Protein Nanopatterning on Self-Assembled Polymer Thin Film Templates and Their Application as Substrates for Cell Adhesion

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May 2010

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Abstract

This work used a hydrophobic polymer pair, i.e. polystyrene (PS) and polyisoprene (PI), to construct well-organized polymer templates for the patterning of proteins through a selective adsorption process. This is a bottom-up method to pattern proteins. The nanopatterned protein surfaces were used as substrates to investigate cell adhesion behavior.

PS-b-PI copolymer ultrathin films formed well-ordered two-dimensional surface structures after spin-coating because of the confinement of substrate-polymer and polymer-air interfaces. A symmetric diblock copolymer film with an 18 nm thickness formed a structure with PI dots dispersed in a PS matrix, and an asymmetric diblock copolymer film of the same thickness formed a stripe-like structure. After incubating these templates in bovine serum albumin (BSA) solution, the ring-like and stripe-like protein nanopatterns were prepared, which resembled their underlying copolymer templates. ToF-SIMS confirmed that there is more BSA adsorption on the PS-b-PI template surface when there is more PS component exposed on the surface. Further, AFM and SIMS analysis confirmed that BSA molecules were localized on the PS domains rather than on the PI domains. The protein’s selective adsorption is attributed to the great mobility of PI chains at room temperature.

The PS, PI, PS-b-PI binary and ternary blends also formed a variety of structures. For thick films, the free surfaces of films are entirely covered by a thin PI layer because its surface energy is lower than PS. When the film thickness is less than 15 nm, both PS and PI components were exposed on the free surface. The resulting complicated surface structures also patterned BSA molecules.

After an extracellular matrix protein, fibronectin (FN), was adsorbed on copolymer substrates, the ring-like and stripe-like FN nanopatterns were incubated in CHO cell suspensions. The ring-like FN pattern adhered more cells than the stripe-like and the control surfaces. The cells on the ring-like FN surface formed more actin fibers and spread better. This can be explained by the ring-like pattern increasing the FN ligand local density and further increasing the integrin clusters and focal adhesion. The ECM protein nanopattern has relevance for tissue engineering.
Acknowledgement

The past three years for my PhD study at the University of Surrey has been memorable in my life. I spent a happy and pleasant time here in Guildford. There are many people that I would like to thank. Firstly, I would like to thank my supervisor Prof Joe Keddie, for instructing and guiding me in the academic field. His passion in science and hard working influenced me a lot in the past three years and will continue to influence me in my future life. Joe is also a nice person and friend in life. I am grateful that I can undertake my PhD study under his supervision. I also thank co-supervisor Dr Alan Dalton for his support. Many thanks to Dr Richard Sear for his help in guiding the cell culture investigation as part of this thesis. Richard exposed a large amount of biology knowledge to me and provided insightful discussion for the experimental results.

I also would like to acknowledge my appreciation to many others. Thanks to Mrs. Che Azura Che Abdullah (University of Surrey) who joined me with the cell culture study. She worked together with me to incubate cells on my protein patterns and use confocal laser scanning microscopy to analyze cell adhesion. Thanks to Dr. Chunhong Lei and Dr. Ibraheem Bushnak (University of Surrey) for sharing with me their laboratory skills and instructing me in lab techniques. Thanks to Mrs Violeta Doukova, our lab technician, for her support and help in everyday laboratory activities. Life has been much easier with her help. Thanks to Dr. Steve Hinder (University of Surrey) for helping with the ToF-SIMS experiments, and Prof. John Watts and Ms. Naoko Sano (University of Surrey) for discussing with me the SIMS data analysis.

I also acknowledge financial support from the Kwan Trust at the University of Surrey and funding from Angiotech BioCoatings for my PhD study.

At last, I would like to thank my husband Dr Tao Wang (University of Surrey). He contributed a lot in my project from basic ideas to detailed experiments. He also drove and encouraged me when I was stuck in the research. Thanks to my family for their forever love.
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List of Abbreviations and Symbols

A - Oscillation amplitude
AFM - Atomic force microscopy
BCN - Block copolymer nanolithography
BSA - Bovine serum albumin
Cs - Solvent concentration
CHO - Chinese hamster ovary
CLSM - Confocal laser scanning microscopy
ECM - Extracellular matrix
Ed - Energy dissipation
F - Tapping force
FA - Focal adhesion
FFT - Fast fourier transformation
FL - Full surface-parallel lamellar morphology
FN - Fibronectin
h - Film thickness
HL - Surface-parallel half-lamellar
HY - Hybrid structure
kB - Boltzmann constant
L - Domain period of polymer bulk
Mn - Number-average molecular weight
Mw - Weight-average molecular weight
N - Degree of polymerization
NSB - Non-specific binding
ODT - Order-disorder temperature
PBS - Phosphate buffer saline
PL - Perpendicular lamellae
PET - Poly(ethylene terephthalate)
PI - Polysoprene
PMMA - Poly(methyl methacrylate)
PP - Polypropylene
PS - Polystyrene
PS-b-PI - Poly(styrene-b-isoprene)
PS-b-PLMA - Polystyrene-b-poly(lauryl methacrylate)
R - Fresnel overall reflection coefficients
RGD - Arginine-Glycine-Aspartic acid
RPI - Relative peak intensity
S - Surface energy
SCF - Self-Consistent-Field
Tc - Critical temperature
Tg - Glass transition temperature
Tm - Melting temperature
ToF-SIMS - Time-of-flight secondary ion mass spectrometry
VASE - Variable angle spectroscopic ellipsometry
WCAA - Water contact angle analysis

θ - Water contact angle
φm - Mass fraction
γ - Interfacial energy
ϕ - Volume fraction
δ - Hildebrand solubility parameter
λ - Wavelength
ΔGmix - Gibbs free energy of mixing
ΔHmix - Enthalpy of mixing
ΔSmix - Entropy of mixing
χ - Flory-Huggins interaction parameter
Chapter 1

Introduction to polymer self-organization, protein nanopatterning and cell adhesion

1.1 Polymer self-organization

Self-organization is a process where the order of a system spontaneously increases without being controlled by the environment or any external force. In condensed matter, self-organization occurs from the nanoscopic to macroscopic length scales and includes, for example, phase separation, microphase separation, mesophase formation, adsorption, and crystallization.¹

1.1.1 Basics of polymers

Polymers, also called macromolecules, are giant molecules made up of many repeating units covalently joined together in the form of a long chain.² The degree of polymerization, \( N \), is the number of the repeating units integrated into the long chain by a polymerization process. Rather than leading to polymers with a unique \( N \), polymerization results in macromolecules with a distribution of \( N \). Synthetic polymers typical have their \( N \) ranges from one hundred to a few ten thousands. In general, the backbones of the chains consist of carbon atoms. In some cases, silicon, nitrogen or oxygen atoms are also incorporated into the backbone.³

One way to classify polymers is based on the chemical type of the monomers:⁴ Homopolymers consist of monomers of the same type, while copolymers have two or more types of repeating units. Furthermore, depending on the arrangement of the different types of monomers, there are random copolymers with repeating units distributed randomly, alternating copolymers with alternating...
sequences of different monomers, block copolymers with a long sequence of one
monomer followed by a long sequence of another monomer, and graft copolymers
with the main chain branched by other long chains.

Polymer architecture describes the shape of a polymer molecule. A linear
polymer consists of a long chain of monomers. A branched polymer has branches
covalently attached to the main chain. Cross–linked polymers have their chains
covalently bonded with monomers of another chain. Cross–linking results in a three-
dimensional network.

Polymers in a solution or in the solid state are not stretched out; they are
folded into a coil. The bonds connecting the repeating units are generally flexible
enough to permit a degree of rotational freedom around the bonds. Therefore, a
polymer can have many possible spatial conformations. Two limits are the completely
stretched chain and the random coil. A completely stretched polymer chain,
corresponding to the lowest entropy of the chain, is the most improbable
conformation. The entropy increase with chain coiling leads to the large number of
possible conformations. The most probable conformation is a Gaussian coil.

Many properties of polymeric materials depend on the microscopic
arrangement of their molecules. Polymers can have an amorphous or semicrystalline
(partially crystalline) structure. Amorphous polymers lack order and are arranged in a
random manner, while semicrystalline polymers are partially organized in ordered
crystalline structures. Two further important parameters that define a polymer
material are the melting temperature, $T_m$ and the glass transition temperature, $T_g$. The
melting temperature is characteristic that applies to those polymers that can crystallize
and define the transition from the liquid to crystalline phase. On the other hand, the
random stereochemistry nature of a polymer will prevent the crystallization. Upon
cooling, this type of polymer will form an amorphous phase similar to a glass. The
temperature that defines this transition is called the glass transition temperature.

1.1.2 Phase separation of polymer blends

In the case of a polymer blend consisting of chains of type A and B, the main
concern for the mixture is the thermodynamic compatibility or incompatibility of the
components. Compatibility depends, however, on many factors, of which the most crucial are temperature and composition. Heating or cooling the sample can shift the blend from the mixed state to a completely demixed one. Therefore, we can define a critical temperature and a critical composition that define the boundary between the mixed state and the demixed one.

Whether the system remains homogeneous or separates into two phases can be predicted from the Gibbs free energy of mixing ($\Delta G_{mix}$). The thermodynamic equation for the Gibbs free energy change accompanying mixing is stated as:

$$\Delta G_{mix} = \Delta H_{mix} - T\Delta S_{mix}$$

(1.1)

where $\Delta H_{mix}$ and $\Delta S_{mix}$ are the enthalpy and entropy of mixing, respectively, associated with the mixing process, and $T$ is the absolute temperature. Flory and Huggins developed a general mean-field theory that provides a basic understanding of the occurrence of different types of phases as a function of temperature and molecular weight. The result obtained by Flory and Huggins is:

$$\frac{\Delta G_{mix}}{k_BT} = \frac{\phi_A}{N_A} \ln(\phi_A) + \frac{\phi_B}{N_B} \ln(\phi_B) + \phi_A \phi_B \chi$$

(1.2)

where $k_B$ is the Boltzmann constant, $N$ is the degree of polymerization, $\phi_i$ is the volume fraction of the $i$ component, and $\chi$ is the A–B Flory–Huggins interaction parameter. The first two terms correspond to the combinational entropy of mixing. Because mixing increases the randomness, it naturally increases $\Delta S_{mix}$ and thereby decreases the free energy of mixing. It can be regulated via the polymerization to change the relative lengths of the chains and fractions of A versus B polymer. The third term represents the enthalpy of mixing and can also increase or decrease $\Delta G_{mix}$ depending on the sign of $\chi$, which is associated with the non-ideal penalty of A–B monomer contacts and is a function of both the chemistry of the molecules and the temperature. In general,

$$\chi = \frac{a}{T} + b$$

(1.3)

where $a$ and $b$ are experimentally obtained constants for a given composition of a particular blend pair. Experimentally, $\chi$ can be controlled through temperature.
The phase behaviour of a mixture can be understood by looking at the free energy against composition change with varying values of $\chi$. Examples of these curves are shown in Fig 1.1. For $\chi \leq 2$, the curve has a single minimum at $\phi_A = \phi_B = 0.5$. While for values of $\chi \geq 2$, there are two minima and a maximum at $\phi_A = \phi_B = 0.5$.

![Figure 1.1. The Gibbs free energy of mixing divided by $k_B T$, as a function of the interaction parameter $\chi$, as given by equation 1.2. (Redrawn from Ref 8)](image)

Figure 1.1. The Gibbs free energy of mixing divided by $k_B T$, as a function of the interaction parameter $\chi$, as given by equation 1.2. (Redrawn from Ref 8)

To understand the physical significance of this, two possible situations need to be considered in more details. In Fig 1.2, the left diagram shows the free energy as a function of composition at a temperature where the mixture is stable over all compositions. In this concave curve, the free energy, resulting from phase separation into any pair of volume fractions $\phi_1$ and $\phi_2$, is always higher than the free energy of the starting composition, so the mixture is stable. In the right diagram, in contrast, if the system starts out at any composition between $\phi_1$ and $\phi_2$, the total free energy is lowered if it splits into two phases with these compositions. The compositions defined by the common tangent construction are called the coexisting compositions, and the locus of these compositions as the concentration is changed is known as the coexistence curve. 

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Figure 1.2. The Gibbs free energy of mixing as a function of composition for one-phase and two-phase mixtures. (a) An initial composition $\phi_0$ phase separated into two phases $\phi_1$ and $\phi_2$; the total free energy of these two phases is always higher than the free energy of the starting composition. (b) Mixtures with compositions between $\phi_1$ and $\phi_2$ can lower their free energy by separating into two phases at these compositions. (Redrawn from Ref 8)

Looking in more detail at the free energy curve for compositions that fall within the coexistence curve, there is another important distinction: the curvature of the free energy function $\frac{d^2 \Delta G}{d\phi^2}$ may be either positive or negative. As shown in Fig 1.3, at a composition $\phi_0$, phase separation to two compositions close to $\phi_0$ results in a lowering of free energy from $G_b$ to $G_b'$. At this composition the system is unstable with respect to small fluctuations in composition, and will immediately begin to phase separate. This composition is unstable. However at composition $\phi_0$, a similar small change in composition leads to an increase in free energy from $G_a$ to $G_a'$; the system is locally stable with respect to such small composition fluctuations, even though it is still globally unstable with respect to separation into the two coexisting phases. There is an energy barrier which needs to be surmounted in order to achieve the global energy minimum associated with phase separation, and as a result this composition is metastable. Clearly the limit of local stability is defined by the condition that the curvature $\frac{d^2 \Delta G}{d\phi^2} = 0$; the locus of these points is known as the spinodal line.
Figure 1.3. The Gibbs free energy of mixing as a function of composition, showing the distinction between compositions that are metastable, such as $\phi_a$, and compositions that are unstable, such as $\phi_0$. (Redrawn from Ref 8)

The critical temperature $T_c$ separates the two types of situation for which mixtures are always stable, and situations for which there are compositions which will phase separate. In the former, the curvature of the free energy function $d^2\Delta G / dp^2$ is always positive, while in the latter $d^2\Delta G / dp^2$ is negative within a certain range of $p$. The critical point is thus defined by the condition $d^3\Delta G / d\phi^3 = 0$, and it is the point at which the coexistence curve and spinodal line meet each other.

Collecting together the definitions in terms of the free energy function of the critical point, the spinodal line, and the coexistence curve, it is possible to calculate the phase diagram. The coexistence curve, spinodal line and critical point can be predicted with Eq. 1.2 based on the standard criteria for equilibrium, and the limits of stability. Fig 1.4 is the theoretical phase diagram for a symmetric ($N_A = N_B = N$) polymer mixture, which presents the mixing-demixing transition of polymer blends depending on these three parameters: $N$, $\chi$ and $\phi$. By decreasing $N$ or $\chi$, the polymers are mixed when $\chi N \leq 2$. For combinations of $\chi N$ and $\phi$ lying inside the coexistence curve, a mixture separates into two phases with compositions $\phi'_A$ and $\phi''_A$. The mixture is thermodynamically metastable between the coexistence curve and the spinodal line. While inside the spinodal line the mixture is unstable and presents spinodal decomposition. In spinodal decomposition, material flows from regions of low concentration to regions of high concentration. This is a reversal of the normal
situation, in which one would expect material to diffuse from regions of high concentration to regions of low concentration.\textsuperscript{10}

![Figure 1.4. Phase diagram of a symmetric ($N_A = N_B = N$) binary mixture of linear homopolymers. The two-phase region is enclosed within the coexistence curve. The blend is stable in the one-phase state below the critical point. (Redrawn from Ref 9.)](image)

1.1.3 Micro-phase separation of block copolymers

The particular chemical structure of block copolymer materials is reflected in the most fundamental and interesting way by incompatibility effects. These effects give block copolymers a number of specific, new morphologies and original physical and mechanical properties that have led to valuable technological applications, such as nanolithography,\textsuperscript{11} controlled drug delivery,\textsuperscript{12} organic photovoltaic,\textsuperscript{13} and organic light emitting diodes.\textsuperscript{14}

In a simple system of an amorphous diblock copolymer (A-B), the most characteristic feature of a block copolymer is the strong repulsion between different segments. Polymer molecules, with their repeating units covalently connected together to form a long molecular chain, can change from one configuration to another. The molecular packing, and thus thermodynamically stable nanodomain patterns, of block copolymers in the bulk state are governed by the mixing enthalpy and entropy of the component segments. Because of the covalent bonding between the segments, the system cannot macroscopically phase separate, and so it minimizes the interfacial energy by adopting well-defined nanodomain patterns with constant
interfacial curvature and stretched interfacial chain configurations. The self-organization or self-assembly of block copolymers can form a variety of well-ordered nanostructures with tunable scales from several to hundreds of nanometers.

Block copolymers are known to segregate into ordered morphological structures in the bulk state. These regular structures can be spherical, cylindrical, bicontinuous 'gyroid', or lamellar, depending on the volume fraction of the blocks. The strength of segregation of the two blocks is proportional to $\chi N$. A symmetric diblock copolymer is predicted to be disordered when $\chi N < 10$ or the temperature is over the order–disorder temperature (ODT). But there are well-ordered structures when the temperature is below the ODT and $\chi N > 10$. When the volume fraction of block A ($\phi_A$) is quite small, it forms spheres in a body-centered cubic (BCC) lattice surrounded by the matrix of B. As $\phi_A$ is increased towards 0.5, the minority nanodomains will form firstly cylinders in a hexagonal lattice, then a bicontinuous double gyroid structure, and finally lamellae, as shown in Fig 1.5.

Figure 1.5. Diblock copolymers are predicted to self-assemble according to a phase diagram predicted by self-consistent mean field theory. (Redrawn from Ref 18) A schematic phase diagram shows the various ‘classical’ morphologies adopted by non-crystalline, linear diblock copolymers. (Reprinted from Ref 17)
1.1.4 Morphology formation of block copolymer films

Over the past decade, block copolymer thin films have become of great interest for high-resolution patterning because of their simplicity and high throughput. The uniform nanostructures that result from organized macromolecular packing, the so-called ‘self-assembly’ process, have been applied as templates for fabricating various nanomaterials and nanodevices with high fidelity.\textsuperscript{20,21}

In addition to the parameters described in the bulk state, there are three additional factors that govern block copolymer thin film patterns: (1) the polymer-air interfacial energy, (2) polymer-substrate interactions, and (3) the film thickness $h$ relative to the bulk states natural domain period $L$. For example, in a simple symmetrical diblock copolymer system, although both of the two blocks have similar volume ratios and the system favours the formation of a lamellar mesophase in the bulk, a very sophisticated structure is revealed in the thin-film state, as illustrated by Fasolka et al.\textsuperscript{22}

1.1.4.1 Surface energy effect

The process of self-assembly of block copolymers is driven by the total free energy minimization, with both enthalpic and entropic contributions. In block copolymer thin films, however, there are additional contributions to the overall free energy: (1) polymer-air surface energy and (2) the interfacial energy between the blocks and the substrate. Block copolymers have also been observed to assemble into two- and three-dimensional structures in thin films.

In the past two decades, the groups of Russell\textsuperscript{23,24} and Thomas\textsuperscript{25,26} have focused plenty of attention on the research of the copolymer morphology near surfaces and interfaces. It was found that the morphology near surfaces can be dramatically affected by the difference of the surface free energies of the two blocks and their affinity to the substrate. After annealing treatments above the glass transition temperature, the block copolymer films obey the thermodynamic requirements: the component block with a lower surface energy covers the external surface.\textsuperscript{27}

In substrate-supported films, where the substrate affords different interfacial energies to two blocks, a symmetric diblock copolymer forms the parallel lamellar
structure in the equilibrium state. But when the boundary energies to each block species are equal, the perpendicular lamellae (PL) can form. Several researchers have verified the PL structure from theoretical and experimental aspects.\textsuperscript{28,29} When a film is confined by two parallel hard walls and the film thickness $h \neq nL$ or $(n+1/2)L$, the PL gain stability (as shown in Fig 1.6a). When the boundaries have neutral surface energies for the two blocks, the PL structure is stable for all film thicknesses. Kellogg et al.\textsuperscript{30} used random-copolymer-coated walls (the random copolymer has the same components as the block copolymer) for the neutral boundaries to generate the PL ordering, so that both block components are present at the walls (as shown in Fig 1.6b). The cylindrical morphology under neutral boundary conditions is also considered. The surface-perpendicular cylindrical domains were observed in Thurn-Albrecht et al.'s research.\textsuperscript{31}

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure1_6}
\caption{(a) Perpendicular lamellar structure in a film confined by two parallel hard walls and the thickness $h \neq nL$ or $(n+1/2)L$. (b) thin films of a symmetric diblock copolymer with a random copolymer (pink region) anchored to two interfaces.}
\end{figure}

In the thin film state, the surface energy effects on the block copolymer nanodomains tend to form a particular orientation to the substrate surface. Parallel and perpendicular lamellae and cylinders, as well as spheres dispersed in another component matrix, are all possible, as shown in Fig 1.7. All of these film structures are useful for many applications. Cylinders lying parallel to the surface and lamellae standing perpendicular may each be of interest in the patterning of nanowires. Upright cylinders and spheres may be of interest in the hexagonal arrays for data storage.
Figure 1.7. When confined to a thin film, various orientations of block copolymer domains can form: (a) Lamellae lying parallel to the substrate, (b) lamellae aligned perpendicular, (c) cylinders lying parallel, (d) cylinders lying perpendicular, and (e) spheres.  

1.1.4.2 Film thickness effect

For thick films \((h > L)\), a symmetric diblock copolymer typically forms a structure with the lamella oriented parallel with the substrate surface, while an asymmetric diblock copolymer forms cylinders aligned parallel to the substrate. More complicated behaviour is exhibited by diblock copolymer thin film systems with a film thickness \(h < L\). In this case, the substrate and the free surface afford significant impact on the morphology formation. Such constrained systems sometimes adopt the morphology where lamellae or cylinders are oriented perpendicularly to the film surface plane.

Research about the thickness dependence of diblock copolymer thin films has been theoretically and experimentally conducted in the past decades. These studies reveal the trend that the diblock copolymer domain orientation changes with film thickness variation. In particular, the substrate perpendicular domain orientation occurs when \(h < L\) or if the film is confined between two hard walls. For example, Morkved and co-workers\(^3\) found the morphology change depends on the thickness in lamellar diblock copolymer ultrathin films. Suh’s group\(^3\) presents a theoretical result
that the thickness, \( h \), has been proven to greatly affect the cylinder domain orientation of the thin films. Fasolka and Mayes\textsuperscript{34} completed a comprehensive Self-Consistent-Field (SCF) analysis of the morphological behaviour of compositional symmetric, substrate-supported diblock films in the thickness regime \( h \leq L \). In their research, one block is favoured to reside at both the free surface and adjacent to the substrate in an A-B diblock copolymer system. Figure 1.8 shows an example phase diagram constructed from an SCF calculation in Fasolka and Mayes’ research. In this figure, \( N = 200 \) and \( \chi_{AB} = 0.1 \). The ordinate is reduced film thickness \( (h/L) \). The abscissa gives the ratio \( R = S_2^B / S_1^B \) (where \( S_1 \) is the surface energy of the top air surface boundary and \( S_2 \) is the surface energy of the bottom substrate boundary, and B means the B segment), a measure of surface energy symmetry. The phase fields are labelled with the abbreviations designating the conditions under which each morphology is predicted to be stable. SCF calculations present four ordered morphologies. When the thickness decreases from \( h \approx L \) to \( h < 1/2 \, L \), there are four morphologies: full surface-parallel lamellar morphology (FL), a surface-parallel half-lamellar (HL), a hybrid structure (HY), and perpendicular lamellae (PL). These morphology structures are also displayed in Fig 1.8 as the insets.

