UNIQUE SURFACE ADSORPTION BEHAVIORS OF SERUM PROTEINS ON CHEMICALLY UNIFORM AND ALTERNATING SURFACES

A dissertation submitted to the Faculty of the Graduate School of Arts and Sciences of Georgetown University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry

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Washington, DC

April 22, 2015
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Abstract

With increasing interests of studying proteins adsorption on the surfaces with nanoscale features in biomedical field, it is crucial to have fundamental understandings on how the proteins are adsorbed on such a surface and what factors contribute to the driving forces of adsorption. Besides, exploring more available nanoscale templates would greatly offer more possibilities one could design surface bio-detection methods with favorable protein-surface interactions. Thus, to fulfill the purpose, the work in this dissertation has been made into three major sections. First, to probe the intermediate states which possibly exist between stable and unstable phases described in mean-field theory diagram, a solvent vapor annealing method is chosen to slowly induce the copolymer polystyrene-block-polyvinylpyridine (PS-b-PVP)’s both blocks undergoing micro-phase separations from initial spherical nanodomains into terminal cylindrical nanodomains. During this process, real time atomic force microscopy (AFM) has been conducted to capture other six intermediate states with different morphologies on the polymeric film surfaces. Secondly, upon recognizing each intermediate state, the solution of immunoglobulin gamma (IgG) proteins has been deposited on the surface and been rinsed off with buffer solution before the protein-bounded surface is imaged by AFM. It
has been found IgG showing a strong adsorption preference on PS over P4VP block. Among all the six intermediate states, the proteins are almost exclusively adsorbed on PS nanodomains regardless the concentration and deposition time. Thirdly, a trinodular shape protein fibrinogen (Fg) is selected for investigating how geometry and surface charge of proteins would interplay with cylindrical nanodomains on a surface developed from Polystyrene –block-Poly-(methyl methacrylate) PS-b-PMMA. Also, Fg adsorptions on chemically homogeneous surfaces are included here to have a better contrast of showing how much difference it can make by using it on a nanoscale surface. Interestingly, higher concentration of protein solution promotes the occurrences of single phase packed Fg on the PS domain. The densely packed network has formed where each Fg keeps its main body in PS domain and leaves its two alpha C chains on nearby PMMA domain. We believe this conformation and orientation would maximize both the hydrophobic and electrostatic interactions between Fg and the underlying surface.
Acknowledgements

First and foremost, I sincerely appreciate my research advisor Prof. Jong-in Hahm. It is her bringing me into an amazing and interesting field - nanosciences; it is her who is always available and helpful for guiding me to a correct direction when I am doing the research; it is still her objectively and critically pointing out how much room I can improve myself. No more words could be written down to address all my gratitude for both essential advice she gave to me in both academic and personal perspectives during the days we have been working together. It is my honor to be her first graduate student in Georgetown.

Meanwhile, I would extend my thanks to the thesis defense committee members, who are willing to give me substantial suggestions on my thesis: Prof. Jennifer Swift, Prof. Daniel Blair and Prof. Rodrigo Maillad.

Besides, I indeed appreciated past and current group members who always supported me along my graduate life and work, especially to Sonia Manpreet, Daniel Choi and Tian Xie. The every advice you gave me in group meetings and paper edits is hearted valued and will be remembered by me.

Furthermore, I shall surely thank all the practical and constructive supports from Prof. Steven Metallo and Prof. Christian Wolf, Director of Graduate Studies. Also, I want to express my thanks for our department’s administration staff, Ms. Mattie Inez Traylor and Ms. Kathleen Bayne and Dr. Mo Itani who constantly assisted me not only during my thesis work but also throughout of all my PhD degree study.

Lastly, for my dearest wife Ping, without your support, patience and understanding, my PhD and this work’s completion would not be possible in time. You
are always there for me, taking care of our home and our little one. Your sacrifice to me and this family honors me. I also thank my parents deeply. As your only son of the family, I want to tell you how much happy and lucky I am to have the wonderful parents as you two. You are the ones supporting and encouraging me to move on no matter what happened as my study and life goes in USA.

Bless all.
Dedication

This work is dedicated to my family: my wife Ping, my son Albert, my dad Shu-qi and my mom Bei-bei, for their endless love, support and encouragement. I hope this work makes them proud.
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Chapter One: Introductions

1.1 Protein-Surface Interaction

There is a long-lasting and also continuously growing effort to understand protein adsorption on solid surfaces. Merely over a decade ago, Dr. Nakanishi described proteins absorbing on a solid surface as “a common but very complicated phenomenon”. Indeed, it is these two elements, common and complex, which stimulate various scientific communities to pursue and reveal the truths behind the phenomena. Spanning a number of disciplines, such as medicine, pharmaceutical sciences, biotechnology, tissue engineering and biophysics, studying protein adsorption behaviors has been playing a crucial role in understanding and addressing other important subjects. Among those topics, many common questions were being raised: Why and how are proteins adsorbed? What parameters and mechanisms control the adsorption process? Are the proteins still bioactive when they get adsorbed to a sorbent? Is this adsorption reversible or irreversible? Under what condition do they undergo conformational change? Are they adsorbing as individual molecules independently or as an ensemble?

A great deal of work has been published in format of reports, reviews, and books in an attempt to address those questions, in the last two decades. However, the principle behind the adsorption behavior still not fully understood. The difficulty in finding a unified rule to explain this topic is not only from possible misinterpretations of experimental observations, comparing different detection methods, but also from the natural complexity of proteins and interfacial interactions. Rather than rigid particle, the protein is made from thousands of amino acids with various substituent groups which
make the protein overall amphiphilic and “soft”. Because of this, changing micro-environments around the protein means potentially adding more variables into this underdeveloped equation, like the pH, temperature, ionic strength of buffer solution, the properties of the protein and the surface. Given a condition that protein adsorption is thermodynamically favored and spontaneous, a slight condition change may affect its pathway to be adsorbed on the surface kinetically, which is a huge factor one should consider when designing an experiment. In the end, the resulting observation may seem similar, but the important kinetic information of how the adsorption is progressing from point of view in individual protein molecule may be missed at that given condition and method.

**Figure 1.** Demonstration of protein adsorption process. A protein molecule in solution ($P$) adsorbs to the material surface $P$, forming $P \cdot S$ in a reversible manner. The adsorbed protein then may transition to an irreversibly adsorbed state $\overline{P} \cdot \overline{S}$ as a result of orientational or conformational changes that further reduce the free energy of the system. The release of surface water because of the protein adsorption and conformational changes (expanding footprint) to replace water molecules gives entropic gains that greatly facilitate overall adsorption processes.

Protein adsorption is driven by different protein-surface forces, including van der Waals, hydrophobic and electrostatic forces. They are all key aspects that govern the
adsorption kinetic pathways and thermodynamic outcomes. Initially, as shown in Figure 1, the protein diffuses towards the surface through Brownian motion, and reaches its free energy minimum by releasing solvent molecules or counter ions from the surface. Later on, these adsorbed proteins usually go through conformational re-arrangement and re-orientation on the surface to maximize their footprints and hydrophobic interactions, when more protein-surface interactions are established because of additional free proteins diffusing from solution. Subsequently, with increasing density of the adsorbed proteins on the surface, the energetically unfavorable repulsion force created by nearby protein-protein electrostatic interactions is no longer negligible since the distance of the surrounding nearby proteins is getting closer than that at the initial low concentration. In the end, the entropy gains from unfolding/extending of protein molecules and release of solvent molecules would lead to macroscopically and experimentally observable results, such as proteins size and shape changes, as well as amount of proteins adsorbed.\(^{11}\) By proper material selection and surface fabrication, one could alter and manipulate protein adsorption behavior in one’s favor by controlling the properties of the surface.\(^9\)

1.2 Biomaterials

The interaction of proteins with solid surfaces is not only a fundamental phenomenon but is also a key to several important and novel applications. One of the biggest interests in biotechnology for studying protein-surface interactions is to develop better biomaterials and improve biocompatibility of medical devices.\(^{13,14,15}\) The reason is that proteins are the first and direct responsible units that are interacting with biomaterials. For example, when a biomedical device (a catheter, contact lens, stent, hip}
joint replacement, or tissue engineering substrate) makes a contact with a body fluid such as blood and interstitial fluid, proteins in those systems quickly adsorb on the surface of the materials. Thus, when a cell approaches to the biomaterial, it is the pre-adsorbed protein layer on the biomaterial that contacts the cell, instead of the material’s surface. Then, since there are membrane proteins residing on cell surfaces, which are actually interacting with proteins on biomaterials, specific receptor-protein binding may be stimulated which activates the cell’s “hot” spots and transduces the signal through the cell membrane. Figure 2 provides an illustration of how a cell responds to the surface-bound proteins when it interacts with the surface.

Figure 2. Cell adhesion proceeds when its membrane proteins receptors are interacting with ligands on the biomaterial surface. The properties and arrangement of surface bound ligands dictate how the membrane proteins adsorbed and thereafter decide how the cell responds to the surface. Remake from ref.16

So, to design a better engineered tissue or implant material, one should first consider how to control the types, amounts and orientations of proteins that would
potentially pre-adsorb on the materials before the surface meets the cell. In that, these pre-adsorbed proteins would selectively bind to the active sites on the cell membrane and induce potentially favorable cell responses. Therefore the question of how studying protein-surface interaction can serve for improved biomaterial-cell interactions comes down to how to efficiently regulate the proteins’ packing density, arrangement, orientation, and conformational stability, by changing the properties of contacting surfaces. Previous studies have extensively shown that proteins on di-block copolymer film’s nanostructures would have very unique self-assembled pattern and adsorption preferences on one block over another. These studies broadened the possibilities one can design to gain different protein assembling patterns and orientations on the nanostructured surface by changing the chemical components of di-block copolymer film. More details of how to develop the nanostructured film will be extensively discussed in later chapters.

1.3 Solid State Protein Assays

Differing from conventional solution based immunoassay, solid state protein microarrays offered distinct advantages in various fields, such as high throughput investigation of proteomic expression, protein-protein/ligand interactions, drug screening and lower detection/diagnosis limits. In Figure 3, there is an example showing how the target proteins have been immobilized through specific antibody-antigen binding and detected on a microarray chip. There is an increasing desire that is to make microarrays smaller to detect more signal, with smaller amount of samples. This ultrasensitive measurement can be achieved through
miniaturization of the surface by nano-patterning. The current detection limits of micro- and nano-arrays for protein analytes have reached atto Molar \((10^{-18}\text{M})\) \(^{26,27}\) and zepto Molar \((10^{-21}\text{M})\) \(^{28}\) range, respectively. The key challenges to achieve lower detection limits, smaller size and accurate quantification ability, would be: 1) increasing array spot density per area, 2) ease of developing patterned surface, 3) long-range orderness over the surface, 4) retaining protein biological activity after adsorption.

**Figure 3.** A scheme shows how the protein micro-array works. The target protein was immobilized on a solid state protein assay. The samples are recognized by specific primary antibodies, and visualized with a near infrared dye-conjugated secondary antibody, detected by a NIR fluorescence scanner.\(^{25}\) Remake from Ref 25.

Great effort has been put into printing proteins on surface with controlled periodicity, density, and uniformity, as summarized in Table 1. Traditional microfabrication technologies such as soft-lithographic techniques,\(^{29}\) micro contact printing (MCP),\(^{30,31}\) nanoimprint lithography (NIL),\(^{32}\) nanosphere lithography (NSL),\(^{33}\) electron beam lithography (EBL),\(^{34}\) and focused ion-beam lithography (FIBL)\(^{35}\) are able to provide micro and nanostructured interfaces where proteins can be subsequently

6
immobilized on, by either physisorption, or chemical surface functionalization. Among these high resolution nanofabrication techniques, like EBL and FIBL, the common concept remains very much similar: using high energy irradiation input to do the serial “writing” of patterns on the surface. Therefore, three technical limitations are still pending to be solved: possibility of damaging sample, compatibility with proteins printing reagents, and functionalization of the individual features with different proteins

<table>
<thead>
<tr>
<th>Transfer Type</th>
<th>Micrometer scale</th>
<th>Nanometer scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parallel</td>
<td>Manual and robotic delivery$^{36}$</td>
<td>Nanoimprint Lithography$^{42}$</td>
</tr>
<tr>
<td>Processing</td>
<td>Micro-contact printing$^{37,31}$</td>
<td>Particle lithography$^{40}$</td>
</tr>
<tr>
<td></td>
<td>Imprint lithography$^{38}$</td>
<td>Self-assembly$^{41}$</td>
</tr>
<tr>
<td></td>
<td>Capillary force lithography$^{19}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Microfluidic channel network$^{39}$</td>
<td></td>
</tr>
<tr>
<td>Serial</td>
<td>Inkjet deposition$^{42}$</td>
<td>Dip-pen lithography$^{43}$</td>
</tr>
<tr>
<td>Processing</td>
<td>Focused ion beam patterning$^{35}$</td>
<td>Scanning probe lithography$^{44}$</td>
</tr>
</tbody>
</table>

**Table 1.** Parallel and serial transfer methods used to create protein patterns on surfaces. Remake from Ref$^{10}$ with permission.

without compromising the entire surface. For nanoscale patterning, nanodispensing provides a means for local delivery of the receptor molecule to its spot on the array with nanosized pipette. Scanning probe lithography,$^{44}$ dip-pen nanolithography,$^{43}$ native protein nanolithography,$^{45}$ as well as nanografting,$^{46}$ have been successfully used for fabricating and patterning arrays of proteins with nanoscale resolution in native-like conditions. However, the inevitably high costs of these required equipment and the exquisite expertise to operate them are commonly recognized as limitations, not to mention tedious sample preparation courses and time-consuming operation. Most recently, a bottom-up method based on self-assembly of di-block copolymer has been utilized as a method to develop protein arrays on nanopatterned structures.$^{23,47,48,41,49}$
Different from those aforementioned top-down lithography and printing methods, forming periodically repeating nanodomains on surface does not require any external energy field, prefabricated mask, sophisticated lithographic machine and highly demanding clean room environment. More details of di-block copolymer and self-assembly will be provided in the following chapter.

Polymers are macromolecules chemically synthesized and covalently bonded from a number of repeating subunits, called monomers, under certain thermodynamically favored conditions. Block copolymers consist of two or more types of chemically dissimilar and physically immiscible polymer chains (or blocks), which are joined together by covalent bonds. As one member of a big family in soft materials, these di-block copolymers are not only used heavily in our daily life for their inherent mechanical properties, like viscoelasticity and plasticity, but they have also been found very functional in fabricating patterned structures on the nanometer scale due to their intrinsic tendencies to self-assemble. Therefore, there has been a growing interest to study these exceptional properties using experimental and theoretical approaches. For example, in the semiconductor industry, where input radiation wavelength are getting close to the diffraction limit, the common way of making nano-spaced patterns on a chip is challenged since the cost of radiation lithography and risk of damaging the parental mask are inevitably high. With recent advancements in synthesizing nanoparticles in nano-pattern, self-assembled di-block polymer has been utilized as a template to grow semiconducting nanorods under external fields over three dimensions with long range orderness.\textsuperscript{50,51} Hence, those mentioned studies certainly provide insights and clues for
one to possibly control self-assembly towards perfection which offers more accurate and quantitative measurements for protein nanoarrays.

The di-block copolymer A-B blocks polystyrene-poly-4-vinyl-pyridine (PS-P4VP) and Polystyrene Poly-(methyl methacrylate) PS-b-PMMA, two of the simplest and most studied systems, are this work’s template materials, shown in Figure 4. Different from the mixture of homopolymer blends going through a macro-phase separation due to a preference of reducing the interfacial areas like oil droplets aggregating into a layer over the water phase, the A-B di-block copolymers undergo an entropic driven micro-phase segregation (also referred to as separation) with a purpose: to maximize the favorable contacts of similar blocks and to minimize the unfavorable contacts of dissimilar blocks. This results in self-assembly where the free energy dissipates because of the fluctuations of copolymer chains’ positions and configurations, due to Brownian motion.

Figure 4. A chemical structure of (Left) PS-b-P4VP; (Right) PS-b-PMMA.

