently internalize 3 μm PS spheres (MP: Macrophage, BP: Backpack, PS-S: 3 μm polystyrene sphere). The backpack is not internalized during the entire duration of the time lapse sequence. (b) Differences in migration distance of macrophages with and without backpacks 3 hours were found to be insignificant. (c) MTT assay results in terms of proliferation potential, where 1 indicates healthy proliferation capacity and 0 indicates complete cell toxicity.

Backpack Cytotoxicity

An MTT assay was performed to determine whether the attachment of backpacks adversely affects the health and proliferation of macrophages. The proliferation index of backpack-associated macrophages was not statistically different from the native population (Figure 7-5c), indicating that backpacks are not acutely toxic to the cells and they do not interfere with a cell’s ability to reproduce. As a control, macrophages exposed to 6 μm spheres also did not induce any noticeable toxicity.
Drug Release from Backpacks

The backpacks reported in this study have significant biomedical potential as multi-modal therapeutic and diagnostic platforms that may carry a variety of drugs, imaging/diagnostic agents, or nanoparticles. Specifically, backpacks can be loaded with drugs which can be released in a controlled manner. As a proof of concept, we loaded the backpacks with fluorescein conjugated Bovine Serum Albumin (FITC-BSA) within a poly(lactic-co-glycolic acid) (PLGA) polymer matrix and measured subsequent protein release.

The fabrication method for drug releasing backpacks was slightly different from previously described due to the inclusion of PLGA (Figure 7-6a), but the backpack still presented the same cell-adhesive and pH labile release regions as backpacks used in the toxicity and mobility studies. FITC-BSA was chosen since it is commonly used as a model protein and it could be replaced with a variety of therapeutic or diagnostic materials. We see that the backpacks released FITC-BSA in a burst manner with ~40% released by 2 hours and >90% released by 24 hours (Figure 7-6b). Also of note is that very similar release profiles were seen independent of the weight percentages of FITC-BSA within the polymer solution before spraying.
H-bonded release and PEM LbL deposited

Spray PLGA/FITC-BSA colloid
Lyophilize overnight
LbL deposit PEM and cell-adhesive region

Film deposition complete
Sonicate in acetone
Release and incubate in PBS

Measure FITC signal of supernatant

(b)

Normalized release of FITC-BSA

Hours

0 20 40 60 80 100 120 140
Figure 7-6: Controlled release of FITC-BSA from therapeutic backpacks. (a) Fabrication schematic (b) Controlled release profile of FITC-BSA in-vitro. The normalized release of FITC-BSA occurred over multiple hours and was not dependent on the loading percentage of the spray emulsion used. Red and blue lines correspond to 7.5 wt% and 25 wt%, respectively.

7.2.3 Discussion

Macrophages are pervasive cells in the mammalian immune system, capable of identifying and neutralizing non-native agents found in the body. In addition, macrophages are routinely recruited to diseased sites, potentially making them highly efficient targeting devices. If non-native entities could be attached to these macrophages without being phagocytosed, these cells could serve as ideal chaperones for delivering drugs or other therapeutic tools to various pathological tissues. This is particularly attractive for the treatment of cancer and inflammatory disorders, where macrophages are very strongly recruited (124-126). This approach would offer much greater spatial resolution and selective uptake of therapeutic or diagnostic components, essentially using an individual cell as a cargo-carrying device.

Cellular backpacks are nanoscale-thickness microparticles fabricated using photolithography where polyelectrolyte multi-layers are assembled using layer by layer technique, one of the most versatile methods to incorporate a variety of materials within film structures for biochemical applications (127, 128). The backpack are engineered to (1) contain therapeutic or diagnostic materials, such as small molecule drugs, proteins, nanoparticles, or functional polymers, and (2) attach to the surface of a cell using a non-toxic mechanism relying upon a natural ligand-receptor interaction such as the HA-CD44-mediated adhesion used in this study (129, 130). Because of their unique geometry and capacity to contain therapeutic and diagnostic materials, cellular backpacks present unique opportunities as a functional phagocytosis-resistant microparticle.
Long-term immobilization of any particle on the surface of phagocytic cells like macrophages is extremely difficult. Recently, Matthias et al. showed the therapeutic potential of covalently attaching nano-particles to the surface of cells, but phagocytic immature dendritic cells posed an internalization problem(131, 132). The primary challenge of attachment to phagocytic cells, like macrophages, is their propensity to engulf any material attached to their surface. Various properties of particles, particularly their size, surface chemistry, shape and mechanical flexibility have been shown to play a critical role in phagocytosis(24, 121, 133-136). Particles in the range of 2-3 μm are known to exhibit high attachment to macrophages(24, 133, 137), and particles in this size range are very efficiently phagocytosed. Although the rate of internalization depends on the size of the particle, most particles with 2-3 μm diameters are internalized within 30 minutes of attachment to the macrophage surface.

Recent literature has shown that the local shape, orientation, and mechanical properties of a particle significantly influence its susceptibility to be internalized by phagocytosis(134). Elongated, high aspect ratio particles and very flat disk-shaped particles are shown to exhibit reduced phagocytosis compared to spherical particles(138). The low internalization rate of disk-shaped backpacks reported here is consistent with these observations. Though not considered in this study, a backpack’s flexibility and low modulus may also contribute to low internalization frequency, a phenomenon seen in previous work(139). Indeed, the right blend of three factors (shape, orientation, and mechanical properties) mentioned may make backpacks an ideal system for avoiding the phagocytosis behavior of macrophages.

Avoiding internalization by macrophages is a key feature of the backpack, but equally important is the observation that backpack attachment does not interfere with the macrophage’s native cellular functions. The results of the MTT proliferation assay suggest that backpacks are
not toxic and do not affect cell proliferation capacity. Furthermore, backpack attachment did not adversely affect a macrophage’s ability to internalize particles that are otherwise phagocytosable. Further investigation is needed to understand whether or not backpack attachment affects other macrophage functions, including the release of chemokines and cytokines.

Cellular backpacks provide a unique opportunity for drug delivery and therapy since the polyelectrolyte multilayers which comprise much of the backpack are well studied for their biomedical applications(140) and drug delivery properties(28). In particular, polyelectrolyte multilayers have shown the ability to deliver DNA(114), vaccines(141), proteins(6) and small molecules(7). The results presented here show that cellular backpacks are capable of delivering a model protein in a controlled and sustained manner in vitro. Another strategy for finely controlled drug release from therapeutic backpacks could be to trigger release by thermal ablation. Chemotherapy agents or particles capable of ablation via RF heating (i.e., gold or iron oxide) may be loaded into a backpack, which can then ride on a monocyte being recruited into a solid tumor. Using a recruited monocyte as an active therapeutic-carrying device may allow access to the relatively inaccessible hypoxic region of a tumor(142), offering clear advantages over passive particle uptake through “leaky” vasculatures. Such a strategy has been adopted for gold nanoparticles with promising results(120).

7.2.4 Experimental Section

Backpack Fabrication

The backpacks were formed through a combination of photolithography and polyelectrolyte multilayers (PEMs). Photolithographically patterned (PDAC4.0/SPS4.0)15.5 (where PDAC is poly(diallyl dimethyl ammonium chloride) and SPS is poly(styrene sulfonate) coated glass substrates
were sequentially dipped in dilute polymer or nanoparticle solution using an automated Zeiss programmable slide stainer or nanoStrata dipping unit, the details for which have been previously described (23, 130, 143). The fully-charged polyelectrolyte and hydrogen-bonded release PEMs were built in the Zeiss dipper; the cell adhesive region was built in the nanoStrata unit. The formula for backpacks used in internalization studies is 

\[(PMAA3.0/PNIPAAM3.0)_{80.5}(FITC-PAH3.0/MNP4.0)_{10}(PAH3.0/SPS4.0)_{30}\]

where the number following each species abbreviation indicates the solution pH and subscripts are the number of bilayers (where a half bilayer is indicated as 0.5). PMAA stands for poly(methacrylic acid), PNIPAAM for poly(N-isopropylacrylamide), FITC-PAH for fluorescein poly(allylamine hydrochloride) and MNP for magnetic nanoparticles. An optional \((CHI3.0/HA3.0)_{3}\) (where CHI is chitosan and HA is hyaluronic acid) region was used as the cell adhesive region for both backpacks and PS spheres. The purpose of each region is described in the backpack fabrication results section. See Supporting Information for backpack compositions in toxicity and migration studies.