**Figure 1.8.** An example of a phase diagram that comes from a SCF calculation. Phase fields are labelled with abbreviations. (Redrawn from Ref 34) The corresponding morphologies at different thicknesses are shown in their regions.
1.2 Protein nanopatterning

Protein nanopatterning on surfaces has been of growing interest in recent years, from both scientific and technological points of view. The controlled positioning of proteins and biomolecules onto surfaces is crucial to diverse biological and medical applications, including nanobiochips, nanobiosensors, tissue engineering, drug screening, and fundamental studies of molecular and cell biology.\textsuperscript{35,36,37}

Over the past few decades, techniques to produce nanoscale features on surfaces have emerged. A variety of methods have been developed to immobilize proteins onto specific sites on surfaces with resolutions at the nanoscale via the fusion of biology and nanotechnology. Surface deposition techniques for biomaterials at the nanoscale have been summarized by Mendes\textsuperscript{38} and Christman\textsuperscript{39}, including scanning probe patterning methods (dip-pen nanolithography, nanografting), soft lithography (nanoimprint lithography, nanocontact printing), photolithography, electron beam patterning, focused ion beam patterning, particle lithography and polymer chain arrangement. The protein nanopatterning technique using polymer chain arrangement is simpler than other techniques.

Protein nanopatterning is a physically defined form of protein immobilization. The simplest method for immobilizing protein on surfaces is physical adsorption through mutual attraction between the solid surface and the protein molecules. Protein adsorption results from attractive forces such as ionic, hydrophobic, or van der Waals forces.\textsuperscript{40} A more stable means of protein immobilization is to covalently link a protein to the surface via a chemical bond between the molecules of the solid and the protein. An example is the use of bifunctional crosslinkers such as silane-based groups.\textsuperscript{41}

There is a strong interest in producing substrates that possess spatially defined bio-adhesive patterns, which promote protein attachment, on a background that resists protein adsorption.\textsuperscript{42} There is a broad range of biological and medical applications that present many challenges for materials design. The design concept includes: (1) position distinct biomolecules within designated nanoregions on a substrate with well-defined feature size, shape, and spacing, while retaining their native biological features and properties and (2) high biomolecule resistance by the other regions of the substrate.\textsuperscript{43} The most difficult issue to address for protein patterning on an organized
polymer surface is the non-specific binding (NSB) of protein to the surface. NSB refers to the indiscriminate adhesion of proteins, i.e., proteins will not only adhere on the desired location, but will attach to all regions of the patterned surface. One solution to the NSB problem is to use surface repulsion chemistry, such as polyethylene glycol (PEG) groups, on the areas apart from the desired locations for protein adsorption.44

Some kinds of polymers have been used as bio-antifouling coatings in biotechnology and science areas. Polymer coatings have been used on stents, heart valves and heart implants to resist the protein adsorption on biomaterial surfaces.45 These polymers with different protein-resistance properties can be used to define protein location. The proteins are expected to be selectively adsorbed on a specific polymer domain rather than the other one in a two-component system. Once a protein preference happens on a well-ordered polymer substrate with spatially and chemically heterogeneous properties, the substrate can be used to pattern the proteins. The present work will use self-organised polymer films with two domains to create protein nanopatterns through selective adsorption.

1.3 Cell adhesion

Mammalian cells have strict requirements for maintenance in culture. Both normal and transformed cells are propagated in specialized culture media that contain a balance of salts, glucose, amino acids, vitamins, and growth factors. In addition to being maintained at the appropriate temperature (37°C), humidity (100%), and pH (7.2), most cells from solid tissues grow as adherent monolayers, and need to attach and spread out on the substrate before they will start to thrive and proliferate.46

The interaction of a cell with the substrate can be strongly influenced by the binding of cell receptors with ligand proteins that are adsorbed on the substrate. There are several classes of cell surface receptors, for example: integrins and selectins. Although there are many types of cell receptors, cell-substrate adhesion is primarily mediated by members of the integrin family. Integrins consist of two non-covalently associated transmembrane subunits, termed α and β. A recent survey of the human genome shows that there are at least 24 α and 9 β subunits existing. However, only 18 α and 8 β subunits among them are identified to form 24 heterodimers.47
Most integrins bind to proteins belonging to the extracellular matrix (ECM). In biology, the ECM is the extracellular part of animal tissue that usually provides structural support to the animal cells in addition to performing various other important functions. Many of the ECM proteins are large (>100 kD) proteins that carry multiple functional sites (e.g. fibronectin, vitronectin, collagens, and laminins), the assembly of which into matrices and matrix fibrils is tightly controlled by cells. The most widely recognized and characterized ligand peptide motif is the RGD (Arginine-Glycine-Aspartic acid) sequence found in a variety of ECM proteins. Integrin recognition of this ligand occurs in almost all cell surfaces and acts to promote cell adhesion.

Cell adhesion occurs at specific areas at which the actin cytoskeleton on the inside of the cell is linked via transmembrane receptors (integrins) to the extracellular matrix (ECM) on the outside (Fig 1.9).

**Figure 1.9.** Cell adhesion is mediated by interactions between cell-surface receptors and ECM molecules. Transmembrane integrins bind to extracellular matrix ligands on the outside of the cell, and to adaptors or signalling molecules (cytoplasmic proteins) inside the cell that link to actin filaments (actin cytoskeleton). The diameter of an actin filament is 6-10 nm, hence the bundle of filaments is 50-100 nm.
These adhesion sites are composed of complexes of more than 50 different cellular proteins. A sufficiently large and stable adhesion site is called a focal adhesion (FA). Focal adhesion is specific types of large macromolecular assemblies through which both mechanical force and regulatory signals are transmitted.50

FA mediates strong cell-substrate interaction. The action of integrin-ligand binding induces complexing of proteins on the cytoplasmic side of the membrane. The cytoplasmic proteins include talin, vinculin, and paxillin, and link integrins with actin filaments in the cell. This complexation triggers the reorganization of the actin cytoskeleton. The assembly and organization of actin filaments promotes more integrin clustering. The result of these events is the formation of aggregates of ECM proteins, integrins and cytoskeletal proteins on either side of cell membranes. Tension in the bundle of actin filaments is required to maintain the clustering of integrins and the integrity of focal adhesions.51,52 These focal adhesions are scattered across the cell surface and are typically 0.25–0.50 μm wide and 2–10 μm long. Their formation, development, and disassembly are not only key activities in cell spreading and migration, but also appear to modulate many cellular functions, such as cell proliferation and differentiation (owing to their ability to modulate intracellular signaling).53 There is strong evidence that the mechanism for focal adhesion assembly and disassembly is force-mediated due to the coupling between focal adhesions and actomyosin contractility machinery of the cytoskeleton.54,55

The function of integrins is not only to act as cell surface receptors for ECM ligands, but also to mediate interactions between the cell cytoskeleton and the ECM. Both α and β subunits form the binding site of an ECM ligand, giving the different integrin heterodimers specificity for different ligands. For example, α1β1 and α5β1 both bind to collagen and laminin, while α5β1 binds to fibronectin. Most of the different integrins are expressed in a variety of cell types, and most cells express several integrins, allowing them to adhere to many different ECM molecules.56 Cell-ECM adhesion is both dynamic and tightly regulated. Integrins have relatively low affinities (~8.6 x 10^7 liters/mole) for their ligands, but most cells express relatively a large number of these molecules (5 x 10^5 receptors/cell).57 Because of their positions at the surface, integrins form an important bi-directional link between cells and their environments. That is, cells use integrins to detect changes in the composition of
ECM on the culture surface and respond by modifying cell behaviour. Cells can also change the levels of integrins displayed at the cell surface to alter cell adhesion. For example, cells must dynamically regulate integrin expression in order to transform from being firmly adhered to moving across the substrate.\textsuperscript{58}

1.4 Motivation and aims of present work

Block copolymers have been suggested for many applications based on their ability to form regular nanoscale patterns. These self-assembled patterns have been considered as nanolithographic masks as well as templates for the further synthesis of inorganic or organic structures.\textsuperscript{59,60} These uses depend on the extremely regular self-assembly of block copolymers over a macroscopic area. Block copolymer thin films are of particular interest because of the possibility of obtaining two-dimensional patterns with very high regularity. These well-ordered polymer films can be used as templates to nanopattern proteins or other bio-molecules via a variety of nanotechniques.

The main requirement for protein nanopatterning is the selective attachment of protein at the desired regions and high protein resistivity by other regions on a substrate. When the protein is a cell adhesive protein, e.g. fibronectin, collagen or vitronectin, the patterned protein substrate can be used to generate a cell pattern. The areas with adhesive protein will allow the selective attachment of cells.

A modern scientific field known as tissue engineering has been developed to design artificial biocompatible materials to substitute irreversibly damaged tissues and organs. In addition, biomaterials are important for fundamental scientific research as relatively simple and physicochemically well-defined artificial templates of ECM. The normal cells respond to various micro and nano-scale environmental signals within the ECM, which eventually alters cellular function and tissue structure.\textsuperscript{61,62}

The aim of this work is to study the protein adsorption and nanopatterning on two component block copolymer templates and followed by study of cell adhesion on the ECM protein patterning substrate. One of the protein patterning techniques, which is of interest in this work, is to use a polymer pair as the substrate for localizing the proteins. Each component of the polymer pair has different adhesive properties.
originating from the interactive forces or geometry between the polymer blocks and the protein molecules. The key point of this work is to find a component pair which has different protein adsorption properties. When this kind of polymer pair is confirmed, the surface morphology of polymer film is regulated by adjusting a few affecting factors, such as film thickness and block ratio. After self-organization of block copolymers, thin films with a variety of morphologies will be used to pattern proteins on the nanoscale. The selective adsorption of protein molecules and the resulting nanopattern will be determined using image capture (atomic force microscopy) and surface analysis techniques. Then the nanopatterned ECM protein surface is used to affect cell function and behaviour via cell adhesion.

This thesis is organised as follows. Firstly, in Chapter 3, soft polymer films are investigated to determine their suitability as coatings for protein templating. The stability and dewetting of the thin film in water are evaluated. Secondly, in Chapter 4, a well-ordered block copolymer film substrate with different protein adhesive properties will be developed as a template. To exploit extensively characterized and precisely controlled surface morphology in the biomaterial field, these polymeric surfaces will serve as two-dimensional nanoscale templates for spontaneously constructed protein nanopatterns that feature high density and stable protein conformation. Thirdly, in Chapter 5, the self-organized polymer blends will develop two-dimensional or three-dimensional film structures and the films are further used as templates to construct protein arrays. Finally, in Chapter 6, the constructed ECM protein substrates with nanoscale patterns will be incubated in cell suspensions to study the cell attachment and spreading behaviour influenced by the lateral spacing of ECM protein.
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Chapter 2

Experimental techniques

This chapter will describe the techniques and methods used to deposit polymer films and to characterise their thickness, morphology and surface energy. Techniques to characterise proteins and cells will then be presented.

2.1 Spin-coating technique

Spin-coating is a technique which has been used for several decades to prepare thin films as illustrated in Fig 2.1. A droplet of a polymer solution is put on a flat substrate. Then this substrate is rotated at a fixed rate. This rotation spreads the liquid over the substrate. During the evaporation of the solvent, the polymer solidifies and forms a smooth film. The thickness of the resulting film depends on the viscosity, the polymer concentration, and the spin-frequency. Using polymer concentrations ranging from 0.1 to 5 wt%, and spin rates from 1000 to 10,000 rpm, film thicknesses ranging from a few Angstroms up to a few micrometers can be formed. The nature of the solvent is very important to determine the quality of the coating. The solvent needs to evaporate within a proper period to ensure the polymer has enough time to form a smooth surface. The solvent must also be volatile enough so that it evaporates within a reasonable time frame. With well chosen parameters, spin-coating results in extremely homogeneous polymer films.1

Figure 2.1. A scheme of the spin-coating process used to produce smooth, homogeneous polymer films.
In this work, diblock copolymers were dissolved in a suitable solvent. Solutions were deposited on silicon (100) crystal substrates and rotated at a certain rate. During the evaporation of the solvent, the micro-phase separation occurred.\textsuperscript{2} Generally, the block copolymer films can be found to be in a well-organized morphology, as shown schematically in Fig 2.2.

\textbf{Solvent evaporation}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{Solvent_evaporation.png}
\caption{Scheme of the micro-phase separation of a block copolymer during the spin-coating process.}
\end{figure}

\textbf{2.2 Variable angle spectroscopic ellipsometry (VASE)}

Ellipsometry is a very sensitive optical technique, which has been used extensively for measuring the thickness and refractive index of thin films at interfaces. It is based on the fact that, in general, a thin film affects the change in the polarization state of an elliptically polarized light beam reflected by an interface. For many samples, ellipsometry is sensitive to film thickness on a submonolayer level. Ellipsometry has also been proven to be the premier technique for determining optical constants in the near-UV, visible, and near-IR wavelength ranges.\textsuperscript{3,4}

The theory underlying ellipsometry can be summarized as follows.\textsuperscript{5} The polarization state of a light beam is characterized by the amplitude ratio, $A_p/A_s$, and the phase difference, $\delta_p-\delta_s$, of the two components of the electric vector, $E$, parallel (p) and normal (s) to the plane of incidence (viewing from Fig. 2.3). The change in the polarization state due to reflection at an interface is then defined by

$$\tan \psi = \frac{A_p^r / A_s^r}{A_p^i / A_s^i}$$ \hspace{1cm} (2.1)
\[ \Delta = (\delta_p' - \delta_i') - (\delta_p' - \delta_i') \]  

(2.2)

where \( \tan \psi \) is the change in the amplitude ratio due to reflection and \( \Delta \) is the change in the phase difference, which can be determined by experiment. The superscripts \( r \) and \( i \) denote the reflected and the incident light beams, respectively. The total effect caused by reflection may then be written as:

\[ \frac{R_p}{R_i} = \tan \psi \exp(i\Delta) = f(n_i, k_i, d_i) \]  

(2.3)

where \( R_p \) and \( R_i \) are the Fresnel overall reflection coefficients of the respective parallel and normal components of the light. Where \( n \) is the real part of refractive index, \( k \) is the imaginary part of refractive index, \( d \) is the thickness of the film, and \( i \) means the number of the layer being measured. Since Eq. 2.3 relates the optical properties of the reflecting system to the measurable parameters \( \psi \) and \( \Delta \), it is regarded as the basic ellipsometric equation.

**Figure 2.3.** Measurement geometry for an ellipsometric experiment: the incident light interacts with the sample and is partially reflected. The interaction of the light with the sample causes a change in polarization, from linear to elliptical polarization.

Film thickness and optical constants cannot be measured directly but can be extracted through a mode-based analysis using optical physics. After obtaining optical measurements which are a function of the physical parameters we are interested in, a model can be constructed which contains both known and unknown physical parameters, such as thicknesses and optical constants. The unknown physical
parameters are varied and the ellipsometry parameters are calculated using the model until the simulation is very close to the experimental data. This process is shown in Fig 2.4.

![Diagram](image)

**Figure 2.4.** The schematic process of ellipsometric data analysis.

Variable angle spectroscopic ellipsometry (VASE) is a type of ellipsometry that combines variable angle of incidence and spectroscopic measurements, which allows the acquisition of a large amount of data from a given sample. As a result, it has the power to characterize many complex structures, which single angle and/or single wavelength ellipsometry cannot do. In this work, the ellipsometry was used to determine the thickness and refractive index of spin-coated polymer films and adsorbed protein layers on the polymers.

### 2.3 Water contact angle analysis (WCAA)

Contact angle, $\theta$, is a quantitative measurement of the wetting of a solid by a liquid. It is the angle formed by the liquid at the three-phase boundary where a liquid, gas (or a second immiscible liquid) and solid intersect. It is a direct measure of interaction taking place between the participating phases. The contact angle is determined by drawing a tangent at the contact place where the liquid and the solid intersect (see the angle $\theta$ in Fig. 2.5). When water is used for the liquid phase in this system, it is called the water contact angle.
The shapes of the drop and the magnitude of the contact angle are controlled by the interfacial energy of each participating phase (gas, liquid and solid). In an ideal situation the relation between the interfacial energy and the contact angle can be described by the Young’s equation:

\[ \gamma_{lv} \cos \theta = \gamma_{sv} - \gamma_{sl} \]  

where \( \gamma_{lv} \), \( \gamma_{sv} \) and \( \gamma_{sl} \) refer to the interfacial energies of the liquid/vapour, solid/vapour and solid/liquid interfaces, respectively. Eq. 2.4 is obtained by a simple force balance of the three surface tensions. The vertical components are balanced by the substrate rigidity. This equation is valid for small drops in which the effect of gravity can be neglected.

In our experiments, a drop shape analysis system is used to characterise the solid surfaces. In order to measure the water contact angle, a water drop is placed on a sample located on a moveable sample table. The drop is illuminated from one side and a camera at the opposite side records the image of the drop, as shown in the schematic diagram of water contact angle measurement in Fig. 2.5. The drop image is recorded with a camera and analysed with computer software.

Taking a liquid drop on a solid surface as example, if the liquid is strongly attracted to the solid surface (for example, water on a strongly hydrophilic solid) the droplet will completely spread out on the solid surface and the contact angle will be close to 0°. Less hydrophilic solids will have a contact angle up to 90°. If the solid
surface is hydrophobic, the contact angle will be larger than 90°. Drawing on the ability of water contact angles to distinguish between the hydrophilic or hydrophobic properties of solid surfaces, it can be used to compare the relative surface energies depending on the different compositions. In addition, the protein adsorption layer can also be examined using this technique. A protein will have a different surface energy than the underlying polymer, and hence $\theta$ will be modified.

2.4 Atomic force microscopy (AFM)

Atomic Force Microscopy (AFM) has evolved rapidly as an imaging tool with high spatial resolution for material surfaces.\textsuperscript{11,12} AFM has the advantage of imaging almost any type of surface, including polymers, ceramics, composites, glass, and biological samples. Unlike conventional microscopes, the AFM does not rely on radiation, such as by photon or electron beams, to create an image. AFM is an instrument that images the three dimensional topography based on mechanical interactions between the tip and the material surface as shown in Fig. 2.6.

Figure 2.6. In the AFM, a sharp probe scans across a surface (top), and by monitoring the motion of the probe pass across the surface, a 2-D line profile is generated. Then the line profiles are combined to create a three dimensional image of the surface (bottom).

AFM uses various surface properties (e.g. topography, friction, hardness, etc.) to generate an image. It works in a similar way as our fingers touching and probing the environment when we cannot see it. By using a finger to “visualize” an object while touching it, our brain is able to deduce its topography. The resolution of the method is determined by the radius of the fingertip. To achieve atomic scale
resolution, a sharp stylus tip attached to a cantilever is used to scan an object point by point, and thereby images its contour. The tip and cantilever come with a wide range of properties. The conical probes used in my experiments had a typical curvature radius of \( \approx 10 \text{ nm} \) and a length between 14 and 16 \( \mu \text{m} \).

The basic principle of the technique can be understood in the following way: A cone or pyramid-shaped tip usually made from silicon or silicon nitride is used as a probe. The probe itself is attached to a cantilever with a certain spring constant \( k \) (typically \( k \approx 1-100 \text{ N/m} \)). When the tip is brought into the proximity of a sample surface, forces between the tip and the sample lead to a deflection of the cantilever. The force is not measured directly, but calculated by measuring the deflection of the cantilever according to Hooke’s law. Hooke’s law gives \( F = -kz \), where \( F \) is the force, and \( z \) is the displacement of the cantilever. The forces that can be measured in AFM include Van der Waals forces, capillary forces, electrostatic interactions, chemical bonding etc. As a result of the various interactions, the probe is exposed to an effective, distance-dependent force. A common technique to measure the cantilever displacement is the deflection of a laser beam (Fig. 2.7). Typically, the deflection is measured using a laser spot reflected from the top of the cantilever into an array of photodiodes. The voltage difference of the upper and lower quadrants of the detector is proportional to the deflection. After calibration, absolute forces with a resolution on the order of \( 10^{-11} \text{ N} \) can be measured in this way.

![Figure 2.7. Beam deflection system, using a laser and photodiodes to measure the beam position.](image)

The AFM can be operated in a number of modes, depending on the application. In general, three commonly modes: contact mode, non-contact mode and
intermittent contact mode can be used to produce topographic images of sample surfaces.\textsuperscript{13}

Contact mode, in which the tip scans the sample in close contact with the surface, is a common mode used in force microscopy. As the tip is scanned across the surface, the cantilever is deflected by the surface corrugation. If the tip was scanned at a constant height, there would be a risk that the tip would collide with the surface, causing damage. Hence, in most cases a feedback mechanism is employed to adjust the tip-to-sample distance to maintain a constant force between the tip and the sample. A record of the adjustment to the tip-sample distances during a scan is used to generate a topographic image. Because the tip is in hard contact with the surface (as illustrated in the repulsive regime in Fig. 2.8), the stiffness of the cantilever should have a spring constant of $< 1 \text{ N/m}$.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig2.8.png}
\caption{AFM force-distance curve between the tip and the sample surface.}
\end{figure}

Non-contact mode is used in situations when the tip contact with the material surface might alter the real surface topography, especially on a soft or loose surface. In this mode, a stiff cantilever is oscillated in the attractive regime (as illustrated in Fig. 2.8), meaning that the tip is quite close to the sample (50-150 Å above the surface) but not touching it. Attractive Van der Waals forces acting between the tip and the sample are detected, and the topographic images are constructed by scanning the tip above the surface. The forces between the tip and sample are quite low, on the order of pN ($10^{-12} \text{ N}$). The detection scheme is based on measuring changes to the
resonant frequency or amplitude of the oscillating cantilever in response to force gradients from the sample.\textsuperscript{14}

Intermittent contact mode is a key advance in AFM. It is also referred to by the trade name of “tapping mode”. This technique allows high resolution topographic imaging of sample surfaces that are easily damaged, loosely held to the substrate, or difficult to image by other AFM techniques.\textsuperscript{15} Tapping mode method alternately places the tip in contact with the surface to provide high resolution information and then lifts the tip off the surface to avoid dragging the tip laterally across the surface. A stiff cantilever is oscillated near its resonant frequency ($f_0$) close to the sample and part of the oscillation extends into the repulsive regime. In tapping mode, the “free” oscillation amplitude ($A_0$) of the tip is much larger than for non-contact mode, often in the range of 20 nm to 200 nm, and the tip makes contact with the sample for a short duration in each oscillation cycle. During scanning, the amplitude at the operating frequency ($f$) (slightly lower than $f_0$) is maintained at a constant level, called the set-point amplitude ($A_{sp}$), by adjusting the relative position of the tip with respect to the sample. A map of the adjustment to the tip-sample distance is used to create an image of the surface topography. In a previous study, it is concluded that decreasing the set point leads to an increase of the tapping force ($F_{av}$). The average tapping force $F_{av}$ can be described with a semiempirical formula:\textsuperscript{16}

$$F_{av} = \frac{1}{2} k \left[1 - \frac{A_{sp}}{A_0(f_0)}\right] A_0(f) \beta$$

(2.5)

with the off-resonance parameter $\beta$ given as:

$$\beta = \frac{A_0(f)}{A_0(f_0)}$$

(2.6)

where $Q$ is the quality factor of the cantilever. Following from the above statements, $\beta < 1$ in all cases. $F_{av}$ is determined by the ratio of $A_{sp}$ to $A_0$ rather than the individual value of $A_{sp}$ alone.

One recent development in tapping mode is the use of the changes in phase angle ($\phi$) of the cantilever probe to produce a second image, called a phase image
phase contrast image. This image often provides significantly more contrast than the topographic image and has been shown to be sensitive to material surface properties, such as stiffness, viscoelasticity, and chemical composition. In general, changes in phase angle during scanning are related to energy dissipation \((E_D)\) during tip-sample interaction. The correlation is described as:\textsuperscript{16}

\[
\sin \phi = \left( \frac{f}{f_0} \frac{A_p}{A_0} \right) + \frac{Q E_D}{\pi k A_p A_0}
\]

(2.7)

where all the variables are already defined. During a scan, all of the parameters in Eq. 2.7 are fixed. Then the phase angle \((\phi)\) is positively correlated with the energy dissipation \((E_D)\). When a tip interacts with a higher viscous area, then more energy will be dissipated. The phase angle will be greater than other areas. In the phase images presented in this work, the darker regions correspond to larger value of \(\phi\). Hence more energy dissipative regions will appear darker.