This thermal incompatibility of A and B seeks an energetic equilibrium in the nanostructure with the lowest configuration entropy by stretching the block chain and minimizing interfacial free energy of unfavorable contacts between the dissimilar A and
B. However this competition between interfacial and stretching energy does not lead to macroscopic separation due to the strong covalent bond. Mean field theory was developed by Flory and Huggins nearly 60 years ago to estimate the free energy change per polymer chain associating with random walk. The mean field theory for phase behavior is determined by the following equation:

\[
\frac{\Delta G_{\text{mix}}}{k_B T} = \frac{1}{N_A} \ln (f_A) + \frac{1}{N_B} \ln (f_B) + f_A f_B \chi
\]

where \(\Delta G_{\text{mix}}\) is the decreased free energy of mixing; \(k_B\) is the Boltzmann constant at temperature \(T\); \(N\) is degree of polymerization (number density of monomers); \(f_A\) is the overall volume fraction of A component. \(N\) and \(f_A\) are tunable in synthetic stoichiometry and determine the translational and configurational entropy. The value of \(\chi = aT^{-1} + b\) is the temperature-dependent Flory-Huggins interaction parameter where \(a\) and \(b\) are

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**Figure 5.** Theoretical (a) and experimental (b) phase diagrams of general A-B diblock copolymer poly-isoprene-styrene. \(f_A\) is the volume fraction of block A in the diblock copolymer. (S) - spherical, (C) - cylindrical, (G) - gyroidal and (L) lamellar. Reproduced without permission from reference 52, 53
experimental constants when the given N and $f_A$ (copolymer) are chosen. Based on self-consistent mean field theory, the phase diagram has been accurately predicted in the previous theoretical reports and a comparable experimental phase diagram of polyisoprene-styrene (PIS) strikingly matches the predicted diagram (Figure 5).\textsuperscript{54, 55, 56} For $\chi N < 10$, the system is disordered and featureless but when $\chi N > 10$, above the order-disorder transition (ODT), there are four ordered microphases and their inverted forms observed with an ascending $f_A$ which are spheres, cylinders, gyroids and lamellae. That means with a chosen copolymer with fixed $f_A$, it is very predictable to control its surface morphology by tuning the $\chi$ (temperature) and N (concentration of BCPs).

Among these possible structures, the thin films with spherical, cylindrical and lamellar nanostructures are highly applicable in fabricating templates to direct the synthesis and growth of nanoparticles on the thin films, instead of employing conventional lithographic methods. Therefore, the self-assembly method has been used to develop the nanostructures on polymeric thin film as protein deposition templates. Since there are tunable parameters $\chi$ (determined by temperature) and N (determined by concentration of BCPs), and wide choices of different polymeric blocks, self-assembly method can offer great versatility and flexibility for one to control nanopattern’s spacings, morphologies, and periodicity over a two dimensional surface.

1.4 Atomic Force Microscopy (AFM) Characterization

Since the AFM was developed from scanning tunnelling microscopy (STM) by Binnig, Quate and Gerter in 1986, it has been used in detecting atomic scale features of various surfaces.\textsuperscript{57, 58} As an advancement to conventional protein detection methods, such
as QCM (quartz crystal microbalance), SPR (surface plasmon resonance), MALDI-MS (Matrix-assisted laser desorption/ionization), and TIRF (total internal reflection fluorescence). AFM allows non-invasive single molecule recognition with minimal sample preparation and label free detection. In other spectroscopic methods, the signals given from the samples and surrounding environments, all are collected and averaged together, being converted into a digital signal for computer program to visualize. What is missed during this ensemble averaging is the details about how each individual protein molecule behaves independently and how they contributed to the collective phenomena as a whole during the protein-surface interactions. Without those information, it is hard to track the trajectory how they are changing from one phase to another. Generally, depending on the study purpose and tip-sample interactions, AFM has been divided into two most popular imaging types, contact mode and tapping mode. In contact mode, a cantilever tip is constantly keeping in contact with surface and scanning over the surface. This is usually used for hard surface, inorganic materials for example. Another one is called tapping mode where the tip is constantly oscillating at its resonant frequency on the surface, which is more preferred for probing soft materials and chemically complexing surfaces.

In tapping mode shown in the upper panel of Figure 6, a tuned cantilever tip is oscillating at its resonant frequency with a constant oscillation amplitude which is used as a feedback to maintain a setpoint root mean square (RMS) of the reflection signal reflected by the split photodiode detector. So, the piezo scanner will extend or contract its length in the z direction to accommodate the tip-sample interactions without breaking the tip, which maintains a constant RMS setpoint at each (x,y) point to sustain that set
feedback. At the same time, the piezo scanner is also scanning laterally over the surface. The distances of a scanner moving along the z direction due to the roughness of the sample surface or the height differences of a sample are recorded at each (x,y) points and then transmitted into topographic signals, being visualized by the computer.

Along with topographic variations due to height changes over the surface, the differences in adhesion and viscoelasticity from the presence of heterogenous substances are included in the phase image as an observable contrast\(^6\). In the lower panel of Figure 6, when the tip scanning from material A to material B, usually there will be a phase lagging shift happening. Because in a condition that the oscillation amplitude and resonant frequency are retained as constants as the feedback signals, the only thing can

**Figure 6.** AFM working diagram and phase shift imaging mode. Upper panel: a scheme shows how tapping mode AFM is generating signals from variations of surface feature and converting them to images. Lower panel: Phase imaging uses the Extender Electronics Module to measure the phase lag of the cantilever oscillation (solid wave) relative to the piezo drive (dashed wave). Reproduced from Digital Instruments Manual.
change is the wave’s phase. The phase imaging in tapping mode provides supplemental information such as mechanical properties and chemical compositions, especially when the boundaries of two mixed materials are ambiguous and indeterminable from the topographic images. Due to the small nature of protein sizes and potential impurities on the surfaces, addition of this phase difference information is a great plus for studying protein-surface interactions, particularly important for the case when surface roughness or particle size is creating non-negligible signal-to-noise ratio from topological channel.

The phase imaging with AFM is extremely necessary for studying the protein adsorption on copolymer films. During the polymeric annealing phases, the microdomains of two different blocks are gradually evolving from one phase to another, but the differences in topography is not phenomenal enough for one to distinguish their chemical identity from the surface structure. However, the differences of mechanical and viscoelastic properties in two different polymer blocks are prominent. It would lead to different phase shifting values when a tip is in contact with them. More importantly, with confirmation of chemical identity of different polymer blocks from using phase imaging, once could track how proteins adsorbed differently at different annealing time on different nanostructures. Therefore, in this thesis, the tapping mode of AFM was used with topological and phase imaging channels enabled to investigate model serum proteins’ adsorption behaviors on various types of surfaces.

AFM can visualize each molecule from a group at each target time frame. In this way, each captured molecule will be treated and analyzed individually. So, with enough sizable imaging data, it is completely a validated method to statistically evaluate proteins’ behaviors as a group, but still with single molecule resolution. The information will
include the surface protein density, the protein (subdomain) size variations and how each conformation distributed at different surfaces. This particularly fit for my study purpose to understand how proteins subdomain would interact with underlying commensurate nanostructures and how different chemical blocks would influence the packing density and configurations of adsorbed proteins.
Chapter Two: Elucidation of Novel Nanostructures by Time-Lapse Monitoring of Polystyrene-block-Poly-4-vinylpyridine under Vapor Treatment

Abstract

Nanoscale micellar structures of polystyrene-\textit{block}-poly-4-vinylpyridine (PS-\textit{b}-P4VP) diblock copolymers have proven their effectiveness in lithography and biological detection by serving as a choice material to produce nanoscale guides and delivery systems in a straightforward and rapid manner through self-assembly. Such applications can greatly benefit from having high versatility for the selection of template sizes (pattern repeat spacing) and shapes (pattern geometry), especially when reaching a size regime where conventional top-down fabrication techniques may not readily be able to provide the desired feature dimensions. Selective chemical treatments of the diblock copolymers are one of the useful methods yielding a rich set of nanoscale features on PS-\textit{b}-P4VP. Exposure to selective vapor can induce reorganization of the polymeric chains of PS-\textit{b}-4PVP and alter the original micellar nanostructures. In this chapter, I identified for the first time a host of new nanostructures formed at different stages of chloroform vapor annealing by performing time-lapse atomic force microscopy measurements. I determined the key, time-dependent, topological parameters defining each nanostructure and presented the likely scenario of polymeric chain reorganization during the morphological evolution of the diblock polymer nanodomains over time. I also ascertained intermediate morphological states containing the characteristic nanostructures from two consecutive phases as well as transition states appearing for a short time in
between two subsequent phases. These research efforts will not only provide insight into the domain evolution steps of the micellar to the cylindrical structures of PS-\textit{b}-PVP but also be technologically advantageous for subwavelength mask design in nanolithography and high-density array fabrication in high throughput bio-detection.

2.1 Background and Introduction

Supramolecular assembly of block copolymers has been a subject of numerous investigations in the past due to their unique bottom-up assembling process which, in turn, yields well-organized features with regularly varying chemical compositions covering a large surface on a substrate. This intriguing assembly of block copolymers occurs whether they are prepared in bulk, in thin film, or in solution. Characteristic domains with a repeat spacing on the order of tens of nanometers are formed in their phase separation process.\textsuperscript{55,56,64} In recent years, diblock copolymers drew even greater attention as functional nanomaterials, especially with the realization of possible contribution of nanoscale polymeric domains to many areas of nanotechnology.\textsuperscript{65} In addition to the capability of rapidly producing periodic nanoscale features through self-assembly at large scale, many other advantages of diblock copolymers are recognized. The ability to predict and control the length scale of their periodicity, the configuration of repeated nanostructures, and the geometric shape of the recurring pattern makes these surfaces highly desirable for many technologically important applications in electronics, energy, and biomedical detection. Thus, diblock copolymers have been previously demonstrated as candidate systems serving the role of lithography templates in microelectronics and photovoltaics\textsuperscript{66,67,68,69} as well as array platforms in protein
measurements. Characteristic and predictable block copolymer domains formed in bulk and in thin films are used more extensively in electronics and energy devices whereas those in solution phase are employed greatly in biomedical delivery and detection.

The self-assembling behavior of block copolymers in thin films is largely controlled by the interfacial energies and size commensurability. Therefore, changes to self-assembled block copolymer domains can be effectively attained by altering these key parameters. Many methods to achieve variations of those nanodomains have been explored for two main reasons. One is to reduce defects in the nanodomains of such micellar copolymers to promote their seamless applications into effective templates at the nanoscale. The other reason is to increase the variety of the sizes and shapes of nanodomains for providing flexibility in their usage. Methods to accomplish the latter goal include thermal, electrical, mechanical, and chemical treatments of block copolymers. For example, topological defects in assembled domains of half-cylinders can be annihilated by thermally annealing diblock copolymers above their glass transition temperatures. The use of electric and shear fields can also increase the degree of correlation associated with the orientation and length of diblock copolymer domains, thereby providing topologically defect-free templates.

Chemical treatment methods can be applied either directly on the diblock copolymers by exposing them to various chemical environments. Recently, many studies have effectively demonstrated solvent annealing approaches to change the shape of polymeric nanostructures that exhibit long-range order. In these cases, the interfacial energies of diblock constituents are adjusted due to the presence of solvent vapor at the
air/polymer interface and the resulting self-assembled nanodomains are manipulated by solvent selectivity. Block copolymers such as polystyrene-block-polymethylmethacrylate (PS-\textit{b-PMMA}), polystyrene-\textit{block}-polybutadiene (PS-\textit{b-PB}), and polystyrene-\textit{block}-polyvinylpyridine (PS-\textit{b-PVP}) are investigated in these studies as their phase-separated domains have proven to be useful as self-organizing templates. Perforated lamella with ringed structures are obtained in a PS-\textit{b-PMMA} film when spun cast from a mixture of two solvents showing different preference to the two polymeric blocks.\textsuperscript{84} New features such as circular multipacked domains and in-plane core–shell cylindrical structures are also found on acetone-treated blend films consisting of PS-\textit{b-PMMA} with different PS volume fraction.\textsuperscript{85} In another study, the authors combined atomic force microscopy (AFM) and transmission electron microscope study investigates the block length and composition dependence on the cyclohexane-induced morphology of neat PS-\textit{b-PB} as well as blends of symmetric and asymmetric PS-\textit{b-PB} with various length/composition.\textsuperscript{86} When introducing an additional variable of solvent type, even more complex structures such as spheres-between-cylinders emerge in the blend films with different PS-\textit{b-PB} chain length/composition.\textsuperscript{87} These research efforts have identified new types of domain structures in diblock copolymer blends and, in addition, addressed complex and difficult-to-control parameters associated with solvent annealing such as physical and chemical compositions of solvents and polymers. The interplay between polymeric volume fraction, chain length, chain composition, and different solvents in a given system contributes to the formation of various domain structures.

In this paper, we focus on reporting a series of novel nanostructures in a homogeneous sample of PS-\textit{b-PVP} diblock copolymer by providing time-lapse findings
of its morphological changes at the air/polymer interface. We demonstrate that periodic arrays of polymeric micelles with novel nanoscale geometries can be easily and straightforwardly achieved from a homogeneous system involving a preset volume fraction, length, and composition of a phase-separated diblock copolymer sample under the vapor of a single solvent. In our time-lapse measurements, I also capture the intermediate or transition states of morphological transformations due to solvent annealing that have never been observed before. In particular, we choose PS-\textit{b}-PVP amphiphilic diblock copolymer micelles formed above the critical micelle concentration for our time-lapse study as their nanostructures can be useful for many applications such as carriers in drug delivery and guides in magnetic storage.\textsuperscript{88–91} In addition to the extensive investigation of these original micellar structures and their applications, convenient solvent-annealing routes to alter the micellar domains of PS-\textit{b}-PVP from the standard spherical geometry to cylinders have been reported.\textsuperscript{80,92,93} Despite these efforts, the exact process of morphological evolution that can shed light on the transformation process of the original PS-\textit{b}-PVP micelles to the modified cylinders is still not known. Possible nanotemplate morphologies that may have developed in various stages during chemical annealing have not been previously identified. Yet, having a detailed understanding of these characteristic stages can be beneficial due to the rich potential of these nanotemplates in the areas of nanofabrication and nanobiotechnology. Research efforts on this front will be especially advantageous for subwavelength mask design and high-density array fabrication by providing high versatility in the selection of template sizes (pattern repeat spacing) and shapes (pattern geometry) that are assembled at the nanoscale utilizing this bottom-up approach. Nanoscale patterns are more difficult, time-
consuming, and costly to produce when using alternative, top-down, techniques such as electron beam lithography or subwavelength photolithography. In this chapter, I identified, for the first time, different nanostructures of polystyrene-\textit{block}-poly(4-vinylpyridine) (PS-\textit{b}-P4VP) formed at different stages of solvent annealing, ascertain various morphologies of the diblock polymer nanodomains over time, and present the likely scenario of polymeric chain reorganization by obtaining time-lapse AFM measurements.

\subsection*{2.2 Methods and Experiments}

Asymmetric PS-\textit{b}-PVP diblock copolymer with an average molecular weight of 68,500 Da was obtained from Polymer Source Inc. (Montreal, Canada). The diblock contains 70\% PS by weight with a polydispersity of 1.14. Upon dissolution of 0.5\% (w/v) PS-\textit{b}-PVP in toluene, micelles consisting of a PVP core and a PS corona readily form in toluene which is a preferential solvent for PS. Silicon substrates, obtained from Silicon Inc. (Boise, Idaho), were cleaned with ethanol, acetone, and toluene and spun dry before spin-casting ultrathin PS-\textit{b}-P4VP films. This micellar solution was then spun on silicon substrates at 3500 rpm for 1 min. The resulting film on the substrate surface was an ultrathin film of PS-\textit{b}-P4VP with a thickness of 22 nm. The film thickness was determined by carrying out ellipsometry measurements using a Stokes Ellipsometer LSE (Gaertner Scientific, Skokie, IL).

Subsequent chemical treatment of the original PS-\textit{b}-P4VP micellar nanostructures was carried out under the vapor of chloroform (CHCl\textsubscript{3}) prepared in a small sealed chamber. The following Figure 7 shows a scheme of how the copolymer chains are being
induced by chemical vapor under the chemical chamber. Due to mild inducing effects from chloroform, both two polymer chains are gradually relaxing and untangled. This process was slow enough for me to capture the intermediate states between the spherical and cylindrical phases, when I removed the vapor away from the film.

Figure 7. Illustration of solvent vapor annealing of diblock copolymer thin film

Solvent-annealed sample surfaces were then imaged at various time intervals ranging from 30 minutes to 30 hours. AFM measurements were carried out using a Digital Instruments Multimode Nanoscope IIIa instrument in tapping mode at a scan speed of 1 Hz or lower. Silicon tips with range of resonant frequency from 65 to 83 kHz and a spring constant of 5 N/m were used in our measurements.

2.3 Results: Morphology Evolutions of Nanostructure

Upon spin-casting, hexagonal arrays of PS-\textit{b}-P4VP micelles spontaneously form on the silicon substrate with an average repeat spacing of 50 nm. For these micelles formed in toluene, the PS rich area is found in the outer region whereas the inner region is P4VP rich. The AFM line profile along these micelles displays an average height difference of 2.43 nm between the P4VP and PS rich areas. The formation of the periodic micellar structures on the surface is driven by a micro-phase separation process due to the
incompatibility and solvent selectivity of the two blocks. Since the surface free energy of 
PS is lower than that of P4VP, PS chains are exposed to the air/polymer interface. In the 
corresponding AFM topography image shown in Figure 8, this tendency is reflected in 
the higher (lighter contrast) P4VP-rich core that is embedded in the middle of a lower 
(darker contrast) PS-rich matrix.