**MTT assay**

J774 mouse macrophages were seeded at 10⁴ cells/well onto a 96-well plate. Backpacks and 6 μm spheres at a concentration of 10⁵/well (200 μl media) were exposed to the cells for 4 hours. Cells not exposed to any particles were used as negative control. Ten microliters of reagent from an MTT kit (Invitrogen, Carlsbad, CA) was applied to each well for 5 hours. The liquid from each well was then replaced by DMSO and allowed to incubate in the dark at room temperature for about 10 minutes to lyse the cells. Absorbance was read at 570 nm (λ<sub>max</sub> of MTT dye) and proliferation index was calculated as the ratio of absorbance of the sample to the absorbance of
negative control. The proliferation index values range from 0 to 1, with 0 indicating maximum mitochondrial toxicity and 1 representing high proliferation capacity and healthy cells.

**Controlled Release of BSA from Backpacks**

Backpacks for drug release studies were slightly different in structure to enable the inclusion of a layer comprised of PLGA and FITC-BSA. The layer was applied by pressure spraying a dilute sonicated mixture of PLGA solubilized in chloroform and FITC-BSA solubilized in water. (See Figure 3a and Supporting Information for details). Backpacks were released from the surface using PBS pH 7.4. BSA release was performed in pH 7.4 PBS in static conditions at 37°C using fluorescence analysis (see Supporting Information for details). Prior to analysis, the sample was centrifuged at 10,000 relative centrifugal force and 0.8 mL of the supernatant was removed for analysis. Protein concentration was determined using a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon, Japan) exciting at 494nm and the emission was measured at 520nm. Immediately following, 0.8mL of fresh PBS was added to replace the removed solution. Data was measured in triplicate and each sample had one microscope slide of backpacks present. Approximately 13 cm² per slide was covered with backpacks with an approximate backpack density of $2 \times 10^5$/cm². After 5 days of elution the backpacks were isolated into 200 μL of PBS, frozen and lyophilized overnight. The samples were then dissolved in 250 μL of dichloromethane and 200 μL of DI water and 800 μL of PBS were added. The samples were then sonicated for 90 minutes to increase extraction surface area. Finally, samples were centrifuged as before and the aqueous phase was removed for fluorescence analysis.

**Materials**

Poly(methacrylic acid) (PMAA, PolySciences, PA, USA M=100kDa), poly(allylamine hydrochloride) (PAH, Aldrich, MO, USA M=70kDa), poly(N-isopropylacrylamide) (PNIPAAm,
Polymer Source, Quebec, Canada M=258kDa), poly(vinylpyrrolidone) (PVPON, Aldrich, M=1.3MDa), fluorescein-labeled poly(allyl amine hydrochloride) (FITC-PAH, Aldrich, M=70kDa), poly (diallyl dimethyl ammonium chloride) (PDAC, Aldrich, M=200-350kDa in 20% aqueous solution), poly(styrene sulfonate) (SPS, Aldrich, M=70kDa), hyaluronic acid (HA, from Streptococcus equi, Sigma, MO, USA, M~1.58 × 10^6 Da), and low MW chitosan (CHI, Aldrich, DS=.75-.85, M~5 × 10^4 Da) were used without purification. Iron oxide magnetic nanoparticles stabilized with a proprietary anionic surfactant were used (MNP, Fe_3O_4, 10nm diameter, Ferrotec EMG 705, NH, USA). 3 μm and 6 μm polystyrene spheres were purchased from Polysciences. FITC-Bovine serum albumin (Sigma), Chloroform (Sigma), poly(DL-lactide-co-glycolide) ester terminated (50:50) M_w 7,000 to 17,000, RESOMER® RG 502 (Sigma) was used.

Cells

The J774 mouse monocyte macrophage cell line (ECACC products, Sigma Aldrich, MO, USA) was used in this study. Cells were cultured with Dulbecco Eagle media (ATCC, VA, USA) which was supplemented with 1% penicillin/streptavidin (Sigma Aldrich) and 10% fetal bovine serum (Invitrogen, CA, USA). Cells were grown and passaged in standard culture conditions (37 °C and 5% CO_2).

Backpack release from fabrication substrate

Backpacks were fabricated with a thermally-controlled release region to give control over the number of backpacks attached to each macrophage. Briefly, this system is based upon the lower critical solution temperature (~29C) of PNIPAAm, above which the PMAA/PNIPAAm film is insoluble and cells may be attached, and below which the film is unstable and the backpacks
delaminate from the fabrication substrate. These thermally controlled backpacks were used for all internalization studies, where ensuring a single backpack per macrophage was critical.

For all other studies, including toxicity and mobility, non-thermally released backpacks with a PMAA/PVPON release region were used. Here, a change in solution pH above 6.0 is sufficient to trigger release region dissolution and concomitant backpack release from the surface. The composition of these backpacks is similar to the thermally-released backpacks except for the release region, and may be represented as: (PMAA2.0/PVPON2.0)_{20.5}(FITC-PAH3.0/MNP4.0)_{10}(PAH3.0/SPS4.0)_{30}, with an optional (HA3.0/CHI3.0)_{3} cell-adhesive region. These backpacks were harvested in PBS and added to a macrophage suspension, where cells randomly associate with backpacks. While this method does not afford control over the association between backpacks and cells (ie, more than 1 backpack per cell is possible), post-exposure processing may be used to adjust this ratio.

**Backpacks for drug release**

In chronological order of deposition (starting at the lithographically patterned substrate), films used were: (PMAA2.0/PVPON2.0)_{40.5}(PDAC4.0/SPS4.0)_{10.5}(PLGA + FITC-BSA)_{1}(PDAC4.0/SPS4.0)_{10.5} (HA3.0/CHI3.0)_{2.5}. A BSA solution in water (40 mg/mL) was added to a PLGA solution in chloroform (2 mg/mL) for the final BSA concentration, and the mixture was sonicated for 10 minutes. The solution was sprayed using an air brush (Badger, IL, USA) at 15cm with a N₂ pressure of 50 psi and a flow rate of ~7 mL/min. After spraying, the film was lyophilized overnight before any further dipping. Next, the final layers of the backpack were deposited and the sample was sonicated.

**Optical and Fluorescence Microscopy**
Cells were cultured overnight in glass bottom delta T dishes (Bioptechs, PA, USA) at a density of around $10^5$ cells per dish (3.8 cm$^2$). The cells were washed with PBS the following day and the medium was replaced with HEPES (Sigma Aldrich) medium (DMEM + 25mM HEPES) to maintain pH regardless of atmospheric carbon dioxide. Experiments were performed with various particle types, concentrations, and length of incubation. Cells were imaged on an Axiovert 25 microscope (Zeiss, NY, USA) fitted with a Delta T temperature controller (Bioptechs) that maintains 37°C and observed using a Neo-Fluor oil immersion, 100x, 1.3 NA objective (Zeiss). Brightfield images were taken using a cooled CCD camera (CoolSnapHQ, Roper Scientific, GA, USA).
7.3 Monocyte Hitchhiking: Delivery of Phagocyte Resistant Backpacks to Lungs

Building on the success of backpack’s resistance to phagocytosis presented in section 7.2 we desired to test the *in vivo* application of the backpack. In collaboration with Aaron Anselmo at UCSB, we determined that a new attachment method was needed since the HA/CHI topped backpacks did not interact with monocytes. As a result, I developed antibody labeled backpacks and validated their monocyte attachment. The *in vitro* study of BPs effects on monocyte differentiation and transmigration as well as the *in vivo* biodistribution study were performed by Aaron at UCSB. The following text was written in collaboration with Aaron Anselmo as equal contributors and is currently in the process of being submitted for publication.

Authors: Aaron C. Anselmo¹*, Jonathan B. Gilbert²*, Sunny Kumar¹, Vivek Gupta¹, Robert E. Cohen², Michael F. Rubner³, Samir Mitragotrî¹

¹ Department of Chemical Engineering, University of California Santa Barbara, CA 93106
² Department of Chemical Engineering ³ Department of Materials Science and Engineering, Massachusetts Institute of Technology, Cambridge MA 02139

The demand for the controlled delivery of therapeutics to desired locations in the body has resulted in tremendous advances in particle technology. These advances include the use of particles with various surface chemistries, targeting ligands, shapes and sizes to direct polymeric drug carriers to the desired location in the body.(21, 144, 145) Despite these advances, polymeric drug carriers have seen limited success in the clinic due to poor circulation times, stemming from the rapid clearance of foreign particles by the immune system, and to the overall inefficient targeting to diseased tissues.(146, 147) While polymeric carriers have been unable to overcome
these challenges, circulating cells can remain in the blood for extended periods and can target and accumulate at diseased tissues in cases of cancer,(148) atherosclerosis,(149) and arthritis,(150) to name a few. Recent studies have combined polymeric particles with mammalian cells to transfer the natural delivery capabilities of circulatory cells to these particles. Indeed, it has been shown that circulatory cells functionalized with polymeric particles, either by internalization or by surface attachment, have been able to improve both the circulation and delivery of polymeric carriers to disease sites.(118, 151) In this work, we have developed phagocytosis resistant polymeric cell backpacks that attach to the surface of monocytes through antibody interactions. Following attachment to monocytes, essential cell functions such as differentiability and extravasation through an endothelial cell layer are preserved. Backpack functionalized monocytes were then tested in vivo and shown to target backpacks to sites of inflammation. Cellular backpacks represent a new type of polymeric carrier that is designed to work synergistically with cells to improve the targeted delivery of therapeutic carriers.