### 2.5 Time-of-flight secondary ion mass spectrometry (ToF-SIMS)

ToF-SIMS is a surface analytical technique used for obtaining elemental and molecular chemical information about surfaces. It is a surface-sensitive spectroscopy technique that uses a pulsed ion beam to create molecular fragments and produces positive and negative mass spectra from the very outermost surface of a specimen.\textsuperscript{17, 18, 19}

ToF-SIMS uses a focused, pulsed ion particle beam (typically Cs or Ga) to dislodge chemical species on a material surface. Particles produced closer to the site of impact tend to be atomic ions (positive or negative). Secondary particles generated farther from the impact site tend to be molecular fragments. These particles are then accelerated into a flight path on their way towards a detector (see Fig. 2.9). The "time-of-flight" of an ion is proportional to the square root of its mass, so that all of the different masses are separated during the flight and can be detected individually.
The ToF-SIMS is widely used in materials science mostly in one of three modes: (I) The mass spectrum identifies the elemental and ion composition of the uppermost 10 to 20 Å of an analyzed surface from the distribution of the masses of fragments. Positively-charged and negatively-charged fragments are analyzed separately. The spectra are recorded as the number of particular mass-over-charge ratios. The high resolution of the ToF analyzer can distinguish species whose masses differ by only a few millimass units. (II) Secondary ion mapping measures the lateral distribution of elements and molecules on a surface. To obtain a SIMS map, a highly focused primary ion beam is rastered across the sample surface, and the secondary ions are collected at specific points. Image brightness at each point is a function of the relative concentration of the mapped element or molecule. Lateral resolution is less than 100 nm for elements. (III) In a depth profile, the sample surface is slowly sputtered away. Continuous analysis obtains composition information as a function of depth. Depth resolution of a few Å is possible. High sensitivity mass spectra can be recorded or reconstructed at any depth of the depth profile.
In the past 20 years, the advances in SIMS have resulted in numerous applications in biomaterial surface analysis. The combination of high mass resolution, sensitivity, mass range, and spatial resolution of static ToF-SIMS is capable of generating an enormous amount of information about the composition and molecular structure of biomaterial surfaces (especially polymer and protein surfaces in this work).

2.5 Confocal laser scanning microscopy (CLSM)

CLSM is widely-used in numerous biological science disciplines, from cell biology and genetics to microbiology and developmental biology. CLSM is a technique for obtaining high-resolution optical images with depth selectivity. The key feature of confocal microscopy is its ability to acquire in-focus images from selected depths. Images are acquired point-by-point and reconstructed with a computer, allowing three-dimensional reconstructions of topologically-complex objects.

The confocal principle in fluorescence laser scanning microscopy is schematically presented in Fig. 2.10. A microscope objective lens is used to focus a laser beam into a small focal volume within or on the surface of a specimen. In biological applications especially, the specimen may be fluorescent. Scattered and reflected laser light, as well as any fluorescent light from the illuminated spot, is then re-collected by the objective lens. A beam splitter separates off some portion of the light into the detection apparatus. The interesting wavelength range of the fluorescence spectrum is selected by an emission filter, which also acts as a barrier blocking the excitation laser line. The pinhole is arranged in front of the detector, on a plane conjugate to the focal plane of the objective. Light coming from planes above or below the focal plane is out of focus when it hits the pinhole (termed Out-of-Focus Light Rays in Fig. 2.10), so most of it cannot pass through the pinhole and therefore does not contribute to forming the image. The light intensity is detected by a photodetection device (usually a photomultiplier tube (PMT)), transforming the light signal into an electrical one that is recorded by a computer.
Confocal microscopy offers several advantages over conventional widefield optical microscopy, including the ability to control the depth of field, elimination or reduction of background information away from the focal plane, and the capability to collect serial optical sections from thick specimens.  

The fluorescence microscopy requires the objects to be fluorescent. Fluorescence is the emission of light that occurs within nanoseconds after the adsorption of light that is typically of shorter wavelengths. The difference between the exciting and emitted wavelength is the critical property that makes fluorescence so powerful. In a fluorescence microscopy, a dichroic mirror is used to separate the excitation and emission light paths. Excitation filter selects the excitation wavelength. Emission filter is used to specifically select the emission wavelength of the light emitted from the sample and to remove traces of excitation light. By completely filtering out the exiting light without blocking the emitted fluorescence, it is possible to see only the objects that are fluorescent. In conventional fluorescence
microscopy, the image of a thick biological specimen will only be in focus if its Z dimension is not greater than the wave-optical depth of focus specified for the respective objective. Unless this condition is satisfied, the in-focus image information from the object plane of interest is mixed with out-of-focus image information from planes outside the focal plane. This reduces image contrast and increases the share of stray light detected. If multiple fluorescences are observed, there will be a colour mix of the image information obtained from the channels involved.

The basic key to the confocal approach is the use of spatial filtering techniques to eliminate out-of-focus light or glare in specimens whose thickness exceeds the immediate plane of focus. With a CLSM it is therefore possible to exclusively image a thin optical slice out of a thick specimen (typically, up to 100 µm), a method known as optical sectioning. The fundamental advantage of the confocal LSM over a conventional microscopy is obvious: A confocal LSM can therefore be used to advantage especially where thick specimens (such as biological cells in tissue) have to be examined by fluorescence. The possibility of optical sectioning eliminates the drawbacks attached to the observation of such specimens by conventional fluorescence microscopy. Through the use of multiply-labeled specimens, the various channels are satisfactorily separated and can simultaneously identify several target molecules simultaneously, both in fixed specimens and living cells and tissues.31 On the other hand, depending on the diameter of the pinhole, light coming from object points outside the focal plane is more-or-less obstructed and thus excluded from detection. As the corresponding object areas are invisible in the image, the confocal microscope can be understood as an inherently depth-discriminating optical system. By varying the pinhole diameter, the degree of confocality can be adapted to practical requirements. As an added advantage, the pinhole suppresses stray light, which improves image contrast. The confocal microscope is often capable of revealing the presence of a single molecule.32

In the research presented here, thin films are deposited by spin coating. Thicknesses are measured by ellipsometry. The surface structure is determined by AFM, the surface hydrophilicity is determined by WCAA, and the surface composition is determined by ToF-SIMS. CLSM is used for the imaging of mammalian cells.
References


Chapter 3

Dewetting of PI films under water incubation

3.1 Introduction

This Chapter describes the dewetting phenomena of PI film on Si wafers under water incubation. As will be shown in subsequent chapters, PI resists protein adsorption. Hence, the stability of PI thin films in aqueous solution is of interest.

Thin polymer films play an important role in modern technology. The film stability is a basic problem still unresolved. If a thin film of one material is forced to coat a non-wetting surface using spin-coating or dip coating, the film can be unstable and dewetting of the substrate can occur. A familiar example is the breakup of a water film on a freshly waxed automobile. In the past several decades, experimental and theoretical studies\textsuperscript{1,2,3,4,5,6,7,8,9,10} of polystyrene thin films dewetting above the glass transition temperature ($T_g$) presented a general dewetting process which suggested the scheme shown in Figure 3.1. Dewetting begins with a nucleation event which leads to the formation of holes. The holes proceed to grow by the transport of material away from the nucleation site to a retreating rim surrounding them. As the hole continues to grow it eventually impinges on adjacent holes until a polygonal "cellular" structure resembling a 2-D foam is created. Complete dewetting occurs when the polymer ribbons forming the polygonal edges further decay into spherical drops, which may be due to the Rayleigh instability\textsuperscript{11} of cylindrical threads.
Figure 3.1. Scheme shows the stages of thermal dewetting of a thin liquid film in air. Dewetting begins with (a) the nucleation of holes in the film, followed by (b) the growth of these holes. A narrow size distribution of holes is typically observed. As the holes continue to grow they impinge on each other, and form a ribbon between them. (c) The onset of the hole coalescence distinguishes advanced dewetting. (d) Complete dewetting results in the formation of cellular patterns composed of liquid droplets. This pattern is formed by the coalescence of the holes followed by the breakup of the ribbons into isolated droplets.

The dewetting mechanism has been studied extensively. It became an almost "religious" question if thin films are destabilized by defects or by the so-called "spinodal dewetting" mechanism. On the spinodal dewetting side, Reiter\textsuperscript{2,3,12,13} has characterized the progression of dewetting from the early stage to complete dewetting using polystyrene films heated above $T_g$. The process of dewetting comes into the category of a general class of phenomena, spinodal dewetting. Spinodal dewetting is formally very similar to the process of spinodal demixing. For thick films, weak gravitational forces may stabilize the film. But in thin films below 100 nm, van der Waals force contributions play an important role. Depending on the dielectric constants of the substrate and the liquid, both forces can either stabilize or destabilize...
the film. If the van der Waals contribution is negative, the second derivative of the effective interface potential is positive, the film gains free energy by undulating the surface. Fig 3.2 describes the undulation of film. The gain of forming “valleys” compensates the cost of forming “mountains”. Assuming a small periodic undulation of the film surface, a single wavelength is amplified to a large extent. The preferred wavelength $\lambda$ is determined by the viscosity of the liquid and the surface tension of the liquid (because undulation increases the surface area). $\lambda$ is quadratic in thickness, $h$, and the characteristic time constant for this instability scales as $h^5$.

\[ \lambda \]

\[ h \]

**Figure 3.2.** A small modulation of the film surface with wavelength $\lambda$ leads to spinodal dewetting.

On the other hand, Jacobs et al.\textsuperscript{7,14,15,16} concluded that spinodal dewetting does not play any significant role. They demonstrated that rupture is dominated by nucleation from defects. Furthermore, strong evidence was given that these defects are intrinsic to the polymer due to its specific molecular structure. No matter which mechanism, i.e. spinodal break-up, heterogeneous or thermal nucleation, determines the dewetting scenario when the film is not stable, the observation that dewetting forms a series of patterns at different stages (as in Fig. 3.1) is identical.

Most studies on dewetting are through thermal dewetting processes, solvent-induced dewetting has also received much attention.\textsuperscript{17,18,19,20} In this mechanism, exposure of a thin film to a solvent induces a dewetting phenomenon. Several recent studies demonstrated that polyelectrolyte can undergo large-scale changes in the internal structure or surface morphology upon exposure to an aqueous medium.\textsuperscript{21,22} The patterns and structures generated during this transformation, e.g., nucleation and growth of holes, coalescence of holes, formation of cell-type structures, and the subsequent breakup of these features into droplets, are similar in many ways to those
observed in the dewetting of thin films of conventional polymers, such as polystyrene, on non-wetting surfaces. The nonsolvent-induced dewetting of thin, glassy polystyrene films at room temperature was also studied.\textsuperscript{23,24} A mechanism of nonsolvent-induced dewetting is deduced in the sequence of penetration, replacement, and coalescence, which is different from other previous dewetting mechanisms. There are no reports of the dewetting of molten polymer films in non-solvents. PI is a rubbery polymer at room temperature and is studied here.

In this chapter, the dewetting of PI films in a non-solvent (water) is studied. Because the polymer films were incubated in protein solutions in my project, stability of the polymer films under aqueous conditions is necessary. It is important to analyze the PI film morphology after protein incubation.

3.2 Experiments

3.2.1 PI thin film dewetting on different substrates

A 1 wt\% solution of PI ($M_w = 100$ kg/mol, Sigma-Aldrich) in toluene was spin-coated onto four different substrates, i.e. Si wafer with a 2.5 nm native silicon oxide layer, polypropylene (PP) sheets, silicone-coated paper and poly(ethylene terephthalate) (PET) sheets. All substrates are used in their as-received state, without any surface treatment. After film formation, the PI films on the four substrates were stored in a desiccator with silica gel at room temperature. After the desired number of days, the film morphologies were checked by optical microscopy.

The substrate surface energies were compared through water contact angle analysis (WCAA). WCAA used a sessile drop method (Easy Drop, Krüss GmbH, Germany) to deposit DI-water onto a sample surface. After capturing the drop images, the angles were measured using the commercial image analysis software. For every sample, the average value was obtained from three drops which were deposited at different areas.

3.2.2 PI thin film dewetting at different times

A 1 wt\% solution of PI in toluene was spin-coated onto Si wafers to prepare PI films with a thickness of ca. 50 nm determined by a variable-angle spectroscopic
ellipsometry (J.A. Woollam Co., Inc., Lincoln, NE, USA). The PI films were incubated in water for 1 min, 5 min, 15 min and 20 min. After taking out from DI-water and allowing to dry, the film morphologies were determined by an atomic force microscope (AFM).

The AFM (NTEGRA, NT-MDT, Moscow, Russia) was used in an intermittent-contact mode (tapping mode) to determine the pattern of the PI films after incubation in DI-water. Silicon cantilevers with gold coating (NT-MDT, Moscow, Russia) were used in the measurements. The spring constant is ca. 5 N/m and the resonant frequency is ca. 130 KHz.

3.2.3 Dewetting of PI thin film with different thickness

PI solutions with different concentrations were spin-coated on as received Si wafers to prepare PI thin films with their thickness of 23 nm, 46 nm, 85 nm and 182 nm, determined by ellipsometry. All films were incubated in DI-water for 15 min. After drying, film morphologies were observed by optical microscopy and tapping mode AFM.

3.3 Results and Discussion

3.3.1 PI thin film dewetting on different substrates

Spin-coating can form smooth but unstable films. PI films that can keep stable at room temperature are crucial for the study on protein adsorption, presented in the following chapters. Figure 3.3 are the optical images of PI films spin-coated on different substrates stored in air for six or more days. After spin-coating, PI film wets the SiOx surface very well. The AFM phase image (shown as the inset in Fig 3.3a) of the PI film surface is very smooth and the roughness is only 0.5 nm. After storing at room temperature for 35 days, the PI film was still smooth and did not dewet the Si wafer substrate, as shown in Fig 3.3a. Compared with PI film on SiOx, films on other substrates became dewetted after six days stored at room temperature. PI film formed holes (or drops) on the silicone sheet but formed big cracks on the PET and PP substrates. The fact that PI can stay stable on the Si wafer makes the study of PI films dewetting in a water environment possible.
Figure 3.3. PI films stored for 35 days on (a) Si wafer, and stored for 6 days on (b) PET, (c) silicone, and (d) PP substrates. White Scale bar is 10 μm. Inset image is AFM phase image of a PI film after spin-coating.

The surface energy of a substrate is critical to achieve good wetting and adhesion of inks, coatings, and adhesives. Non-wetting occurs when a drop of material (liquid or molten polymer) makes a finite contact angle upon being placed on a substrate. The substrate's surface energy must be higher than the surface tension of the liquid being laid down in order to get good wetting. The literature values of the substrates and PI film surface energies, have been listed in Table 3.1.

**Table 3.1.** Surface energies of different substrates.

<table>
<thead>
<tr>
<th>Substrates or films</th>
<th>Surface energy (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI</td>
<td>30–31</td>
</tr>
<tr>
<td>Si wafer</td>
<td>50–51</td>
</tr>
<tr>
<td>PET</td>
<td>41–44</td>
</tr>
<tr>
<td>PP</td>
<td>29–31</td>
</tr>
<tr>
<td>Silicone</td>
<td>22–24</td>
</tr>
</tbody>
</table>
The silicone surface has a lower surface energy than PI as summarized in Table 3.1. The PI film should dewet rather than wet to reduce its contact area with the silicone substrate. In Fig 3.3c, the PI film formed non-uniform holes or beads on silicone substrate after 6 days. PET sheet has a higher surface energy compared with PI. Theoretically, therefore, the PI film should wet the PET substrate. Nevertheless, the $T_g$ of PI with -65°C and the high chain mobility of PI molecules at room temperature makes a PI film unstable (Fig. 3.3b). The PP film has a similar surface energy as the PI film. With the high mobility of PI chains, PI cannot be stable on the PP sheet and dewets to create cracks, as seen in Fig 3.3d. The spin-coated PI film was stable on Si wafer for a month. It is expected that the Si wafer with a thin SiO$_x$ layer has a much higher surface energy than PI and can be well wetted by PI liquid melt.

3.3.2 PI thin film dewetting at different times

We next consider the dewetting of PI films on SiO$_x$ substrate after incubation in water. Fig 3.4 illustrates the AFM height and phase images of PI films (ca. 50 nm thick) dewetted for different times.

In Fig. 3.4a, the nucleation makes the PI film form non-uniform holes. The nucleation process is very fast and the isolated holes begin to touch each other after only 1 minute. AFM section views (the middle column) show the relative height dimensions during the AFM tip scanning. The valley in Fig 3.4(a) is 5 µm wide and 50 nm deep with a 23 nm high rim around it. The 50 nm is close to the original PI film thickness. The right column shows phase images of film patterns. The domains of the holes present bright spots. These areas dissipate less energy compared with PI during AFM tip scanning. We can deduce that the PI film ruptures to form holes which totally reach the Si substrate. There is not a thin wetting PI layer staying on the substrate in the hole. After 5 min. of incubation, these holes expand gradually until they touch each other to form the ribbons between every neighbouring hole. The valley in Fig 3.4(b) is 6.5 µm wide and 60 nm deep with the same 23 nm height rim. The PI film thickness increases about 10 nm. After a 15 min. of incubation, upon meeting ('coalescence'), the rims of the two holes overlap and form a straight common ribbon, which in cross-section is a portion of a cylinder. Such a liquid cylinder is dynamically unstable and easily decays into single droplets. In Fig 3.4(c), the adjacent holes coalesced to a typical polygonal cellular pattern and some areas
began to decay to the drops. The final stage is therefore a polygonal network made up of droplets, as illustrated in Fig 3.4(d). AFM scans reveal, however, that droplets indeed exhibit the shape of a spherical cap, and the drop height is around 280 nm.

Figure 3.4. PI film (with initial thickness ca. 50 nm) patterns after being incubated in DI-water for (a) 1 min, (b) 5 min, (c) 15 min, and (d) 20 min. The left column is AFM height images, the middle column is the cross-section of the marked lines in the height image, and the right column is the AFM phase images. Image sizes are 50 × 50 μm.
The dewetting mechanism of PI thin films at room temperature should be similar with PS dewetting under the thermal annealing because they all happen above their T_g. It could be expected that water has an influence on the wetting of PI because the less polar PI would be replaced by polar water at the SiO_x surface. The scheme in Fig. 3.5 illustrates the PI dewetting process incubated in a water environment. First of all, water molecules penetrate to the silicon oxide surface, perhaps through film defects, and nucleate small droplets. Nucleation happens at roughly the same time at relatively few nucleation sites. When water molecules reach the substrate, they will replace the nonpolar PI from the substrate, as water wets SiO_x better than PI.23 (See section 3.3.4 for a deeper discussion.). It is thermodynamically favorable for the holes to grow in size and to increase the area of the solid/water interface, if its interfacial energy is lower than the polymer/solid interfacial energy. In this case, the water droplets grow and spread, pushing the PI in a radial direction to the periphery. Then the impinging holes coalesce to form the polygon structure. Finally, the polygonal PI edges decay into spherical droplets. It is noteworthy that the water droplets increase in size and displace the PI film. The remaining polymer film, being displaced by the water, becomes thicker.

![Scheme of PI pattern formation through dewetting on silicon oxide surface under water incubation. (Redrawn from Ref 23)](image)

**Figure 3.5.** Scheme of PI pattern formation through dewetting on silicon oxide surface under water incubation. (Redrawn from Ref 23)
In previous research\textsuperscript{24} on the dewetting of thin, glassy PS films in water, an irregular structure appears on the PS film in the process of hole coalescence. The process differed from the usual thermal and solvent-induced dewetting process. This result may be because PS is not in its viscous state under the reported experimental conditions. It was concluded that the mobility of polymer chains is an important factor in influencing the morphology of the film in the nonsolvent-induced dewetting process. In these experiments, the PI film is viscous at room temperature. The high chain mobility of PI molecules makes water molecules more able to penetrate and displace the PI. The PI dewetting patterns of all processes followed the stages in Fig. 3.1.

![AFM images](image_url)

**Figure 3.6.** AFM images show that holes are nucleated in a PI film. (a) is the height image, (b) is the 3-D image corresponding with 3.6a, (c) is the magnified height image of area circled using a green square in 3.6a, and (d) is the scheme of the cross-section of the hole structure. Image sizes of 3.6a and 3.6b are 50 μm × 50 μm, image size of 3.6c is 2 μm × 2 μm.

Figure 3.6 shows the dewetted hole structure obtained from the water nucleation and displacement. There are bright rings around the holes in the height image. From the 3-D image (in Fig. 3.6b), it is very clear that the viscous PI that
previously was located in the interior of the hole is accumulated in a surrounding rim. The capillary forces push the polymer thin film away from the dewetted area, in a similar way to how a layer of snow of certain thickness is pushed with a shovel, resulting in the buildup of a rim in front of the shovel. Profiles of a hole taken at different times look quite similar. The profiles are typically asymmetric, as sketched in Fig. 3.6d and, as can be seen in Fig 3.4a, at the interior side of the hole there is a higher slope than on the outer side of the profile, where it meets the undisturbed film.

3.3.3 Dewetting of PI thin film with different thickness

A previous study about PS film dewetting under thermo-annealing conditions concludes that an increase of film thickness decreases the number density of initial holes, increases the polygon diameter, and increases the drop diameter. Experiments were conducted to see if PI films dewetting in water followed this trend. Fig. 3.7 shows the PI film patterns with four different original film thicknesses incubated in DI-water for 15 min. The thinnest PI film of 23 nm already begins to show a broken-up polygon cellular structure after 15 min as shown in Fig. 3.7a. The fast Fourier transform is shown as an inset, and it can be used to confirm that there is a characteristic length associated with the structure. The mechanism for the dewetting is not known with certainty, but its appearance suggests that spinodal dewetting could be occurring. The 46 nm PI film forms a bigger cellular pattern and begins to break up into ribbons. In a thicker film (85 nm), the PI forms dense holes and some holes have impinged to each other to form ribbons. Whereas, the thickest 182 nm PI film only goes through the first step, hole formation, and the hole density is less than in a thinner 85 nm film. The thickness-dependent result reveals that the thicker the film, then the slower is the dewetting processes. This experimental result agrees with the description of PS film dewetting under thermo-annealing conditions, although the dewetting-forming condition (PI dewets under water incubation but PS dewets under thermal annealing) and mechanism (PI dewetting initiates by the water molecule penetrating and nucleating but PS dewetting nucleates from the spinodal decomposition or the defects in films) are different. In the absence of water, these thickness variations are insignificant, because the film is stable in air. However, when water is introduced, the film thickness variation becomes a crucial factor.
Figure 3.7. PI film dewetting patterns after incubating in water for 15 minutes. Film thicknesses are (a) 23 nm, (b) 46 nm, (c) 85 nm, and (d) 182 nm. The left column shows the optical images, scale bar is 10 μm. The right column shows the AFM height images, image sizes are 50 μm × 50 μm. A fast Fourier transform of image (a) is shown as an inset.
3.3.4 Discussion

Young's Equation provides a relation of equilibrium between the contact angle of a liquid drop on a solid substrate and the interfacial energies between the liquid, substrate and vapour. Can we deduce the dewetting of PI on SiO$_x$ in water using this theory? Fig. 3.8 is the three-phase state of solid, liquid and ambient air or another liquid for experiments carried out in this work. Each case has different phases.

![Diagram of Young's Equation](image)

**Figure 3.8.** Three-phase state for Young's Equation. (a) Water drop on SiO$_x$ in the air, (b) water drop on PI film in the air, (c) PI film on SiO$_x$ in the air, (d) PI film on SiO$_x$ in the water ambient.