Subsequent chemical treatment of the original PS-b-P4VP micellar nanostructures 
was carried out under the vapor of chloroform (CHCl₃) prepared in a small chamber. 
When exposed to the chloroform vapor, the micellar PS-b-P4VP nanostructures undergo 
polymeric chain rearrangement and develop into unique, time-dependent morphologies. 
These distinctive nanostructures appearing at different solvent-annealing stages can be 
easily correlated with the time of vapor exposure. We identified eight different 
morphological stages between the initial spherical micelles to the final cylinders, Figures 
8 and 9. Schemes illustrating polymeric chain organizations and AFM results of line 
analysis are summarized in Figure 10.

After 3 hours of additional CHCl₃ treatment, the surface morphology changes to 
hexagonally arranged “holes” phase II with a similar repeat spacing to its original 
template, 49 nm. However, the height difference between the higher (matrix) and the 
lower (center of the hole) areas is reduced to more than 50% of the original template to 
1.05 nm. CHCl₃ is generally considered as a good, nonselective solvent for both PS and
Figure 8. AFM topography displaying the four distinctive, self-assembled, nanostructures of PS-b-P4VP monitored in the first four stages out of the total eight phases that are identified during CHCl₃ vapor annealing. Panels shown on the left and right are 1 × 1 μm² and 500 × 500 nm² in scan size, respectively, and the image size of the insets is 125 × 125 nm². Roman numerals indicated next to each set of images represent the four distinctive phases which are characteristic of the solvent annealing time, (I) original spheres, (II) holes, (III) reformed spheres, and (IV) embedded spheres.
P4VP blocks. However, due to differences in the chemical makeup of the benzene versus pyridine rings in PS and P4VP, the solvent is slightly more selective to P4VP. After the initial annealing, PS chains near the air/polymer surface tend to move toward the outside regions of the micelle, uncovering the underlying P4VP core. This chain rearrangement allows more P4VP chains to make contact with the CHCl₃ vapor.

Further treatment with CHCl₃ yields a series of intriguing nanoscale surface structures which have never been observed before. For subsequent CHCl₃ treatment up to 9 hours, continuing rearrangement of polymeric chains leads to “reformed spheres” with a slightly reduced periodic spacing of 47 nm. This geometry is due to the template reversal of holes to original spherical templates. The height of the reformed spheres is 1.43 nm, suggesting the formation of more flattened spheres than the original. The transition from the hole structure in the earlier stage to the reformed sphere structure seems anomalous as the PS segment on the surface of the reformed sphere is less favorable to the solvent. The use of chloroform as the annealing vapor permits this careful balance due to its only slightly more favorable interaction with P4VP relative to PS. Both chains tolerate contact with the vapor to some extent, and the formation energetics of specific nanostructures are determined by the careful balance between PS chain frustration and slight solvent preference toward P4VP. It may be likely that the exposed P4VP chains in the hole structure further extend toward the surface during this transition. However, the film is under frustration as P4VP is the minority block which prohibits the complete exposure of P4VP to the surface, that is, an inversion of the micelle. Simultaneously, the PS chains have a tendency to avoid contact with the solvent vapor. This process may result in a nanostructure in which the pocket of P4VP chains is
situated close to the surface of the micelle (instead of the center) and the majority of PS chains are buried underneath the surface, leaving a small volume of PS chains covering the top of the P4VP pocket. Although frustrated, this configuration of the reformed sphere may be energetically favorable relative to leaving a large volume of unfavored PS chains in contact with the solvent as expected from the hole.

After an additional 7 hours of CHCl₃ treatment, the film topography changes to hexagonally packed “embedded spheres” whose unit structure is defined by a small sphere nesting at the center of a micelle. The periodic spacing is increased back to 49 nm as a result of this morphological transition. The height difference between the center spheres to the neighboring lower region is 0.59 nm and the outer micelle wall to the same lower region encapsulating the center sphere is 0.34 nm. The origin of this structure is likely due the redistribution of PS chains near the air/polymer interface. The evenly distributed PS chains closer to the center of the micelle in the reformed sphere are rearranged to expose a ring of PS-free area at the interface, thus creating the lower region around the nested sphere in the micelle. The ringed regions where PS chains are pulled away expose the P4VP cores of the micelles to CHCl₃.

Figure 8 displays AFM topography images of the first four stages of annealing: original spheres (stage I), holes (stage II), reformed spheres (stage III), and embedded spheres (stage IV). The transition between the reformed spheres to the embedded sphere state is an attempt to release the frustrated PS chains covering the entirety of the micelle surface in the reformed sphere. The geometry of the embedded spheres allows more exposure of the favorable P4VP chains to the micelle surface, while maintaining minimized contact of the solvent with the major component, PS.
Figure 9. AFM topography displaying the four distinctive, self-assembled, nanostructures identified in the second series of CHCl₃ vapor annealing of PS-b-P4VP. Panels shown on the left and right are 1 x 1 μm² and 500 x 500 nm² in scan size, respectively, whereas the insets are 125 x 125 nm². Roman numerals denoted next to each set of images represent the four characteristic, time-dependent, phases of (V) enlarged spheres, (VI) cylinder precursors, (VII) enlarged holes, and (VIII) cylinders.
Figure 9 shows AFM topography images of the ensuing series of morphological changes that the PS-b-P4VP film undergo when exposed to the CHCl₃ vapor. The next stage of the solvent annealing up to 25 hours yields the formation of ‘enlarged spheres’ with an average repeat spacing of 48 nm. These spheres mimic those of the original spheres but the volume ratio of the sphere to the surrounding matrix is higher than that of the original template. The height difference between the elevated and depressed regions of the enlarged spheres is 1.23 nm. Reconfiguration pathways of the polymeric chains responsible for this transformation are not as straightforward to deduce as other steps, when solely based on the two corresponding panels in Figure 9. However, the transition state from the embedded spheres to the enlarged spheres captured with our time-lapse measurements can provide some insight into possible pathways. These aspects are discussed in detail in later sections of this chapter.

Further CHCl₃ treatment up to 29 hours results in a more complex morphology of ‘cylinder precursors’. The repeat unit of these nanostructures mimics the shape of two concentric rings, which are hexagonally packed in space with a repeat spacing of 49 nm. A height difference of 0.2 nm and 0.5 nm is measured from the inner ring to the center depression and from the outer ring to the neighboring lower region, respectively. The likely driving force of this transformation is to increase the preferred interaction between P4VP and CHCl₃ by exposing the P4VP chains to the air/polymer interface at multiple locations. The resulting micelles in the cylinder precursor phase exhibit P4VP cores exposed to the interface at the micelle center, in contrast to the similar nanostructures found in the embedded sphere stage of the stage IV. The latter structures contain a single ring surrounding the sphere formed by PS chains at the micelle center, whereas surface
PS chains in the cylinder precursor nanostructures are configured into two concentric rings on the film surface in order to provide more interaction between P4VP chains and CHCl₃. Therefore, cylinder precursor nanostructures may be explained by the rearrangement of the PS chains at the air/polymer interface in a manner which enables multiple exposure points of the P4VP cores.

I also observed small areas of the sample that underwent further transformation from cylinder precursors to ‘enlarged holes’. A relatively large degree of chain reorganization is needed for this change to occur as the average repeat spacing and the depth of the enlarged holes are 47 nm and 1.58 nm, respectively. Although the formation of enlarged holes is not a favored pathway due to such a reason, they are stable once formed. Even after annealing up to 100 h, only an extremely slow structural change is yielded merging several neighboring enlarged holes into short grooves. However their formation is limited to less than 10% of the total cylinder precursors and does not result in cylinders even after the extended vapor treatment.

In contrast, the majority of the cylinder precursors will readily proceed to become short rods and then progress to cylinders. Cylinders formed in this way exhibit major and minor repeats in which minor peaks repeatedly appear in the middle of two neighboring major cylinders with the same average repeat spacing. The additional cylinders consisting of the minor peaks between the major cylindrical peaks are due to the joining of nearby cylinder precursors whose unique nanostructures contain two concentric rings per precursor. A detailed discussion of the cylinder formation process involving transition frames is provided in a later section. The nanoscale structural changes from the original sphere to the cylinder formation are highly reproducible.
Figure 10. Schematic cartoons of the nanoscale structures are provided for each identified stage and possible chain configurations in the corresponding polymeric structures leading up to cylinders are proposed. Key topological features are determined by carrying out AFM line measurements and summarized for each nanostructure.
Figure 10 displays the proposed model for chain rearrangements involving each nanostructure in the eight stable stages of solvent annealing leading up to cylinder formation. Schematic cartoons corresponding to each nanostructure are also displayed for clarity in Figure 10. The figure also summarizes the results of AFM line measurements carried out on key topological features of the nanostructures from each annealing stage. As the characteristic nanostructure in each solvent-annealing stage can be conveniently correlated with vapor exposure time, each unique nanostructure can be conveniently and reproducibly obtained by exposing the original PS-\textit{b}-P4VP film for the specified amount of time as discussed above.

The polymeric chain evolution observed in stages between the (I) original sphere, (IV) embedded sphere, and (VI) cylinder precursor can be rationalized by applying a simple model based on 2D geometrical shapes of the surface-exposed PS and P4VP blocks. The radii of the regions corresponding to the two polymeric segments in each nanostructure are determined by AFM analysis, and the exposed surface areas for the two polymeric blocks are calculated accordingly. The surface areas taken up by PS and P4VP in the three stages are (I) PS: 625\(\pi\) nm\(^2\) and P4VP: 0, (IV) PS: 550\(\pi\) nm\(^2\) and P4VP: 75\(\pi\) nm\(^2\), and (VI) PS: 375\(\pi\) nm\(^2\) and P4VP: 250\(\pi\) nm\(^2\). As annealing progresses over time, the nanostructures transition into configurations which expose more PMMA toward the air/polymer interface while minimizing the complete inversion of the micelle.

2.4 Discussions

In this chapter, I presented an effective method to expand the flexibility and complexity of the PS-\textit{b}-P4VP nanostructures that can be applicable particularly for
creating lithographic and bio-array templates. However, the nature of the new set of intricate nanopatterns displayed in Figures 8 and 9 also poses significant challenges in determining the chemical identities corresponding to the smaller substructures within a given nanostructure. The complex subdomains within these individual nanofeatures cannot be effectively probed with conventional scattering or spectroscopic tools. This difficulty is partly due to the lack of surface characterization techniques that can simultaneously provide high-enough spatial and chemical resolution. For example, to probe the spatial and chemical characteristics of a single nanostructure within the embedded sphere shown in Figure 9 (VI), a chemically sensitive surface tool with a horizontal and vertical resolution down to several angstroms and nanometers is required. In order to bypass this difficulty, a small-enough marker element which can serve as a faithful marker for one of the two diblock copolymer segments can be added to the surface of these individual nanofeatures in order to distinguish the two chemical components of the diblock copolymer. In addition to the series of time-lapse images of CHCl₃-annealed PS-b-P4VP nanostructures, I found, for the first time, the transient and intermediate morphological images of the various nanostructures which can provide insight into the evolution process between the eight identified states.

From a subset of the eight annealing phases, the two morphologies belonging to two consecutive stages were observed in the same panel of the time-lapse AFM measurements, as presented in Figure 11. We defined these panels displaying the presence of both morphologies from neighboring stages as intermediate states. We identify two intermediate stages. The subset pairs showing intermediate states included embedded spheres and enlarged spheres in Figure 11(a), as well as enlarged spheres and
Figure 11. AFM panels showing intermediate and transient states monitored from a subset of annealing phases. a. The 300 x 300 nm$^2$ topography panel shows the presence of embedded spheres (IV) and enlarged spheres (V). Arrows mark the corresponding structures in the intermediate stage. The phase images (50 nm in diameter) of the characteristic nanostructures are also presented on top of the topography frame to discern each structure unambiguously. b. The 300 x 300 nm$^2$ topography displays the existence of cylinder precursors (VI) and enlarged holes (VII) in the same panel. The phase images (50 nm in diameter) of the characteristic nanostructures are also presented on top of the topography panel. c. The top three panels are topography images acquired from the IV stage of embedded spheres (left) and the V stage of enlarged spheres (right). The middle panel show the transient morphology between the two stages where the center spheres in embedded spheres have grown in size before turning into the enlarged spheres in the following stage. All panels are 300 x 300 nm$^2$ in scan size.
cylinder precursors in Figure 11(b). Repeated observation of these frames in our AFM measurements suggests that the configuration of the polymeric chains in these subsets may be easily reversible within the pairs, and therefore, the transformation process involving the two specific pairs has lower activation energy than that for the necessary chain rearrangements in other stages. By decreasing the time interval of the vapor annealing, we collect AFM panels that capture transition from one state to the other.

Similar to what is observed in the evolution process from holes to reformed spheres, the energy penalty to be paid by the surface exposure of PS chains in enlarged spheres seems to be compensated by the drive to release the PS chain frustration as the PS block occupies a much larger volume fraction in the diblock.

Progression of embedded spheres to enlarged spheres is captured in the transient topography panel, the top three images in Figure 11(c), showing the transformation pathway between the two annealing states. Changeover to the enlarged spheres is driven by the growth of the center regions in the embedded spheres. The nested spheres in the middle of the micelles grow in size reaching up to 25 nm in diameter in the matrix before developing into enlarged spheres in the next stage. The bottom three images in Figure 11c display the transition from cylinder precursors to enlarged holes and the subsequent morphological change of the enlarged holes from extended vapor treatment. The last image shows some merging of the enlarged holes observed after 100 h annealing.

In addition, the transition states from cylinder precursors to cylinders (Figure 12a and b) and from short to long cylinders (Figure 12c) are observed in our experiment which can shed light on the formation process of the cylinders as well as the progression
Figure 12. Transition states from cylinder precursors to cylinders. (a-d). AFM topography panels are (a) 75 x 75 nm², (b and c) 300 x 300 nm² and (d) 210 x 210 nm² in scan size. Formation of short rods via adjoining of two neighboring cylinder precursors in the hexagonally packed geometry is seen clearly in the white boxes inserted in the AFM panel (a). Transition states from cylinder precursors to short rods (b) and short rods to long cylinders (c) are also identified in the AFM panels. (d). Line measurement along the inserted white line in the AFM image clearly shows that a periodic minor peak appears in the middle of two neighboring major peaks. The minor peaks have the same repeat spacing as the major peaks with an approximately half-reduced height between peak and valley. (e). Schematic cartoons display the likely chain reconfiguration of the cylinder precursors to finally form cylinders with the major and minor repeats.
of the polymeric chains rearrangement to reduce topological defects in cylinders. The curvature of the stretched polymeric chains in the outer perimeters of the cylinder precursors experiences high surface energy. In order to minimize the strain due to the curvature, cylinder precursors tend to join and form short rods instead.

Subsequently, these short rods turn gradually into longer rods to eliminate topological defects such as disclamations’s and dislocations, similar to what has been observed from thermal annealing of cylindrical PS-b-PMMA domains in ultrathin films. The major and minor cylindrical peaks are observed in all evolution stages of cylinders with different persistence lengths. The major peaks have a height difference of 1.5 nm between the lower and higher areas of the nanodomains, whereas the minor peaks show approximately one-half of that height difference, as seen in Figure 12(d). The average repeat spacing of the long cylinders is 43 nm, while shorter cylinders tend to exhibit a slightly larger repeat spacing. For example, the short cylinders shown in Figure 12(b) display a repeat spacing of 45 nm. This observation suggests that polymeric chains pack tightly in space as they increase the correlation length of the cylinders. The likely pathway of chain reorganization from cylinder precursors to cylinders is illustrated in Figure 12(e) where the formations of the repeating major and minor peaks are rationalized during the transition.