Monocytes are a type of white blood cell that can target, and cross the endothelium of inflamed tissues before terminally differentiating into macrophages. One example of this is in the case of tumors, where monocyte-differentiated macrophages potentially make up 50% of the cell tumor mass,(148) illustrating the potential of monocytes to carry drug containing particles into inflamed tissues. Macrophages have already been used for a variety of cellular hitchhiking therapies, including reducing HIV replication in the brain,(119) preventing tumor growth,(152) targeting brain metastases(153) and reducing inflammation by providing neuroprotective capabilities in a Parkinson’s disease model.(154) While similar, macrophages and monocytes are distinct cells. Unlike macrophages which are terminally differentiated and reside in the tissue, monocytes are circulatory cells and thus can navigate the vasculature and target typically elusive
diseased tissues. However, monocytes are also capable of internalizing foreign materials. Avoiding this internalization for cell-mediated therapies is of paramount importance since internalization can potentially lead to endosomal degradation and subsequent loss of therapeutic use. However by engineering the backpack shape using recently discovered design parameters as a guideline, we developed polymeric particles capable of avoiding phagocytosis. (24, 133, 138) By taking advantage of a monocyte’s natural targeting abilities, monocyte hitchhiked backpacks can potentially target tissues that backpacks, on their own, would find challenging.

Cellular backpacks are phagocytosis resistant particles designed to attach to the surface of immune cells. (23, 29) These flat, anisotropic polymeric particles are fabricated by layer-by-layer (LbL) processing and are hundreds of nanometers thick and microns wide. The LbL technique has the ability to constrain the location of the desired materials within the backpack simply by controlling the order of fabrication; (92) thus allowing the backpack to have a distinct region designed for cell attachment and other regions for functional materials. Cellular backpacks are fabricated using a combination of photolithography and polyelectrolyte multilayers as described previously. (23, 29) Photolithographically patterned substrates control the shape and size, while polyelectrolyte multilayers allow for control over the composition, nanostructure (92) and stimuli responsiveness of the cellular backpack. (155) Backpacks are ~7μm in diameter and are made of three polyelectrolyte multilayer regions with a total thickness less than 500nm (Figure 7-7a). The region shown in green is hydrogen bonded and consists of the donor poly(methacrylic acid) (PMAA) and the acceptor poly(vinylpyrrolidone) (PVPON). This region attaches the backpack to the fabrication substrate and when this layer is exposed to a solution with a pH > 6.5 the region dissolves by ionizing the acid. (16) Thus by controlling pH, the backpacks can be released from the surface when desired. The next region, shown in yellow, consisting of electrostatically
interacting poly(allylamine hydrochloride) (PAH) and anionic iron oxide magnetic nanoparticles (MNP, 10 nm diameter) provides both mechanical rigidity for fabrication and functional activity in the magnetic responsiveness of the backpack. (23) The final region, shown in blue, is the attachment region consisting of electrostatically interacting poly(acrylic acid) (PAA) and PAH functionalized with biotin (PAH-b). The attachment region is exposed to streptavidin before a biotinylated antibody of choice is used to attach to the desired cell. For this work, we used biotinylated mouse IgG to ensure interaction between the backpack and cell (Figure 7-10). The activity of the mouse-IgG present on the surface of the backpack, was validated using secondary antibody labeling (Figure 7-7 b, c and method show in Figure 7-11). The resultant backpack is chemically anisotropic with an attachment region found only on one side and thus can control its orientation on the surface of living cells. (113)

Figure 7-7: Design of cellular backpacks and confirmation of antibody presentation. (a) Schematic of a cellular backpack. (b) TRITC-Streptavidin and mouse-IgG-biotin are localized to the backpack and activity is shown by secondary antibody FITC-anti-mouse-IgG labeling. (c) If mouse-IgG-biotin is not added to the backpack, no specific FITC-anti-mouse-IgG labeling is seen.

Backpacks (BP) were then removed from glass slides and attached to WEHI-265.1 murine monocytes by incubation at a 1:1 (BP:monocyte) ratio. BP attachment was mediated by IgG-Fc receptor interactions. (156) The attachment was confirmed by confocal microscopy in
solution (Figure 7-8ai) and on the surface of backpack arrays without the pH sensitive (PMAA/PVPON) region (Figure 7-8aii). Attachment was quantified using fluorescent activated cell sorting (FACS) (Figure 7-12). At a BP:monocyte ratio of 1:1, ~50% of monocytes carried at least 1 BP. MTT assays determined that BP attachment to monocytes does not impair health or proliferation of macrophages. The proliferation index of BP-conjugated monocytes at 24 hours was not statistically different than that of monocytes interacting with plain 3μm polystyrene particles or 3μm polystyrene particles coated with IgG-b, which offer roughly the same surface area of the IgG-b functionalized BP surface (Figure 7-13).

Figure 7-8: Attachment of cellular backpacks to WEHI-265.1 monocytes and effect on monocyte ability to differentiate into macrophages. (a) Confocal and brightfield overlay images of: (i) a BP (green) wrapping around a stained monocyte (red) and (ii) monocytes (unstained) attaching to an array of patterned BPs (green). (b) Brightfield images of: (i) WEHI-265.1 monocytes in normal culture media at 48 hours, (ii) WEHI-265.1 monocytes differentiated into macrophages via incubation with phorbol 12-myristate 13-acetate
(PMA) treated culture media at 48 hours, (iii) FACS sorted monocytes with BPs (green) attached prior to incubation for 48 hours with 50nM PMA, and (iv) J774 macrophages. Scale bar = 10 μm.

To determine whether monocyte’s ability to cross an endothelial monolayer was affected by BP attachment, we evaluated the transmigration of BP-conjugated monocytes through a confluent human umbilical vein endothelial cells (HUVEC) monolayer. HUVECs were grown to a stable monolayer (30 ± 5 Ωcm²) and ability of monocytes alone, BPs alone, and BP-conjugated monocytes was assessed for transmigration through the monolayer. BP-conjugated monocytes and unconjugated monocytes passed through the endothelial layer in similar amounts at both 24 and 48 hours (Figure 7-9a). Independently using the BP fluorescence the percentage of BPs transmigrated for BP-conjugated monocytes and free BPs was then assessed. At 24 and 48 hours, ~35% and ~60%, respectively, of BPs attached to monocytes were able to cross the monolayer while free BPs were unable to cross the monolayer (Figure 7-9b). Altogether, the in vitro experiments show that monocytes maintain many of their important functions following BP attachment.

Monocytes were next investigated for their ability to home to, and carry BPs to, inflamed tissues in vivo in an acute lipopolysaccharide (LPS) lung inflammation model. This widely established model(157) results in increased lung expression of markers VCAM(158) and ICAM.(159) Together, VCAM which mediates the initial endothelial adhesion(160) and ICAM which mediates leukocyte spreading across the endothelium,(161) should enhance adhesion of monocytes in the inflamed lungs (Figure 7-14). Inflammation was confirmed by measuring increased expression of ICAM-1 in lungs (Figure 7-15). For in vivo studies, WEHI-3 monocyte-like cells were used as they have been proven to circulate and migrate to tissues in BALB/c mice when injected intravenously, or even intraperitoneally. 3H-labeled BPs were attached to monocytes and were intravenously injected in both LPS lung inflamed mice and healthy mice. At
6 hours, backpacks accumulated ~2-fold higher in the lung tissue of mice that were challenged with LPS (Figure 7-9c). All other organs and blood showed no statistical differences.

Figure 7-9: Transmigration of WEHI-265.1 monocytes and *in vivo* targeting. (a) Analysis of monocyte migration (MCP-1 initiated) through HUVEC layer for monocytes alone (black squares), monocytes with backpacks attached (black circles) and negative control of media alone without monocytes (black triangles). Values represent mean ± SD (n = 3). (b) Analysis of BP migration through HUVEC layer when attached to monocytes (black) and in free solution without monocytes present (hatched) at 24 and 48 hours. Values represent mean ± SD (n = 3). (c) Percent of injectable dose per gram (%ID/g) 3H-backpacks attached to monocytes in normal mice (black bars) and LPS induced lung inflamed mice (hatched...
bars) at 6 hours. Values represent mean ± SEM (n = 4). Statistical difference (P < 0.01) is seen only in lungs.