Every state in Fig 3.8 can be described using Young's Equation as shown here:

\[
\cos \theta_a = \frac{\gamma_{sv} - \gamma_{sw}}{\gamma_{sv}} \quad (3.1) \quad \cos \theta_b = \frac{\gamma_{pv} - \gamma_{pw}}{\gamma_{pw}} \quad (3.2)
\]

\[
\cos \theta_c = \frac{\gamma_{sv} - \gamma_{sw}}{\gamma_{sv}} \quad (3.3) \quad \cos \theta_d = \frac{\gamma_{sv} - \gamma_{sp}}{\gamma_{sv}} \quad (3.4)
\]

where, $\gamma$ is the interfacial energy, and the capitals in the subscripts in the equations are described as: S=Silica, W=Water, P=PI, and V=Vapour. $\theta_a$, $\theta_b$, $\theta_c$, and $\theta_d$ are the contact angles in Fig. 3.8a, 3.8b, 3.8c, and 3.8d, respectively.
The tested water contact angles, $\theta$, of different substrates and PI film are listed in Table 3.2.

**Table 3.2. Water contact angles of different substrates.**

<table>
<thead>
<tr>
<th>Substrates or film</th>
<th>Water contact angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI film</td>
<td>100.5±1.3</td>
</tr>
<tr>
<td>Si wafer</td>
<td>30.0±2.4</td>
</tr>
<tr>
<td>PET</td>
<td>76.2±1.6</td>
</tr>
<tr>
<td>PP</td>
<td>102.1±1.1</td>
</tr>
<tr>
<td>Silicone</td>
<td>113.4±1.0</td>
</tr>
</tbody>
</table>

From Equation 3.2, $\gamma_{PW} = \gamma_{PV} - \gamma_{WV}(\cos \theta_b)$. Using values given in Table 3.1 and 3.2, the interfacial energy of PI in air, ($\gamma_{PV}$), is 30.5 mN/m, the surface tension of water, ($\gamma_{WV}$), is 72 mN/m, and the contact angle of water on the PI film, $\theta_b$, is 100.5°. Then the $\gamma_{PW}$ is calculated to be 43.6 mN/m.

From Equation 3.1, $\gamma_{SW} = \gamma_{SV} - \gamma_{WV}(\cos \theta_n)$. The contact angle of water on the SiO$_x$ substrate, $\theta_n$, is 30°. Then $\gamma_{SW} = \gamma_{SV} - 62.354$ (mN/m).

From Equation 3.3, $\gamma_{SP} = \gamma_{SV} - \gamma_{PV}(\cos \theta_c)$. We already confirmed the PI film can be stable in air for a month and did not dewet on the SiO$_x$ substrate; the angle of the PI on SiO$_x$ substrate can be taken to be ca. 0°. Then $\gamma_{SP} = \gamma_{SV} - 30.5$ (mN/m).

Plugging in values of $\gamma_{PW}$, $\gamma_{SW}$, and $\gamma_{SP}$ in Equation 3.4, we find that:

$$\cos \theta_d = \frac{\gamma_{SW} - \gamma_{SP}}{\gamma_{PW}} = -0.73 \quad \Rightarrow \quad \theta_d = 137°.$$  

This result means that the PI film on SiO$_x$ substrate in water ambient is not expected to be stable. Young’s equation predicts that the PI film will go through a dewetting process under water incubation.
3.4 Conclusion

Whereas there are numerous studies of the dewetting of polymer films in air (or vacuum) at elevated temperatures, there are fewer studies of non-solvent-induced dewetting. This is the first report of a molten polymer film dewetting in a non-solvent, to the best of our knowledge. Spin-coated PI films on Si wafers were found to dewet under a water incubation environment. The dewetting process consists of nucleation and growth of holes, coalescence of holes, formation of cell-type structures, and the subsequent breakup of these features into droplets. This process is very similar to the conventional thermo-dewetting process of PS films. The proposed mechanism is that the water molecules penetrate to the polar silicon oxide surface and nucleate water beads. As the water spreads, PI holes coalesce and form ribbons. In addition, the PI film dewetting patterns are dependent on the original film thickness. Larger patterns are found in thicker films. Increasing the film thickness can slow down the PI dewetting process.
References

http://www.accudynetest.com/surface_energy_materials.html
Chapter 4

Protein nanopatterning on self-organized PS-b-PI copolymer thin film templates

This chapter will describe the creation of well-organized PS-b-PI thin film templates, and the templates will be used to nanopattern a protein through the selective adsorption on glassy PS domains.

4.1 Introduction

4.1.1 Control of diblock copolymer film morphology

In the bulk, the equilibrium morphology depends largely on the relative volume fractions of the components. It is known that a symmetric diblock copolymer forms the lamellar structure and an asymmetric diblock copolymer forms the cylindrical or spherical structures, depending on the volume fractions of the two blocks. But in thin films, additional effects have been observed and some questions remain as regards the morphology and topography of the free surface. For example, there has been a recent interest concerning: (1) the influence of the bulk morphology on the arrangements of surface microdomains; (2) the effect of surface energies of copolymer blocks; (3) the effect of the substrate; (4) the dependence on the film thickness; and (5) the effects of casting solvents and evaporation rate.

The role of film thickness on the morphology of amorphous diblock copolymer films has been a topic of concern in the past decades. Some experimental and theoretical works on the fundamental physics of these systems were conducted by a few research groups. Decreasing the thickness of the film such that it approaches or is less than one domain period \( L \) places further constraint on the copolymer

\footnote{Portions of this chapter have been published in Liu D. et al, Langmuir, 2009, 25, 4526.}
molecules. Even without an actual confining hard wall at the top surface, the films with thickness $h \leq L$ are effectively confined.\(^6\) Henkee et al\(^7\) also reported that, in this case, the normal microdomain morphology is inhibited. Nonequilibrium morphologies of thin films are easily obtained on the laboratory scale by freezing morphological patterns with quick evaporation of solvents.\(^8\) Since the morphology of the nonequilibrium state is reproducible and is long-time metastable, this current study of the surface morphology dependence on the film thickness of PS-PI diblock copolymers was carried out on the as-spun state.

As we know, the as-spun film is a nonequilibrium state although it is stable for a very long time. A previous study\(^9\) showed that in the thick copolymer films the most-observed structure is the parallel ordering in the equilibrium state. In the case of symmetric diblock copolymers, the preferential interaction of one block with the substrate and the difference in the surface energies of the blocks can cause the formation of a multilayer structure, when the film is heated above the glass transition temperature.\(^10\) The microphase separation of a block copolymer results in a quantised film thickness. The thickness depends on the number of lamellar layers. If a film is spin-coated slightly thicker than the thickness to form $n$ layers, this thickness constraint will force the creation of an $n+1$ 'islands' layer on the top of a uniform film with $n$ layers. Conversely, if a film is spin-coated slightly thinner than the thickness to form $n$ layers, this constraint will produce 'holes', which are regions of the $n-1$ layer in a thicker background of $n$ layers.\(^11\) Green and co-workers\(^12,13\) also probed the patterning dynamics of block copolymer ultrathin films and reported that the resultant surface topology was likewise dependent on film thickness and annealing time. The average size and spatial distribution of these islands or holes were found to vary with the annealing time. The appearance of the free surface is reminiscent of a two-dimensional phase coarsening process.

In this chapter, the above-discussed influencing factors on the surface structures of diblock copolymer films will be investigated. It includes the weight ratio effect, the thickness influence, and the annealing treatment effect.
4.1.2 Protein nanopatterning on self-assembled copolymer templates

Biomolecule patterning is of great interest both for applications in biosensors and tissue engineering. The patterning requires a straightforward method to immobilize biomolecules on a solid surface with a precise and sub-micrometer spatial control. Various top-down methods, e.g. optical lithography, electron lithography, dip-pen lithography, and imprint lithography, have already been employed to pattern biomolecules, especially proteins. These lithographic techniques have been developed to reduce the size of biomolecular patterns from the micrometer to the sub-micrometer length scale.

Bottom-up methods are an important alternative approach that have been developed to immobilize proteins onto specific sites on surfaces with resolution on the nano-scale. For the bottom-up method, a substrate that possesses spatially-defined bio-adhesive patterns on a background that resists protein adsorption can be employed to define the protein positions. With selective adsorption of the protein on one component, the substrate can be used to pattern proteins. Of particular interest here, pairs of polymers with differing protein-adhesive/resistant properties can be used in the bottom-up method to create a template to define the protein location on a surface.

Diblock copolymers have many applications based principally on their ability to form regular nanoscale patterns over a macroscopic area via a self-assembly process. These self-assembled patterns have been applied as nanolithographic masks and as templates for the synthesis of inorganic or organic structures. Extensive work has been done to control the self-assembly and orientation of block copolymer thin films. Block copolymer thin films are of particular interest for protein patterning because of the possibility of obtaining two-dimensional templates with very high regularity.

In the past several years, some researchers have focused their attention on a direct and facile approach for protein patterning. They directly adsorbed proteins onto hydrophobic-hydrophilic diblock copolymer film templates. For example, Kumar et al. used amphiphilic diblock copolymer templates to control the spatial resolution of adsorbed proteins on the nanometer scale by site-selective adsorption. Their results indicate that the protein selectively localizes on the hydrophobic
component, which guides the spontaneous construction of protein arrays. Knoll et al.\textsuperscript{32} have likewise recently reported protein nanoarrays on templates made from a diblock copolymer of poly(styrene) and poly(methyl methacrylate), in which the latter block is hydrophilic (having a water contact angle less than 90°). Other groups\textsuperscript{33, 34} have used polymer blend systems to study the selective protein adsorption on phase-separated polymer surfaces. Meanwhile, researchers\textsuperscript{35, 36} have patterned proteins on templates created by the controlled dewetting of the top film in a bilayer consisting of a hydrophobic polymer and a hydrophilic polymer. The protein spatial array on a well-ordered polymer template is illustrated in Fig. 4.1

![Self-organized diblock copolymer film](image)

**Figure 4.1.** Schematic diagram of the protein nanopatterning on self-assembled diblock copolymer template.

Hydrophobic and hydrophilic polymers both adsorb proteins, but there is less protein adsorption on the hydrophilic polymer surface.\textsuperscript{37} When a material is brought into contact with a biological fluid, the first event that occurs is usually the adsorption of proteins onto its surface. The broad assumption in discussions of these interactions is that the initial stage ends in non-biospecific adsorption involve hydrophobic interactions between the surfaces of the material and hydrophobic patches of the protein. The initial adsorption is probably weak and reversible. Following the initial stage, proteins may denature and unfold to expose more hydrophobic groups, and ultimately attach irreversibly to the surface.\textsuperscript{38, 39} Then proteins are preferentially adsorbed on hydrophobic polymer surfaces. Hence, the differences in hydrophilicity have been used in previous reports to explain observed protein templating on hydrophobic/hydrophilic diblock copolymers.\textsuperscript{32} (However, there are a variety of physical and chemical factors that can influence protein adsorption beyond the
hydrophilicity of the substrate.) In previously reported work, the successful use of a hydrophobic-hydrophilic diblock copolymer system to pattern proteins employed a very low protein concentration of 4-20 μg/ml and a very short protein adsorption time of 20-60 s. We are not aware of any reports of the formation of protein patterns at higher protein concentrations or for longer adsorption times using the copolymer templating method.

In this work, a diblock copolymer with two hydrophobic components was explored to nanopattern proteins. One block has a glass transition temperature (T_g) above room temperature, making it glassy ("hard") during application. The other block has a lower T_g, making it rubbery ("soft"). The protein nanopatterning is achieved even in a relatively high protein concentration solution incubated over relatively long times. Specifically, bovine serum albumin (BSA) protein adsorption and nanopatterning is reported on a template made from self-organized poly(styrene-b-isoprene) (PS-b-PI) diblock copolymer thin films in their non-equilibrated state. These particular structures have not been previously reported for ultrathin PS-b-PI films without prior thermal or solvent annealing. Atomic force microscopy (AFM), Time-of-flight secondary ion mass spectrometry (ToF-SIMS) and water contact angle analysis (WCAA) are complementarily used to confirm the protein preferential adsorption and nanopatterning on the diblock copolymer templates.

4.2 Experiments

4.2.1 Film preparation of PS-b-PI with different weight ratios

Two poly(styrene)-block-poly(isoprene) (1,4 addition) (PS-b-PI) block copolymers purchased from Polymer Source Inc (Montreal, Canada) with the same overall number molecular weight but with different weight ratios were used to compare the morphologies in ultra-thin films. Both copolymers have the number-average molecular weight of \( Mn = 91 \) kg/mol. One symmetric PS-b-PI possesses a PS component with the weight of 45 kg/mol and PI component with the weight of 46 kg/mol. It is presented here as PS(45)-b-PI(46). Another asymmetric PS-b-PI has a PS component with the weight of 65 kg/mol and PI component with the weight of 26 kg/mol. It is described as PS(65)-b-PI(26).
Both PS-b-PI copolymers were dissolved in toluene solvent. The concentration was 0.5 wt% in the two solutions. The PS-b-PI solutions were spin-coated onto 1×1 cm², (100) single-crystal silicon wafers with a spin rate of 2000 rpm. The thicknesses of the as-spun films were measured with spectroscopic ellipsometry over a wavelength range from 400 to 800 nm. The thicknesses are 18.2 nm of PS(45)-b-PI(46) and 18 nm of PS(65)-b-PI(26), respectively.

4.2.2 Film preparation of PS-b-PI with varying thicknesses

The symmetric PS(45)-b-PI(46) and asymmetric PS(65)-b-PI(26) were dissolved in toluene. Thin films were prepared by spin-coating solution onto silicon substrates with a spin rate of 2000 rpm. The film thicknesses of PS(45)-b-PI(46) were adjusted by changing the solution concentration over a range from 0.3 to 3 wt%. The film thicknesses of PS(65)-b-PI(26) were controlled by adjusting the solution concentrations from 0.3 to 1 wt%. After drying in a desiccator at room temperature, all film thicknesses were measured with ellipsometry.

4.2.3 Film preparation of PS-b-PI and annealing treatment

It is possible to get various topographies of copolymer films by adjusting the annealing conditions. We chose 125 °C as the annealing temperature, a temperature which is over the glass transition temperature of PS (the higher $T_g$ in SI system, $T_g$ of PS is 100°C and $T_g$ of PI is -65°C.) and below the $T_{ODT}$ (order-disorder transition temperature) of our SI diblock copolymers. It should be realized that within seconds the temperature of the film is below the glass transition temperature of the copolymer when it is moved out from the annealing condition. Within this time no significant changes in the state of the copolymer could occur; therefore, the structure at the annealing temperature is effectively frozen-in.

Thick PS(45)-b-PI(46) films were prepared by spin-coating toluene solution onto silicon substrates with a spin rate of 2000 rpm. The film thicknesses of PS(45)-b-PI(46) were adjusted by changing the solution concentration from 1 wt% to 3 wt%. Films with various thicknesses were annealed in vacuum oven at 125 °C. The different annealing times (as-spun state, annealing for 4 h and 20 h) were used to explore the thermodynamics of polymer thin film structure development.
4.2.4 PS-b-PI, PS and PI film preparation for BSA adsorption

PS(45)-b-PI(46) was dissolved in toluene to obtain 0.5 wt% and 3 wt% concentrations, respectively. The spin-cast films (2000 rpm and 10 s) were 18.2 nm and 199 nm thick as measured by ellipsometry. The 199 nm PS(45)-b-PI(46) film was annealed for 24 hr at 125 °C until reaching the equilibrium state. PS(65)-b-PI(26) 0.5 wt% toluene solution was spin-cast onto Si wafer and obtained a 17.6 nm thick film. A 1 wt% solution of PS (M_w = 257.9 kg/mol, purchased from Polymer Source Inc.) in toluene was spin-coated to obtain a 42 nm thick film. A toluene solution of PI with molecular weight of 100 kg/mol (ca. 4 wt%) was spin-coated onto the Si wafer substrate to deposit two 285 nm thick PI films for different uses.

4.2.5 Incubation of polymer films in BSA solution

BSA (>98%, lyophilized powder, 66 kDa) was used as received from Sigma-Aldrich. The PS-b-PI, PS and PI films were incubated at room temperature in a BSA solution (500 µg/ml in a phosphate buffer (PB) solution, pH=7.2) for 1 h. Another PI film was incubated for 15 min. Upon removal from the BSA solution, the samples were rinsed thoroughly with flowing PB solution and DI water to remove non-adsorbed BSA molecules and residual salt from the buffer, respectively. The samples were dried in a desiccator (containing silica gel) for 24 hr prior to AFM, ToF-SIMS and WCAA. In some later experiments, the PS-b-PI templates were incubated in a higher concentration of 1 mg/ml for a longer time of 2 hr.

4.2.6 Atomic force microscopy

AFM was used in an intermittent-contact mode to determine the morphology of the templates before and after BSA incubation. Silicon cantilevers with a spring constant of ca. 5 N/m and a resonant frequency of around 130 KHz were used in the measurements. The setpoint amplitudes were kept at similar values when analysing the same type of surface in order to ensure consistency and reproducibility.

4.2.7 Water contact angle analysis

WCAA used the sessile drop method. A 5 µl drop of de-ionized water was deposited onto the sample surface. The drop shapes were captured immediately, and
the water contact angles were determined from high resolution images using commercial image analysis software. For every sample, four drops were deposited at different areas, and the average value was obtained.

4.2.8 Time-of-flight secondary ion mass spectrometry

ToF-SIMS was employed to characterize the relative intensity of surface components before and after the protein adsorption. ToF-SIMS analyses were carried out on an ION-TOF GmbH (Münster, Germany) TOF-SIMS 5 system. The instrument is equipped with a reflection type analyser and microchannel detector. SIMS spectra were acquired using a Bi$_3^+$ cluster ion beam; data acquisition was performed by raster scanning the Bi$_3^+$ primary ion beam over a 100 $\mu$m $\times$ 100 $\mu$m area at a resolution of 64 $\times$ 64 pixels. Every sample was probed three times in three different areas, and averages were obtained. The probe depth was around 10-20 Å from the film surfaces. The positive and negative ions from the sample’s outermost surfaces were collected and converted to the m/z = 0-500 mass spectra.

4.3 Results and discussion

4.3.1 Changes of diblock copolymer film morphologies with varying factors

4.3.1.1 Thin film morphologies with different weight ratios

The ultrathin films of around 20 nm thickness were selected to compare their surface morphologies. Their height and phase images of AFM were shown in Fig. 4.2. The contact angle of water on PS films was found to be 95±0.7°, whereas it had only a slightly higher value of 101±1.2° for the PI films. As both contact angles are greater than 90°, both blocks of PS-b-PI are classed as hydrophobic, in comparison to the hydrophilic/hydrophobic combinations used in previous work on protein templates. The $T_g$ of PS is 100 °C,$^{40}$ and hence PS molecules are in a glassy state at a room temperature of 20 °C. The $T_g$ of PI is -65 °C,$^{40}$ and PI molecules are in a rubbery state at room temperature. Hence, this copolymer can be referred to as having a “hard-soft” pair of blocks. The glass transition temperature of PI is much below the normal room temperature. PI domains are more viscous and dissipate more energy than PS
when in contact with the AFM tip.\footnote{41} Hence PI domains are presented as the darker areas in the phase image.\footnote{42} The very soft material can be indented down to the substrate and presents a lower surface compared to the hard material. So PI also presents the dark area in the height image. Observing the height and phase images of PS(45)-b-PI(46) film surface, the height roughness is only 5 nm but the phase difference is 25 degrees. This means that the PI domains on the free surface were indented a little, but the energy dissipations of PS and PI are quite different.

![AFM images](image_url)

**Figure 4.2.** (a) and (b) are AFM height and phase images of a PS(45)-b-PI(46) film of 18.2 nm, (c) and (d) are AFM height and phase images of a PS(65)-b-PI(26) film of 18 nm thick; image sizes are 2 μm x 2 μm.

In bulk, the *symmetric* diblock copolymer should form the lamellar structure and the *asymmetric* one (71% PS) should create PI cylinders which are dispersed in a PS matrix.\footnote{43} In block copolymer thin films, however, there are additional contributions of the surface free energy and the substrate-polymer interaction to the morphology formation and therefore the morphology of a thin film is different than in the bulk. The images in Fig. 4.2 further proved the morphology difference in thin films. The *symmetric* PS(45)-b-PI(46) film showed the PI spheres in the PS connected
network. The PI component on the surface is smaller than the 50% ratio in the bulk. It is because the PI component has a strong interaction with the Si substrate, which has been pointed out by others. The strong affinity of PI with the substrate makes PI prefer to form a thin layer on the substrate. The lower surface energy of PI also makes PI domains easily segregate to the free surface to minimize the film's surface free energy. There are several reports in the literature for the nanostructure of PS-b-PI films after they have been annealed to achieve an equilibrium structure. PI creates a wetting layer at the air interface, and etching has been used to reveal the sub-surface phase structure. But the images in Fig. 4.2 are from films that have been frozen in nonequilibrium states in the spin-casting process. So PS domains are also presented on the free surface to create the spheres structure. The coexistence of PS and PI components on the free surface will be confirmed later through SIMS analysis. The asymmetric PS(65)-b-PI(26) with less PI component formed the PS cylinders parallel to the surface and only a few PI domains (the dark spots in Fig 4.2d) were observed at the free surface. This structure result is also because the PI component prefers to form a PI layer with a thickness of several nanometres to satisfy covering the substrate. Then PI, the component with the lower surface energy, further tries to appear on the free surface. But there is a lower PI ratio in PS(65)-b-PI(26) compared to the PS(45)-b-PI(46), so less PI component segregates at the free surface. This is reflected in the AFM phase images, where there are less dark areas on the free surface.

4.3.1.2 Thickness-induced morphology changes

The symmetric PS(45)-b-PI(46) case is shown in Fig. 4.3. In Fig. 4.3a, a stripe-like pattern is formed with the minimum thickness of 12 nm that we made. With the thickness increasing, the PI nanodomains become shorter at the surface and tend to form the spheres, shown in Fig. 4.3b. At a thickness of 19 nm, almost all the PI domains form the spheres dispersed in a PS network matrix. As the thickness continues to increase, the PS network disappears, and a continuous PS phase with PI separated domains is created. At a 28 nm film surface, the PI domains form spherical and cylindrical complex structures. The morphology of a “patchwork quilt” is also shown in the thickness of 60 nm. When the thickness is 200 nm, a similar “nanoflower” structure is created, and this structure is found over a wide thickness range.
The complicated behaviour is exhibited for films of thickness $h < L$. The morphology of the symmetric PS(45)-b-PI(46) film of a thickness near 20 nm is a similar hexagonal structure. The magnified hexagonal order is presented in Fig. 4.4a. The fast Fourier transformation (FFT) analysis (Fig. 4.4b) shows an elongated hexagonal pattern of the domains. Such morphologies are particularly interesting because they offer the possibility of designing nanometer scale chemically-patterned surfaces.
Figure 4.4. (a) Phase images of PS(45)-b-PI(46) as-spun films with 19 nm thickness, image was magnified until one ordered hexagon filled the entire area. (b) Fast Fourier transformation of the phase image shown in Fig. 4.3c.

The striped surface structure in Fig. 4.5a is consistent with an edge-on lamellae structure, which is the lamellae oriented perpendicularly to the substrate, as shown schematically in Fig. 4.5c. This morphology has been noted in previous theoretical and experimental studies\textsuperscript{47,48} for polystyrene-b-poly(lauryl methacrylate) (PS-b-PLMA) ultrathin film in their equilibrium state. As the film thickness increases, the PI nanodomains become shorter at the surface and tend to form circular dots. At a thickness of 19 nm, almost all of the PI surface domains form uniform dots surrounded by the PS matrix (Fig. 4.5b). Drawing on the structures reported for PS-b-PLMA films, which was called a hybrid structure,\textsuperscript{48} we propose that the PS surface shown here in Fig. 4.5b lies above a layer of PI at the substrate, with perpendicular PI protrusions extending to the free surface, perforating the upper PS layer, as illustrated in Fig. 4.5d.