2.5 Conclusion

In summary, I have successfully monitored time-lapse morphology changes of the ultrathin films of PS-b-P4VP induced by exposing it to discriminatingly interacting CHCl₃ vapor and identified for the first time various nanostructures that are associated
uniquely with different annealing time. Eight stable nanostructures are found at various annealing stages, spanning from the original micellar phase to the cylindrical phase. We also ascertain intermediate states containing characteristic nanostructures from two consecutive phases and transition states appearing for a short time in between two subsequent phases. In addition, we discuss the likely scenarios of polymeric chain reorganization in order to explain the diblock polymer transformation from one stage to the next based on our time-lapse AFM observation of the nanostructures. These efforts provide insight into the evolutionary steps of the original PS-$b$-P4VP micellar to the cylindrical template which has not been elucidated before through previous studies. Self-assembled nanostructures of PS-$b$-P4VP have been demonstrated for many technologically important applications in nanolithography and nanobiotechnology. The rich set of novel nanostructures identified in this paper can be highly beneficial to these fields by providing much needed versatility and flexibility to meet the increasing demand for self-assembled, nanoscale guides and platforms for such applications.
Chapter Three: Spontaneous Nanoscale Protein Assembly on Novel Nanostructures of Polystyrene-block-Polyvinylpyridine

Abstract

Well-controlled assembly of proteins on supramolecular templates of block copolymers can be extremely useful for high-throughput biodetection due to its inherent capability associated with high packing density and diversity. Therefore, the development of a novel method to increase versatility in the repeat spacing and shape of periodic nanoscale templates can facilitate surface assembly of proteins which, in turn, can be beneficial to their application as high-density, high-payload, protein arrays. In this chapter, the adsorption and assembly characteristics of a model antibody protein on various polystyrene-block-poly(4-vinylpyridine) templates have been investigated whose distinctive nanoscale structures were obtained through time-regulated exposure to chloroform vapor. Strong adsorption preference of the protein to the segment of polystyrene in the diblock copolymer templates leads to an easily predictable, controllable, rich set of nanoscale protein morphologies through self-assembly. The result also demonstrated that the chemical identities of various subareas within individual nanostructures can be readily elucidated by investigating the corresponding protein adsorption behavior. The chemical compositions of polymeric blocks in these complex nanoscale structures could not be easily ascertained otherwise, due to the lack of techniques simultaneously providing high enough spatial resolution which simultaneously correlate information on nanoscale topological details and their corresponding chemical compositions. In this approach, the specific arrangement of
polymeric chains and their chemical identities associated with each nanoregion can be easily ascertained due to the high discriminating tendency of proteins to one of the two block components. Together with the ease of fabrication in producing polymeric templates with well-defined nanoscale structures of two dimensional degrees of freedom, self-assembled arrays of proteins created using our approach can contribute a high degree of versatility to acquire intricate nanoscale geometry and spatial distribution of proteins in an array. These advantages can be highly beneficial both to fundamental biology research and biomedical detection, especially in the areas of solid-state based, high-throughput protein sensing and screening.

3.1 Introduction

Proteins assembled on supramolecular templates of block copolymers can be extremely useful in the area of proteomics and protein sensors due to the highly dense packing density and self-passivation capability demonstrated by various biomolecules on these substrates.95,49,70 Both the formation of the underlying nanoscale polymeric guides as well as the biomolecular arrangements on the substrates are driven by self-assembly. In the former case, the immiscibility and degree of polymerization determine the phase separation behavior of polymeric guides, whereas in the latter case, chemical and physical interaction parameters between polymers and proteins govern the specific protein arrangements on the polymer of preferred composition. This bottom-up assembly process, in turn, yields well-organized protein arrays whose individual features are periodically arranged nanostructures. Since block copolymers produce characteristic domains with a repeat spacing on the order of tens of nanometers after their phase
separation process, the spatial resolution of the individually addressable units in the resulting protein arrays is also on the order of nanometers. Creating nanoscale features through conventional lithography techniques can be costly and time-consuming as they require either specially engineered photomasks for a parallel fabrication process or the use of electron beam writing for a serial process. Even with such techniques, fabrication of intricate surface patterns below tens of nanometers cannot be easily and rapidly accomplished. These difficulties are circumvented in the case of spontaneous nanoscale organization through the self-assembly of block copolymers demonstrated in our previous studies.

In addition to the capability of rapidly producing periodic nanoscale features through self-organization on a large scale, protein arrays created via diblock copolymer nanodomains can be effectively tuned by controlling the phase separation behavior of the underlying diblock copolymers. A variety of important parameters, such as desired length scale in periodicity, spatial arrangement in repeated nanostructures, and geometric shape in individually addressable features, can be modified. As an additional degree of freedom, a given diblock copolymer template initially produced by controlling the aforementioned variables can be further modified with a post-phase separation process. Recently, it has been shown that chemical treatment methods can be applied directly to the diblock copolymer and other polymers by exposing the surfaces to various chemical environments for modifying surface morphology. Solvent annealing approaches effectively adjust the interfacial energies of diblock constituents through chemical selectivity toward one of the two polymeric components, thus resulting in changes of the original size and shape of polymeric nanostructures. Nanostructures of
useful block copolymers such as polystyrene-*block*-polymethylmethacrylate (PS-*b*-PMMA) and polystyrene-*block*-polyvinylpyridine (PS-*b*-PVP) are often investigated by various solvent annealing methods.\textsuperscript{80,81,82,83, 98,99}

Understanding protein adsorption behavior on various polymeric surfaces is crucial, for there are the rising demands for highly miniaturized, small-volume detection platforms for examining proteins both in laboratory and clinical diagnosis settings.\textsuperscript{97,100}

Such solid-phase assays involving proteins on polymeric array or plate surfaces have the advantage of requiring only a very small amount (a few microliter or smaller) of assay reagents in most detection settings.\textsuperscript{49,95} Solid-phase approaches also enable a large number of biosamples to be assayed rapidly and simultaneously. The nanostructures resulting from polystyrene-*block*-poly(4-vinylpyridine) (PS-*b*-P4VP) are particularly useful as protein arrays. This is because the control of size, shape, and spacing of individually addressable units in phase-separated PS-*b*-P4VP can be achieved with two-dimensional degrees of freedom as compared to those of PS-*b*-PMMA nanotemplates, which provide only a one-dimensional degree of spatial periodicity. Increased versatility in template sizes (pattern repeat spacing) and shapes (pattern geometry) that are bottom-up assembled on the nanoscale can allow the use of protein arrays in basic biology research and biomedical detection. These advancements may also have the potential to allow truly nanoscale fabrication of protein arrays, which could facilitate non-diffraction-limited optical detection.

Being motivated by the aforementioned importance, we reported here for the first time the adsorption behavior of a model protein on a newly identified set of chemically modified PS-*b*-P4VP micellar nanotemplates. Detailed evolution processes and
mechanisms of the solvent-induced, nanoscale morphologies of PS-\(b\)-P4VP had been discussed in chapter two. In this chapter, the effort is focused on elucidating the unique protein adsorption behavior of anti-bovine immunoglobulin G antibody (anti-IgG) on various PS-\(b\)-P4VP nanostructures. This study can provide insight into the evolution and modification process of the underlying polymeric chains under solvent annealing retrospectively. This can be achieved with a spatial resolution down to the nanometer range while providing information on the nanoscale subarea-specific chemical composition simultaneously. We demonstrated that the specific arrangement of the polymeric chains and their chemical identities can be easily ascertained for each nanostructure in the new set of PS-\(b\)-P4VP templates by analyzing preferential interactions between proteins and their favored polymeric block. Resolving the exact spatial distribution of particular polymeric chains in various substructures within each individual micelle cannot be readily attained using other methods. This difficulty is due to the lack of straightforward experimental techniques that can simultaneously assess the complex individual nanostructures with topological and chemical resolution on the required nanometer level. In addition, the level of morphological sophistication and the nanometer size regime observed in the new set of solvent-induced PS-\(b\)-P4VP nanostructures cannot be created in a straightforward manner by using conventional fabrication techniques. The bottom-up method used in our approach to yield highly structured and efficiently assembled proteins eliminates the need for employing time-consuming and costly steps for producing sophisticated nanotemplates via techniques such as electron beam writing and sub-wavelength photolithography.\(^{93,101}\) In addition to providing valuable information on nanoscale spatial maps and the corresponding
chemical compositions of the intricate polymeric structures, our efforts could be highly beneficial for realizing the rich potential of high-density, diblock copolymer-assisted, protein array-based detection platforms in the areas of nanofabrication and nanobiotechnology.

3.2 Methods and Experiments

Anti-IgG was chosen as a model protein system for assembly on PS-b-P4VP because it is widely used in a variety of protein detection settings to monitor antigen–antibody complex formation, enzymatic activity, and affinity-based purification. In addition, the size of the individual protein is smaller than the size of the nanostructures found in PS-b-P4VP thin films and the spherical shape of the protein matches closely with those of the polymer nanotemplates.

Periodic arrays of self-assembled proteins are readily formed on various PS-b-P4VP templates that are pretreated with the selective vapor of CHCl₃. The highly discriminating nature of proteins against the P4VP polymeric block drives the extremely selective spatial organization of the proteins to PS-rich areas only. Hydrophobic interaction plays an important role as the driving force of the selective adsorption of the anti-IgG proteins to the PS phase. In a buffer environment, the interaction of anti-IgG proteins to water is unfavorable, similar to that of another globular protein, bovine serum albumin.¹⁰² The enthalpy is small and negative, and there is a large contribution from the negative entropy. When a hydrophobic solid surface is present, the entropic effect becomes largely positive. As the protein surface near the solid substrate can exclude
water molecules which in turn, yields a negative Gibbs free energy. The presence of a hydrophilic surface such as P4VP leads to an opposite effect.

In this process of protein adsorption, surface regions consisting of the P4VP block are left completely free of adsorbed proteins, serving as naturally generated areas of a passivated surface. The exclusive adsorption of proteins on the chemically competing nanoscale templates can eliminate the need for the 'surface passivation' often required for producing protein arrays. The strong tendency of many proteins to adsorb randomly on various surfaces can hinder the sensitivity and specificity of protein arrays and, therefore, treatments such as passivating certain surface areas of protein arrays chemically or biologically have been conventionally used in order to avoid this problem. In our approach, the chemically heterogeneous nature of the block copolymer nanotemplates serves as a 'built-in' self-passivating array where the facilitation and prevention of protein adsorption can be controlled precisely on the nanoscale by varying the size and shape of the polymeric nanotemplates.

Asymmetric PS-b-P4VP diblock copolymer with an average molecular weight of 68,500 Da was obtained from Polymer Source Inc. (Montreal, Canada). Silicon substrates, obtained from Silicon Inc. (Boise, Idaho), were cleaned with ethanol, acetone and toluene, then spun dry before coating ultrathin PS-b-P4VP films. The diblock contains 70% of PS by weight with a polydispersity of 1.14. Upon dissolution of 0.5% (w/v) PS-b-P4VP in toluene, micelles consisting of a P4VP core and a PS corona readily form in toluene, a preferential solvent for PS. The amphiphilic PS-b-P4VP diblock copolymer with the specified volume composition is known to form micelles when dissolved in toluene. This micellar solution was then spun on silicon substrates.
at a speed of 3500–4000 rpm for 1 min, in order to produce an ultrathin film of \( \text{PS-}b\text{-P4VP} \) with a thickness of 21 nm. The film thickness is determined by carrying out ellipsometry measurements. Upon spin-casting, hexagonal arrays of \( \text{PS-}b\text{-P4VP} \) micelles spontaneously form on the silicon substrate with an average repeat spacing of 50 nm. Subsequent chemical treatment of the original \( \text{PS-}b\text{-P4VP} \) micellar nanostructures was carried out under a vapor of chloroform, \( \text{CHCl}_3 \), prepared in a small sealed chamber. Solvent-annealed samples were then imaged at various time intervals and the morphologies of the polymeric templates at each temporal stage recorded by Atomic Force Microscopy (AFM). AFM measurements are carried out using a Digital Instruments Multimode Nanoscope IIIa in tapping mode at a scan speed of 1 Hz or lower. Silicon tips with a resonant frequency of 71.5 kHz and a spring constant of 5 N m\(^{-1}\) are used in our ambient AFM measurements. Homopolymers of PS and P4VP were purchased from Polymer Source, Inc. and dissolved in toluene and a 1:1 (v/v) mixture of acetone and ethanol, respectively, to make a 0.5% (w/v) solution. The homopolymer solution was spun-cast on silicon substrates using the same spin coating condition described above for the diblock copolymer.

Dichlorotriazinylaminofluorescein-conjugated, anti-bovine immunoglobulin G antibody (anti-IgG) was received from Rockland Immunochemicals (Gilbertsville, PA) and the lyophilized powder of the proteins was reconstituted in a PBS buffer (10 mM mixture of \( \text{Na}_2\text{HPO}_4 \) and \( \text{NaH}_2\text{PO}_4 \), 140 mM NaCl, 3 mM KCl, pH 7.4) to varying concentrations ranging from 0.1 to 1 \( \mu \text{g/ml} \). A total of 8 \( \mu \text{l} \) of desired protein solution was then deposited onto various chemically treated, ultrathin \( \text{PS-}b\text{-PVP} \) micelle films for
2 min at room temperature. The sample surface was then thoroughly rinsed with PBS buffer followed by blow-drying under a gentle stream of compressed air.

### 3.3 Results and Discussions: Protein Adsorption on Evolved Nanostructures

The vapor treatment of the micellar PS-\(b\)-P4VP template leads to a time-dependent topology which can serve effectively as self-assembling guides for other inorganic or biological nanomaterials. Various, time-specific, surface features can be generated at large scale via self-assembly using this method.\(^{48}\) Figure 13 shows the diverse polymeric morphologies of PS-\(b\)-P4VP obtained by the aforementioned method that was used specifically in this protein adsorption study. The unique topological characteristics of the polymeric templates can be precisely controlled by varying solvent annealing time, where the series of PS-\(b\)-P4VP templates shown in Figure 13 is referred hereto as (a) original spheres, (b) holes, (c) reformed spheres, (d) embedded spheres, (e) enlarged spheres and (f) cylinder precursors, respectively.

The AFM results demonstrate that the same type of diblock, PS-\(b\)-P4VP, can be successfully modified and utilized to create an assortment of highly intricate, nanoscale protein patterns that are otherwise difficult to produce. Figures 14 and 15 display 0.1 \(\mu\)g/ml of anti-IgG molecules deposited on the series of chemically treated PS-\(b\)-P4VP micellar templates. Proteins tend to decorate the outer edges of the micelles while leaving the centers of the spheres devoid of proteins. Our previous studies on similar diblock copolymer templates confirm that proteins such as immunoglobulin G and serum albumins show an extremely high affinity for PS domains.\(^ {41,20,106}\)
Figure 13. AFM panels of the unique PS-b-P4VP nanostructures monitored during time-dependent exposure to CHCl3 vapor; (a) original spheres, (b) holes, (c) reformed spheres, (d) embedded spheres, (e) enlarged spheres and (f) cylinder precursors. AFM images clearly demonstrate periodic two-dimensional arrays consisting of well-defined nanostructures at each time period of annealing for (a) 0 h, (b) 3 h, (c) 9 h, (d) 16 h, (e) 25 h, and (f) 29 h. The scan sizes of the images shown on the left and right column are 500 nm × 500 nm and 140 nm × 140 nm, respectively. As a guide, illustrations showing possible polymeric chain distributions in each micelle are inserted next to each AFM panel for each time-dependent PS-b-P4VP nanostructure. Red and yellow chains in the cartoons represent PS and P4VP, respectively.
If a micelle in the film consists of a P4VP core and a PS crown, as expected in a solution where PS is uniformly encapsulating the inner P4VP core, the adsorption tendency of the proteins should show no inclination for a particular sub-region within a micelle and proteins should evenly distribute themselves on the entire surface of the micelle. However, our AFM results from anti-IgG adsorption repeatedly confirm that at both low (less than monolayer coverage) and high (saturation coverage) concentrations, proteins evade the center surface areas of the micelles during adsorption, in Figures 14 and 15. This observation, in conjunction with the known adsorption tendency of the protein, demonstrates that the center of the sphere is P4VP rich at the air/polymer interface. This phenomenon suggests that PS-\textit{b}-P4VP micelles tend to adjust their isotropic spherical configuration when prepared on a solid substrate. Due to the presence of the underlying silicon surface, each micelle is likely to be flattened in the vertical direction and take the form of a collapsed micelle. Unlike its concentric core/corona micellar counterpart of PS-\textit{b}-P4VP found in solution, our data indicate that the center region of the collapsed micelle prepared on a substrate is devoid of PS blocks in its corona structure near the surface and that the PS chains are likely to have moved down toward the substrate. As a result of this rearrangement of PS chains, some P4VP fragments close to the surface of the micelle, which would have otherwise been buried in the inner core, are now exposed to the air/polymer interface.

Another interesting behavior of protein adsorption occurs at low protein coverage resulting from 0.1 \( \mu g/mL \) deposition. In these instances, proteins occupy less than a monolayer of the available PS-\textit{b}-P4VP surface sites. Anti-IgG assembly favors fully saturating a subset of micelles with proteins rather than partially filling in all available
Figure 14. AFM images displaying the protein adsorption characteristics on each of the six polymeric nanotemplates indicated in Figure 13. The scan sizes of the topography images shown on the left and right column are 250 nm × 250 nm and 140 nm × 140 nm, respectively. The concentration of protein solution deposited onto various PS-b-P4VP templates is 0.1 μg/mL. This concentration is well below the saturation coverage, i.e. not all available PS areas on the polymeric film surface are covered by anti-IgG at this concentration. As a guide to understand the complex structures after protein adsorption, side-view illustrations of the AFM data are added. The proteins adsorbed on various surface locations of each polymer nanostructure are depicted in the illustrations as red (PS), yellow (P4VP), and blue (anti-IgG) areas.
micelles with a few proteins per micelle, see Figure 14(a). Each saturated micellar template hosts four to five individual protein molecules around its center. When a group of saturated micelles is nearby, a network of loops is observed, where each loop consists of four to five beads of protein molecules. The maximum number of proteins that can decorate each micelle is determined by the size of the protein and the micellar template. The proteins are neither found at the center areas of the loops nor at the shared areas between neighboring loops. The relatively high presence of P4VP chains at the air/polymer interface is attributed to the lack of adsorbed proteins at the center of the loops, whereas steric hindrance from proteins forming the loops is responsible for limiting protein adsorption in the neighboring areas.