Attachment of backpacks to the surface of monocytes for cellular hitchhiking delivery presents a promising method to achieve targeted drug delivery. The results presented here demonstrate the design and synthesis of a novel particle-based system with the ability to target inflamed endothelium via monocyte-mediated hitchhiking. To accomplish this intricate task, the cell backpack was designed as a multifunctional material that binds to monocytes strongly, contains a payload and resists phagocytosis. Given the high material requirements the LbL technique was chosen since it allows for the precise control over the shape and structure of the backpacks as well as flexibility in the backpack composition. Alternate methods including PRINT(162, 163), particle stretching(24) and template based methods(164, 165) have fabricated non-spherical polymeric particles for drug delivery purposes, but it is difficult to match the inherent material flexibility found with LbL. By combining lift-off photolithography and conformal coatings a variety of shapes could be explored but given the proven phagocytosis resistance of circular backpacks, we chose to maintain the design from our previous work.(29) Monocyte hitchhiked backpacks represent one of many cellular hitchhiking formulations that can utilize the backpack platform. Furthermore, due to the freedom afforded by LbL fabrication, these backpacks can be modified to present any biotin functionalized antibody for the adhesion to the appropriate circulatory cell. Cellular hitchhiking of drug carrying particles can potentially address the two main limitations preventing the widespread use of nanoparticles in the clinic, notably the poor circulation times and limited targeting to diseased tissues. This is further evidenced by the fact that there are currently no active targeting nanoparticle drug delivery systems in the clinic, as all rely on passive targeting to diseased tissue. Future research will be focused on incorporating drug containing backpacks for therapeutic treatment and utilizing
primary monocytes. Collectively, these results display the huge potential of monocyte mediated backpack delivery for the treatment of a numerous inflammation based diseases.

7.3.1 Supporting Figures

Figure 7-10: Preparation of Cellular Backpacks i) incubation of Cell Backpacks with streptavidin (can be TRITC labeled) ii) Exposure to mouse IgG-biotin to dock onto streptavidin iii) Backpacks washed and ready for attachment.

Figure 7-11: Secondary Labeling to Check Activity of the Antibody on Backpack Array (no pH sensitive release region). i) and ii) show backpacks exposed to IgG-b or not respectively. iii) After exposure to anti-mouse IgG-FITC, if the antibody is active and present the sample will fluoresce (iii), if not there will be no signal (iv).
Figure 7-12: FACS plots and approximate gating for backpack attachment to WEHI-265.1 monocytes. (i) Representative FACS output for WEHI-265.1 monocytes alone with approximate gate. (ii) Representative FACS output for backpacks alone with approximate gate. (iii) Representative FACS output for solution containing conjugates, unmodified monocytes and unattached backpacks with approximate gates. (iv) Representative FACS data of monocyte-bound BPs via FACS, showing unmodified monocytes (red), unattached BPs (purple), and conjugates (green) showing both >50% BPs attached to monocytes and >50% of monocytes with at least 1 BP attached at a 1:1 (cell:BP) incubation ratio. Inset shows representative histogram as a function of fluorescent intensity from the same experiment.
Figure 7-13: Toxicity assay for backpacks and WEHI-265.1 monocytes. 24 hour MTT assay showing limited toxicity to monocytes incubated with BPs functionalized with IgG-b. Values represent mean ± SD (n = 3).

Figure 7-14: Schematic of Monocyte Targeting in vivo Experiment. Acute inflammation caused by LPS will cause the monocytes to localize to the lung (left), while in the non-inflamed case (right) minimal accumulation will occur.
Figure 7-15: Expression of ICAM-1 following LPS induction in BALB/c mice. ICAM-1 expression in lungs 24 hours after intranasal administration of 0 µg, 5 µg, and 10 µg LPS.

7.3.2 Experimental Section

Materials:

PMAA (Aldrich, M=100kDa) PVPON (Sigma, M=1300kDa), PAH (Polysciences, M=150kDa), EMG 705 anionic MNP (Ferrotec), PAA (Polysciences, M=450kDa) N-hydroxysuccinimide-biotin (Sigma) was used as received. To fabricate PAH-biotin, 25mg of PAH was dissolved in 50mM MES buffer at pH 8. 15.3mg of NHS-biotin was dissolved in DMSO at 22mg/ml before adding to the PAH solution resulting in ~15% amine substitution. The solution reacted for 4 hours before it was purified by dialysis overnight into water.

Backpack Fabrication:

The nomenclature for LbL follows (poly1X/poly2Y)$_z$, where X and Y are the pH of the polymer solutions and z is the number of bilayers deposited (1 bilayer = poly1+poly2). A non-integer value of z indicates the assembly was terminated with poly1. Backpacks are formed through a combination of photolithography and polyelectrolyte multilayers as previously described.(23) All
backpacks tested had the structure of (PMAA2/PVPON2)$_{30.5}$ (PAH3/MNP4)$_{10.5}$ (PAA4/PAH-biotin4)$_{8}$. The attachment region (PAA4/PAH-biotin4) had 150mM NaCl added to the polymer solutions.

**Backpack Preparation**

Fluorescent (TRITC, FITC) streptavidin (Pierce Thermo) was diluted with 0.1 M (pH 5) sodium acetate to 100ug/ml. 600ul streptavidin solution was pipetted and spread uniformly onto glass slides containing BPs for 30 minutes at room temperature. Glass slides were then washed in 0.1 M sodium acetate (pH 5) for 30s to 1 min and allowed to air dry. Mouse IgG functionalized with biotin, IgG-B (Santa Cruz Biotechnologies), was diluted to 100ug/ml with PBS and pipetted onto glass slide containing BPs as above. For 3H studies, 3H-labeled biotin (American Radiolabeled Chemicals) was mixed with IgG-B to radiolabel BPs. After 30 minutes, BPs (in PBS) were gently scraped off of the glass slide surface and centrifuged at 5000g for 10 minutes. IgG-B containing supernatant was removed and BPs were washed once in PBS.

**Attachment of BPs to Monocytes**

A known number of BPs, in suspension, were incubated with monocytes at 37°C for 30 minutes in PBS. Unbound BPs were separated from conjugates via centrifugation at 200g. Conjugates were imaged on an Olympus Fluoview 500 (Olympus America Inc.).

**Differentiation of Monocytes**

WEHI-265.1 monocytes without BPs attached were incubated in 6 well plates with 50nM phorbol 12-myristate 13-acetate (Sigma), PMA, in standard cell culture media. In parallel,
WEHI-265.1 monocytes were incubated with FITC labeled BPs and sorted for using FACS. FACS sorted BP-monocyte conjugates were incubated in 6 well plates at identical conditions.

**Transmigration Assay**

HUVECs were plated in the upper chamber of Corning Transwell plates (3μm, 6 well) at 6x10^5 cells per well in M200 media and allowed to attach and grow to form a complete monolayer (confirmed with TEER measurements), after which media was changed to DMEM in all chambers. 6x10^5 WEHI-265.1 monocytes (also in DMEM), either with none or a known number of FITC-labeled BPs, were pipetted on top of the HUVEC monolayer in the top chambers, which was then suspended above the below chamber containing DMEM with 10mM Monocyte Chemoattractant Protein-1 (Fisher). Monocytes which passed through the HUVEC monolayer were counted manually on a hemocytometer. BP migration was quantified via fluorescent reading (normalized to applied dose) of BPs that had migrated into the lower chamber.

**Inflammation Model and Biodistribution**

Healthy female BALB/c mice (18-20g) were administered 10μg of lipopolysaccharide (LPS) in 60ul saline intranasally. 3x10^6 WEHI-3 monocytes conjugated with 1.5x10^6 3H-labeled backpacks (2:1 cell:BP ratio) in saline were then intravenously injected into either healthy female BALB/c mice (18-20g; n=4) or LPS challenged female BALB/c mice (18-20g; n=4). At 6 hours, mice were sacrificed via CO₂ overdose and known organ weights were harvested and dissolved at 60°C in 5ml Solvable (Perkin Elmer) overnight. 5ml Ultima Gold was then added to each sample, allowed to cool, and measured for 3H content in a Packard TriCarb 2100TR

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scintillation counter. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Santa Barbara.

**Cell culture**

WEHI-265.1 monocytes (ATCC) were incubated at 37°C in 5% CO₂ in high glucose DMEM, 10% FBS, 1% penicillin/streptomycin, and 0.05 M 2-mercaptoethanol. HUVECs (Invitrogen) were incubated at 37°C in 5% CO₂ in Medium 200 with low serum growth supplement (LSGS) and 1% penicillin/streptomycin. J774 macrophages were incubated at 37°C in 5% CO₂ in high glucose DMEM, 10% FBS, 1% penicillin/streptomycin. WEHI-3 monocytes were incubated at 37°C in 5% CO₂ in Iscove’s modified DMEM, 10% FBS, 1% penicillin/streptomycin, and 0.05 M 2-mercaptoethanol.