Figure 4.5. A proposed diagram of PS(45)-b-PI(46) as-spun thin films in cross-sectional view. The left one corresponds to 12 nm thick film and the right is for a 19 nm thick film.
The ultrathin films show the well-ordered patterns on the free surface because of the strong substrate-block interactions. But the thicker films (over 1/2 lamellar period) present disordered structures on the surfaces. In the as-spun state, these metastable films allow more PS component to form the continuous matrix at the free surface and a few PI domains to locate perpendicular (spheres viewed from the top) or parallel (cylinders viewed from the top) to the free surface. This result can be explained by the influences of solvent selection and solvent evaporation rate. In general, toluene is considered to be a mutual solvent for PS and PI. However, a careful analysis of several Hildebrand solubility parameter ($\delta$) values reported in the literature$^{49,50}$ shows that the $\delta$ values are 16.3, 18.6 and 18.2 (MPa)$^{1/2}$ for 1,4-PI, PS and toluene, respectively. The values of $\delta$ must be similar for a solvent to dissolve a polymer. There is also evidence that toluene is a slightly selective solvent to PS over PI. It has been shown that the selective solvents do cause surface enrichment of the most soluble species.$^{51}$ It is shown elsewhere that the solvent evaporation rate affected the surface morphology. A fast solvent evaporation formed an inverted morphology at the free surface rather than a thermodynamic equilibrium film.$^{52}$ In our results, the films are solidified by fast solvent evaporation (likely in the spin-coating procedure), and the PS component is favourable to reside on the free surface of the thicker films.

The asymmetric PS(65)-b-PI(26) case about thickness-induced film surface morphology changes was shown in Fig. 4.6. Theoretically, the polymer molecules of PS(65)-b-PI(26) are expected to form a cylindrical structure in the bulk.$^{43}$ It will be different in a thin film because of the substrate and free surface confinements. At the minimum thickness of 11.5 nm in our samples, the morphology appears to be PI spheres dispersed in a PS matrix. There is less PI component residing at the free surface compared with the 12 nm PS(45)-b-PI(46) film surface. This result can be explained by the PI segregation to the substrate, and the film surface morphologies being dependent on the block weight ratios in the same thickness. When the thickness is increased to 19 nm, a striped structure emerges at the surface, which is interpreted as a cylindrical structure parallel to the substrate. With increasing film thickness, it was found that the stripe length decreases. A short ribbon structure is seen in the 25 nm film (Fig. 4.6c). With the film thickness continuing to increase, the lengths of the stripes decrease. At a thickness of 39 nm (Fig. 4.6d), the morphology becomes a
complex structure of spheres and short ribbons. Previous theoretical\textsuperscript{53} and experimental\textsuperscript{54} study of the parallel and vertical morphologies of cylindrical domains likewise reported such a thickness dependence. Based on their conclusion, one interpretation of the structure transition in Fig 4.6 is that copolymer cylinders are transferred from being parallel to the substrate to being perpendicular to the substrate as the film thickness, $h$, approaches the lamellar domain period, $L$.

![Figure 4.6. Phase images of PS(65)-b-PI(26) as-spun film surfaces on silicon wafers. (a) 11.5 nm, (b) 19 nm, (c) 25 nm (d) 39nm, Image sizes are 2 $\mu$m x 2 $\mu$m.](image)

To the best of our knowledge, there are no previous studies of the effects of confinement on the non-equilibrium (freshly-cast) structures of PS-b-PI films, as previous work has concerned the equilibrium structures obtained after thermal annealing.\textsuperscript{44,45,46} The results in Fig. 4.3 and Fig. 4.6 show that the surface structure of nonequilibrium PS-b-PI films is profoundly affected by $h$ when $h < L$. In future applications of this copolymer as a protein template, a variety of nanostructures can be achieved through selection of the diblock ratio and adjustment of $h$. But there are not ordered structures and little regularity when the film thickness is bigger than $L/2$ in the as-spun state. So the heat treatment becomes crucial in thicker films.
4.3.1.3 Annealing-induced morphology changes

As an example, the results of the 75 nm film are presented in Fig. 4.7. The morphologies of the three processing conditions (as-spun, 4h annealing and 20h annealing) are compared. The structure of the as-spun film is PI spheres and worm-like short cylinders dispersed in the PS matrix. The PI domain size is around 50 nm, as indicated by the scale bar. This is a nonequilibrium state because of the fast solidification of the chain movement during spin-coating. During the heat treatment, the rearrangement of chains occurs. This will relax the nonequilibrium morphology formed by the fast spin-coating process. The PI, a lower surface energy component, will move to the free surface. After 4h annealing, the microphase-separated nanostructure has been destroyed. More PI domains move from the inner interface of the film to polymer/air interface through the network channels. During the moving process, PI domains also aggregate together to form a large sphere and short ribbon structure. The domain size of PI is around 500 nm, as observed from the scale bar, and the size is 10 times that of the non-annealed state. After 20h annealing, all of the PI component segregates to the free surface and forms a very flat surface. The roughness and phase contrast decrease a lot compared with the other two images. In the 2 × 2 μm image of Fig 4.7c, it shows that only one phase is located at the free surface. The three images of this film show the thermodynamic process going from the nonequilibrium to the equilibrium state.

Figure 4.7. Phase images of the surface of PS(45)-b-PI(46) 75nm film. (a) as-spun, (b) after 4 h annealing at 125 °C, (c) after 20 h annealing at 125 °C. The scale bar is 1 μm in each phase image.
The PS(45)-b-PI(46) film morphologies of two thicknesses, 75 nm and 182 nm, after 20 h annealing are compared in Fig. 4.8. It is clear that the 75 nm film has a totally flat surface and only one component segregates to the free surface. But the 182 nm film forms a structure of holes and the size of the holes is around 1 μm. There is a 50 nm roughness of the annealed 182 nm film viewed from the height image. The phase contrast is lower than seen in some of the previous images. One explanation could be the presence of a PI wetting layer. This result further proves that the film topography after annealing treatment will be different when we vary the thickness of the initial as-cast films.

Figure 4.8. Height and phase images of PS(45)-b-PI(46) films after 20 h annealing, (a) height image of 75 nm film, (b) phase image of 75 nm film, (c) height image of 182 nm film, (d) phase image of 182 nm film. Image sizes are 10 μm x 10 μm.

Figure 4.9 compares the morphologies of the PS(45)-b-PI(46) films with three different thicknesses after a 4 h annealing treatment. This time is not enough for the thin film to reach its equilibrium state. But we can explore the dynamic process from the series of morphologies. The 75 nm film presents a structure of PI spheres or ribbons dispersed in a PS matrix. There is a spinodal-like structure in the 107 nm film.
after 4 h annealing. The 182 nm film shows a hole structure and the size of the holes is around 3 μm, which is six times that in the 75 nm film. From the results in Fig 4.9, we conclude that the process of the chain rearrangement as driven by thermodynamics is also dependent on the film thickness.

![Figure 4.9](image1.png)

**Figure 4.9.** Phase images of the surface of PS(45)-b-PI(46) films after 4 h annealing. (a) 75 nm film, (b) 107 nm film, (c) 182 nm film. Image sizes are 10 μm × 10 μm.

This study presents that copolymer organization in thin films develops over time. During the heating, the local rearrangement of chains occurs, relaxing the nonequilibrium microphase-separated morphology formed during the film formation by spin-coating. In previous studies,\textsuperscript{55,56} it was found that different morphologies are encountered depending on the annealing time and the thickness. Our results are consistent with that of other research groups.\textsuperscript{33,36} The thermodynamic process of a copolymer film upon thermal annealing was summarized as below: The resulting three-dimensional segregated network provides percolating pathways for low-resistance diffusion of chains along the PS-PI interface. Under 125 °C, an oriented multilayer front travels from the substrate upwards, fed by these channels which perforate the lamellae. As the lamellar domains reach the equilibrium thickness, the channels become unstable and dissolve into the lamellar structure. At the film/air interface, PI blocks gradually wet the PS at the surface, causing height fluctuations which ultimately decay or develop into steps, depending on the initial film thickness.

### 4.3.2 BSA adsorption and nanopatterning on PS-b-PI templates

The surface morphologies of all samples (polymers and BSA layers) were scanned by AFM. After BSA incubation and rinsing, the surface morphologies of PS and PI homopolymers are shown in Figure 4.10. As viewed from the upper row in Fig
4.10, the PS surface is densely coated by BSA molecules, which forms a "carpet" with a few visible packing defects or "holes". Complementary ellipsometry measurements on the same sample, before and after BSA incubation, were performed. This analysis used a refractive index for the BSA that was consistent with the literature\textsuperscript{57} to model the data. Ellipsometry indicated that the average BSA layer thickness on PS is approximately 3 nm. This thickness is comparable to the BSA's molecular dimension of 4 nm.\textsuperscript{58} We conclude from the complementary use of AFM and ellipsometry that the PS surface is almost entirely covered by a monolayer of BSA molecules.

For the PI surface after BSA adsorption, several white spots in topographic AFM image and dark spots at the corresponding positions in the phase image. These spots might be BSA molecules located on the PI surface. The image after BSA incubation is quite similar to the PI homopolymer film surface before protein adsorption (not shown). This AFM analysis indicates that PI is perhaps a protein-resistant material, but further analysis by ToF-SIMS and WCAA were carried out to validate this interpretation.

![Image of AFM images](image_url)

Figure 4.10. AFM (a) height and (b) phase images of a 42 nm PS film after BSA incubation for 1h; and AFM (c) height and (d) phase images of a 285 nm PI film after being soaked in BSA for 15 min; image sizes are 1 \( \mu \text{m} \times 1 \mu \text{m} \).
From the survey just presented, two nanostructures were chosen for subsequent application as protein templates: a PI dot pattern (as in Fig 4.3c) and a striped pattern (as in Fig 4.6b). We first consider an 18.2 nm thick film of the PS(45)-b-PI(46) symmetric copolymer, which forms a surface pattern of PI circular dots dispersed across a PS surface (Fig. 4.11a and b). After BSA adsorption, the surface presents a very similar pattern. The image is interpreted as showing that BSA molecules are localized onto the bright PS blocks (as observed in Figure 4.11d and e) in a connected ring-like pattern. In support of this interpretation, the surface roughness values before and after BSA adsorption are compared from the topographical traces across a 1 μm distance. The peak-to-valley roughness of the height profile increases from 5 nm to 10 nm after the BSA adsorption. An increase in 5 nm is roughly consistent with the adsorption of at least a single layer of BSA molecules. The pitch of the profile after BSA adsorption matches that of the original copolymer surface.

![Figure 4.11](image_url)

**Figure 4.11.** Evidence for selective BSA adsorption. The upper row shows AFM (a) height and (b) phase images and (c) the corresponding height profile of the PS(45)-b-PI(46) 18.2 nm thick film. The bottom row shows AFM (d) height and (e) phase images and (f) corresponding height profile of a BSA layer adsorbed on the polymer film surface. Image sizes are 2 μm × 2 μm.
We next consider a 17.6 nm film of the asymmetric PS(65)-b-PI(26) copolymer, which presents a striped pattern (Figure 4.12a and b). As seen in the upper images in Figure 4.12, the PS cylinders are oriented in the plane of the film with their long axis parallel to the substrate. A few regions of PI blocks dispersed in among the PS cylinders are seen as dark spots in the phase image. After BSA adsorption, the images indicate that the BSA molecules adsorb in a striped pattern resembling the underlying polymer structure (as viewed in Figure 4.12d and e). The peak-to-valley height profile increases from 3 nm to 8 nm after BSA adsorption, but the pitch is not changed, which is consistent with at least one monolayer of BSA being adsorbed on the PS. There is still a strong contrast in the phase image, which is consistent with a preferential adsorption of the BSA rather than having a blanket coverage.

**Figure 4.12.** Evidence for selective BSA adsorption. The upper row shows AFM (a) height and (b) phase images and (c) a height profile for the PS(65)-b-PI(26) 17.6nm thick film. The bottom row shows AFM (d) height and (e) phase images and (f) a height profile of a BSA layer adsorbed on the polymer. Image sizes are 2μm x 2μm.
The incubations of the PS-b-PI templates were repeated at a higher BSA concentration of 1 mg/ml BSA and for a longer time of 2 hr. The morphologies of the PS-b-PI dotted and striped templates before and after incubation were determined by AFM. The images (not shown) are quite similar to those of the adsorbed BSA layers presented in Fig. 4.11 and Fig. 4.12. The well-ordered protein nanopatterns maintain stability independent on the circumstance of protein solution concentration and incubation time. In previous reports on other templates, much shorter times (20 – 60 s) and lower protein concentrations (4-20 µg/ml) were used. Thus, the PS-PI templates have a different range of process parameters over which they have been shown to be applicable.

Although the two components of PS and PI are both exposed at the surface of the very thin PS-b-PI film \( (h < L) \), thicker PS-b-PI films after an annealing treatment are known to undergo thermodynamic process to reach an equilibrium state, according to previous studies by other researchers. The lower surface energy of PI block will reside at the surface at equilibrium. The surface topography of a thicker PS(45)-b-PI(46) film with a thickness of 199 nm after 24 hours annealing at 125 °C was viewed by AFM. It is a smooth surface, and there is no evidence for a phase separated structure across the surface according to the upper images in Figure 4.13. It is proposed that after thermal-annealing treatment, a thin PI layer moves to the free surface of the copolymer film. After BSA adsorption, the surface presents a complicated break-up structure (seen in the bottom images in Figure 4.13). This “break-up” phenomenon occurred when the film was soaked in BSA buffer solution. This structure is a type of dewetting of PI film. Although this is a PS-PI copolymer film, the PI layer of several nanometres thick located on the top surface underwent a dewetting process because of the strong mobility of PI block at room temperature. This detailed dewetting phenomenon has been explained in Chapter 3. With AFM images alone, we cannot judge if BSA molecules are adsorbed onto the copolymer surface. Correlation with ToF-SIMS and WCAA results is required to reach a firm conclusion.
Figure 4.13. The upper row shows AFM (a) height and (b) phase images of the smooth PS(45)-b-PI(46) 199nm thick film surface, and the bottom row shows AFM (c) height and (d) phase images after 1 h BSA incubation. Image sizes are 2μm × 2μm.

Taken together, the AFM images in Figures 4.10-4.13 indicate that the BSA molecules could be selectively adsorbed onto the PS site rather than on the PI site. However, AFM images cannot be interpreted with full confidence, and complementary analysis is required. Hence, SIMS and WCAA were performed to provide additional information about the surface compositions, before and after BSA incubation. The primary aim was to determine whether BSA adsorbed selectively onto one of the copolymer blocks.
4.3.3 Surface analysis to verify preferential adsorption

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) has a high molecular specificity and extreme surface sensitivity, and it is well-suited for the analysis of organic and polymer surfaces.\textsuperscript{59} Although ToF-SIMS is not absolutely quantitative, the chemical specificity of the technique can be used in a quantitative manner by measuring the relative peak intensities of secondary ions unique to each material type.\textsuperscript{60,61,62}

Pure PS, PI, BSA surfaces and PS-b-PI copolymer surfaces and BSA surfaces on polymers were checked by ToF-SIMS. Their positive and negative ion spectra were collected. Since negative spectra have fewer peaks and are quite similar when comparing PS and PI mass spectra, the positive ion spectra were chosen to analyze the PS-PI and BSA surfaces. The aim was to determine whether BSA adsorbed selectively on one of the copolymer blocks.

Figure 4.14 shows the low-mass-range spectra for PS and PI homopolymers and the pure BSA film on Si substrate over the mass range $m/z = 0-200$ Da. These so-called “fingerprint” spectra contain peak intensity patterns which are dependent on the material type. PS and PI are all hydrocarbon materials and only provide hydrocarbon secondary ions. Although they share peaks at almost all positions in the spectra, they can be distinguished at some characteristic peaks which are much higher than each other. For example, the PS homopolymer has characteristic peaks at $m/z = 63, 89, 91, 103, 115, 117, 128, 165, 178$ and $193$ Da, which are significantly more intense than the peaks found in PI at the same positions. The PI homopolymer has characteristic peaks at $m/z = 41, 68, 69, 81, 93, 95, 119$ and $121$ Da, which are significantly more intense than the peaks of PS. These peaks will be used to compare different PS-PI copolymers with varying components through further calculating the peak intensity. Meanwhile, BSA has unique peaks corresponding to nitrogen-containing fragments at $m/z = 18, 30, 44, 60, 70$ and $86$ Da, none of which are found in the spectra of PS-PI hydrocarbon polymer fragments.
Figure 4.14. Positive ToF-SIMS spectra in the mass range m/z 0-200 Da for (a) PS on Si substrate, (b) PI on Si substrate, (c) BSA on Si substrate.
Because PS and PI are both hydrocarbon polymers, the PS-PI copolymer is a complicated system to analyse. Analysis of the relative peak intensity is a more reliable method for the quantitative analysis. Although this method is a semi-quantitative way, in the relative sense, a two-phase copolymer surface composition and preferential protein adsorption can be determined. The relative peak intensity (RPI) was expressed by

\[
RPI = \frac{I_{\text{indv}}}{I_{\text{total}}}
\]

where \(I_{\text{indv}}\) is the intensity of an individual peak of interest in the ToF-SIMS spectrum and \(I_{\text{total}}\) is the intensity of all ion peaks in the same spectrum over the entire mass range (i.e. the total yield). The sum of the RPIs of all peaks (characteristic and common peaks) in a spectrum should be 1.

Only the characteristic peaks labelled in Fig 4.14 are useful in this work. These characteristic peaks are selected to calculate the relative intensities, with the large amount of common peaks being neglected. Then the summed RPI of these characteristic peaks in one specimen are much less than 1. Table 4.1 gives the summed RPI of the characteristic PS and PI peaks for each of the polymer samples. The pure PS has a PS relative peak intensity of \(182.8 \times 10^{-3}\) and a PI relative peak intensity of \(9.3 \times 10^{-3}\). The pure PI has a PI relative peak intensity of \(196.8 \times 10^{-3}\) and a PS relative peak intensity of \(40.5 \times 10^{-3}\). For the pure PS film, although the intensities of the selected PS characteristic peaks are much higher than the peaks found in the same position of the PI spectrum, they are not unique compared with PI spectrum. Then, the pure PS also gives a weak intensity at the positions of characteristic PI peaks. It is vice versa for the pure PI film. The 18.2 nm thick PS(45)-b-PI(46) copolymer sample gives a PI relative intensity of \(89.8 \times 10^{-3}\), whereas the PS(65)-b-PI(26) sample has a PI relative intensity of only \(18.4 \times 10^{-3}\). On the contrary, the PS(45)-b-PI(46) sample gives a PS relative intensity of \(76.5 \times 10^{-3}\) but PS(65)-b-PI(26) sample has a PS relative intensity of \(122 \times 10^{-3}\). It is clear that the PS(65)-b-PI(26) sample with a 17.6 nm thickness has a greater fraction of PS block and a lower fraction of PI blocks on the surface compared with the PS(45)-b-PI(46) sample at a 18.2 nm thickness. This result matches the AFM image results showing that the PS(65)-b-PI(26) sample had a smaller amount of the PI component in the cylinder.
structure. Furthermore, these SIMS spectra indicate that a PI wetting layer has not formed in the nonequilibrium structures of the spin-coating copolymer films.

Additionally, the summed RPI of PI peaks of the PS(45)-b-PI(46) sample with a 199 nm thickness is similar to that of the PI homopolymer. Also, the RPI of PS peaks of this thick copolymer are much lower in the spectrum when compared to the thinner films. It means that after an annealing treatment above its glass transition temperature, a diblock copolymer film obeys the thermodynamic requirements: the component block with a lower interfacial energy segregates at that interface. Recall the AFM result (Fig 4.13), the image of this sample showed that only one block segregated on the top surface. Considering the SIMS data, we conclude that only PI occupies the surface of the thick annealed copolymer film.

Table 4.1. Relative peak intensities (RPI) of characteristic PS and PI ion peaks (the uncertainty is in the range of $1 \times 10^{-3} - 6 \times 10^{-3}$).

<table>
<thead>
<tr>
<th>m/z</th>
<th>PI</th>
<th>PS(45)-b-PI(46), 199nm</th>
<th>PS(45)-b-PI(46), 18.2nm</th>
<th>PS(65)-b-PI(26), 17.6nm</th>
<th>PS</th>
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<tr>
<td></td>
<td></td>
<td>Polyisoprene peaks ($\times 10^{-3}$)</td>
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<td></td>
</tr>
<tr>
<td>68</td>
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<td>76.5</td>
<td>122.0</td>
<td>182.8</td>
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Table 4.2 gives the summed RPI of the characteristic BSA peaks on each polymer samples after protein adsorption. The summed RPI of BSA on the PS and PS(65)-b-PI(26) substrates are only a little lower than the pure BSA. So, it is concluded that the BSA molecules almost fully covered these polymer surfaces. PS(45)-b-PI(46) sample with 18.2 nm thickness, which has a lower PS component on its surface, has a BSA RPI value only ca. half of the pure PS film. This result means only part of this sample surface was covered by BSA molecules. The pure PI sample also adsorbed a few BSA molecules but less than any thin copolymer samples. Finally, there is evidence that the thick (199 nm) PS(45)-b-PI(46) copolymer adsorbs nearly as little BSA as the pure PI sample. This result is consistent with the finding that the thick PS-b-PI film was enriched with the PI blocks on the surface after annealing and that PI reduces BSA adsorption compared to PS.

**Table 4.2.** Relative peak intensities (RPI) \(\times 10^3\) of characteristic BSA ion peaks (the uncertainty is in the range of \(1 \times 10^3\) - \(4 \times 10^3\)).

<table>
<thead>
<tr>
<th>BSA peaks (m/z)</th>
<th>BSA on PS</th>
<th>BSA on PS(65)-b-PI(26) 17.6 nm</th>
<th>BSA on PS(45)-b-PI(46) 18.2 nm</th>
<th>BSA on PS(45)-b-PI(46) 199 nm</th>
<th>BSA on PI (15min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>11.1</td>
<td>7.4</td>
<td>7.6</td>
<td>2.8</td>
<td>1.7</td>
</tr>
<tr>
<td>30</td>
<td>35.6</td>
<td>24.1</td>
<td>25.7</td>
<td>10.3</td>
<td>7.0</td>
</tr>
<tr>
<td>44</td>
<td>14.4</td>
<td>14.7</td>
<td>13.1</td>
<td>10.4</td>
<td>8.4</td>
</tr>
<tr>
<td>60</td>
<td>14.6</td>
<td>9.1</td>
<td>9.4</td>
<td>10.4</td>
<td>8.4</td>
</tr>
<tr>
<td>70</td>
<td>19.6</td>
<td>20.7</td>
<td>19.5</td>
<td>15.7</td>
<td>14.8</td>
</tr>
<tr>
<td>86</td>
<td>14.8</td>
<td>12.3</td>
<td>9.3</td>
<td>5.0</td>
<td>3.7</td>
</tr>
<tr>
<td>Totals</td>
<td>110.1</td>
<td>88.3</td>
<td>84.6</td>
<td>48.9</td>
<td>39.6</td>
</tr>
</tbody>
</table>

The RPI for the characteristic PI peaks is assumed here to be proportional to the fraction of PI on the free copolymer surface. The RPI for the characteristic BSA peaks is likewise proportional to the fraction of the surface that is covered with adsorbed BSA. Using the data of Table 4.2, Figure 4.15 was drawn to show the relationship between the adsorbed BSA’s RPI and the PI’s RPI in the underlying polymer templates before BSA adsorption. The amount of PI fraction on the polymer film surface was adjusted with the film thickness and diblock ratios (17.6 nm PS(65)-
b-PI(26), 18.2 nm PS(45)-b-PI(46), 199 nm PS(45)-b-PI(46)) and compared to the two homopolymers.