Because the sizes of the polymeric templates and proteins are well-defined, it is possible to predict and control the maximum number of proteins that can be gathered on each template. The size of the micellar templates can be controlled in order to fine-tune the adsorption density of proteins per given surface area of the substrate. A micellar template of approximately 50 nm in diameter will host three globular proteins with a diameter of ~15 nm on average if the proteins tend to occupy the top of the micellar template. The same polymeric template will be able to accommodate approximately six protein molecules of the same size around the perimeter of the micelle if protein adsorption favors the outer periphery of the template.

Quantitative analysis and comparison of detection signals from multiple locations on the templates are often necessary in protein array measurements. However, the task of obtaining a well-controlled density of proteins on arrays is difficult to achieve using conventional protein printing methods. They require either additional fabrication steps for
array production or user-calibration processes for the normalization of background signal between all spots in the arrays prior to the intended protein measurements. The unique ability of the protein arrays prepared by our method in controlling the exact protein density on surfaces can overcome the drawbacks of conventional arrays and, therefore, is extremely beneficial for quantitatively comparable protein detection.

Exposing spherical PS-\(b\)-P4VP micelles in the original template to chloroform vapor for up to 3 hours leads to a hole geometry due to the favored interaction between the P4VP block and chloroform, Figure 13(b). Figure 14 (b) displays 0.1 \(\mu\)g/ml anti-IgG deposited on the hole template, where both bare holes as well as those decorated with proteins are observed at this low deposition concentration. It is evident that anti-IgG proteins deposited onto this hole template completely avoid the depressed regions in the polymer film where the dominant polymeric phase is the surface-exposed P4VP. This arrangement of the proteins onto the hole template is, in part, similar to the process observed on the original template. At the same time, the assembled proteins on holes exhibit an additional grouping geometry that is not seen on the original template. Strings of proteins are found to be placed into distinctive lines emanating from the protein loops around the holes. This assembly behavior results in a unique windmill-like pattern of proteins at this surface coverage, as seen in figure 14(b). The formation of these distinctive lines may indicate that there is a dominant morphological pathway for PS chains to fold away from the center of the original sphere during the chemically-induced rearrangement process of the polymeric chains into holes. Similar to the observation in the original templates, not all available hole templates are occupied by proteins at this concentration. Proteins also show the same tendency to complete assembling around a
Figure 15. Comparison of Anti-IgG on nanotemplates of PS-b-PVP (a) Anti-IgG assembled on the PS-b-P4VP nanotemplates of original spheres (left) and holes (right), showing the protein adsorption behavior on the ultrathin film on a large scale. AFM panels of 1 μm in diameter are obtained after depositing 0.1 μg ml⁻¹ (top row) and 0.5 μg ml⁻¹ (bottom row) protein solutions. (b) Zoomed-in, 60 nm × 60 nm, AFM topography of anti-IgG proteins decorating original micellar (top) and hole (bottom) templates. Height profiles along the inserted white lines are displayed on the right to clearly show the morphology of anti-IgG upon adsorption to each template.

subset of hole templates, instead of incompletely distributing among all available hole templates with only a few protein molecules per hole. This behavior is seen clearly in
Figure 15 which displays AFM panels acquired from a larger scan area. The original micelles in Figure 15(a) exhibit a repeat spacing and height profile of 50 nm and 2.43 nm, respectively. The width and height of a single anti-IgG protein adsorbed on top of the sphere are measured as 14.8 nm and 0.59 nm, respectively. The protein presents no net charge under the specified deposition condition. The Y-shaped IgG antibody is known to exhibit widths along the short and long axes and a height of 14.5 nm, 8.5 nm, 4.0 nm, respectively. The height of the observed IgG antibody is much smaller than expected due to the tapping of the Si tip onto the surface of the soft protein sample during AFM imaging. This observation is similar to previously reported AFM measurements. However, a strikingly different outcome is revealed when carrying out a line analysis of the same protein on the hole template. Anti-IgG proteins in Figure 15(b) adsorbed on the hole nanostructures, showing an average repeat spacing of 49 nm and depth of 2.5 nm, are measured to have a greatly reduced width and an increased height of 9.2 nm and 1.9 nm, respectively. This observation indicates that the anti-IgG proteins near the more hydrophilic chains of P4VP sit tighter on the adhesion area of the surface while pushing the protein domains upward and away from the polymer.

Further exposure of the hole template to the chloroform vapor for up to 9 hours yields reformed spheres, Figure 13(c). These structures are similar to the original templates but exhibit much shallower height variations within micelles. Anti-IgG proteins prefer to gather on the tops of spheres, Figure 14(c). However, no pronounced tendency to resist protein adsorption in the middle of the sphere is seen on the reformed spheres. This observation indicates that PS chains in the reformed spheres sweep back fully toward the center of the sphere when transitioning from holes to the reformed spheres.
This process ends up burying the unfavored P4VP chains deep into the film. On average, each reformed sphere accommodates three protein molecules on its surface.

The adsorption behavior of proteins on embedded spheres obtained by vapor annealing of the template for up to 16 hours shows two dominant adsorption pathways in the 0.1 \( \mu g/ml \) concentration regime, Figure 14(d). One adsorption route is for the protein molecules to engage in the center lobes of the embedded spheres. The other path is for the proteins to encircle around the outer edges of the embedded spheres. The surface-assembled proteins serve as a faithful indicator of the chemical phase difference of the underlying polymeric blocks of PS and P4VP. The protein molecules adsorb onto the embedded sphere at its center whose dominant surface phase is PS. At the same time, the nested sphere in the center is encircled by a circular band of depressed area where no proteins are found. The lack of protein adsorption in this area suggests that P4VP chains are either exposed to or lie very close to the air/polymer interface. In addition, the proteins tend to gather outside the depressed band regions, indicating that the surface areas outside of the circular bands are predominantly PS. Anti-IgG proteins show no strong preference to take one of the two available adsorption paths at this concentration as each case is represented by approximately half of the templates. Assembled proteins on the enlarged spheres created by annealing the polymeric template for up to 25 hours closely mimic the adsorption behavior observed on the reformed spheres. Proteins prefer to adsorb on top of the enlarged spheres at a deposition concentration of 0.1 \( \mu g/ml \), Figure 14(e).

The next chloroform annealing stage to 29 hours yields cylinder precursor templates whose individual nanostructures can be viewed as two concentric rings of
alternating PS (brighter rings in the AFM topography) and P4VP (darker depressed areas between the two rings in the AFM topography), see Figure 14(f). At the same protein concentration of 0.1 μg/mL, proteins gather on top of the two concentric rings, as shown in Figure 14(f). A striking adsorption behavior of the proteins is monitored on these cylinder precursors; the proteins tend to assemble in much higher density than what is observed from prior templates. This tendency is confirmed with repeated measurements, although the exact reason for the increase in protein density on cylinder precursors is not yet clear. From our earlier study, which was carried out on a series of polystyrene and polymethylmethacrylate homopolymers, blends, and diblock copolymers, it was demonstrated that the chemical heterogeneity of template surfaces plays a very important role in protein adsorption, especially with regards to achieving high protein density. Similar effects may be seen in our current study, where the density of adsorbed proteins is highest in the polymeric nanotemplates whose degree of chemical heterogeneity on surfaces is the largest: i.e. when using identical protein deposition conditions, protein density is the highest in the cylinder precursor templates amongst the various vapor-annealed PS-\(b\)-PVP templates. When comparing the most plausible polymeric chain compositions between the six templates used in our protein experiment, the heterogeneity in chemical composition of a given surface area is expected to be the highest in cylinder precursors. This may explain why the unusually high protein density is achieved on cylinder precursor templates.

At a higher protein concentration of 0.5 μg/ml, the proteins engage all available template nanostructures and reach close to a monolayer-forming surface coverage of the proteins. We define monolayer coverage as the surface coverage where all available and
preferred surface adsorption sites of PS are taken by proteins. Protein molecules adsorbed on the original micelles before any vapor annealing favor the PS domains on the spheres while avoiding the center surfaces of the micellar spheres at this concentration. This observation is identical to the protein adsorption behavior monitored at the lower concentration of 0.1 μg/ml. The resulting network of adsorbed proteins is formed by the complex link between protein bands on all available original micelles, where each band consists of four to five individual protein molecules, see Figure 16(a). Protein assembly on the hole template after deposition of the 0.5 μg ml⁻¹ solution also leads to the involvement of all existing nanotemplates. Every available PS area encircling the holes is engaged with proteins. The windmill patterns of the assembled proteins originating from each hole are monitored as in the previous case of the lower concentration, and, at this higher concentration, they are intricately interlaced with each other in Figure 16(b). On reformed spheres, proteins deposited at a concentration of 0.5 μg/ml fill all available areas on the tops of the spheres and, on some places of the nanotemplates, begin to spread out into the regions between the reformed spheres. This result is displayed in the AFM panel of Figure 16(c) as merged patches of various sizes and shapes, derived from the amalgamation of protein domains from neighboring templates of reformed spheres. No strong resistance against protein adsorption is seen in the center region of the reformed sphere, a phenomenon similar to that of the assembly on reformed spheres at the lower concentration. Proteins deposited on the embedded spheres from the 0.5 μg/ml solution primarily adsorb on the outside perimeter of the nanostructure. Five to six protein molecules circle around each embedded sphere in Figure 16(d). Unlike the assembly behavior of the lower concentration of deposited protein, no embedded spheres show IgG
Figure 16. AFM images displaying protein adsorption characteristics on each of the six polymeric PS-b-P4VP nanotemplates. The concentration of protein solution used is 0.5 μg/ml, which is approximately at the saturation coverage. The scan sizes of AFM panels displayed in the left and right columns are 500 nm × 500 nm and 140 nm × 140 nm, respectively.
proteins positioned on the nested sphere (center lobe) of the template and proteins preferentially adsorb to PS domains located outside the embedded spheres at this increased concentration. Proteins on enlarged spheres closely resemble the adsorption behavior of protein on the reformed spheres at the same concentration. At a deposition concentration of 0.5 μg/ml, proteins occupy all available sites on the tops of the enlarged spheres and they additionally start to fill up surface regions between enlarged spheres, see Figure 16(e). Assembled proteins on cylinder precursors at the same solution concentration Figure 16(f), pack all areas encircling the nanostructures as well as neighboring areas between nanostructures. This supramolecular assembly behavior leads to the formation of two concentric protein bands, which are connected together into large-scale protein networks, as shown in the zoomed-in AFM topography panel. The distinctively assembled protein structures described in this paper are observed consistently on the template substrate, whose typical size was kept as 0.5 × 0.5 cm² in our experiments.

The characteristic adsorption behavior of proteins on each polymeric nanotemplate displayed in Figures 14 through 16 occurs throughout the sample where the assembly of the proteins takes place instantaneously at large scale. The reproducibility of the unique protein assembly behavior is confirmed by repeated measurements on at least four sets of CHCl₃-annealed template series and the height/width data of the adsorbed proteins are obtained from analyzing at least 50 unit structures per template. High-magnification images are provided in Figure 14 through 16 in order to show both the configurations of the proteins and the underlying polymeric nanotemplates clearly. Fluorescence images Figures 17 (a) and (b) were then taken with a Zeiss A2M optical
Figure 17. 150 μm × 150 μm fluorescence panels obtained from self-assembled proteins on PS-b-P4VP templates and 1.5 μm × 1.5 μm AFM phase panels collected from DTAF-antiIgG molecules treated identically on the two homopolymer surfaces of P4VP and PS. (a) Fluorescence signal obtained from 0.5 μg/ml DTAF-antiIgG molecules adsorbed on the cylinder precursor PS-b-P4VP template. (b) Fluorescence signal from IgG/DTAF-antiIgG proteins interacting on the original PS-b-P4VP micellar template. A layer of 0.4 μg/ml IgG molecules was deposited onto the polymeric surface and a solution of 4 μg ml⁻¹ DTAF-antiIgG was subsequently added to the sample, allowing protein–protein interaction. Before the fluorescence measurement, the sample was rinsed with an ample amount of PBS to remove any unbound proteins after the reaction. When 0.5 μg/ml of DTAF-antiIgG was identically applied on the two homopolymer surfaces of (c) P4VP and (d) PS, the strong adsorption preference of the protein to PS over P4VP was confirmed from the much higher surface coverage of the proteins on PS homopolymer.
microscope in order to assess the functionalities of adsorbed proteins on PS-\textit{b}-P4VP templates. Due to the optical diffraction limit, the fluorescence signal from individual nanoscale templates cannot be resolved. However, the strong fluorescence signal combined with the AFM data indicates that a large portion of the protein molecules retain their bioactivity upon their surface adsorption onto the polymer film. More systematic and quantitative measurements are currently underway in order to investigate the degree of denaturation and biofunctionality of the proteins upon surface adsorption to all the aforementioned PS-\textit{b}-P4VP nanotemplates. When ultrathin homopolymer films of PS and P4VP are used instead of the diblock copolymer, the strong adsorption preference of the protein to PS is unambiguously determined by the significant differences in protein density on the two homopolymer films. The phase AFM panels displayed in Figures 17(c) and (d) show that DTAF-antiIgG density is much higher on the PS homopolymer film than on the P4VP homopolymer surface, even though identical biodeposition conditions are used to prepare the two samples.

3.4 Conclusion

In summary, we have determined the adsorption and assembly characteristics of anti-IgG on various PS-\textit{b}-P4VP templates, whose unique and intricate nanoscale structures are produced by time-dependent annealing of the polymeric surfaces under a selective solvent vapor. Strong adsorption preference of the proteins to certain segments in the diblock copolymer templates leads to an easily predictable, controllable, and rich set of nanoscale protein arrays through self-assembly. In our approach, a rich set of intricate nanoscale morphologies of protein arrays is successfully produced via self-
assembly of proteins on chemically treated diblock copolymer surfaces, without the use of clean-room-based fabrication tools. These morphologies cannot be easily accessed by the well-known microphase separation of block copolymers or any other lithographic means. Therefore, our new method can offer a high degree of flexibility and versatility for the use of block copolymer-based protein arrays in biodetection.

We also demonstrate that polymer compositions of hard-to-probe, sub-domain features within individual nanostructures can be readily elucidated by investigating the corresponding protein adsorption behavior. Due to the lack of available techniques simultaneously providing site-specific and high vertical and lateral resolution, correlating sub-nanometer scale topological details within individual nanostructures to their surface chemical compositions cannot be readily achieved. In our approach, the specific arrangement of polymeric chains and the chemical identities associated with each nanostructure can be easily determined by utilizing the high discriminating power of the proteins during surface adsorption. Immediate applications of these protein nanoarrays will most likely involve the use of currently available microsample handling and conventional detection apparatuses. In this case, a known number of protein molecules in a group of nanoscale micelles will serve as an independently addressable, quantitative microscale unit in a given protein array. However, ideal applications of our protein arrays should address each micelle as an independent nanoscale assay unit that can be further utilized for protein detection apparatuses with nanoscale detection resolution.

Together with the ease of fabrication in producing polymeric templates with well-defined nanoscale structures via self-assembly, the diverse arrays of proteins created using our approach can promote a high degree of versatility in creating high-density,
high-payload, protein arrays. These advantages can be extremely beneficial to both miniaturized and low-cost fundamental biology research as well as biomedical detection, especially in the areas of high-throughput protein sensing and screening.
Chapter Four: Distinct Adsorption Configurations and Self-Assembly

Characteristics of Fibrinogen on Chemically Uniform and Alternating Surfaces including Block Copolymer Nanodomains

Abstract

Understanding protein-surface interactions are crucial to solid-state biomedical applications whose functionality is directly correlated with the precise control of the adsorption configuration, surface packing, loading density, and bioactivity of protein molecules. Due to the small dimensions and highly amphiphilic nature of proteins, investigation of protein adsorption performed on nanoscale topology can shed light on protein subdomain interaction preferences. In this study, we examine the adsorption and assembly behavior of a highly elongated protein, fibrinogen, on both chemically uniform (as-is and buffered HF-treated SiO$_2$/Si, and homopolymers of polystyrene and polymethylmethacrylate) and varying (polystyrene-block-polymethylmethacrylate) surfaces. By focusing on high resolution imaging of individual protein molecules whose configurations are influenced by protein-surface rather than protein-protein interactions, fibrinogen conformations characteristic to each surface are identified and statistically analyzed for structural similarities and/or differences in key protein domains. By exploiting block copolymer nanodomains whose repeat distance is commensurate with the length of the individual protein, we determine that fibrinogen exhibits a more neutral tendency for interaction with both polystyrene and polymethylmethacrylate blocks relative to the case of common globular proteins. Factors affecting fibrinogen-polymer interactions are discussed in terms of hydrophobic and electrostatic interactions. 