**Fluorescence-activated cell sorting (FACS)**

BPs, monocytes, and conjugates were suspended in PBS. A FACSAría Flow Cytomer with a 70µm nozzle was used to quantify attachment of BPs to monocytes. Mammalian cells were filtered prior to analyzing sample. BD FACSDiva software 6.0 was used to analyze data.

**MTT Assay**

WEHI-265.1 monocytes were seeded in a 96-well plate at 1.5x10⁴ cells per well for 24 hours. BPs, 3µm polystyrene (PS) particles (Polysciences), and 3µm IgG-B coated PS particles were incubated with monocytes at a 1:1 (Cell:Particle) ratio. 10µl of MTT reagent was added to each well and allowed to incubate in standard cell culture conditions for 2 hours. 100µl of Detergent
Reagent was then added and the plate was incubated in the dark at room temperature for 2 hours. Absorbance was read at 570nm using a Tecan Safire plate reader.

Inflammation Model

To induce lung inflammation, healthy female BALB/c mice (18-20g) were administered 0-10μg of lipopolysaccharide (LPS) in 60ul saline intranasally. 24 hours later, the lungs were harvested and mechanically homogenized and the expression of ICAM-1 was measured using an ELISA kit (Ray Biotech).
7.4 Future Applications

Due to the unique phagocytosis resistant property of the backpack, the applications of this material for cell-mediated drug delivery is exciting. We believe this could be applied to areas ranging from delivering material across the blood-brain barrier to responding to chronic or acute inflammation in the lungs. It is very difficult for nanoparticles or free floating drugs to cross the blood-brain barrier but it has been found that macrophages traffic to inflammation regions in the brain and they may help alleviate diseases like Parkinson’s disease (166). Currently macrophages are loaded with enzymes that are designed to escape endosomes and be excreted by the macrophage near the desired location (166). However, a macrophage with an attached phagocytosis resistant backpack could allow for efficient delivery of proteins to the brain and other previously inaccessible regions of the body.
7.5 Tissue Engineering Applications

This section will discuss how the size of immune cell aggregates depend on the backpack size and ratio of cells to backpacks in solution. This section will also discuss methods to create controllable and stable bilayers of living cells.

7.5.1 Controlled Aggregate Size using Cell Backpacks


We investigated whether by simply controlling the size of the backpack, and the ratio of cells to backpacks, we could control the size of the resultant cell clusters. Controlling cell cluster size is of value for creating aggregates of reproducible and consistent size for tissue engineering. One could potentially imagine injecting cells and backpacks into a tissue void and using the design rules discovered to create aggregates of controllable size. Alternatively for drug delivery applications a single cell and single backpack would be desired and thus since this interaction occurs in solution, the design rules presented here can help design the material. This work was primarily completed by Albert Swiston, but relevant to the future of backpacks for tissue engineering or drug delivery. In this work we have shown through a systematic study that the backpack size and the ratio of cells to backpacks determines how many cells attach to create a cluster(167).
Figure 7-16: Cluster Size Dependence on Patch Diameter A) Using a 7μm backpack B) Using a 15μm backpack. \( R = \frac{\text{# cells}}{\text{# Backpacks}} \) (167)

In Figure 7-16 we show that the ratio of cells to backpacks and also the backpack diameter influences the size of the small aggregates that occur(167). At lower R, meaning more backpacks per cell, the CH27 B-cells will start to form multiple cells per backpack clumps or multi-backpack, multi-cell aggregates. This effect is particularly seen with 15μm backpacks. We believe that this close proximity of multiple immune cells might have uses in tissue engineering areas such as creating lymphoid-like tissue(168).

7.5.2 Immune Cell Bilayers Assembled Using Layer-by-Layer Processing

*This work was done in collaboration with Girma Endale. Girma completed the experiments while I helped image the results and design the experiments.
The specific hyaluronic acid-CD44 cell receptor interaction that attaches the backpack to a cell can also create bilayers of non-adherent immune cells on a surface. These multilayers of B-cells are stable, fabricated from biopolymers and relatively simple to produce. They are exciting since the surface immobilization of immune cells such as T-cells and B-cells in close proximity is extremely important for development of biosensors(169). Furthermore this may allow for the creation of free-floating films of immune cells.

Figure 7-17: Bilayers of B-cells stabilized by HA and CHI on a (HA3/CHI3) substrate A) First layer of the cell structure and the structure is primarily green. B) The second layer of the cellular bilayer and the structure is primarily red. Scale bar is 20 μm.

To build a bilayer of immune cells, the first step is to settle single layer of green labeled B-cells onto a substrate of (HA3/CHI3)_{3,5}. The fabrication conditions of this layer have been previously optimized(10). The second layer of red labeled cells were added after an intermediate CHI, HA or (HA/CHI), polyelectrolyte layer. The resulting bilayer of cells was imaged with confocal microscopy. Figure 7-17 shows the first layer is primarily composed of green labeled B-cells and the second layer is primarily composed of red labeled B-cells. It was found that intermediate CHI and HA/CHI were able to form a bilayer of cells, while intermediate HA was
unable to form a second layer. This result suggests that HA alone was antagonistic to the first layer of cells and competitively bound the B-cell surface receptor, inhibiting the creation of a second layer.

Furthermore, immune cell bilayers that were built with an intermediate (HA/CHI) polyelectrolyte layer showed strong resistance to detachment during incubation at 100 and 200rpm in HBSS for 20 minutes. On the other hand, immune cell bilayers assembled on an intermediate CHI layer were unable to resist agitation at 200rpm (data not shown). Bilayers assembled only with an intermediate CHI layer fell off during the robustness test due to a weaker electrostatic bond and the absence of CD44 based interactions. In contrast, the HA/CHI intermediate based bilayers resisted detachment during the robustness test due to the combination of electrostatic and CD44 based interactions. One role of CHI may be to stop soluble HA from saturating the B-cell surface receptor of the already deposited cells and thus allow for strong adhesion of the second layer of B-cells. The result suggests that robust cellular bilayers need both HA-CD44 interactions as well as electrostatic attractions driven by CHI.

**Experimental Details**

Hyaluornic acid (HA, Fluka, from Streptococcus equi, MW 150kDa), chitosan (CHI, sigma, deacelated 0.85, MW~390KDa) 0.1%CHI (w/V) and 0.1% HA(w/V) solution were dissolved in deionized water. Poly (diallyldimethylammonium chloride (PDAC, molecular weight 200-300 kDa), in 20 wt % in water solution), poly (styrene sulfonate) (SPS, MW 70 kDa) were purchased from Sigma-Aldrich and used without purification. Immune Cells, CH27 B lymphocyte cell lines, were passage and maintained in RPMI cell culture media with L-glutamine,
streptomycin/penicillin (P/S), and Fetal calf serum were purchased from mediatech. Hank’s balanced salt solution 1x was purchased from Gibco. Clean glass slides (VWR) were used as substrates. All polymer and biopolymer solutions were prepared with deionized water.

Glass Substrate Preparation

For single layer HA and CHI deposition, glass slides were sonicated in a 3% detergent aqueous solution for 15 min followed by a 10 min wash in 1 M NaOH and two consecutive 5 min rinses in deionized water and then air-dried before use.

Polyelectrolyte Solutions

Poly (diallyldimethylammonium chloride) (PDAC) and poly (styrene sulfonate) (SPS) solutions (10⁻²M) were prepared by dissolving the polymer in water with 100mM NaCl at concentrations of 1 mg/ml and adjusted at pH 4.0. The biopolymers CHI and HA solution were also prepared with 100 mM NaCl and adjusted at pH 3.0.

Polyelectrolyte Multilayer Film Assembly

Polyelectrolyte multilayers (PDAC4/SPS4)15.5 and (HA3/CHI3)3.5 were deposited on clean glass substrates using an automatic dipping procedure. (PDAC4/SPS4)15.5 bilayers were deposited with a Zeiss HMS Series Programmable Slide Stainer by immersing the substrate alternatively in the polymer solution for 10 min followed by 2min and 1 min pH 4.0 water rinse.

The biopolymers (HA3/CHI3)3.5 bilayers were deposited on the (PDAC4/SPS4)15.5 layers with spinning dipper by immersing the substrate alternately in the polymer solution for 10 min, followed by 2,1,1 min pH 3.0 water rinse. For good immune cell adhesion, the prepared samples were stored overnight at ambient conditions before performing immune cell adhesion.
Cell Culture

The immune cells (CH27 B lymphocytes) were maintained at 37 °C, 5% CO2 and passaged 1:10 once every 3 days in RPMI-1640 culture media containing 10% fetal calf serum (FCS), and 1% penicillin/ streptomycin (mediatech).