In Figure 4.15, the PS(45)-b-PI(46) sample with a thickness of 18.2 nm, which has a higher fraction of PI at its surface, shows dramatically less protein adsorption compared with the PS(65)-b-PI(26) sample. The PI homopolymer sample has a lower BSA coverage in comparison to any thin film copolymer samples. Finally, the thick (199 nm) PS(45)-b-PI(46) copolymer adsorbs nearly as little BSA as the PI homopolymer film. Then, there is a negative correlation between adsorbed BSA’s RPI and PI’s RPI of the underlying copolymer templates. Also in Figure 4.15, the dependence of the BSA’s RPI is shown as a function of the PS’s RPI before BSA adsorption. A positive correlation is observed, which is the opposite of the inverse trend for PI. We conclude that there are fewer BSA molecules adsorbed onto the polymer surfaces when a greater fraction of PI blocks segregate to the free surface and when a lower fraction of PS blocks are present.

![Graph showing the dependence of BSA's RPI after adsorption on the PI's RPI before adsorption.](image)

**Figure 4.15.** The dependence of the BSA’s RPI after BSA adsorption on the PI’s RPI before BSA adsorption for different polymer film surfaces (open symbols). On the same axes, the dependence of the BSA’s RPI on the PS’s RPI before BSA adsorption is also shown (filled symbols). The solid lines show the general trends.

With further analysis, presented next, we can obtain more information from the SIMS spectra. This analysis will identify the segments on which the BSA is
adsorbed. The relative intensities of characteristic PS and PI ion peaks after BSA adsorption on PS, PI and PS-b-PI copolymer films were collected. The ion peak at m/z = 68 u has been removed from consideration because BSA also provides strong intensities at this value because of a hydrogen-carbon fragment. After BSA adsorption, this will affect the PI’s RPI analysis.

Table 4.3 gives a comparison of the summed RPI of the PI and PS characteristic peaks before and after BSA adsorption. The idea behind this analysis is that preferential protein adsorption will cause a decrease in the yield from the block on which the protein adsorbs, as it will cover its surface. (The probe depth of SIMS is ca. 1 nm. In the case of adsorbed BSA, therefore, the technique will not detect the underlying polymer substrate.) The yield from the other block, however, should not change if there is no adsorption on it. The magnitude of the drop in the summed RPI for a particular polymer block is assumed to be proportional to the fraction of its surface covered by adsorbed protein. For the PI homopolymer film and the 199 nm PS(45)-b-PI(46) film (which has PI segregating to the free surface), the RPI of the characteristic PI peaks of both film surfaces decrease by only about 30x10^{-3} after the BSA adsorption, which is a relatively small change. (We estimate the uncertainty to be less that 5 x 10^{-3} in the RPI in Table 4.3.) For the samples in the right three columns of Table 4.3, all the RPI of characteristic PI peaks do not decrease but increase a little after the BSA adsorption. There is an increase because the BSA SIMS spectra also provide some counts at the exact same positions as the characteristic PI peaks, although these peaks are not the characteristic peaks for the BSA SIMS spectra.

Clear evidence for the preferential adsorption of BSA on PS is given by the RPI of the characteristic PS peaks in the lower half of Table 4.3. After BSA adsorption, the PS’s RPI for the PS homopolymer is decreased by 126.8x10^{-3}. For the 18.2nm PS(45)-b-PI(46) film and the 17.6nm PS(65)-b-PI(26) film, the PS’s RPI decreases by 35 x 10^{-3} and 80.9 x 10^{-3}, respectively, whereas the PI’s RPI does not decrease. The amount of BSA adsorption on the PS block, as gauged by the RPI decrease, is found to be proportional to the fraction of PS blocks at the film surface.
Table 4.3. RPI changes of characteristic PS and PI peaks after 1 h BSA adsorption.

<table>
<thead>
<tr>
<th></th>
<th>PI</th>
<th>PS(45)-b-PI(46)</th>
<th>PS(45)-b-PI(46)</th>
<th>PS(65)-b-PI(26)</th>
<th>PS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Poly(isoprene) peaks ($\times 10^3$)</td>
<td>Poly(styrene) peaks ($\times 10^3$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before BSA Ads.</td>
<td>184.1</td>
<td>161.6</td>
<td>85.0</td>
<td>17.6</td>
<td>9.0</td>
</tr>
<tr>
<td>After BSA Ads.</td>
<td>153.8</td>
<td>133.4</td>
<td>98.5</td>
<td>22.9</td>
<td>11.8</td>
</tr>
<tr>
<td>Difference</td>
<td>-30.3</td>
<td>-28.2</td>
<td>13.5</td>
<td>5.3</td>
<td>2.8</td>
</tr>
</tbody>
</table>

The data in Table 4.3 are presented graphically in Figure 4.16. The RPI for the PS and PI polymers after BSA adsorption are plotted as a function of the RPI for the same polymer before BSA adsorption. The graph indicates the extent to which the PI and PS blocks are “covered up” by the adsorbed BSA molecules. The gradient of the data will be unity if there is no BSA adsorption on one of the blocks, whereas it will be zero if there is full BSA coverage. (In these plots, the RPI of the PI has been “corrected” by subtracting the small RPI for PS ($9 \times 10^3$); likewise, the RPI of the PS has been corrected by subtracting the RPI for PI ($40.5 \times 10^3$).) It is seen that the PI data points lie close to a line with a gradient of 1, indicating that there is limited adsorption of BSA on the PI blocks. In comparison, the data points for PS lie on a shallow trend-line, indicating much stronger BSA adsorption on that block. From this analysis (coupled with the AFM analysis), there is a strong evidence that BSA molecules are adsorbed on the PS blocks preferentially over the PI blocks. It has been demonstrated that the copolymer acts as a template.
Changes in the relative hydrophilicity of the surfaces were determined to provide further evidence for BSA adsorption and to test the conclusions regarding templating. Table 4.4 gives a collection of WCAA measurements for the polymer surfaces, with and without adsorbed BSA. The observed decreases in contact angle after BSA adsorption are attributed to the hydrophilicity of the protein. It is hypothesized that there will be a greater reduction in the contact angle when there is a greater fraction of surface coverage by the BSA.

The water contact angles are similar (~101°) when comparing the thick PS-b-PI copolymer with the PI homopolymer sample, as both surfaces are PI rich. After protein adsorption, the contact angles on PS and PS(65)-b-PI(26) surfaces decrease by around 30°. The reduction of the contact angle on the 18.2 nm PS(45)-b-PI(46) film surface is ca. 9° less than seen for the PS(65)-b-PI(26) film, which was found by SIMS to have more PI at its surface, leading to less BSA adsorption. On the other hand, the contact angle on the PI surface only decreases by 2° after BSA incubation, indicating that only a small amount of BSA was adsorbed. There is a clear trend of a smaller contact angle reduction after BSA adsorption with an increase in the fraction
of PI located on the surface. This trend is agreement with conclusions of the SIMS analysis and further confirms that PI surface segregation prevents protein from being adsorbed.

Table 4.4. Water contact angles of polymer and adsorbed BSA layers and angle differences before and after BSA adsorption.

<table>
<thead>
<tr>
<th></th>
<th>PS</th>
<th>PS(65)-b-PI(26) 17.6 nm</th>
<th>PS(45)-b-PI(46) 18.2 nm</th>
<th>PS(45)-b-PI(46) 199 nm</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>WCA of initial polymers (°)</td>
<td>95.0±0.7</td>
<td>96.9±0.9</td>
<td>99.8±1.0</td>
<td>101.1±1.2</td>
<td>101.5±1.2</td>
</tr>
<tr>
<td>WCA after BSA 1h ads. (°)</td>
<td>67.0±1.5</td>
<td>68.0±1.7</td>
<td>79.3±1.4</td>
<td>93.8±1.9</td>
<td>99.5±1.5</td>
</tr>
<tr>
<td>WCA differences before and after BSA ads. (°)</td>
<td>-29</td>
<td>-28.9</td>
<td>-20.5</td>
<td>-7.3</td>
<td>-2</td>
</tr>
</tbody>
</table>

In the work presented here, the more hydrophobic block (PI) is resistant to BSA adsorption, whereas usually hydrophilic blocks are found to resist protein adsorption. There must be an alternative explanation, such as a physical characteristic, leading to the preferential adsorption on PS over PI domains. Previous studies on protein adsorption onto polymer brushes have led to a hypothesis that the polymer chain mobility can influence the amount of protein adsorption. The protein adsorption was found to decrease with increasing polymer molecular mobility. In our work, PS-b-PI is a hard-soft diblock copolymer, with the PI segments presenting a high molecular mobility at the room temperature. The high mobility of PI chains might resist the non-specific protein adsorption.

4.4 Conclusion

In PS-b-PI copolymer, the ultrathin films ($h<L$) form well-ordered patterns (perpendicular lamella, parallel cylinder and similar hexagonal spheres) on the Si substrates because of the confinement between the substrate-polymer and polymer-air interfaces. All these patterns can be controlled by adjusting some parameters such as weight ratios, film thickness and annealing treatment.
With the same thickness, the film surface morphologies are different when the weight ratios are different. For about 20 nm films, the symmetric PS(45)-b-PI(46) film forms the PI dots structure in the surface but the asymmetric PS(65)-b-PI(26) film forms the striped structure in the surface. The film surface morphologies also vary with the thickness changes. In a given thickness, the film can form a specially well-organized structure and the surface presents a spatially and chemically ordered pattern. For the thicker films ($h>L$), the as-spun films showed disordered morphologies on the free surface. After an annealing treatment, the lower surface energy component, PI, moves to the free surface and forms a thin PI layer. The resulting surface topography in the equilibrium is dependent on the initial film thickness. Flat, holey or island structures will be present on the surface with different thicknesses. In the thermodynamic process, the morphology and topography of film surfaces also depend on the annealing time and thickness.

The use of self-assembled PS-b-PI (a copolymer with two hydrophobic blocks) has been found to provide a precise, two-dimensional template for the nanopatterning of proteins. The preferential adsorption of BSA on PS blocks rather than on PI induces the BSA molecules to create a well-ordered pattern resembling the underlying block copolymer nanoscale template. Preferential adsorption was confirmed through the complementary use of AFM, ToF-SIMS and contact angle analysis. The selective protein adsorption might be explained by the greater molecular mobility of the rubbery PI block in comparison to the glassy PS block.

The highly-organized PS-b-PI copolymer template offers a versatile and simple approach for the nanopatterning of proteins. Crucially, the dotted and striped patterned templates were used to nanopattern BSA molecules at relatively long incubation times (2 hr) and in relatively high concentrations (1 mg/ml) of BSA solution. Hence, the template is suitable for bioengineering applications where increased deposition times from higher protein concentrations are required. There is a broad window of applicability for the templating process. Furthermore, the templates are created from the non-equilibrium copolymer structure. As their creation does not require annealing, the templates can be deposited on fragile, heat-sensitive substrates. In my work, these templates will be used to explore cell adhesion behaviour showing in Chapter 6.
References


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Chapter 5

Thin films of binary or ternary polymer blends and their applications as templates for protein patterning

5.1 Introduction

This chapter will study the film structures of binary or ternary blends of PS, PI, and PS(45)-b-PI(46). Compared to the block copolymer templates with patterns on the nanoscale, the polymer blend films can form patterns with structures from one hundred nanometers to several micrometers. The chemically-heterogeneous polymer blend templates will be used to pattern the proteins.

In Chapter 1, the theory of phase separation of polymer blends was described. Although the mechanisms of phase separation of two homopolymers in the bulk are in general well understood, the situation in thin polymer films is complicated because of the presence of the substrate-polymer interface and polymer-air interface. The presence of two interfaces imposes boundary conditions which give rise to complex morphologies both parallel and perpendicular to the surface. In thin blend films these structures are very sensitive to a variety of parameters, such as the polymer-substrate interaction, the surface and interfacial energies of the individual polymers, and the processing method of the thin film.\textsuperscript{1,2,3,4}

Typically, the thin films are prepared by spin coating of a solution of one or more polymers in a common solvent. Due to the intrinsic immiscibility of most macromolecular blends, polymer mixtures will demix during the rapid solvent-casting process. The resulting phase-separated morphology may be far from thermodynamic
equilibrium, and relaxation toward equilibrium may be hindered by kinetic barriers formed by the non-equilibrium phase morphology. Then films prepared by spin-coating may lead to long-lived metastable phase morphologies. During the rapid evaporation of the solvent, the thin film forms from a concentration quench into the two phase region in the phase diagram.\textsuperscript{5, 6} Although this evaporation is a fast process, it is often not fast enough to suppress any structure formation completely. It is, however, a quite gentle process, as compared to the more classical thermal quench.

The phase separation during a spin-coating process is shown in Fig. 5.1. Unlike the phase diagram in Fig. 1.1 in which the vertical axis is inversely proportional to temperature, the axis in Fig. 1.5 represents solvent concentration. A polymer blend solution with low polymer concentration (typically several percent polymer content by mass) is dropped onto the spinning substrate. During the initial stage of the spin-coating process, most of the solution is cast off, leaving a thin layer on the substrate. As the layer thins due to fluid flow, the evaporation of the solvent becomes important. The solvent evaporation will increase the polymer concentration, and consequently dramatically increases the viscosity of the polymer solution and slows the shear thinning of the film.\textsuperscript{7} Phase separation of the polymer blends occurs during this later stage of the spin-coating process. This process produces a rapid quench of the polymer blends, which “freezes in” a non-equilibrium phase separation morphology. Since eventually all solvent will have evaporated, the final composition of the polymer blend lies on the $\phi_h$ axis of the schematic phase diagram in Fig. 5.1.
Figure 5.1. Schematic phase diagram for the solvent quench experiment. $\phi_m$ is the mass fraction of one component in a polymer blend and $C_s$ is the solvent concentration. Phase separation process begins as $C_s$ decreases below the concentration $C_{bin}$ (corresponding to binodal line), and occurs effectively only after $C_s$ decreases below the concentration $C_{spin}$ (corresponding to the spinodal line). Below the $C_{sf}$ value (stiff concentration), phase separation stops because the molecules of one polymer are no longer mobile. (Redrawn from Ref 5)

The addition of homopolymers into a copolymer has received interest because the desired phase structure orientation$^{8,9}$ and periodicity$^{10}$ can be obtained by simply adjusting the amount of the incorporated homopolymer. In such blends there is an interplay between macrophase separation of the homopolymers and microphase separation of the block copolymer. Which effect dominates depends on the relative lengths of the polymers and on the composition of the blends. This ternary system shows blend-like behavior at low diblock concentrations and diblock-like behavior at low homopolymer concentrations.$^{11,12}$

In this study, PS, PI and symmetric PS-b-PI were mixed to form binary or ternary blends. By adjusting the total solution concentration or the compositional mass ratios, the spin-cast films can form different morphologies with various spacing dimensions and domain sizes. The low energy phase wets the surface. The objective of this work is to create templates with both PS and PI. It is expected that such films can be used as various templates to pattern the proteins through selective adsorption.
AFM and ToF-SIMS were used to characterize the surface topography and composition of a series of blends.

5.2 Experimental details

5.2.1 Films preparation of PS/PI binary blends

PS ($M_w = 114$ kg/mol) and PI ($M_w = 100$ kg/mol) with different composition ratios were dissolved in toluene (a good solvent for both polymers). The total polymer concentration was fixed at 2 wt% and the PI mass fractions ($\phi_{PI}$) were chosen as 0.3, 0.4, 0.5, 0.6 and 0.7. The PS mass fraction is then $\phi_{PS} = 1 - \phi_{PI}$. Thin films were prepared by spin-coating solution droplets on Si wafers with a 2.5 nm thin SiO$_x$ top layer at room temperature. The spin-coating was at 3000 rpm for 10 s. The as-received film average thicknesses are 70 to 100 nm as measured by ellipsometry, varying with $\phi_{w}$. The film surface topographies were determined by tapping-mode AFM and the surface composition analysis was determined by ToF-SIMS. Details of the techniques were given in Chapter 4. The PI area fractions in the film morphologies were determined by ImageJ program (developed by National Institutes of Health). The average minority domain areas of circular PI or PS were determined using the Nova software of the AFM (NT-MDT). A threshold level in the images was set. Dark regions are assigned to PI-rich phase, and bright regions are assigned to PS-rich phase.

All these polymer blend solutions were diluted to 0.4 wt% concentration with PI mass fractions fixed. The film thicknesses from the dilutions are 14 to 20 nm. Their topographies and surface compositions were also determined by AFM and ToF-SIMS, respectively.

5.2.2 Films preparation of PI/PS(45)-b-PI(46) binary blends

PI ($M_w = 100$ kg/mol) and PS(45)-b-PI(46) ($M_{w,PS} = 45$ kg/mol, $M_{w,PI} = 46$ kg/mol) were dissolved in toluene. The total polymer concentration was fixed at 0.5 wt% and the PI mass fractions ($\phi_{PI}$) were chosen as 0.2, 0.5, 0.6, 0.8 and 0.9. Thin films were prepared by spin-coating of solution droplets on Si wafers with a 2.5 nm thin SiO$_x$ top layer at room temperature. After 2000 rpm and 10 s spinning, films with a thickness of ca. 20 nm were prepared. The film surface morphologies were
determined by tapping-mode AFM and the surface composition analysis was determined by ToF-SIMS.

5.2.3 Films preparation of PS/PI/PS(45)-b-PI(46) ternary blends

PS ($M_w = 114$ kg/mol), PI ($M_w = 100$ kg/mol) and PS(45)-b-PI(46) with different composition mass ratios were dissolved in toluene. The total polymer concentration was fixed at 2 wt% and the composition mass ratios of PS: PI: PS(45)-b-PI(46) were 23: 38: 39, 25: 25: 50, and 40: 40: 20. Thin films were prepared by spin-coating solution droplets on Si wafers with a rate of 3000 rpm and 10 s spinning. Film thicknesses were ca. 80 nm measured by ellipsometry. The surface topographies were determined by tapping-mode AFM and the surface composition analysis was determined by ToF-SIMS.

All these blend polymer solutions were diluted to 0.4 wt% concentration with the composition mass ratios fixed. After 2500 rpm and 10 s spinning, films with their thickness 14 to 16 nm were prepared. Their topographies and surface compositions were determined by AFM and ToF-SIMS, respectively.

5.2.4 BSA incubation

Some spin-cast films with suitable morphologies were incubated in 0.5 mg/ml BSA (PB buffer) solution. After 1 h incubation, all films were rinsed five times by PB buffer and once by DI-water. Films were put in a desiccator for 24 hours before the surface analysis by AFM and SIMS.

5.2.5 UV-ozonation

Ozone treatment has been used to form masks for nanolithography from polydiene-containing block copolymer structures. Harrison et al. applied ozone treatment to thin films of PS–PI and PS–PB diblock copolymers. After coating the surface with a diblock template, the polydiene component was selectively removed by exploiting the chemical differences between the blocks. Polydiene chains are rapidly cleaved with ozone ($O_3$) at a rate one million times faster than the scission rate of PS chains. Ozone cleaves carbon-carbon double bounds, producing low molecular weight products that can be removed by water. At the same time, PS is
cross-linked by ozone. As a result, ozonated cylindrical or spherical voids surrounded by un-etched PS matrix can be obtained. The authors showed that such masks are highly suitable for lithographic processes, using the block copolymer films as templates for semiconductor growth. Hamley et al.\textsuperscript{17,18} used moderate ozone doses to investigate ozone etching of thin films of a triblock PS–PI–PS block copolymer having a majority PI phase. The changes in morphology as a function of ozone treatment time were examined and the ozone process was concluded with two stages for the ozonation. UV ozone was used to etch films with nanoporous cylindrical domains as well.\textsuperscript{19} UV ozone shows the same mechanism with the normal ozone etching to break down the long polymer chains.

The purpose of our experiment is to etch off the polyisoprene component of the copolymer films to study the detailed information of the interior structures of the film morphology. UV ozone treatment was performed in a UV ozone cleaner from Bioforce Nanosciences company. It uses a UV lamp to generate ozone from oxygen. After a 1 h ozone treatment, the blend film cast from a 2 wt\% concentration with $\phi_{PI} = 0.6$ was soaked in DI-water and shaken for 24 h to completely remove the scissored PI fragments.

5.3 Results and discussion

5.3.1 Film topography and surface composition of PS/PI binary blends

5.3.1.1 Thick films spin cast from 2 wt\% PS/PI binary blend solution

Fig. 5.2 shows typical morphologies of films spin-coated from 2 wt\% PS/PI binary blend solutions. PS is in its glassy state at room temperature but PI is in its rubbery state. So PS dissipates less energy compared with PI when interacting with an AFM tip during scanning and presents brighter areas in an AFM phase image. The \textit{ca.} 30 degree phase contrast of bright and dark areas presents the compositional difference. Circular dark PI domains were dispersed in a PS matrix for $\phi_{PI} = 0.3$ and 0.4. As the mass fraction increased to $\phi_{PI} = 0.5$, the PI-rich domains grow and form the elongated spherical domains, as shown in Fig. 5.2c. Further increase of $\phi_{PI}$ results
in the formation of circular PS-rich domains dispersed in a PI-rich matrix. The circular diameter is much smaller for $\phi_{PI} = 0.7$ than for $\phi_{PI} = 0.6$.

**Figure 5.2.** Phase images of spin-coated films from 2 wt% PS/PI binary blend solutions. PI mass fractions $\phi_{PI}$ are (a) 0.3, (b) 0.4, (c) 0.5, (d) 0.6, and (e) 0.7. Image sizes are 20 μm × 20 μm. The phase angle difference from dark to bright is $30^\circ$.

The results of the PI area fractions and the minority average domain areas ($\mu m^2$) of circular PI or PS rich phases are plotted in Fig. 5.3. The surface PI area fraction and overall PI mass fraction in solution has a linear relationship. The gradient from the linear fit is $0.8\pm0.05$ and the intercept is $20\pm1.5$ (%). The phenomenon of the fitting line being away from a gradient of 1 and an intercept of 0 will be explained later in this section.

Since the dark domain area fraction increases with the increasing PI bulk fraction, it seems reasonable to conclude that the dark domains are composed of a PI-rich phase. Almost all points stay on the line except the point of 0.5 mass fraction. Viewing the AFM phase image of Fig 5.2c, a more complex structural feature is noticeable. In addition to the large dark structures discussed above, many smaller
domains can be detected inside the PI-rich phases. These small domains appear bright compared with their surroundings, so they should be PS-rich domains and come from the secondary spinodal process. It is attributed to a deep quench during rapid solvent evaporation.\textsuperscript{20} These small PS domains are included in the large PI domains when calculating the PI area fraction. Therefore, the observation that the area fraction at 0.5 $\phi_{PI}$ is slightly above the fit line in Fig. 5.3 seems reasonable.

The measured average minority domain area versus PI mass fraction $\phi_{PI}$ is also shown in Fig. 5.3. The five data points correspond to the AFM images of the observed morphologies shown in Fig 5.2. As $\phi_{PI}$ is increased, the PI-rich circular domain areas increase from 6.2 to 6.6 $\mu m^2$, and a very sharp increase to 13.4 $\mu m^2$ occurs at $\phi_{PI} = 0.5$. When $\phi_{PI}$ is increased further, the interconnected PS-rich domains break up and the smaller PS-rich domains are dispersed in the PI-rich matrix. The PS circular domain areas decrease sharply to 0.3 $\mu m^2$ with $\phi_{PI} = 0.6$ and further decrease to 0.1 $\mu m^2$ with $\phi_{PI} = 0.7$. In other research, there is a bicontinuous morphology near the critical mass fraction $\phi_{mc}$ within a narrow range.\textsuperscript{5, 6} This morphology should be formed at the $\phi_m$ range from 0.5 to 0.55 in this blend system. The morphology at $\phi_{PI} = 0.5$ is already very close to the bicontinuous morphology at $\phi_{mc}$ and the maximum domain areas should happen in this critical position.

![Figure 5.3](image)

**Figure 5.3.** The black straight line is PI area fraction versus PI mass fraction $\phi_{PI}$ for 2 wt% PS/PI blend films on Si-SiO$_x$ substrates. The blue curved line is average domain area of circular PI or PS domains versus PI mass fraction $\phi_{PI}$. 

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If these films are flat without any surface topography and there is no preferential segregation of both phases on two interfaces (substrate-polymer and polymer-air interfaces), the correlation line of area fraction and mass fraction should have a gradient of 1 and an intercept of 0. A slope of 0.8 and an intercept of 20 tell us that there may be an undulating surface instead of a smooth flat surface. The height images of AFM confirm that these films undulate (Fig. 5.4).