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addition, assembly and packing attributes of fibrinogen are determined at different loading conditions. Primary orientations of fibrinogen and its rearrangements with respect to the underlying diblock nanodomains associated with different surface coverage are explained by pertinent protein interaction mechanisms. Based on two-dimensional stacking behavior, a protein assembly model is proposed for the formation of an extended fibrinogen network on the diblock copolymer.

4.1 A Brief Review of Fibrinogen Study with AFM

Human fibrinogen (Fg), as showed in Figure 18, is a protein with a highly elongated shape and composed of three interweaved polypeptide chains of Aα, Bβ and γ that are connected together by 29 disulfide bonds. The structures of the 340 kDa dimeric protein were first imaged by Electron Microscopy (EM), revealing the molecular length of 47.5 nm with roughly spherical D and E domains.107

Figure 18. Model of a fibrinogen molecule deduced from electron microscope imaging observations and molecular volume from physical chemical data. Remake from references107,108 without permissions.

Later, more complex depictions of Fg such as 47 nm-long heptanodular and octanodular models were reported based on EM and crystallographic observations.
In more recent years, Atomic Force Microscopy (AFM) operated in air/liquid has been extensively used to visualize directly Fg in less invasive sampling conditions involving a biological buffer with no need of staining, crystallization, or vacuum drying. The use of surface-sensitive AFM techniques has been demonstrated for structural investigations of Fg adsorbed on surfaces such as mica, highly oriented pyrolytic graphite, gold, and glass. In some of those cases, original substrates have been further treated with modifying layers such as silane, poly-l-lysine, and self-assembled monolayer compounds before Fg adsorption. AFM has also been extensively employed to obtain force-distance relationships of Fg interacting with various surfaces. In this chapter, I put my focus on using high resolution imaging to investigate surface-bound Fg’s conformational and orientational changes using AFM.

Although variations in the protein length, width, and height are reported depending on the underlying substrates and sampling conditions, a trinodular Fg configuration is predominantly reported in the aforementioned AFM studies. In contrast, the more complex Fg conformations previously reported as the hepta and octanodular models have rarely been identified via AFM and, therefore, their topographic profiles have not been systematically examined in previous AFM studies. Protein imaging parameters such as the size of AFM scan, the surface loading degree of Fg, and the choice of substrates for Fg adsorption can affect the successful identification of topographic details on the single protein and sub-protein level. High resolution structural imaging can be valuable in identifying new protein configurations and protein-surface interaction preferences. Therefore, more AFM efforts with the parameters optimized for imaging individual proteins are warranted to resolve fine topographic
features within a single protein. In addition, statistical analyses of the resolved features are needed for the meaningful comparison and correlation of newly observed protein configurations via AFM with the chemical/physical properties of the substrates.

One of the major biomedical significances of Fg pertains to its role in thrombosis and how it affects blood coagulation and adhesion of platelets to biomaterial surfaces. Polymers are increasingly employed as promising alternatives to existing metallic and ceramic biomaterials as they offer a wide variety of chemical structures and properties. In addition, the application of polymers as coating layers of existing biomaterials has been steadily growing to improve the biocompatibility of metals and ceramics. As such, Fg adsorption studies on polymers whose surfaces are seldom explored in previous AFM investigations of the plasma protein may be highly beneficial to the field of polymer-based biomaterial design and development.

4.2 Methodology for Studying Fg Surface Adsorption

Herein, we carried out topographical examinations to identify surface-dependent Fg conformations at the single protein and subprotein level. My efforts in this chapter focus on high-resolution structural investigations of individual Fg molecules whose configurations are influenced by protein-surface interactions rather than protein-protein interactions in most cases. I then obtained statistical data on Fg subdomain dimensions on both chemically uniform and alternating surfaces. Based on high-resolution AFM imaging, I described in detail the dominant configurations of Fg molecules on five different surfaces that are silicon- and polymer-based. I compared the preferred protein conformations on each surface based on protein-surface interaction mechanisms. Native
oxide-terminated silicon (n-SiO₂, n denoting for the native oxide) and buffered HF-treated silicon (m-SiO₂, m denoting for the modified oxide layer) are employed as atomically flat surfaces that provide chemically uniform environments for Fg. In addition, two types of widely employed bio-substrates, polystyrene (PS) and polymethylmethacrylate (PMMA), are used for Fg adsorption onto chemically uniform, polymeric platforms. Upon surface adsorption, Fg displays distinctively different configurations on these substrates and the subdomain profiles of the protein are systematically catalogued. Observed protein conformations are then discussed with regard to the trinodular and complex models of Fg taken from the EM and crystallographic results.

A diblock copolymer of polystyrene-block-polymethylmethacrylate (PS-b-PMMA) is chosen as a chemically alternating surface model. I exploited the unique physical and chemical properties of PS-b-PMMA nanodomains offering self-assembled, periodically repeating, chemical heterogeneity on the surface.⁹⁴,¹²⁸,¹²⁹ I resolved novel Fg adsorption and assembly behavior on the chemically alternating polymeric domains, that specifically present a length scale commensurate with the size of a single Fg. It also showed that Fg on PS-b-PMMA favors configurations similar to the hepta/octanodular models. In addition, specific adsorption preferences were identified between different subdomains within individual Fg molecules and particular polymeric blocks. The significance of our efforts lies in providing insights to preferential interactions between key Fg domains/chains to specific polymeric nanodomains that has never been observed before. We demonstrate that Fg adsorption behavior onto the PS-b-PMMA nanodomains is fairly different from the adsorption behavior of common globular proteins such as
immunoglobulin G and bovine serum albumin that were reported earlier on the same polymeric surface.\textsuperscript{49,95} Instead of exhibiting strongly biased and complete segregation to the PS block of PS-b-PMMA as seen for the globular proteins, Fg displays adsorption events more neutral to both blocks, involving not only PS but also PMMA phases at specific conditions. Fg assembly and orientation into large networks using intermediate and high protein concentration regimes tuned for mono- and multi-layer forming conditions on PS-b-PMMA. I further concluded the preferred surface packing configurations and the molecular orientation of Fg with respect to the polymer nanodomain axis under intermediate to high loading conditions.

4.3 Materials and Experiments

Silicon-based substrates of n-SiO\textsubscript{2} and m-SiO\textsubscript{2} are prepared by using Si wafers (resistivity < 1 \( \Omega \) cm, thickness: 0.017 inch) obtained from Silicon Quest International, Inc (San Jose, CA). The surface of n-SiO\textsubscript{2} contains a natively formed oxide layer on top of Si and is used as is after cleaning with deionized water (DI) and ethanol. The surface of m-SiO\textsubscript{2} is prepared by immersing the pre-cleaned substrate in a buffered HF etching medium (6:1 volume ratio of 40% NH\textsubscript{4}F : 49% HF in deionized water) for 2 min and then rinsing with an ample amount of DI. The substrates are then dried under a gentle stream of N\textsubscript{2} before use. Polymer-based substrates of PS, PMMA, and PS-b-PMMA are prepared from the homopolymer and diblock copolymer granules received from Polymer Source Inc. (Montreal, Canada). The average molecular weight of PS, PMMA, and PS-b-PMMA (71% PS by weight) is 152 kDa, 120 kDa and 71.4 kDa, respectively, and their polydispersity is 1.06. Both chemically uniform (PS and PMMA homopolymers) and
alternating (PS-b-PMMA diblock copolymer) surfaces are made by spin coating 2 % (w/v) polymeric solutions at 3500 rpm for 1 min on Si which is pre-cleaned with a series of solvents using ethanol, acetone, and toluene. Phase separation of PS-b-PMMA is subsequently achieved via thermal annealing in an Ar atmosphere at 240 °C for 6 hours with a transient ramp-up rate of 5 K/min and a cooling rate of 2 K/min. This process yields periodically alternating and chemically varying nanodomains which are known as half-cylinders and exposes repeating stripes of PS and PMMA blocks at the air/polymer interface with repeat units of 45 nm (PS to PS distance). Fg, human plasma fibrinogen, is purchased from VWR Scientific Inc. (West Chester, PA). The lyophilized powder of Fg is reconstituted in PBS buffer (10 mM mixture of Na2HPO4 and NaH2PO4, 140 mM NaCl, 3 mM KCl, pH 7.4) and diluted to varying concentrations ranging from 5 to 1000 μg/mL. A volume of 10 to 100 μL of Fg solution is applied to the desired substrate and placed in a humidity-controlled chamber for time spans of 20 sec to 5 min. The deposition conditions such as protein concentration and incubation time are controlled to vary the surface density of adsorbed proteins from sparsely to densely populated cases, as needed. The sample surfaces are then carefully rinsed with 40ml PBS multiple times, followed by a thorough rinsing with DI and a gentle drying under a stream of N2 before protein characterization. Water contact angles of all substrates are measured using a Tantec contact angle meter (Chemsultants International, Inc, Fairfield, OH) with a half-angle method prior to protein deposition. Surface roughness of the substrates prior to protein deposition and characteristic Fg conformations on different surfaces are profiled and analyzed by performing high resolution AFM imaging focusing on individual Fg molecules. Topography and phase scans are carried out with a MultiMode 8 AFM
interfaced with a Nanoscope V controller (Bruker Corp., Santa Barbara, CA), operated in a soft tapping mode at a scan speed of 1 Hz or lower using silicon tips with a typical resonant frequency of 60-70 kHz and a spring constant of 1-5 N/m. The statistical data for the height, diameter, and length of the key subdomains in Fg are obtained by measuring the topographic profiles of approximately 100 protein molecules on each of different surfaces.

4.4 Results and Discussions: Chemically Homogeneous Surface

AFM data presented in Figures 19(A) through 19(D) display substrate-dependent, conformational variations of Fg upon adsorption onto the chemically uniform surfaces of n-SiO2 (Figure 19(A)), m-SiO2 (Figure 19(B)), PS (Figure 19(C)), and PMMA (Figure 19(D)). 20μg/ml Fg is used for 3 min deposition onto the two silicon-based surfaces whereas 5μg/ml of Fg is deposited for 5 min on the two homopolymer-based surfaces. Figures 19(E) and 19(F) are schematic representations of Fg based on what have been reported earlier in the literature.108 The two commonly discussed Fg models are depicted as the trinodular model in Figure 19(E) and the complex model in Figure 19(F). In the trinodular model portrayed in Figure 19(E), Fg consists as three spherical nodules embodying the two D as well as the E domains, and the spherical nodules are linked by coiled-coil connectors.1 The middle nodule of E is smaller in size than those of D at the ends. The connector cable is reported to be 0.8 – 1.5 nm in diameter and 16 nm in length.107 In the complex model outlined in Figure 19(F), Fg displays heptanodular domains. In this case, each D domain is divided into two lobes stemming from the independent folding of β and γ chains that are shaped into the βC and γC lobes,
respectively.$^{109,110}$ A small, non-helical globular domain, serving as a binding site to plasmin, is also included in each coiled-coil arm of the complex model. The $\alpha$ chain is longer than the $\beta$ and $\gamma$ chains and extends towards the center of the molecule from the $\beta$C and $\gamma$C lobes, forming a globular $\alpha$C domain at its C-terminus. Experimental conditions such as pH and certain ion species can affect the degree of the physical separation between the C-termini of the $\alpha$ chains and the E domain via the formation of disulfide bonds between the two.$^{108}$

From all Fg on n-SiO$_2$ in our AFM measurements, three distinct nodes encompassing the D and E domains are unambiguously observed, see Figure 19(A). The overall adsorption configuration resembles the trinodular model rather than the complex model. However, no connector cables are visible in our measurements between the three nodules of Fg on n-SiO$_2$. This is similar to many previous AFM results reporting similar trinodular Fg shapes on different surfaces.$^{130}$ The two models with connector cables in Figures 19(E) and 19(F) are based on EM observations. For EM inspections carried out under vacuum, Fg plates are typically prepared on substrates such as thin carbon films using a high pressure spray gun containing Fg in a volatile buffer mixed with glycerol. Subsequently, metal (Pt, Pd, or W) shadowing of the protein is carried out in a vacuum evaporator to increase contrast in electron density. On the other hand, Fg samples for AFM imaging under ambient conditions are usually prepared in physiologically relevant buffers. While these differences in protein sample preparation may result in the morphological disparity involving the connector cable regions observed by AFM and EM, the expected conformations of surface-confined Fg in actual biomaterial applications will mimic what are observed by AFM more closely.
Figure 19. 500 x 500 nm topographic AFM panels clearly displaying different morphologies of adsorbed Fg on the chemically uniform surface of (A) n-SiO₂, (B) m-SiO₂, (C) PS homopolymer, and (D) PMMA homopolymer. Insets show a 90 nm x 90 nm view of an individual Fg molecule on each surface. (E and F) Schematic drawings showing the structure of Fg based on (E) the trinodular model and (F) a more complex model.

Adsorption onto m-SiO₂ leads to complete unraveling of protein chains for the majority of the surface-bound Fg molecules. Remnants of denatured Fg are frequently observed on m-SiO₂ surface and the typical topographic panels of completely unraveled Fg are indicated by a white arrow in Figure 19(B). Buffered HF treatment used to prepare m-SiO₂ surface can result in partial fluorination of silicon atoms by forming
(NH₄)₂SiF₆, and it is well known that Fg adsorption onto fluorinated polymeric surfaces is greatly discouraged. The AFM observation of Fg on SiO₂-based surface agrees with these indications of fluorinated surfaces resisting protein adsorption. The number of adsorbed Fg is significantly reduced on m-SiO₂ to approximately 5% of those found on n-SiO₂. For the intact Fg population, only the nodules centering at the D domains are observed on m-SiO₂. The two D nodules of the intact Fg are connected by a thin connecting thread. In contrast to Fg on n-SiO₂, virtually no node is identified around the E domain, rendering a binodal configuration, Figure 19(B). The binodal shape may be an indication of the denaturation process prone to Fg on m-SiO₂. The swollen nodal features of the D domains may be caused by this unraveling of the protein chains. Further chain unfolding may lead to complete denaturing of one or both D domain. The latter is evidenced by a small population of Fg molecules on m-SiO₂ that are intact with only one of the two D domains accompanied by a short tail of thin lines. A typical example of Fg undergoing this chain unraveling process is marked with an arrow in Figure 19(B).

Fg on PS homopolymer in Figure 19(C) reveals a more elongated shape with 13 ± 0.5% longer than the average length of Fg on PMMA. Fg on PS exhibits a larger number of smaller nodes than what are observed on other chemically uniform surfaces. Both the nodes at the D and E positions are much narrower and thinner in size than those measured on n-SiO₂, m-SiO₂, and PMMA. Connector cables between different nodes are clearly identified from Fg on PS. The elongated conformation and the frequent appearance of small nodes may be attributed to the presence of the αC chains/nodules and plasmin-sensitive sites, similar to what are depicted in the complex model. The typical Fg
configuration on the other chemically uniform PMMA surface is shown in Figure 19(D). Fg chooses quite different conformations on PMMA when compared to those on PS and the trinodular conformation is observed from the majority of Fg population on PMMA, resembling the protein configuration on SiO₂. Similar to those on n-SiO₂, no connector threads between the three nodes are observed from Fg on PMMA. However, when comparing the same protein domains between the two surfaces, the spherical nodules around the D and E domains become smaller in size on PMMA than those on SiO₂. At the same time, the domain size difference between the D and E is larger on n-SiO₂ than that measured between the two domains on PMMA. To recap the preferred conformations of adsorbed Fg on the chemically uniform surfaces, the protein molecules display a binodal configuration on m-SiO₂, a trinodal shape on n-SiO₂ and PMMA, and a more complex configuration on PS. The amount of Fg on the four chemically uniform surfaces is found to be the highest to lowest in the order of PS > PMMA > n-SiO₂ > m-SiO₂.