Immune Cells Multilayer Assembly

The immune cells were washed two times with HBSS and then, the cells resuspended at $10^6$ cell/ml in warm cell culture media (RPMI-1640). Next, gently pipette 2ml of the suspension directly onto the prepared HA/CHI multilayered surfaces in 35mm Petri dishes. Samples were incubated for 15 min, followed by 15min agitation at 100 rpm for 15 min at 37°C and 5% CO2 for a total time of 1 or 2hrs and then samples removed from the incubator and gently washed in warm fresh HBSS to remove unbound cells. Samples were placed in warm RPMI media and analyzed. Prior to the second and subsequent immune cell layer adhesion, the first layer immune cells were incubated either in an intermediate HA/CHI mixture, or only in HA or CHI solution. The above solutions were prepared in Hank’s balanced salt solution 1x (HBSS) at a concentration of 0.01%CHI and 0.1% HA solution or 0.1%CHI and 0.01%HA. In the former case, the first layer immune cells were placed in pH 7.4 HA solution and incubated for 10 min and gently rinsed in HBSS and then transferred into pH 6.3 CHI solution and incubated for 1 min followed by HBSS rinse. Then 2ml of $1 \times 10^6$ cell/ml immune cells were gently pipetted on the sample, and incubated for 15 min followed by a gentle agitation at 100 rpm for 15 min. The above steps were repeated for subsequent immune cell adhesion. After each polyelectrolyte layer deposition, samples were rinsed in warm HBSS.

Robustness Test
The robustness of the immune cells polyelectrolyte multilayers were tested by intensive rinsing and dipping of the sample in HBSS. In addition, samples were placed in HBSS solution in Petri dish and agitated, for 1, 2, 5, 10 and 20 min, at 100 and 200rpm.
7.6 Supplemental Information

Figure 7-18: Inclusion of synthetic RNA into a film: A) Film containing (Poly(I:C)5/LPEI5) was built and is stable through acetone sonication. Thickness of (Poly(I:C)5/LPEI5)20 was 350nm.

In the figure above a possible payload of synthetic double stranded RNA (200-600 basepairs) was incorporated into a film which retained its thickness and UV absorbance even after acetone sonication. Poly(I:C) is an immunostimulant molecule that interacts with the toll receptor 3 of the immune system(170). This could serve to enhance the potency of a vaccine by acting as an adjuvant.
Figure 7-19: 7μm Quantum Dot full BPs as excited by 543nm HeNe Laser A) Backpack Structure (PMAA2/PVPON2)_{30.5} (PAH3/MNP4)_{10.5} (PAA4/PAH-b4)_{8} B) Backpack Structure (PMAA2/PVPON2)_{30.5} (PAH5/QD5)_{30.5} (PAA4/PAH-b4)_{8} C) Backpack Structure (PMAA2/PVPON2)_{30.5} (PAH3/MNP4)_{10.5} (QD5/PAH5)_{30} (PAA4/PAH-b4)_{8} D) Emission of backpacks shown in A-C. Backpacks were released into PBS and excited at 543nm on a spectrofluorometer.
8. ORIENTATION-SPECIFIC ATTACHMENT OF POLYMERIC MICROTIUBES ON CELL SURFACES


This work was profiled in Nature “Materials Science: To bind or not to bind” 502, 313-314 (17 Oct 2013)

8.1 Introduction

Understanding and controlling the interaction of living cells with synthetic micro or nanoparticles is becoming increasingly important for a number of biomedical applications, driven largely by the desire to limit cytotoxicity and produce more efficient drug delivery systems. To enhance drug delivery efficiency, for example, material characteristics such as particle shape and surface chemistry have been manipulated to tune the half-life, biodistribution and cellular uptake of synthetic particles.(20, 135, 162, 164, 171) From such studies it has been established that the local shape or orientation of a particle when it contacts a cell surface drastically alters the internalization rate.(24, 29, 172, 173) Therefore of particular interest are strategies to control the orientation of a particle on a cell surface.(174, 175) Chemically non-uniform, Janus or ‘patchy’ particles have been utilized to control local orientation in colloid systems by presenting heterogeneously defined regions of attractive or repulsive chemistry.(176, 177) These simple building blocks can also self-assemble into a variety of higher order structures depending on the placement and interaction strength of the heterogeneous regions.(25, 178-180)

In this work, we demonstrate a simple method to create functionally heterogeneous hollow...
microtubes whose interactions with living cells exploit both the effects of particle shape and chemistry. The net result is an ability to control the orientation of a tube shaped microparticle on cell surfaces, thus opening up new design space for the fabrication of novel cell-biomaterial hybrids.

In addition to considering the characteristics and potential of synthetic particles as vehicles for drug delivery, the functionalization of a cell surface with synthetic materials to create cell-biomaterial hybrids is an evolving new method to control cell phenotype, fate and function.(22, 29, 111, 131, 181-183) A logical extension of this approach is to attach synthetic particles directly to the surface of living cells to enhance their native functionalities and provide additional capabilities not found in nature.(22, 29, 111, 184) Toward this goal, prior work in our group has investigated the attachment of payload carrying cellular backpacks onto immune system cells for cell-mediated drug delivery, imaging and magnetic manipulation.(23, 29) These disc-shaped cellular backpacks, which are microns wide and a few hundred nanometers thick, are able to resist internalization by macrophages due to their unique shape.(29) The ability to attach functional, cargo carrying backpacks onto living immune cells without inducing phagocytosis provides new opportunities for extracellular targeted drug delivery. In this work, we explore the use of hollow microtubes with a controllable, non-uniform presentation of functional groups as a new type of biomaterial with the means to control particle orientation on cell surfaces.

Controlling the orientation of a microparticle on a cell surface requires the use of an anisotropic particle with distinct surface regions that promote and resist cellular attachment. Surfaces that are highly hydrated are commonly used to resist cell attachment.(185) while surfaces presenting particular ligands promote cell attachment.(162) Polyelectrolyte multilayers (PEMs) can achieve such surface characteristics simply by choice of polyelectrolytes and the
assembly conditions. In this work, poly(acrylic acid)/poly(allylamine hydrochloride) (PAA/PAH) multilayers assembled at pH 3 were used to create microtube surfaces that resist cell interactions due to their highly hydrated nature. To create regions of the microtubes that strongly interact with the lymphocyte B-cells used in this study, multilayers containing the biopolymers hyaluronic acid (HA) and chitosan (CHI) were used. Previous studies have shown that the B-cell CD44 receptor binds strongly and specifically to HA present in a film. HA containing multilayers also could be used to interact with other cell types including macrophages and T-cells. In all cases rhodamine (Rhod) labeled PAH and fluorescein (FITC) labeled CHI were used to identify the two different multilayer systems (depicted as red and green colors respectively in all figures).

8.2 Fabrication of Cellular Tubes

To fabricate anisotropic, chemically non-uniform microtubes, we used a simple template approach involving the use of a sacrificial track-etched membrane. The PEM assemblies were designated as follows: (poly1/poly2)z, where poly1 and poly2 represent the polymers used to assemble the multilayer and z is the total number of bilayers deposited (1 bilayer = poly1+poly2). The fabrication sequence for microtubes is shown in Figure 8-1. To fabricate chemically uniform tubes, the entire membrane, including the pores, was coated conformally with a PEM (Figure 8-1 step i). For completely cell-resistant tubes, multilayers of (PAA/PAH-Rhod) were assembled. The coated membrane was then plasma treated to remove the polymer selectively from the top and bottom surfaces of the membrane; previous work has demonstrated that the polymers within the pores are protected from the plasma etching process (Figure 8-1 step ii). Carbodiimide chemistry was then used to cross-link the PAH amine groups to the PAA carboxylic acid groups to form stable amide bonds. The
membrane was then selectively dissolved and single component cell-resistant tubes filtered out (Figure 8-1 step iii).