**Figure 5.4.** Typical domain cross-sectional profiles of spin-coated films from 2 wt% PS/PI binary blend solutions. The insets are corresponding AFM height images. The pink lines show the regions where the profiles are collected and the profiles of d and e are collected from a single island. PI mass fractions \( \phi_{\text{PI}} \) are (a) 0.3, (b) 0.4, (c) 0.5, (d) 0.6, and (e) 0.7. AFM image sizes are 20 \( \mu \text{m} \times 20 \mu \text{m} \).
All PS domains present themselves higher than PI domains, i.e. PS forms the hill and PI forms the valley. The protruded PS domains make the PS domain area fraction smaller than corresponding PS mass fraction and then the PI reaches a reverse result. This explains the intercept of 20 in this linear fitting line. The PI valleys are ca. 50 nm deep in every hole-structure although their domain sizes are different. In Fig. 5.4c, the small PS domains in surrounding PI matrix are protuberances with a height of 20-30 nm. When the PI fraction further increases, the interconnected PS hills break up to circular and isolated islands. The height of PS islands is 90 nm at $\phi_{PI} = 0.6$ and 80 nm at $\phi_{PI} = 0.7$, respectively. In addition to the phase separation, the undulated surface topography may be related to the modified rate of solvent evaporation from PS- and PI-rich phases due to the varied solubility of both phases in a common solvent.21

The film of $\phi_{PI} = 0.6$ with an island structure was treated by UV-ozone for 1 h. The AFM result is shown in Fig. 5.5. Because the PS can cross-link without being cleaved by ozone, the PS island domains survive and the pitch of the dimensions stays the same (about 1.2 μm) with the previous morphology before UV-ozone, as seen in Fig. 5.4d. There are several observations that will be used to deduce the film overall structure later in this Chapter. First of all, it is apparent from a comparison of Figures 5.4d and 5.5b that the difference in height between PS domains and the PI matrix increases after the surface etching treatment with UV-ozone. The height of the island increases from 90 nm to 120 nm. Secondly, the brightness of the islands and their surroundings in the phase image is quite similar, in contrast to the image of Fig. 5.2d where the surrounding area is much darker than the island domains. Thirdly, the protrusions of islands have a concave shape (Fig 5.5b) but the previous one (Fig 5.4d) is almost flat. All these differences indicate that film compositions have changed and the corresponding topography is changed. The matrix PI, which initially covers the silicon oxide substrate, has been removed by the double bond cleavage and the water rinsing. The elevated and depressed regions in the surface undulations are composed of PS- and PI-rich phases, respectively. This is consistent with the conclusion drawn from Fig. 5.3.
Figure 5.5. (a) AFM height image, (b) cross-sectional profile, and (c) phase image of a spin-cast film for $\phi_{pl} = 0.6$ after 1 h UV-ozone treatment. Image sizes are 20 $\mu$m $\times$ 20 $\mu$m.

As mentioned in previous research, surface energy is an important factor in the segregation of blend components, and the component with a lower surface energy is generally enriched at the surface. At the equilibrium state (normally after thermal or solvent annealing), the low surface energy component must stay on the free surface to minimize the total energy. However, the fast spin-cast film forms non-equilibrium morphology. It is necessary to check out whether the PI component (low surface energy compared to PS) only stays in the PI-rich domains or covers entirely the free surface. For application as protein templates (shown already in Chapter 4), a PI wetting layer must be avoided.

ToF-SIMS is very sensitive to analyze the surface composition of the outmost 1~2 nm. Table 5.1 summarizes the RPI of the characteristic PS and PI peaks for each of the polymer samples. Two typical films (one is a PI hole structure with $\phi_{pl} = 0.4$, and the other is a PS island structure with $\phi_{pl} = 0.6$) were chosen for the surface composition analysis. The spectra of the pure PS and PI films were also determined by SIMS to compare with polymer blends. For the polymer blend films, no matter whether it is the PI characteristic peak intensity or the PS characteristic peak intensity, the RPI results for the blend films are similar to those from pure PI surface and very different from those from PS surface. This further experimental result confirms that PS-rich domains are covered by a thin PI layer at the free surface. The phase images in Fig. 5.2 appear much brighter on the PS-rich domains rather than appear the same darkness with PI domains. It can be explained that the sharp AFM tips penetrate the
ultrathin PI layer and touch the PS surface underneath during scanning. This is reasonable because PI is very soft and has a high chain-mobility at room temperature.

**Table 5.1.** Relative peak intensities (RPI) of characteristic PS and PI ion peaks of spin-cast films with different PI mass fractions.

<table>
<thead>
<tr>
<th>RPI</th>
<th>PI</th>
<th>$\phi_{pl} = 0.4$</th>
<th>$\phi_{pl} = 0.6$</th>
<th>PS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>70 nm</td>
<td>103 nm</td>
<td></td>
</tr>
<tr>
<td>PI peaks ($\times 10^{-3}$)</td>
<td></td>
<td>215.9</td>
<td>202.9</td>
<td></td>
</tr>
<tr>
<td>Summed RPI</td>
<td></td>
<td>214.2</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>PS peaks ($\times 10^{-3}$)</td>
<td></td>
<td>37.1</td>
<td>41.3</td>
<td>186.9</td>
</tr>
<tr>
<td>Summed RPI</td>
<td></td>
<td>37.4</td>
<td>186.9</td>
<td></td>
</tr>
</tbody>
</table>

Combining all results from the aforementioned figures and tables, a schematic illustration of the overall phase domain morphology from a cross-section view is presented in Fig. 5.6a. PS-rich domains form elevated hills no matter whether they form a connected network or isolated islands. On the other hand, PI-rich domains are always present in depressed valleys. The average thickness of spin-cast blend film ($\phi_{pl} = 0.6$) of 2 wt% solution is 103 nm (determined by ellipsometry). Ellipsometry can measure the average thickness of the film but is not sensitive to the pillars. The peak to valley height (from the AFM height profile) is 90 nm, but after UV-ozone etching the height becomes 120 nm. This means that the PS domain height is 120 nm and about 30 nm of the bottom part of the pillar is buried in the PI matrix. PI forms a tens-of-nanometer thick film totally covered the substrate. Simultaneously, the low surface energy component, PI, also forms a very thin layer covering all air surfaces.

![Figure 5.6](image_url)

**Figure 5.6.** Schematic illustration describing overall morphology from a cross-section view. (a) as-spun 2 wt% PS, PI blend film of $\phi_{pl} = 0.6$, (b) after 1 h UV-ozone etching.
The above results also allow us to present and discuss a consistent model (illustrated schematically in Figure 5.7) accounting for the phase domain structure and the free surface undulations formed in the spin-cast blend films. A formation mechanism has been proposed by Budkowski\textsuperscript{1} analyzing the PS/PI system on hydrophilic or hydrophobic self-assembled monolayers. This morphology formation scenario, presented here, is based on an extension of earlier models of blend films.\textsuperscript{22,23,24,20,5} Although the process is not completely resolved due to the multiple effects that cannot be observed directly, its obvious and relevant features have been recognized.

There are three consecutive stages of the spin-coating. First of all, most (ca. 90\%) of the homogeneous fluid, which is composed of PS and PI dissolved in toluene, is spun-off, leaving a uniform film (Figure 5.7a). Secondly, the radial flow of the fluid, which is a balance between centrifugal and viscous forces, decreases film thickness and controls its final average value.\textsuperscript{7} Phase separation takes place during this stage (Figure 5.7b-d), initiated by decreasing solvent concentration, \(c_s\). The preferential attraction of PI to both external surfaces is expected based on earlier observations made for copolymer systems.\textsuperscript{25,26,27,28} Here, the second stage of spin-coating starts very likely with a formation of an unstable trilayer\textsuperscript{29,30} PI-rich/ PS-rich/ PI-rich structure in the blend film with a high solvent concentration \(c_s\) (Figure 5.7b). As solvent continues to evaporate, two instabilities can develop. An instability of the liquid-liquid interface, caused by the increasing unfavorable enthalpic interactions as the solvent concentration decreases, leads to the break up of the film. Alternatively, an instability of the free surface (caused for example by a hydrodynamic instability, which leads to a lateral variation in polymer composition) leads also to a laterally heterogeneous phase morphology.\textsuperscript{31} Both instabilities can take place simultaneously and laterally organized domains are formed (Figure 5.7c). The PI layer decreases its thickness and widens the spacing of PS domains. A trace of this phase arrangement is observed in the fully dried films as a thin PI free surface layer and much thicker PI lamella adjacent to the substrate (Figure 5.7d). This phase rearrangement involves reduction of unfavorable polymer/polymer interfaces.\textsuperscript{32,33} Thirdly, the rearrangement of phase domains is terminated for very low \(c_s\) values, when one of the homopolymers (PS) is no longer mobile.\textsuperscript{5}
Figure 5.7. Schematic illustration describing morphology formation in the PS/PI binary blend film during spin-coating from toluene on Si wafer. Subsequent stages of phase domain arrangement correspond to (a) a homogeneous fluid film, (b) formation of an unstable trilayer PI-rich/PS-rich/PI-rich structure, and (c-d) its reorganization into lateral phase structures. The average film thickness decreases as a function of time (a-d).

5.3.1.2 Ultrathin films spin cast from 0.4 wt% PS/PI binary blend solution

The thick blend films form morphologies with only PI segregating to the free surface. This is not useful for the protein patterning on chemically heterogeneous templates. It was hypothesized that PS could be forced to the film surface if the film thickness was very low. If the film thickness is less than the chain random coil size, it would be entropically unfavorable for the chain not to be near the surface. The diluted 0.4 wt% PS/PI binary blend solutions form thinner films after spin-coating with thicknesses only 14 to 20 nm. These film topographies are shown in Figure 5.8. The depressed PI domains formed holes dispersed in elevated PS network domains when $\phi_{PI}$ was smaller than 0.5. The hole structures changed from circular to elongated spheres and the hole sizes increased gradually with the increase of $\phi_{PI}$. With a further $\phi_{PI}$ increase, the PS network broke up to create round islands and was dispersed in the
depressed PI matrix. Meanwhile, the island radius decreased a lot for the film with $\phi_{\text{PI}} = 0.7$ compared with $\phi_{\text{PI}} = 0.6$. The biggest domain size is viewed at the symmetric composition ratio. This mass fraction is quite close to the critical fraction where the domain size will reach maximum.$^5,6$

Figure 5.8. Film topographies spin-coated from 0.4wt% PS/PI blend solutions. PI mass fractions are (a) 0.3, (b) 0.4, (c) 0.5, (d) 0.6, and (e) 0.7. Image sizes are 20 µm × 20 µm.

The correlations of PI area fractions and circular PI/PS domain areas with PI mass fraction in the bulk are illustrated in Figure 5.9. There is also a linear relationship between PI area fraction and its mass fraction. The best fit line for PI area fraction as a function of PI mass fraction has a slope of 0.97 and an intercept of 4%. These values are close to the expected values of 1 and 0%, respectively. Because the PS domain protrudes to a higher level, its domain area is reduced. The trend for domain areas in thin films is quite similar to what was found for thick films (comparing to Fig. 5.3). The biggest domain area is found at $\phi_{\text{PI}} = 0.5$. This is consistent with the result from AFM images. Although the images seem to exhibit a self-similar domain structure for all composition ratios, with decreasing film thickness
from ca. 75 nm in Figure 5.2 to ca. 15 nm in Figure 5.8, the characteristic size of the phase-separated domains decreases. Other groups have studied the film thickness dependence of the domain size in thin polymer blend films.\textsuperscript{34,35,36} They likewise concluded that the domain size increases with a thickness increase. Furthermore, Ton-That et al.\textsuperscript{36} found that for a given blend composition, the square root of the domain diameter appeared to increase linearly with the film thickness in a PS/PMMA system. Our results demonstrate that the size of feature domains of spin-cast films can be controlled by changing the concentration of the casting solution (both the total and relative concentration of the two polymer components are important). It is possible, therefore, to create films of a desired thickness with a predictable surface feature in a simple, one step process.

\textbf{Figure 5.9.} The black square points are PI area fraction versus PI mass fraction $\phi_{PI}$ from films cast from 0.4 wt\% PS/PI blend on Si-SiO$_x$ substrates. Black line is the linear fit. The blue points and curve line is average domain area of circular PI or PS domains versus PI mass fraction $\phi_{PI}$.

Recall that the thicker films are all covered by an ultrathin PI layer, despite the films having different morphologies. When the film thickness is decreased, it should be examined whether a PI layer also assembles on the free surface and totally covers the PS domains. Table 5.2 shows the chemical compositional result from the RPI of characteristic ion peaks. The 19 nm film with $\phi_{PI} = 0.6$ still has similar data to the
pure PI. It indicates that this blend film has a PI layer on the free surface. But the film with $\phi_{PI} = 0.3$ presents a lower RPI number of characteristic PI peaks and a higher RPI number of characteristic PS peaks, compared with those from the pure PI film. This indicates that with lower $\phi_{PI}$, the PS domains are not totally covered by PI. All or part of the PS domains exist on the free surface. This chemically heterogeneous surface can provide a template with a bigger feature size for the protein patterning in comparison to a diblock pattern.

Table 5.2. Relative peak intensities (RPI) of characteristic PS and PI ion peaks of spin-cast films with different PI mass fractions.

<table>
<thead>
<tr>
<th></th>
<th>PI</th>
<th>$\phi_{PI} = 0.6$</th>
<th>$\phi_{PI} = 0.3$</th>
<th>PS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>19 nm</td>
<td>14 nm</td>
<td></td>
</tr>
<tr>
<td>RPI</td>
<td>PI</td>
<td></td>
<td></td>
<td>PS</td>
</tr>
<tr>
<td>Summed RPI</td>
<td>215.9</td>
<td>209.6</td>
<td>156.8</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>PI</td>
<td></td>
<td></td>
<td>PS</td>
</tr>
<tr>
<td>Summed RPI</td>
<td>37.1</td>
<td>37.8</td>
<td>59.0</td>
<td>186.9</td>
</tr>
</tbody>
</table>

Comparing with the thin films from the diblock copolymer PS-b-PI, the homopolymer blend films can form morphologies with bigger domain sizes of hundreds of nanometers or even a few micrometers through phase separation. Normally, the fast solvent evaporation through spin-coating causes the non-equilibrium film formation. The connectivity of covalent bond in block copolymer restricts the free mobility of one block in a polymer chain and both components can stay on the free surface without annealing treatment. But the two incompatible homopolymers are quite free to separate. The film morphology formation time is enough to allow the low surface energy component to move to the free surface. For the thicker films from homopolymer blends, the PI stays on both the substrate and the free surface. But when the thickness is decreased, PI will first satisfy the substrate because of the stronger interaction of PI with silicon oxide. When the PI mass ratio is as high as 0.6, there is enough PI to cover the free surface. Whereas a low PI mass fraction of 0.3 cannot satisfy this requirement, and some PS domains will be exposed at the surface to form a chemically heterogeneous film.
5.3.1.3 PS/PI binary blend films incubating in BSA solution

Four typical films ($\phi_{PI} = 0.4$ with 70 nm thickness, $\phi_{PI} = 0.6$ with 103 nm thickness, $\phi_{PI} = 0.3$ with 14 nm thickness and $\phi_{PI} = 0.6$ with 19 nm thickness) were chosen to incubate in BSA solutions to study protein adsorbing and patterning. The topography and surface composition of these four samples have been analyzed by AFM and SIMS. The previous analysis in Chapter 4 can help to understand the protein adsorption phenomena.

In Figures 5.10 b and d, the thick and thin PS/PI blend films both form island structures but the domain sizes are different. After BSA adsorption, both island patterns form a network structure on the surface no matter what the PI-rich matrix or the PS-rich domains are. This structure is quite similar to the PI dewetting pattern shown in Chapter 3. In the PI-rich matrix, PI can dewet on the Si/SiO$_x$ surface when incubated in water solution. The mechanism of dewetting was explained in Chapter 3. In the PS-rich domain, the PS domains are covered by a very thin PI layer on the free surface. Then PI layer dewetted the PS surface under the water soaking. Because the thickness of PI layer on PS-rich domains is much thinner than PI-rich domains, the dewetting pattern on PS-rich surface is much smaller than that on PI-rich surface. Meanwhile, there are no or very limited BSA molecules adsorbed on these film surfaces. This further verifies the PI layer stays on the free surface and it prevents protein adsorption. Comparing with the films with hole structures after BSA soaking, the thick film (Fig. 5.10a) also forms a network structure on the entire surface although the network size on the depressed PI-rich area is bigger than the size on the elevated PS-rich area. But, the thinner hole-structure film seems not forming dewetting network structure and be adsorbing BSA molecules and forming an approximate protein ring-like pattern (Fig. 5.10c). This means that the thin hole structure maybe exposes PS component on the free surface and adsorbs BSA molecules on PS domains. This will be verified further using SIMS analysis. Once the film surface of a PS/PI blend is totally covered by a several nanometers thick PI layer, the dewetting network pattern after soaking in water solution will appear. This pattern is a typical structure which will appear often in the following content of this chapter.
Figure 5.10. AFM phase images of PS, PI blend films before (left column) and after (right column) incubating in BSA solution for 1 h. Films have different $\phi_{PI}$ and film thicknesses. (a) $\phi_{PI} = 0.4$ and 70 nm thick, (b) $\phi_{PI} = 0.6$ and 103 nm thick, (c) $\phi_{PI} = 0.3$ and 14 nm thick, (d) $\phi_{PI} = 0.6$ and 19 nm thick. Image sizes are $2 \mu m \times 2 \mu m$. 
Table 5.3 gives the summed ion relative intensities of the characteristic BSA peaks on each polymer sample after protein adsorption. The \( \phi_{PI} = 0.6 \) sample with 19 nm thickness, with its free surface covered by an ultrathin PI film, dramatically decreases the protein adsorption. The RPI data are quite similar to the pure PI film and means it adsorbs as little BSA as the pure PI sample. The \( \phi_{PI} = 0.3 \) sample with 14 nm thickness, which is partially covered by the PS component on its surface (known from SIMS result in Table 5.2), adsorbs some BSA molecules on its surface. The RPI data of BSA is between the data of BSA on pure PS and PI films. This result is consistent with the AFM image in Fig 5.10c.

Table 5.3. RPI \((\times 10^3)\) of BSA peaks on homopolymer or polymer blend substrates.

<table>
<thead>
<tr>
<th>BSA peaks (m/z)</th>
<th>BSA on PS (1h)</th>
<th>BSA on PS/PI blend, ( \phi_{PI} = 0.3 ), 14 nm (1h)</th>
<th>BSA on PS/PI blend, ( \phi_{PI} = 0.6 ), 19 nm (1h)</th>
<th>BSA on PI (15min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>7.9</td>
<td>2.8</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>30</td>
<td>25.1</td>
<td>11.6</td>
<td>5.5</td>
<td>6.1</td>
</tr>
<tr>
<td>44</td>
<td>14.9</td>
<td>16.2</td>
<td>6.2</td>
<td>8.3</td>
</tr>
<tr>
<td>60</td>
<td>9.6</td>
<td>4.1</td>
<td>3.7</td>
<td>3.0</td>
</tr>
<tr>
<td>70</td>
<td>20.9</td>
<td>16.7</td>
<td>12</td>
<td>13.9</td>
</tr>
<tr>
<td>86</td>
<td>9.9</td>
<td>7.2</td>
<td>2.1</td>
<td>4.0</td>
</tr>
<tr>
<td>Totals</td>
<td>88.3</td>
<td>58.6</td>
<td>30.8</td>
<td>36.7</td>
</tr>
</tbody>
</table>

5.3.2 Film topography and surface composition of PI/PS(45)-b-PI(46) blends

Further work was carried out to create a structure that was expected to resist dewetting. In this part, the PI homopolymer was added to PS(45)-b-PI(46) with different PI mass fractions. As we know, the symmetric PS(45)-b-PI(46) can form lamellar structure in the bulk. But for the 19 nm film freshly spin-cast from 0.5 wt% solution, the surface morphology is PI circular dots dispersed in a PS network (as shown in Fig. 5.11a). The formation mechanism of this morphology has been discussed in Chapter 4. In this case, the blended PI plays an important role in the self-assembly of the block copolymer. One might expect the PI to swell the PI phase of the copolymer and to decrease the PS domain size and form PS isolated islands. On the
other hand, the network of glassy PS could provide mechanical resistance to dewetting in water.

Figure 5.11. Phase images of films spin-cast from 0.5 wt% PI/PS(45)-b-PI(46) blend solutions. From (a) to (f), PI mass fractions are 0, 0.2, 0.5, 0.6, 0.8 and 0.9. Image sizes are 2 μm × 2 μm.

All the surface images shown in Fig 5.11 are films made by adding PI into the block copolymer. With φ_{PI} = 0.2, the PI domains become elongated and are still dispersed in a connected PS matrix. When φ_{PI} increases to 0.5, the PI phase forms the continuous phase but PS begins to form big rings or broken cylinders. At φ_{PI} = 0.6, the PS cylinders become shorter and spherical dots begin to appear. When φ_{PI} increases to 0.8, PS is a mixture of short ribbons and spherical dots. At φ_{PI} = 0.9, PS forms spherical dots dispersed in a PI matrix. Although the film surface morphology is not the same as the bulk phase structure, Fig. 5.11 indicates that the addition of homopolymer to the diblock copolymer effectively modifies the phase structure of the system. This result is very close to previous research, where Mykhaylyk et al.\textsuperscript{37} mixed different mass fraction low molecular weight PI in an asymmetric PS-PI-PS triblock (containing 18 wt% PS). In their results, the copolymer surface morphology evolved
from cylinders to spheres by increasing the PI fraction. On the contrary, Lee et al.\textsuperscript{38} mixed PS with different mass ratio and molecular weight in the asymmetric PS-PI-PS (15 wt% PS). Their bulk morphology changed from spheres to cylinders and finally lamellae. The morphological transition from lamellae to spheres on addition of homopolymer is a well-known phenomenon for a bulk mixture of block copolymer and homopolymer.\textsuperscript{39} The added homopolymers electively swells the block chains. The increase in the asymmetry of the molecular volume for the two components causes an increase in the interfacial curvature, resulting in the transition from lamellae to spheres in our system.\textsuperscript{9}

The heights of the hills to valleys in height images of these samples are only 2 to 4 nm (not shown here). Because the AFM tip can indent into PI for several nanometers, this topography might be attributed to AFM artifacts. These films should be topographically smooth. It is important to verify whether various PS domains stay on the free surface for further protein adsorption analysis. SIMS results of the surface compositions are listed in Table 5.4. All samples present a high RPI number of PI characteristic peaks and a low RPI number of PS characteristic peaks. The data are quite similar to the pure PI film. This indicates that an ultrathin PI homopolymer layer segregated at the free surface during the spin-coating process. Therefore, the films are not suitable for protein templates; nevertheless the effects of BSA incubation were investigated.

\textbf{Table 5.4.} RPI of characteristic PS and PI ion peaks of spin-cast PI/PS(45)-b-PI(46) binary blend films with different PI mass fractions.

<table>
<thead>
<tr>
<th>RPI</th>
<th>(\phi_{PI} = 0.2)</th>
<th>(\phi_{PI} = 0.5)</th>
<th>(\phi_{PI} = 0.6)</th>
<th>(\phi_{PI} = 0.9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PI peaks ((x10^3))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summed RPI</td>
<td>211.3</td>
<td>215.0</td>
<td>217.2</td>
<td>213.9</td>
</tr>
<tr>
<td></td>
<td>PS peaks ((x10^3))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summed RPI</td>
<td>36.5</td>
<td>31.2</td>
<td>30.1</td>
<td>32.0</td>
</tr>
</tbody>
</table>

After 1 h incubation in BSA solution, all these film surfaces form ring-like or broken network structures. These structures come from the PI film dewetting under water soaking. When \(\phi_{PI}\) increases from 0.2 to 0.9, the network sizes increase maybe
because the PI layer thickness on the top surface has increased with $\phi_{PI}$. In Fig 5.12d, some spherical dots are also clear under the network layer, which further confirms the PS phase structures are buried under a PI layer.