### 4.5 Results and Discussions: Chemically Heterogeneous Nanostructured Surface

In order to elucidate the differences in Fg configurations on polymeric surfaces with nanoscale chemical homogeneity, Fg adsorption behavior are further extensively investigated on the PS-b-PMMA nanodomains. PS-b-PMMA diblock copolymer nanodomains form upon thermal annealing above its glass transition temperature in an Ar gas atmosphere. This phase separation process results in the self-assembly of a chemically alternating, striped surface of PS and PMMA nanodomains, whose surface structures mimic fingerprint patterns. The nanodomains exhibit a repeat spacing of 45 nm
Figure 20. AFM topography images of Fg on PS-b-PMMA nanostructures. (A) 500 nm x 500 nm AFM images display overlaid views of topography and phase scans to show clearly the morphology of adsorbed Fg as well as the underlying PS-b-PMMA diblock copolymer nanodomains. In all panels, the lighter (yellow) and darker (orange) domains of the underlying polymeric template are composed of PMMA and PS blocks, respectively. Upon surface adsorption, Fg favors two distinctive orientations of SP and TP. Individual Fg molecules are grouped into two sets based on their surface adsorption configuration and marked with black and white dotted circles for SP and TP, respectively. (B) 160 nm x 160 nm magnified views of the two dominant orientations of Fg after their adsorption onto PS-b-PMMA. Typical examples of TP and SP adsorption cases are presented in the left and right panel, respectively. (C) Fg configurations typically observed in the TP and SP adsorption cases are depicted in the cartoon. For SP, the main axis of Fg takes either of the two orientations, parallel and perpendicular with respect to the long axis of the PS domain. They are further categorized as $SP_{\parallel}$ and $SP_{\perp}$ in the diagram. (D) Net charges of key Fg domains under physiological conditions are shown.

while presenting both polymer blocks to the air/polymer interface. This repeat distance is measured along the short axis of the patterns from PS to PS nanodomains (or PMMA to
PMMA) and commensurate with the length scale of individual Fg molecules. The persistence length scale of the nanostripes along the long axis of nanodomains is related to the domain curvature which is much larger than the length of a single Fg. A low Fg concentration of 5μg/ml is deposited for 20 sec on PS-b-PMMA nanodomains. The highest amount of Fg is found on PS-b-PMMA among the five substrates used in this study.

On the periodically repeating polymeric nanotemplates consisting of PS (darker areas in AFM panels) and PMMA (lighter areas) blocks, two orientations are dominantly adopted by Fg as displayed in Figure 20. Adsorption events of the two dominant arrangements on PS-b-PMMA are profiled inside the white and black dotted circles in the overlaid topography and phase AFM images of Figure 20(A). The first adsorption case groups Fg molecules with the main (D-E-D) axis oriented perpendicular to the long axis of the underlying, striped nanodomains. In this case, the protein adsorption engages both PMMA and PS phases. The second adsorption case yields Fg molecules with the main axis oriented within a single polymeric phase of the PS nanodomain. We define these two Fg adsorption conformations as two phase (TP) and single phase (SP) orientations. The zoomed-in AFM data in Figure 20(B) clearly display individual Fg molecules taking the TP and SP orientations in the left and right panels, respectively. The ratio of the adsorption frequencies between the two orientations is approximately TP:SP = 40% : 60%.

The adsorption characteristics of the elongated Fg, especially in the TP orientation, are quite different from what was previously observed from globular proteins on the same types of polymeric surfaces. We and others have previously reported strong adsorption
preferences of proteins such as immunoglobulin G (IgG), bovine serum albumin (BSA), fibronectin (Fn), horseradish peroxidase (HRP), mushroom tyrosinase (MT), and protein G (PG) on the PS block. In the earlier studies carried out by Kumar et al. and Lau et al., their adsorption onto PS-b-PMMA nanodomains occurs exclusively to the PS domains and the PMMA domains are left completely devoid of the proteins below a monolayer surface coverage. In contrast to the strongly biased adsorption of the globular proteins, adsorption of Fg on PS-b-PMMA tends to be shared by the PS and PMMA blocks.

On the diblock copolymer nanodomains, Fg exhibits an elongated length along its main axis and small multiple nodes on its body, signifying the presence of αC chains. In both TP and SP, Fg adsorption onto PS-b-PMMA nanodomains leads to configurations analogous to the complex model of Figure 19(F), yielding results similar to the PS case. In TP, the center E domain of Fg lies in the middle of a PMMA domain, positioning the protein main axis vertical to the polymer nanodomain direction. The two D domains at the protein ends are found in the neighboring PS domains. In this adsorption geometry, αC chains are kept spread apart from the center E domain rather than folding towards it. The C-termini of the αC chains are placed near the PMMA domains closest to either end of the protein. In SP, the center E domain is located at an interfacial region of PS:PMMA, instead of the middle of a PMMA domain as found in the TP case. In SP, the protein adapts a bow-shaped curvature while keeping a large section of the Fg main body aligned along the long axis of the underlying nanodomain at the PS:PMMA interface. At the same time, a portion or the entire αC chain folds back towards the protein center in SP, instead of spreading away from the main axis of the protein as in TP. This folding of αC
chain proteins on both ends in SP leads to fuller topographic profiles along the main body of Fg than in the TP case. The bent configurations of Fg in both TP (the bend at the αC chains/domains) and SP (the bow-shaped curvature) are similar to EM and X-ray results in that the subdomains of Fg are not located collinearly and a small bend near the two distal end domains gives a better fit than the colinear model in simulating those experimental data.110

It is not yet clear what factors control the TP and SP orientations of Fg upon adsorption onto PS-b-PMMA. When the protein deposition conditions such as the pH, concentration, and the presence of ions are kept the same in our experimental conditions, factors related to the underlying polymeric templates may dictate the occurrence likelihood of the two orientations rather than those associated with proteins. Fg with the TP orientation tends to appear more on the nanostripe areas of high curvatures as shown in the left panel of Figure 20(A), whereas the SP orientation in the right panel of Figure 20(A) is preferred by regions with straighter nanodomains. In addition, it has been observed that the SP orientation is favored as the protein concentration is increased. The concentration-dependent adsorption behavior will be discussed later in detail with regard to self-assembly of Fg molecules into a large network.

All our AFM data presented in this paper were collected in ambient conditions after gentle N₂ drying of the proteins. When AFM is taking images in air, the configurations and conformations of the proteins observed in this study will closely mimic the conditions of proteins spotted onto polymeric surfaces of protein microarrays/chips and the environments of proteins linked to silicon-based surfaces in lab-on-a-chip protein detection devices. Therefore, our efforts can be beneficial in
developing next-generation nanoprotein arrays and solid-state protein detection devices while providing fundamental insight onto nanoscale adsorption behavior of proteins. It is not clear how proteins adsorb from the solution phase to the surface phase of different polymeric blocks. In buffer, proteins will expose more of their hydrophilic regions to the exterior while burying hydrophobic cores inside. Hence, the initial adsorption of proteins onto PS-b-PMMA is likely to be on the PMMA domains followed by migration to PS. This migration may happen sometime after the initial adsorption while still in buffer or during the drying process. When considering the typical translational diffusion coefficient of Fg $2 \times 10^{-7}$ cm$^2$/sec (in water at 37 °C), Fg can move the characteristic repeat distance of the underlying PS-b-PMMA domains (45 nm) approximately in 20 msec. Although the timescale of Fg adsorption onto the polymeric surfaces is determined to be relatively fast in our experiments as several tens of seconds to several minutes, the diffusion-based timescale provides enough opportunities for Fg to adsorb and migrate to the nearby polymeric nanodomains of more preferred interactions after protein chain unfolding. Work to elucidate the extended process of solution to surface adsorption and rates of Fg adsorption/desorption is currently under progress.

Driving forces such as van der Waals (dispersion), electrostatic, hydrogen bonding, and hydrophobic/hydrophilic interactions can affect protein adsorption onto surfaces. Table 2 lists water contact angle values for the five substrates used for Fg adsorption to compare their hydrophobicity characteristics. Our group members have previously reported the important role of hydrophobic/hydrophilic interactions in various adsorption cases of globular proteins onto polymeric surfaces by carrying out control experiments to rule out the effect of the other forces.
Table 2. Water contact angle and surface roughness values of the five substrates assessed for Fg adsorption. Ra and Rq correspond to the average roughness and root mean square average roughness, respectively. a) A network of thin SiO₂ is formed natively on top of a Si wafer. b) Buffered HF disrupts the extended network of tetrahedral Si(-O)₄ network in the SiO₂ layer and forms (NH₄)₂SiF₆.131 c) The pI value is from a direct HF treatment139 (dipped into a 1:50 HF:deionized water (DI) solution for 15 sec followed by a 1 min DI rinse) instead of a buffered HF etch.

Similar to the case of globular proteins, hydrophobicity of the underlying substrate seems to play an important role in the adsorption of the highly anisotropically shaped Fg. In our combined AFM and contact angle analysis, Fg conformations analogous to the trinodular and complex model are expected on surfaces with lower and higher hydrophobicity, respectively. This tendency agrees with the general trend for Fg conformations reported previously in different hydrophobic and hydrophilic systems. For example, when H₂SO₄/H₂O₂-treated SiO₂ and muscovite mica were used as substrates, trinodular Fg conformations similar to what we have observed on n-SiO₂ and PMMA were detected.130 On the other hand, on a titanium oxide surface with a water contact angle of greater than 85°, a more complex morphology of Fg similar to what we have identified on PS and on PS-b-PMMA was reported.140
From the adsorption results gathered in the TP case, interaction preferences of each protein subdomain in a single Fg molecule can be deduced with respect to the hydrophobicity of the two polymeric blocks in PS-b-PMMA. Two factors in the TP arrangement permit the direct correlation of interaction preferences between the protein subdomains and the polymeric blocks in PS-b-PMMA. The first is contributed by the nanoscale diblock copolymer template with chemical heterogeneity on the size scale comparable to the protein length. The second factor is attributed to the fully elongated $\alpha$C-D-E-D-$\alpha$C conformation of Fg in TP with minimized folding or collapsing of protein chains onto themselves. This unique configuration enables an unambiguous identification of each protein subdomain and its position with respect to PS and PMMA. Our AFM results of Fg in TP indicate that E domains favor the hydrophilic PMMA, whereas D domains prefer the more hydrophobic PS. They also suggest that the plasmin-sensitive domains prefer the amphiphilic interfacial areas defined by PS:PMMA and the bulk of the $\alpha$C chains favors the PS block whereas the $\alpha$C domains show a preference to PMMA.

The isoelectric points of PS and PMMA are 3.5-5 and 2-4, respectively. At the Fg adsorption condition of pH = 7.4, both polymer surfaces have negative charges. PMMA blocks will exhibit more negative character than PS blocks due to the lower pI values. The net charges of each protein subdomain under this pH condition are provided in Figure 20(D). The experimental observations made in the TP case of Fg can be reasoned from the electrostatic interaction point of view. When considering the D and E domains of Fg, PMMA blocks will be preferred by the E domains as they exhibit a less net negative charge (-2 for the E domain versus -4 for the D domain). Between the $\alpha$C chains and the main body of Fg, the negatively charged PS domain will tolerate $\alpha$C.
chains displaying net positive charges more than the negatively charged main body of the protein. These tendencies agree with the protein configuration in TP where the E domain lies in the middle of PMMA while the αC arms are located in PS. The bow shaped Fg configuration in SP may be also reasoned from electrostatic interactions. When αC chains are folded onto the main body of the protein as in the SP arrangement, the overlapping of protein chains will lead to partial cancellation of the net negative charges of the Fg main body by the net positive charges of the αC chains. Specifically, the net negative charges on the D domain (-4) and the coiled-coil region (-3) will be offset mainly by the net positive charge of the αC chain (+1). On the other hand, the E domain can cancel out their charges more effectively than the D domain via its domain-overlapping with the C-terminus of the αC chain (-2 and +2, respectively). In this case, the amphiphilic PS:PMMA interfacial regions may be able to attract the charge-neutralized Fg domains effectively. The characteristic shape of Fg molecules commonly observed in the SP configuration may stem from the Fg adsorption preference onto the PS:PMMA interfacial regions.

4.6 Results and Discussions: Statistical Analysis of Fg Morphologies

The surface-dependent morphological variations of Fg are catalogued by performing statistical AFM line analysis of the key domains on approximately 100 individual Fg molecules adsorbed on each surface. The average surface roughness values of the underlying substrates measured before protein deposition are provided in Table 2. The AFM roughness measurements indicate that silicon-based surfaces (roughness less than 2 Å) are slightly smoother than the polymer-based platforms (2-4 Å). Figure 21
displays the protein height and diameter histograms of the D and E domains as well as the histograms for the D-E-D domain length. The average values of the key Fg domains are summarized along with their standard deviations in Figure 22. The reported D-E-D length in Figures 21 and 22 does not include that of αC chains. The height of the D domain is found to be the highest on m-SiO$_2$ whereas the diameters of both the D and E domains are the largest on n-SiO$_2$. Between polymeric surfaces, the heights of the D and E domains are measured to be similar to one another when comparing the topological profiles of the same Fg domains. When not including αC chains, the protein length is longer on the silicon-based surfaces than on polymeric surfaces. However, when the full
length of Fg is considered including the extended $\alpha$C chains, the most elongated conformation of Fg is found on PS and the second longest on PS-b-PMMA.

![Table and Figure 22]

**Figure 22.** Summary of the AFM section analysis based on the Fg adsorption behavior on the five surfaces. D-h, E-h, D-d, E-d, and D-E-D-l denote the height of the D domain, the height of the E domain, the diameter of the D domain, the diameter of the E domain, and the length spanning the D-E-D domains, respectively. The length of the $\alpha$C domains is not included in the reported D-E-D-l values. The last column provides cartoons of Fg molecules on each surface portraying the characteristic topological features identified by AFM.

As discussed earlier regarding hydrophobic protein interaction mechanisms, the statistical length data in Figures 21 and 22 indicate that the more elongated Fg configurations are expected on polymeric surfaces with a greater hydrophobic character. A body of earlier work by Wertz et. al. has reported extended Fg footprints on a hydrophobic than hydrophilic surface.\textsuperscript{143–145} When bovine Fg adsorption was studied in these efforts via total internal reflectance fluorescence on OH- and C16-surfaces with
their respective contact angle values of 54-56° and 109-111°, it was also found that the Fg footprint was larger on the more hydrophobic surface. While these studies provided valuable insights into the adsorption behaviors of Fg molecules as an ensemble on hydrophobic versus hydrophilic surfaces, our AFM results complemented these earlier work from the adsorption perspective of individual Fg molecules on nanoscale surfaces.

4.7 Results and Discussions: Nanostructured Surface at Higher Concentration

In addition to Fg adsorption at the concentration yielding a low protein number density on the surface of PS-b-PMMA as seen in Figure 20, surface assembly characteristics of the protein in intermediate and high concentration regimes are also examined. Under the deposition condition of 20 sec with an intermediate concentration of 20 μg/ml, SP arrangements of Fg dominate the adsorption events on PS-b-PMMA as displayed in Figure 23. The majority of the protein molecules under this condition are confined to the PS regions of PS-b-PMMA, and TP adsorption events involving both PMMA and PS domains are significantly reduced. Fg molecules in the SP case can exhibit either a perpendicular (SP⊥) or parallel (SP∥) orientation on the PS phase with respect to the long axis of the polymeric nanodomains. Typical Fg configurations observed from the two cases of SP⊥ and SP∥ are depicted in the diagram shown in Figure 19(C). The frequency ratio between the TP : SP adsorption events is 25% : 75%. The occurrence ratio between the perpendicular and parallel orientations in SP is SP⊥ : SP∥ = 20% : 80%.

When increasing the Fg concentration further to 50 μg/ml for the same deposition time, the SP adsorption event shows the same growing trend over TP and the frequency
of the perpendicular orientation rises among Fg molecules taking the $SP$ configuration.

Figure 23. Adsorption behavior of Fg molecules observed on PS-b-PMMA nanodomains at an intermediate protein concentration of 20 μg/ml. (A and B) 1 μm x 1μm AFM topography and phase images are displayed in panels (A) and (B), respectively. (C and D) 200 nm x 200 nm topography and phase channels in (C) and (D) show the preferred $SP$ arrangement of Fg over the $TP$ configuration at this concentration.

At this concentration, the ratio between $TP$: $SP$ changes to 20% : 80% while the occurrence ratio between the perpendicular and parallel orientations in $SP$ switches to $SP\perp : SP\parallel = 35% : 65%$. The exact source of the preferred $SP$ adsorption to $TP$ under the increased protein concentration still needs to be determined. It is likely that $SP$ arrangements may be due to more dominant influence of the hydrophobic interactions between Fg and PS over the electrostatic interactions associated with the net charges of Fg subdomains and the PS and PMMA phases. For systems involving a larger number of proteins, the free energy of the surface-bound proteins can be effectively lowered by driving many protein molecules to the more hydrophobic phase of PS than to PMMA,
although such processes may result in electrostatic energy penalties. When taking both the protein and surface into consideration, the electrostatic penalty in $SP$ is due to the tendency for $\alpha C$ domains/chains to prefer PMMA to PS. However, from the electrostatic considerations of solely the protein not including the polymeric substrate, Fg assembled in $SP$ configuration can minimize the electrostatic repulsions from different protein subdomains more effectively than $TP$. In $SP$, the net negative charges along the main body of Fg are neutralized by the net positive charges of the $\alpha C$ domains placed spatially in close proximity. A more neutralized net charge of Fg can be realized in the $\alpha C$ arm-folded $SP$. When the number of adsorbed proteins increases on PS-b-PMMA, this minimization of the electrostatic repulsion between Fg molecules themselves in $SP$ may enable dense assembly and packing of Fg molecules close to one another on the surface.