Figure 8-1: Fabrication Scheme of Orientation Controlled Microtubes

To fabricate orientation-specific tubes, a cell-adhesive multilayer containing HA and CHI was deposited onto a membrane previously coated with (PAA/PAH-Rhod) (Figure 8-1 step iv). Another plasma treatment step removed the polymer film from the exposed surfaces, leaving the polymers only in the pores (Figure 8-1 step v). The resultant heterostructured tubes in the membrane pores have cell-adhesive multilayers on the inside of the tube and therefore will be largely shielded from interacting with cells. However, as will be shown, when a cell approaches the end of the tube, the polymer from the interior of the tube is sufficiently exposed to participate in cell adhesion. The tubes were then cross-linked before the polycarbonate membrane was dissolved and the tubes filtered out (Figure 8-1 step vi). The fabrication method yields millions of stable tubes per membrane and aqueous suspensions containing around $10^6$ tubes/mL. Scanning electron micrographs of typical microtubes are seen in Figure 8-2.
8.3 Effect of Chemistry on the Cell Surface Orientation

To investigate how the structure of the microtubes affects the orientation on cell surfaces, three microtube groups were fabricated and imaged with confocal microscopy (Figure 8-3). The first group (Figure 8-3a) consists of red labeled cell-resistant (PAA/PAH-Rhod) microtubes that were fabricated by ending at step (iii) in Figure 1. The second group (Figure 8-3b) consists of heterostructured microtubes with red labeled cell-resistant (PAA/PAH-Rhod) multilayers on the outside and green labeled cell-adhesive (HA/FITC-CHI) multilayers on the interior which were fabricated by continuing to step (vi) in Figure 1. The third group (Figure 8-3c) is produced by reversing the order of film deposition such that green labeled cell-adhesive (HA/FITC-CHI) multilayers are on the outside and red labeled cell-resistant (PAA/PAH-Rhod) multilayers are on the interior of the tube. Figure 8-3b shows the green FITC-CHI presence at both ends, and in most cases localized at the ends of the tubes. The clumping at the ends of the tubes is believed to
be caused by the (HA/FITC-CHI) multilayer clogging or plugging the tube during fabrication. In Figure 8-3c the overlay images do not distinctly show the green FITC-CHI around the tube exterior surface since the layer containing FITC labeled chitosan is thin, but as seen in the inset, upon splitting into independent FITC and rhodamine channels, the full coating of chitosan is displayed. Additional images of the three tube types are shown in Figure 8-6.

Figure 8-3: Confocal microscopy images of heterostructured tubes dried on a slide along with cartoons of the structure. Red represents the cell-resistant rhodamine conjugated (PAA/PAH) multilayers and green represents the cell-adhesive FITC conjugated (HA/CHI) multilayers. a) (PAA/PAH-Rhod)$_{40}$ b) (PAA/PAH-Rhod)$_{40}$ outside and (HA/FITC-CHI)$_{40}$ inside c) (HA/FITC-CHI)$_{4}$ outside and (PAA/PAH-Rhod)$_{40}$ inside. Full image scale bars are 10µm and inset scale bars are 5µm.

To study the cell-microtube interaction, tubes suspended in water were added to a solution of B-cells. B-cells were chosen since they are non-adherent lymphocytes that are
integral to the adaptive immune response and thus are therapeutic targets in immune system
diseases. (191, 192) After 1-2 hours confocal imaging was used to analyze attachment
orientation.

The quantitative results shown in Figure 8-4a were obtained by analyzing over 100 tubes
each in at least 3 different experiments and assigning each interaction to one of three possible
categories; i) unattached, ii) end-on attachment or iii) side-on attachment. The first group of
(PAA/PAH-Rhod) tubes minimally associated with cells due to the highly hydrated, non-
adhesive nature of the (PAA/PAH-Rhod) multilayer and therefore >80% of the tubes were
unattached to any cell. The second group of tubes with (HA/FITC-CHI) multilayers on the
interior attached preferentially end-on, consistent with the cell interacting with HA on either end
of the microtubes. The third group of tubes with (HA/FITC-CHI) multilayers on the outside
preferentially attached side-on, consistent with the presentation of cell-adhesive regions on the
outside of the tube. Confocal microscopy images of cell-tube interactions are shown in Figure
8-4b with larger images in Figure 8-5. Approximately 50% of the tubes in groups 2 and 3 did not
attach after 1-2 hours of agitation and incubation. This time was chosen as the analysis point
since longer intervals of agitation and incubation led to increased attachment and the formation
of large aggregates, limiting the ability to determine the attachment orientation. Conditions were
not optimized to achieve the highest levels of tube attachment; future work focuses on
immobilizing cells on a surface to minimize the aggregation potential. Nevertheless, these data
clearly show a preference for microtube attachment based on the location of the cell binding
(HA/FITC-CHI) multilayers. Figure 8-4c compares the ratio of tubes end-on to side-on and
further supports the notion that the presentation of HA on the ends of the tubes favors end-on cell
surface orientation. Some of the side-on orientation observed with the (HA/FITC-CHI)
multilayers on the inside of microtubes may simply be due to the cell binding the HA from both ends of the tube at the same time. We are confident that HA is not diffusing through the (PAA/PAH-Rhod) outside multilayer, since previous work has documented the effective blocking properties of PAH-containing layers.(92) Regarding the issue of cytotoxicity, Figure 8-4d shows that the microtubes did not decrease cellular viability.

Figure 8-4: Controlled Orientation of Microtubes on the Surface of B-cells: a) Samples were analyzed using confocal microscopy and microtubes with cell-adhesive (HA/CHI) on the inside caused more end-on attachment. b) Confocal images of cell tube interactions i) (PAA/PAH-Rhod)$_{40}$ ii) (PAA/PAH-Rhod)$_{40}$ outside and (HA/FITC-CHI)$_{40}$ inside iii) (HA/FITC-CHI)$_{4}$ outside and (PAA/PAH-Rhod)$_{40}$ inside. Scale bar 10 μm. c) Ratio of ‘End-on’ to ‘Side-on’ showing effect of tube structure d) Cellular viability of the B-cells after 24 hr incubation with tubes. Viability was not altered by the addition of (HA/CHI) outside tubes added at a 1:1 cell to tube ratio.
8.4 Conclusion and Future Outlook

In conclusion, we have developed a novel and reproducible fabrication method that allows for the presentation of a selected polymer preferentially on the ends of microtubes. Our method provides a general platform that could be expanded to many applications in biomaterials and responsive materials. In particular, we have shown in the present work that by limiting the cell-adhesive polymer to the end of the microtube, we can control the orientation of an anisotropic microtube on cell surfaces. These results point to the possibility of using microtubes as part of a bottom-up cell scaffold in the future. Controlling the interaction points on the microparticle may allow for structures such as linear or branched ‘polymerized’ chains of cells. In addition, regions of the microtube can be designed to respond to stimuli such as pH, salt or temperature, opening the possibility of stimuli sensitive polymer tubes for drug delivery or controlled assembly.(155, 186, 188, 193-197) In general, we believe that controlling the
orientation of microparticles on the cell surface could open new design space for applications in tissue engineering and drug delivery.

8.5 Experimental Details

**Materials:** PAA (Aldrich, M=450kDa), PAH (Aldrich, M=56kDa), HA (from Streptococcus equip, Fluka, M~1580 kDa), 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, Sigma), N-hydroxysuccinimide (NHS, Sigma), NHS-Rhodamine (Pierce), Nuclepore track-etch polycarbonate membrane 3.0μm (Whatman), Fluorescein isothiocyanate (FITC, Sigma), Millex-LCR 0.45μm hydrophilic PTFE 25mm membranes (Millipore) and low molecular weight CHI (deacetylation 0.9, M=50-190kDa, Sigma) were used as received. FITC-chitosan was made from in a 1% aqueous acetic acid–methanol (1:2 v/v) solution of pH 4.5 at room temperature as described.(198) Rhodamine-PAH was synthesized by reaction between PAH and NHS-rhodamine in 50mM MES buffer at pH 8 for 2hrs, after the solution was dialyzed into water. Detailed tube fabrication steps are given in supporting information.

**Fabrication of Microtubes:** Polymers solutions were made from Milli-Q 18.2MΩ water. Solutions of PAA and PAH-Rhod were 0.01M and solutions of HA and FITC-CHI were 0.1% and 0.05% (w/v) respectively, FITC-CHI had 0.1M of acetic acid added to aid dissolution. HA and CHI solutions had 100mM NaCl added to the solution. All solution pHs were adjusted to pH 3.0 with 1M HCl. Track-etch polycarbonate membranes were immobilized on teflon holders and sequentially dipped in the polymer solutions using a nanoStrata dipping unit. Polymer depositions were done for 10 minutes, followed by three pH3 Milli-Q water rinses: one for 2 minutes and two for 1 minute, while the substrate was rotating at 100rpm. Both sides of the membrane were exposed to plasma etching simultaneously. The chamber was flushed with O₂
twice before igniting the plasma at 150 mTorr. (PAA/PAH-Rhod)$_{40}$ and (HA/FITC-CHI)$_{40}$ layers required 20 min and 60 min of etching respectively. Following plasma etching, the membrane was gently rubbed with a moist q-tip to fully remove the damaged film. The tubes were cross-linked by EDC/NHS at a concentration of 60mM EDC and 24mM NHS in 50mM MES buffer at pH 5 for 15 minutes. The membrane was dissolved in dichloromethane and the tubes filtered with a Millex-LCR filter before rinsing with 10mL of dichloromethane. The filter surface was wet with water and gently rubbed with a cell scraper for 5 minutes to transfer the microtubes. The concentration of tubes were measured using a standard hemocytometer on a fluorescent microscope. If aggregation occurs, light sonication can disperse the tubes.

**Cell Study:** CH27 B-cells were grown in RPMI media with 10% fetal calf serum and 1% penicillin/streptomycin. Before incubation with microtubes the cells were washed with Hanks Buffered Salt Solution and resuspended at $10^6$ cells/mL in complete media. The tubes and cells were iteratively incubated on a shaker plate for 15 minutes at 100rpm and then incubated for 15 minutes for 1-2 hours before analysis. Confocal images were taken on a Zeiss LSM 510 using a 488nm Argon laser for FITC excitation and a 543nm HeNe laser for rhodamine excitation. The images were visually analyzed to determine the orientation of the microtube. A minimum of 100 interactions were analyzed per sample. Cell viability was determined by trypan blue staining.
8.6 Supplemental Figures

Figure 8-6: Confocal microscopy images of heterostructured tubes dried on a slide along with cartoons of the structure. Red represents the cell-resistant rhodamine conjugated (PAA/PAH) multilayers and green represents the cell-adhesive FITC conjugated (HA/CHI) multilayers. a) (PAA/PAH-Rhod)₄₀ outside and (HA/FITC-CHI)₄₀ inside c) (HA/FITC-CHI)₄ outside and (PAA/PAH-Rhod)₄₀ inside. Image scale bars are 10µm.
9. FUTURE WORK

Polymeric nanostructured thin films have a wide variety of applications and through new experimental techniques, we can begin to understand the underlying design principles involved. Depth Profiling XPS using C$_{60}^+$ sputtering is only beginning to be developed for analyzing nanostructured films. I hope future members of the Rubner and Cohen group and many others will continue to exploit its strengths to determine the chemical and atomic structure of polymeric thin films. This technique could help define the necessary characteristics to achieve the desired nanostructure. In particular, the technique is suited to help determine the true mechanism behind exponential film growth by analyzing the diffusion and exchange of material throughout the film during growth. Another area in which I believe depth profiling XPS will be utilized is in lamellar block copolymer films. We have chosen to look at lithium-ion distribution block copolymer films, but given the exceptionally wide ranging applications of block copolymers, I believe that once this technique is clearly displayed, many other areas of investigation will begin.

The biomedical applications of structured thin films are equally broad, and I believe that the surface of the cell presents new ground for scientists to manipulate and combine synthetic materials with natural cell functions. This combination can be applied to a variety of applications including diagnostic and therapeutic uses. Currently, our synthetic backpack material is uninvolved, with no major effect on the attached cell; the cell simply serves as a cargo carrying device. In particular, we saw that one can use the natural homing ability of the cell to carry the therapeutic cargo to a disease site, thus reducing general toxicity and allowing for more efficient therapeutic delivery. The cargo could also be diagnostic since the cell could be labeled and tracked as it migrates through the body. In general, if synthetic materials can be attached to a cell without deleteriously altering it, the material may serve as a platform for a variety of molecules,
particles, ligands and/or responsive devices. Alternatively, the material could be designed to promote artificial homing capabilities, signaling capabilities and other new functionalities that are not biologically natural. These new capabilities could greatly expand the flexibility of the cellular ‘platform’ and open up a new level of cellular engineering. With so many possibilities, I am excited for the many new applications of nanostructured polymeric materials in the future.
10. CITATIONS


11. APPENDIX

11.1 Chemistry of ‘Released’ Side of Free Floating Films

To study the chemistry of the released side of a free floating film, a region of hydrogen bonded polymers, topped with a region of electrostatically bonded polyelectrolytes was created. The hydrogen bonded region was made of poly(acrylic acid) and poly(ethylene glycol) or (PAA3.0/PEG3.0) which has been found to be stable up to pH 3.6(16) and therefore pH 7.4 PBS solution was used to dissolve the region. The electrostatically bonded region was built with the weak cation poly(allylamine hydrochloride) and magnetic nanoparticles coated with an anionic surfactant (PAH3.0/MNP4.0).

Figure 11-1: PEM System studied for analysis of released free floating film chemistry

XPS measurements were performed on films of the following compositions: 1) (PAA3.0/PEG3.0)_{20.5}, 2) (PAA3.0/PAH3.0)_{10} and 3) flipped (PAA3.0/PEG3.0)_{20.5} (PAH3.0/MNP4.0)_{10}. Flipping the third sample is detailed in part A of the figure below. In part B of the figure below, the flipped carbon region is compared to the other two films, a (PAA3.0/PEG3.0)_{20.5} hydrogen-bonded film, and a (PAA3.0/PAH3.0)_{10} electrostatic film.
Figure 11-2: A) Method for flipping of the film prior to XPS, pH 7.4 PBS dissolves the hydrogen bonding region, allowing the film to be flipped. B) C1s XPS spectra of an as-deposited (PAA3.0/PEG3.0)_{20.5}, (PAA3.0/PAH3.0)_{10} and flipped (PAA3.0/PEG3.0)_{20.5} (PAH3.0/MNP4.0)_{10} film. PAA’s carboxyl C1s peak in the flipped film shifts to lower binding energy as would be expected when paired with an amine. PEG’s C1s peak also significantly shrinks in the flipped sample, suggesting the ejection of PEG upon PAH disrupting the PAA-PEG hydrogen bonds.

The PAA/PAH film’s a broad peak in the binding energy of 288eV to 290eV is due to the carboxyl groups. This peak can be deconvoluted into two regions, one peak centered around 289eV that represents a protonated carboxyl group and one peak around 288eV that represents an
ionized carboxyl group that has been electrostatically paired with a primary amine. The lower binding energy displayed when a carboxyl is paired with an amine has been previously seen (199, 200). It has been hypothesized that it is due to the delocalization of the negative charge of the oxygen groups due to the presence of the nitrogen group (199, 200). Thus the carbon’s electrons are not pulled to the oxygen as strongly and have a lower binding energy. Previous studies have shown that (PAA3.0/PAH3.0) films have around 50% of the carboxylic acid groups ionized and the deconvolution of the figure above qualitatively supports this conclusion(9).

We see that the carboxyl groups of the hydrogen bonded film (PAA3.0/PEG3.0) are all in the protonated form since there are no electrostatic interactions occurring. However, the flipped sample has a large amount of ionized groups showing that the carboxyl groups that were once hydrogen bonding are now electrostatically interacting with amine groups of PAH. This percentage is consistent with a prior study since the film was last placed in a pH 7.4 PBS(9).

A second qualitative change is that the PEG O-C-C peak at 286.5 eV(69) significantly shrinks in the flipped film. This is consistent with PAH disrupting PAA-PEG hydrogen bonds, allowing the PEG to exit the film. To detect if PEG remained in the film, a thiolated PEG was used and as seen by the presence of sulfur in Figure 11-3 we see some PEG is still kinetically entangled in the multilayer. Therefore part of the sacrificial region is tethered to the outer face of the released film. These XPS results show that the outer surface of the released film is composed of PAA-PAH electrostatic pairs with a small amount of PEG trapped.
Study Details

To directly measure if the hydrogen bonding region is attached to the released film, hydrogen-bonded films were prepared with a 20kDa MW thiol-end group PEG (PEG-SH/PAA) layers and topped with (PAH3.0/MNP4.0)$_{10}$. The film was scored and released in pH 7.4 PBS, flipped over and placed on a glass slide so that the film’s face originally closest to the glass surface was exposed. The (PAA/PEG-SH) system was chosen over other amine-containing hydrogen-bonded systems so that any N peak must be due to PAH. If the polycation PAH was present in the hydrogen bonding region, a nitrogen peak would be found. Also if part of the hydrogen bonding region remains attached to the film after release, XPS results will show a small sulfur peak. Further, if no iron peak was seen, then the superparamagnetic Fe$_2$O$_3$ nanoparticles are not within ~10nm (the penetration depth of XPS) of the outer face. The figure below shows that both S and N peaks were found, but no magnetic nanoparticle signal, proving that some PEG-SH from the sacrificial region and PAH are present on the released face of the film and that the magnetic nanoparticles are >10nm beneath the surface. It is expected that the PAH has electrostatically paired with the PAA and thus formed a stable region that has entrapped some of the PEG-SH.
Figure 11-3: XPS spectra and atomic percentages for a flipped (PAA3.0/PEG-SH3.0)$_{20.5}$ (PAH3.0/MNP4.0)$_{10}$ film. The peaks for C, N, O, and S are as indicated – all other peaks are due to residual salt from PBS. Inset shows the S peak at 168eV.