A full surface coverage of Fg on all available PS domains of PS-b-PMMA is obtained when the protein concentration is further increased to 100 $\mu$g/ml. We defined this surface loading condition as the monolayer-forming state. Assembled protein patterns under this condition are shown in the topography and phase AFM images in Figure 24 which faithfully reproduce the shape of the underlying polymeric nanodomains. From the physical size of Fg and the available PS area in PS-b-PMMA, we determined that this protein deposition condition yields a loading density of 0.94 mg/m$^2$. With resolved discernable individual Fg molecules at this surface coverage, several interesting observations are made from the self-assembled network of Fg that are different from the previously reported, monolayer-forming patterns of the globular protein, IgG. Two IgG proteins tend to pack side by side along the minor axis of the polymeric nanodomain on PS. In contrast, the monolayer patterns of Fg on PS-b-PMMA contain lobes that usually
Figure 24. Extended Fg self-assembly into monolayer-forming patterns when depositing 100 μg/ml Fg for 20 sec on PS-b-PMMA. AFM images of (A) 1 μm x 1 μm topography, (B) 1 μm x 1 μm phase, (C) 400 nm x 400 nm topography, and (D) 200 nm x 200 nm topography show the packing configuration of self-arranged Fg which faithfully reproduces the underlying PS-b-PMMA nanodomain patterns.

appear as triplets along the short axis of the nanodomain. In addition, periodically spaced thin lines spanning over PMMA domains are identified. The lines connect two closest Fg lobes, each lying on different PS domains. Such structures that have not been observed before in the globular protein self-assembly are discussed in detail in the following section.

Figure 25(A) showed a contrast-adjusted topography image of Fg triplets packing on the PS domains. Three distinctive regions of lobes are observed parallel to the long axis of the polymeric nanodomain, forming a chain of Fg lobes in each region. These areas of chained lobes are marked with dashed white lines in Figure 25(A). The assembly pattern implies that the close surface packing of the bow-shaped Fg molecules is favored
Figure 25. High AFM images of Fg assembly at the monolayer-forming condition. (A) The 90 nm x 90 nm AFM topographic image displays the appearance of Fg lobes on PS domains in three distinctive strips which run parallel to the long axis of the polymeric nanodomain. The brightness and contrast of the image are modified so that only the lobes on PS domains are visible in the frame. Dashed white lines are added to the image in order to classify the three chains of lobes. (B and C) Linker lines connecting Fg lobes on different PS domains are shown in the 2D topography images with the scan size of (B) 150 nm x 150 nm and (C) 90 nm x 90 nm. The image contrast is restored to show the topological features on all area of the surface. (D) A 90 nm x 90 nm 3D topography profile from the same area in the 2D view of panel (C) shows the linker lines on PMMA. To guide the eye, each traversing line on PMMA is highlighted with the inserted white arrow. (E) AFM section analysis along the white dotted line in panel (C) indicates that the periodicity of the linker lines bridging Fg molecules between two neighboring PS domains is approximately 18.8 nm. (F) A plausible Fg assembly model on PS-b-PMMA nanodomains is hypothesized based on the appearance of the triplet lobes and linker lines. The model displays a close-packing geometry of bow-shaped Fg molecules in perpendicular rather than parallel orientation with respect to the long axis of the polymeric nanodomain.
in perpendicular rather than parallel orientation with respect to the long axis of the PS-b-PMMA nanodomain. When the AFM image contrast is restored to show topological features appearing on both polymeric domains, a new topological feature of thin lines traversing over the PMMA domains is clearly visible as displayed in Figure 25(B). Fg lobes located on the PS domains on either side of the PMMA are connected via these lines. The magnified 2D and 3D images of Figures 25(C) and 25(D) display the periodically appearing linkers. They may be attributed to the \( \alpha C \) chains donated from each side by a pair of Fg molecules, each Fg lying on two neighboring PS domains. Packed this way on PS-b-PMMA, the free energy of surface-bound Fg may benefit from both standpoints of hydrophobic and electrostatic interactions. The main bodies of packed protein molecules are located on the PS domains for maximizing hydrophobic interactions. In addition, \( \alpha C \) chains from each Fg pair are unfolded away from the main body of Fg and placed over the PMMA domains, which is more electrostatically favored than having the \( \alpha C \) chains folded on PS. AFM section measurement is performed on the periodic linker lines marked with a white dashed line in Figure 25(C) and a series of white arrows in Figure 25(D). The results in Figure 25(E) reveal that the linker lines exhibit a repeat spacing of 18.8 nm. The characteristic Fg assembling patterns on PS-b-PMMA may be explained by a surface packing model hypothesized in the schematic representation shown in Figure 25(F). The suggested model is based on our AFM topographic results exhibiting the typical appearance of triplet lobes along the short axis of the PS nanodomain. The two close-packing scenarios in the parallel and perpendicular orientations of bow-shaped Fg molecules can lead to two and three chains of lobes along the long axis of the polymer nanodomain, respectively. In the assembly model suggested
in Figure 25(F), the bow-shaped Fg molecules surface-pack on the PS domains via the latter scenario and form a stack of bows whose major protein axis is oriented perpendicular to the long axis of the polymeric nanodomain. The linker lines are established between the D domains of Fg on a PS domain and other closely located D domains from a different Fg on a neighboring PS domain.

Figure 26 displays topography panels of surface-bound Fg on the three polymeric surfaces of PS, PMMA, and PS-b-PMMA when Fg concentrations of 1000 μg/ml for PS and PMMA and 500 μg/ml for PS-b-PMMA are used for 20 sec deposition. We estimate that the respective Fg deposition conditions lead to the protein surface density of 1.6 mg/m², 1.2 mg/m², and 1.8 mg/m² on PS, PMMA, and PS-b-PMMA, respectively. Figures 26(A) and 26(B) show typical topographic panels of Fg adsorbed on PS and PMMA homopolymer, respectively, whereas Figures 26(C) and 26(D) are the adsorption results on the PS-b-PMMA nanodomains. The number density of surface-bound proteins is highest to lowest in the order of PS-b-PMMA, PS, and PMMA similar to the surface density order reported for the globular protein of IgG. Fg forms a dense network on the PS-b-PMMA surface and the striped patterns defined by the underlying polymeric nanodomains are no longer clearly identifiable in Figure 26(C). Bigger grains and interlacing strings appear on the entire surface at this concentration, instead of the smaller lobes seen in Figure 25. It is likely that the grains are formed by a cluster of layered lobes. As profiled in Figure 26(E), AFM line analysis on these grains in the two directions of the blue and red arrows marked in Figure 26(D) shows a shorter and longer, periodic spacing of 20 nm and 40 nm, respectively. These repeat distances are correlated with the intermolecular distances of Fg in two orthogonal directions in the assembly.
Figure 26. Fg adsorptions on PS, PMMA and PS-b-PMMA at higher concentration. (A and B) Fg adsorption behavior is shown in the 350 nm x 350 nm AFM topography panels when a higher protein concentration of 1000 μg/ml is used onto (A) PS and (B) PMMA homopolymer surfaces. (C through F) Fg adsorption morphology on PS-b-PMMA is shown in the (C) 180 nm x 180 nm and (D) 90 nm x 90 nm AFM topography panels when using a 500 μg/ml protein solution. (E) Although the underlying stripe patterns of the diblock copolymer nanodomains are no longer clearly visible under these conditions, the height profiles of the assembled Fg display distinctively periodic spacings in two directions. Results from the height analysis along the blue and red lines in panel (D) are shown. (F) The repeat distances along the red and blue lines are correlated with the intermolecular spacing between relevant protein domains using the same Fg surface assembly model shown in Figure 25(F).

model presented in Figure 26(F). In this model, the short repeat distance is attributed to the spacing between two Fg molecules adsorbed on the same PS domain, whereas the gap
between the periodically arranged Fg molecules on two different PS domains is responsible for the long repeating distance.

4.8 Conclusion

In summary, we systematically examined surface-specific Fg conformations on silicon-based (n-SiO₂, m-SiO₂) and polymer-based (PS, PMMA, PS-b-PMMA) substrates by first performing high resolution AFM imaging focusing on individual proteins and then by statistically analyzing the topological profiles of key subdomains on each surface. I discussed the differences and similarities between the characteristic adsorption behavior of the highly anisotropically shaped Fg molecule in terms of the physical/chemical properties of the substrate, protein-polymer interaction forces, protein surface density, and trinodular/complex conformational models. I investigated Fg subdomain-specific adsorption preferences and orientations to the PS and PMMA block by exploiting PS-b-PMMA diblock copolymer nanodomains which provide a periodically and chemically varying surface on the length scale commensurate with single Fg molecules. Adsorption behavior of the Fg is compared to that of common globular proteins reported earlier on the same nanoscale polymeric domains. Adsorption of Fg molecules is affected less by the exclusive interaction between the protein and the PS block and Fg exhibits a more neutral tendency for shared interaction with both PS and PMMA blocks.

I also examined Fg configurations and molecular orientations during its assembly and surface packing into a larger protein network. I hypothesized Fg assembly and packing model on PS-b-PMMA based on the AFM results, which signifies the first attempt to discern Fg domain-specific interaction preferences to chemically alternating,
nanoscale surfaces. In this assembly model, the bow-shaped Fg molecules in SP configuration surface-pack on the PS domains by forming a stack of bows whose major protein axis are perpendicular to the long axis of the polymeric nanodomain. The linker lines are established via stretched αC chains over PMMA connecting between the D domain of a Fg molecule on a PS domain and a closely located D domain from another Fg molecule situated on a neighboring PS domain. The formed Fg molecules network by connecting two neighboring molecules over the nanopatterned surfaces indicated that the underlying nanostructures with chemical heterogeneity would dictate how the bound Fg arranged and orientated for maximization of free energy. It is majorly contributed by the commensurate dimension and similar chemical properties among the diblock copolymer and the Fg D-E-D nodes.
Chapter Five: Research Significances and Outlooks

5.1 Summary and Significances

In exploration of the common but complicated phenomenon - protein adsorption on a surface, a brief introduction of protein adsorption’s importance in daily life and biomedical research has been given at the beginning, such as tissue engineering and solid state protein array. That being said, to develop a decent solid state protein, one priority is to develop a reliable and low-cost nano-patterning method. Compared to conventional top-down technology such as lithography and serial writing processing, self-assembly of diblock copolymer as bottom-up technology would be an ideal alternative for one to achieve long range and ordered nanostructure with minimum sample treatment and cost. To enable nanoscale detection of protein morphology and density, single molecule detection technique - atomic force microscopy - was introduced and was applied to the overall surface characterization to obtain high resolution protein images. This have concluded chapter one.

In chapter two, since one of biggest advantages of using diblock copolymer is its diversity in morphologies, I tried to use chemical vapor annealing method to develop 7 different nanostructures. That result indicated there would be great potential for one to explore the possibilities of those metastable states’ morphologies in copolymer thin film. As long as the annealing condition is well controlled and planned, some unique surface features are certainly within the reach. The different surface morphologies and tunable chemical composition of diblock copolymer shed a light for designing variable protein surface detection and immobilization methods. Thus, once one can control the surface
chemistry and pattern of the substrate, one certainly can manipulate how proteins adsorbed and what kind of proteins is adsorbed.

Chapter three is follow-up work for chapter two: a direct observation of protein adsorptions on developed intermediate nanostructures. It showed the model globular protein IgG’s preferential adsorption on PS over P4VP for each one of nanostructures discussed in chapter two. Due to its exclusive adsorption preferences, the specific interactions between polymeric chains and proteins would be a key to discern the chemical composition of diblock copolymer in AFM imaging. It would be easy for one to discriminate two different diblocks since proteins exclusively bind to one over another. Moreover, this study has proved that the self-assembled nanostructures from diblock copolymers will be diverse and versatile templates for future solid state protein assay.

Chapter four started with a systematical and statistical method to observe and reveal the elongated trinodular serum protein fibrinogen adsorption behaviors on varied surfaces. First, we are very interested in how protein Fg would adsorb differently on homogeneous surfaces. Thus, two inorganic surfaces: native SiO2 and buffered-HF treated SiO2 have been chosen to compare other two organic surfaces: PS and PMMA film surfaces. Then, protein Fg molecules have been deposited on PS-b-PMMA nanostructured surface, which is chemically heterogeneous. It is first time for one to conduct a systematic and statistical measurement and analysis on such nanoscale surfaces. It turned out the result is very different from the globular proteins IgG on PS-b-P4VP. It is mainly because the interactions between the Fg’s nodes have a strong hydrophobic interaction with the underlying spaced nanopatterns. Also, it has been found that the initial concentration of added proteins would determine how the sequentially
added proteins adsorbed network would form. That indicated the protein adsorption on surfaces is a process that is interplayed and governed by several different factors, such as protein-polymer attraction, protein-protein repulsion, initial proteins’ diffusion, concentration, and water molecules desorption. Overall, it thermodynamically can be explained and predicted by entropy gain (the common part), but kinetically, every adsorption has its own unique traits that should be dealt with individually which makes it a complex process. This fully convinced me why the pioneer biochemists and biophysicists that I quoted in the beginning of introduction chapter, called it a common and complicated phenomenon.

Chapter five here is describing how each chapter would give impactful significance and what the whole research project would bring to the protein-surface study field. It is especially important for the case where proteins are adsorbed on patterned nanostructures with chemical heterogeneity. I also pointed out how the future research would be leading to practical tissue engineering and biomedical sensor design.

5.2 Research Outlook

The unique protein adsorption behaviors on nanostructures proved the hypothesis: it is highly possible that one can gain access to controlling the surface proteins’ type, orientation, conformation and arrangement by careful choosing the diblock copolymers. At this point, I have showed that there were plenty unknown and intrinsic morphologies attainable from known diblock copolymers, and also proved that those would be still decent template for protein adsorption. Nevertheless, fibrinogen on PS-b-PMMA as an example showed how important it is to match the protein chemistry and geometry traits
along with the those of underlying polymeric nanostructures when one is considering how to arrange surface proteins packing for certain specific orientations or network in his/her favor. It is paramount to understand that gaining control of how surface proteins bound to the surface can lead to better control of how well the cell would be binding to the surface. Therefore, it paved a new way to seek novel biocompatible tissue engineered surfaces.

Although we have revealed some interesting phenomena and given our explanations in protein adsorptions on nanostructure, there is still a great need to explore how other cell adhesion related proteins would interact with such a surface. Beside fibrinogen, there are other ECM (extracellular matrix) proteins, including fibronectin, vitronectin, bone sialoprotein, laminin, collagen and elastin, which all possess very similar linear shape but different dimensions. Some research group already reported how nanostructured nanomaterials would promote cell adhesions and proliferation on implanted material surface.\textsuperscript{146,147,148,149,150, 151} These studies probed this topics with various detection methods and protein combinations. However, what is still lacking here is to understand how the nanostructure variations would affect the degree of the cell adhesions and in what degree. The variations mentioned here for a nanostructured surface could mean the surface features with variable roughness, minimum repeating spacing, topographies/morphologies, and chemical compositions. Theoretically, it is the best we can find some types of materials that can give us an optimal condition, considering each of above mentioned features, such as roughness and chemical compositions. But in real life, it is almost impossible to find the perfect one. So, it is essential and practical to find the most influential factor that governs the protein adsorption, from the perspective of
material surface. It could be started from choosing a series of diblock copolymers by carefully varying their key parameters to adjust the surface roughness, minimum repeating spacings, morphologies and chemical composition. For each set of experiments, keeping only one variable but leaving other parameters constant would help isolate their effects individually. By applying this method systematically with a model protein and comparing the adsorption results from varied parameters, it is very possible for us to rank and identify how each variable in terms of changing the surface features would affect the final adsorption behavior. Thus, with this piece of knowledge, one could always have more confidence and better control to engineer the ideal material surface for getting its optimal protein adsorption features, but without compromising too many other properties. Even it is biomaterial that will be applied to our body, we still need to consider other physical properties, such as mechanical strength, durability and viscoelasticity.

Another direction we can take is looking if we can find a way to better control the orientation of protein adsorbed on surfaces. This is important because certain enzyme or antibody only can function well or have a strong binding affinity when certain active sites are orientated toward to the incoming counter-partners. If we can devise a surface that can manipulate how the proteins are orientated, the design of biosensor, such as protein nanoarray would have better detection limit and resolution, because with optimal orientation, the protein-protein interactions would be enhanced by minimizing non-specific binding and inherently give off stronger signals for detection. To realize this degree of orientational control for the proteins, it would better start experimenting with diblock or even triblock copolymer’s surface modifications, such as etching off certain blocks away to create the topological trenches for better fitting of desired proteins.
Bibliography