![AFM phase images of PI/PS(45)-b-PI(46) blend films after incubating in BSA solution for 1 h. (a) $\phi_{PI} = 0.2$, (b) $\phi_{PI} = 0.5$, (c) $\phi_{PI} = 0.6$, (d) $\phi_{PI} = 0.9$. Image sizes are 2 $\mu$m x 2 $\mu$m.](image)

**Figure 5.12.** AFM phase images of PI/PS(45)-b-PI(46) blend films after incubating in BSA solution for 1 h. (a) $\phi_{PI} = 0.2$, (b) $\phi_{PI} = 0.5$, (c) $\phi_{PI} = 0.6$, (d) $\phi_{PI} = 0.9$. Image sizes are 2 $\mu$m x 2 $\mu$m.

### 5.3.3 Film topography and surface composition of PS/PI/PS(45)-b-PI(46) ternary blends

#### 5.3.3.1 Thick films spin-cast from 2 wt% ternary blend solution

Next, ternary blend films were investigated as a possible template. The AFM images of spin-cast films from 2 wt% ternary PS/PI/PS(45)-b-PI(46) blend solutions are shown in Fig. 5.13. The morphology of the film from PS: PI: PS(45)-b-PI(46) = 40: 40: 20 (in the top row) is elongated PI holes dispersed in a connected PS-rich phase. This morphology is quite similar to Fig. 5.2c, which is spin-cast from PS/PI blends with $\phi_{PI} = 0.5$. The domain size of the first-order phase separation is quite big and the lateral distances are one or two micrometers. The distance from hill to valley...
is 40 nm for the bigger feature domains and 20 nm for the smaller domains dispersed in PI holes. The film from PS: PI: PS(45)-b-PI(46) = 23: 38: 39 (middle row in Fig. 5.13) is a PS island structure, which is quite similar to the structure in Fig. 5.2d, except that the island diameter is smaller (about 0.5 μm) and the hill-to-valley height is lower (about 30 nm) than the one in the binary PS/PI system. The film from PS: PI: PS(45)-b-PI(46) = 25: 25: 50 (bottom row) has a very complicated structure. It seems a combination of PS islands and lamellar structures.

**Figure 5.13.** Film height images (left column), cross-sectional profiles of the green line marked in height images (middle column), and phase images (right column) of spin-cast 2 wt% PS/PI/PS(45)-b-PI(46) blend solutions. From top to bottom, PS: PI: PS(45)-b-PI(46) mass ratios are 40: 40: 20, 23: 38: 39, and 25: 25: 50. Image sizes are 5 μm × 5μm.
A phase diagram of a symmetric ternary blend, which consists of a symmetric A-B block copolymer and equal volume fractions of A and B homopolymers, has already been constructed based on previous research of bulk polymer blends.\cite{40,41,42,43}

It was concluded that as $\phi_H$ (homopolymer fraction) increases, the added homopolymer will swell the lamellar domains to reach a transition from the lamellar phase to a microemulsion phase and further to a macrophase state. The morphology transitions in the thin film state from lamellae to microemulsion leading to macrophase separation were also studied in experiments\cite{44} and theory\cite{45}. This film morphology formation can also be used to interpret the phenomena in our study.

In our work, the symmetric copolymer and each homopolymer have similar volume fractions, but the polymerization degree of PI ($N_{PI}$) is slightly higher than the value for PS ($N_{PS}$) and the PS-b-PI copolymer ($N_C$). The ratio of $\alpha_1 = N_{PS}/N_C$ is ca. 1 and the ratio of $\alpha_2 = N_{PI}/N_C$ is ca. 1.3. In this dry brush regime, added homopolymer will be solubilized selectively into the corresponding microdomains and also tend to localize in the middle of it excluded by copolymer brushes.\cite{37} Because the $\alpha$ of $N_H/N_C$ is $\geq 1$, the trend of the microemulsion channel shifts toward a lower $\phi_H$ in the phase diagram. Then, the macrophase separation is easy to happen even with the addition of a small mass fraction of homopolymers.\cite{45} The PS/PI/PS(45)-b-PI(46) with a mass ratio of 40: 40: 20 has 80 % homopolymer, so the film made from this material will undergo macrophase separation. The block copolymer is presumed to reside at the A/B homopolymer interface and results in a minimization of free energy. In addition, the total $\phi_{PI}$ is 0.5 and then the surface morphology is similar to the morphology of $\phi_{PI}$=0.5 PS/PI blend in Fig 5.2c. In the system of PS/PI/PS(45)-b-PI(46) with a mass ratio of 23: 38: 39, the homopolymer mass fraction is 61%. It seems the macrophase separation dominates the phase formation. PS forms islands on the film surface because the mass fraction of the PS composition considering the two homopolymers is only 0.38. When the mass ratio is 25: 25: 50, the film forms a complicated morphology. In this case, the micro- and macro- phase separation will both operate during the solvent evaporation process. The disordered AB copolymer region starts to microphase separate, and the system is in another three-phase coexistence between PS-rich, PI-rich, and lamellar phases. This structure was also investigated using a lattice (real-space) self-consistent field theory for $\alpha = 1$.\cite{45}
These three surfaces were incubated in BSA solutions for one hour, and the results are presented in Fig. 5.14. It is apparent that a thin PI layer covers the free surface and dewets to form network structures under water incubation. No or very few BSA molecules were adsorbed on these surfaces according to the AFM images. Further experiments were carried out to see if PS came to the free surface in thinner films.

Figure 5.14. AFM phase images of 2 wt% PS/PI/PS(45)-b-PI(46) blend films after incubating in BSA solution for 1 h. PS: PI: PS(45)-b-PI(46) mass ratios are (a) 40: 40: 20, (b) 23: 38: 39, and (c) 25: 25: 50. Image sizes are 2 µm × 2 µm.

5.3.3.2 Ultrathin films spin cast from 0.4 wt% ternary blend solution

When the ternary blend solutions were diluted to 0.4 wt%, the morphologies of the spin-cast thin films are totally different from what was found in the thick films. Fig. 5.15 shows the observed structures. The film of PS/PI/PS(45)-b-PI(46) with a mass ratio of 40: 40: 20 forms a “fancy” pattern, with the PS domain looking like a knitted or jigsaw structure. The ternary blends of other two mass ratios form similar discontinuous morphologies. The domain size of the film with a mass ratio of 23:38:39 is much bigger than the one with a mass ratio of 25:25:50. The morphology formation mechanism is very complicated. A lot of factors can affect the morphology, i.e. molecular weight of homopolymer and block copolymer, weight ratios of homo- and co-polymer, weight ratio of A/B block in the block copolymer, substrate surface energy, film thickness and so on. Although the mechanism is unclear, two findings can be concluded: Firstly, decreasing the film thickness decreases the feature size of the ternary blends comparing Fig. 5.15 to Fig. 5.13. Secondly, unlike the binary homopolymer blend, which keeps its morphology pattern but only decreases its
domain size in thinner films, the ternary blend films change their morphologies when decreasing the film thickness.

**Figure 5.15.** Film height (left column) and phase (right column) images of spin-cast 0.4 wt% PS/PI/PS(45)-b-PI(46) blend solutions. PS: PI: PS(45)-b-PI(46) ratios are (a) 40: 40: 20, (b) 23: 38: 39, and (c) 25: 25: 50. Image sizes are 2 μm × 2 μm.

The question arises as to whether there is a PI wetting layer. The surface composition analysis is collected in Table 5.5. The surface compositions of two typical structure films were determined by SIMS. The RPI of characteristic PS and PI peaks of these two thin ternary blend films are all between pure PS and PI films. It indicates that both PS and PI components are present on the free surface. In this case, there is not a PI wetting layer.
Table 5.5. RPI of characteristic PS and PI ion peaks of thin ternary blend films.

<table>
<thead>
<tr>
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<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Summed RPI (×10⁻⁵)</td>
<td>Summed RPI (×10⁻⁵)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Summed RPI</td>
<td>Summed RPI</td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>PE</td>
<td>215.9</td>
<td>163.2</td>
<td>153.1</td>
</tr>
<tr>
<td>PS</td>
<td>PE</td>
<td>37.1</td>
<td>49.4</td>
<td>55.3</td>
</tr>
</tbody>
</table>

Figure 5.16 shows the AFM height and phase images after BSA adsorption for the two samples studied by SIMS. In Fig. 5.16a, the BSA molecules seem to be adsorbed on the PS domains and form a twist pattern in the height image that matches the “knitted” PS structure. In the phase image, it is difficult to interpret and most of the surface appears to be covered. The size of knitted BSA pattern is ca. 200 nm from the height image. Comparing to the diblock copolymer templates in Chapter 4, this template structure is larger. In Fig. 5.16b, it is very clear that BSA molecules were adsorbed on PS domains and form a pattern quite similar to the polymer template.

Figure 5.16. AFM height (left column) and phase (right column) images of 0.4 wt% PS/PI/PS(45)-b-PI(46) blend films after incubating in BSA solution for 1 h. PS: PI: PS(45)-b-PI(46) mass ratios are (a) 40: 40: 20, and (b) 25: 25: 50. Image sizes are 2 μm × 2 μm.
Table 5.6 shows the BSA intensity from SIMS analysis of various polymers after BSA incubation. The intensities from BSA of these two films are between the intensity amount of BSA adsorbed on PS and PI films. It is concluded that there is partial coverage by the BSA in the templates.

**Table 5.6.** RPI ($\times 10^3$) of BSA ion peaks on thin ternary blend films compared to homopolymer films. (The uncertainty is in the range from $0.2 \times 10^3$ to $2 \times 10^3$)

<table>
<thead>
<tr>
<th>BSA peaks (m/z)</th>
<th>BSA on PS (1h incubation)</th>
<th>BSA on PS:PI:PS(45)-b-PI(46) = 40:40:20 (14 nm) (1h incubation)</th>
<th>BSA on PS:PI:PS(45)-b-PI(46) = 25:25:50 (15 nm) (1h incubation)</th>
<th>BSA on PI (15min incubation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>7.9</td>
<td>2.4</td>
<td>3.1</td>
<td>1.4</td>
</tr>
<tr>
<td>30</td>
<td>25.1</td>
<td>9.8</td>
<td>12.5</td>
<td>6.1</td>
</tr>
<tr>
<td>44</td>
<td>14.9</td>
<td>11.3</td>
<td>12.4</td>
<td>8.3</td>
</tr>
<tr>
<td>60</td>
<td>9.6</td>
<td>2.8</td>
<td>1.5</td>
<td>3.0</td>
</tr>
<tr>
<td>70</td>
<td>20.9</td>
<td>18.4</td>
<td>19.4</td>
<td>13.9</td>
</tr>
<tr>
<td>86</td>
<td>9.9</td>
<td>5.9</td>
<td>5.3</td>
<td>4.0</td>
</tr>
<tr>
<td>Totals</td>
<td>88.3</td>
<td>50.6</td>
<td>54.2</td>
<td>36.7</td>
</tr>
</tbody>
</table>

Table 5.7 gives a comparison of the total intensities of the PI and PS characteristic peaks before and after BSA adsorption. After BSA adsorption, the RPI of PS peaks decreases by ca. $23 \times 10^3$ for the sample of PS:PI:PS(45)-b-PI(46) = 40:40:20 and ca. $30 \times 10^3$ for the sample of PS:PI:PS(45)-b-PI(46) = 25:25:50. The PI intensity only slightly decreases, but the PS intensity decreases a lot for these two samples. It appears that more BSA molecules were adsorbed on the PS domains than on the PI domains. This SIMS result further verifies that these two ternary blend films can selectively adsorb proteins and form protein patterns.

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Table 5.7. RPI of characteristic PS and PI ion peaks after 1h BSA adsorption.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40:40:20 (14nm)</td>
<td>25:25:50 (15nm)</td>
</tr>
<tr>
<td>PI peaks ($\times 10^{-3}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before BSA Ads.</td>
<td>163.2</td>
<td>153.1</td>
</tr>
<tr>
<td>After BSA Ads.</td>
<td>155.9</td>
<td>145.3</td>
</tr>
<tr>
<td>Difference</td>
<td>-7.3</td>
<td>-7.8</td>
</tr>
<tr>
<td>PS peaks ($\times 10^{-3}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before BSA Ads.</td>
<td>49.4</td>
<td>55.3</td>
</tr>
<tr>
<td>After BSA Ads.</td>
<td>26.4</td>
<td>25.9</td>
</tr>
<tr>
<td>Difference</td>
<td>-23.0</td>
<td>-29.4</td>
</tr>
</tbody>
</table>

5.4 Conclusion

In this chapter, the film topographies, morphologies and surface compositions of binary and ternary blends of PS, PI and PS(45)-b-PI(46) prepared by spin-coating were studied. In the PS/PI binary blend system, film morphologies vary with changing PI mass fraction ($\phi_{PI}$). The PI forms a depressed-hole structure when $\phi_{PI} \leq 0.5$, and PS forms an elevated circular island structure when $\phi_{PI} \geq 0.6$. The domain size of the minority phase on the surface reaches the maximum when $\phi_{PI}$ is 0.5, which is close to the critical point of the bicontinuous phase. When the thickness is decreased, all films maintain their surface pattern but dramatically decrease their domain size. Using SIMS and UV-ozone analysis, the structures of thicker films, including their interior distribution, were determined through cross-sectional profiling. A PI layer is segregated at the Si interface and the bottom part of elevated PS domains is buried in the PI layer. No matter which morphology is present, the films are all covered by an ultrathin PI layer on the free surface.

The composition on the free surfaces of the ultrathin films depends on $\phi_{PI}$. High $\phi_{PI}$ can provide enough PI to cover the free surface but low $\phi_{PI}$ will leave part PS on the free surface. The ultrathin blend film with low $\phi_{PI}$ can selectively adsorb BSA molecules and form protein patterns with a several hundred nm domain spacing.
In the PI/PS(45)-b-PI(46) binary system, the films undergo microphase separation to form smooth surfaces no matter which mass ratios the blends have. All films have an ultrathin PI layer on the free surface. When increasing the $\phi_{PI}$, the PS domain structure under this PI layer changes from a ring-like network to dispersed PS cylinders and then to spherical dots.

In the PS/PI/PS(45)-b-PI(46) ternary system, the thicker films spin-cast from 2 wt% blend solutions form macrophase-like structures when the homopolymer mass fraction ($\phi_H$) is higher than 0.6. When the $\phi_H$ decreases to 0.5, both macrophase and microphase separations operate during the spin-coating process. The PS macrophase-separated islands and copolymer microphase-separated parallel lamellae are coexistent in the film. The free surfaces of these thicker films are all covered by a PI layer. When the film thickness decreases to ca. 15 nm, a visually striking morphology developed. The film morphologies are totally different from their thicker films with the same mass ratios. Meanwhile, the free surfaces are not entirely covered by PI. Both PS and PI are exposed on the free surface to form a chemically heterogeneous surface. These templates were used to form some complex protein patterns, and they have potential applications in cell cultures.
References


17 Collins S, Hamley IW, Mykhaylyk TA. An atomic force microscopy study of ozone etching of a polystyrene/polyisoprene block copolymer. Polymer 2003, 44, 2403.


Chapter 6

Cell adhesion on fibronectin-nanopatterned substrates

6.1 Introduction

This chapter builds on Chapter 4 by using PS-b-PI templates to create nanopatterns of an extracellular matrix (ECM) protein. The resulting substrates are then used to increase the adhesion of mammalian cells. To grow, cells of most types need to adhere to something. In vivo, the substrate is the ECM or other cells. Thus, to encourage cell growth for tissue engineering, we need to provide substrates that mimic at least some properties of the ECM. Interactions with the ECM, or with artificial mimics of it, play a crucial role in fundamental cellular functions, including cell migration (the movement of cells in particular directions to specific locations, and a central process in the development and maintenance of multicellular organisms), proliferation (an increase in the number of cells as a result of cell growth and cell division), differentiation (a generic cell develops into a specific type of cell in response to specific triggers from the body or the cell itself, and a process which allows a single celled zygote to develop into a multicellular adult organism that can contain hundreds of different types of cells), and apoptosis (a process of programmed cell death that may occur in multicellular organisms).

Cell adhesion is mediated by specific receptors known as integrins on the cell surface that interact with ECM molecules. These trans-membrane receptors have extracellular domains that bind to the ECM and intracellular domains that link to the cytoskeleton. Integrins are approximately 10 nm wide and are 10–100 times more prevalent on the cell’s surface than other types of receptors. In their inactive state, integrins freely diffuse within the cell membrane until they encounter an available
binding domain in the ECM. Upon ligand binding, integrins undergo a conformational change that leads to the recruitment of cytoplasmic “anchor proteins” such as vinculin, talin and paxillin, which bind the actin cytoskeleton to the membrane. Through physical clustering of multiple integrins, more cytoplasmic proteins are recruited to the adhesion site to increase its size, adhesion strength, and biochemical signalling activity. These larger, clustered structures of integrins and cytoplasmic proteins, which are 100s of nm across, are commonly called focal adhesions (FAs). (See Figure 1.5 in Chapter 1 for a schematic figure showing cell structure and FAs.). Focal adhesions are comprised of an extracellular protein (e.g., fibronectin and laminin), a transmembrane protein (integrins), and intracellular proteins (e.g. actin and vinculin). They function as crucial outside-to-inside signalling ports and help cells to function properly.

For the binding interactions between cells and surfaces, it has become increasingly evident that cells detect and respond to numerous features of the ECM, including the structural composition and availability of adhesive ligands, mechanical stiffness, spatial organization of cell recognition sites, and surface topography of these ECM scaffolds at the microscale and nanoscale. The importance of the structural organization of focal adhesions on a molecular length scale has been demonstrated by investigations of cellular responses in the lateral spacing of adhesion-associated ligands (such as ECM proteins). The nanometer- and micrometer-scale organization of surface proteins is expected to play a crucial role in complex adhesion formation and hence on cell behaviour.

Cell adhesion and cellular organization have been widely studied as a function of the available adhesive area and shape on a substrate using micrometer-scale patterns. However, a key event in focal adhesion assembly is the activation and clustering of ligand-occupied integrins. The molecular composition of the ECM determines the recruitment of specific integrins at these adhesion sites. For example, on fibronectin, the major integrin is \( \alpha_5\beta_1 \), on vitronectin, the major receptor is \( \alpha_v\beta_3 \). Patterning methods at the length-scale of tens of nanometres are required to explore how integrin-mediated cell adhesion depends not only on receptor occupancy but also on receptor clustering. With the control of integrin receptor clustering, substrates patterned with ligands at the nanoscale level are suitable for addressing this aspect of
cell-ECM interactions. Recently, there has been considerable work on cell behaviour on nanopatterned substrates of ECM proteins\textsuperscript{25,26} or particular ECM sequence\textsuperscript{27,28} (e.g. arginine-glycine-aspartate (RGD)).

The vast majority of in vitro studies on cell-ECM interactions have been focused on the design of bioactive surface coatings. Well-defined templates of ECM molecules allow studies of cell adhesion, spreading, growth, differentiation and functioning. Protein templates have been fabricated through controlling the placement of whole ECM molecules (or moieties present in the ECM to which integrins bind) on the surface. These nano- or micro-arrayed adhesive regions that encourage integrin binding are surrounded by non-adhesive regions, which typically resist cell adhesion by resisting the adsorption of protein. Protein surface patterning can be realized through various approaches such as microcontact printing,\textsuperscript{29} dip-pen lithography,\textsuperscript{30} electron beam lithography,\textsuperscript{31} and self-assembly.\textsuperscript{32} As an example of self-assembly, a patterning method using block copolymer nanolithography (BCN) has been developed recently. This technique is based on the self-assembly driven deposition of spherical micelles on solid substrates. By using diblock copolymers of different molecular weight, the separation distance between spherical minority phases can be tuned.\textsuperscript{32}

In this chapter, a very simple method was used to pattern protein molecules shown in Chapter 4. The hard-soft self-assembled polymer substrates with well-organized structures were used as templates to form protein nanopatterns through the protein selective adsorption on hard domains. The ECM protein, fibronectin (FN), will be adsorbed on the copolymer templates and form nanopatterned FN substrates to explore the cell adhesion responses. By comparing the cell adhesion phenomena on nanopatterned FN surfaces with varying length scales, we determine how surface patterning and organization at the nanoscale level of the ECM affect cell adhesion and spreading.

6.2 Experimental details

6.2.1 FN nanopattern fabrication

Si (100) wafers and glass coverslips were cleaned by a UV-ozone cleaner. PS, PI and PS-b-PI copolymer solutions were spin-coated onto these cleaned substrates.
The preparation procedures were described in Chapter 4. Homogeneous, dot-like and stripe-like polymer templates were fabricated for protein adsorption.

Fibronectin (1 mg/ml solution from bovine plasma, purchased from Sigma-Aldrich) was dissolved in a universal buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5) and diluted to 50 µg/ml. The polymer-coated Si and glass substrates were incubated with FN solution for one hour at room temperature except PI films were incubated for 10 and 30 min. The incubation times for PI are different from others because they are subject to dewetting at longer times and the dewetting patterns are dependent on the incubation times in water solution. Upon removal from the FN solution, the samples were rinsed thoroughly with flowing Tris buffer solution followed by DI water to remove non-adsorbed FN molecules and residual salt from the buffer, respectively. The samples were dried in a desiccator (containing silica gel) for 24 hr prior to AFM and ToF-SIMS analysis.

6.2.2 FN surface imaging and characterization

AFM images were acquired using an intermittent-contact mode by using a gold-coated silicon cantilever with a constant ranging from 5 to 10 N/m and a resonance frequency ranging from 130 to 180 KHz. All the AFM experiments were performed in air at room temperature, and the images were captured by using a scan speed of 1.2 Hz.

ToF-SIMS was employed to characterize the relative intensity of surface components before and after the FN adsorption. Data acquisition was performed by raster scanning a primary ion beam over a 100 µm × 100 µm area at a resolution of 64 × 64 pixels. Every sample was probed three times in three different areas, and averages were reported. The probe depth was around 10-20 Å from the film surfaces. The positive and negative ions from the sample’s outermost surfaces were collected and converted to the m/z = 0-500 mass spectra. Secondary ion mapping was used for the PI sample because its big feature size of dewetting after soaking in FN solution could be resolved by the technique. The image brightness at each point in a map is a function of the relative concentration of the mapped element or molecule.\(^{33}\)

Contact angle analysis was used to explore the FN surface hydrophilicity at
different areas, which were selected before cell incubation. A 3 x 3 grid was drawn in permanent ink on the backside of the glass substrate to divide the substrate into nine sub-areas. In this approach, the water contact angle in a particular sub-area could be correlated with the cell adhesion at that same position. A 1 μl drop of DI water was deposited onto the sample surface. For every sub-area of a sample, three drops were deposited, and the average value was obtained.

6.2.3 Cell culture

Cells are grown and maintained at an appropriate temperature and gas mixture in a cell incubator. The growth medium, plating density, cell culture protocols, and the "age" of the cell line or the "passage number" are all important parameters to culture cells. Culture conditions vary widely for each cell type and every cell line has its practical culture conditions. Established protocols for the culture and passage of Chinese Hamster Ovary (CHO) cells were followed. Cells were cultured in tissue culture flasks (75 cm²) at 37 °C humidified atmosphere at 5% (by volume) CO₂ in F-12 Ham's medium (Invitrogen) supplemented with 10% (by volume) fetal bovine serum (purchased from Gibco) and 1% (by volume) of antibiotics (penicillin/streptomycin). For maximum yield, cells are kept less than 100% (log phase of growth) but more than 10% confluent. Cells may die if they are too few or too crowded. A 10% confluent plate will reach 100% confluence in two or three days. If no evasive action is taken, the nutrients will be depleted and the cells will die shortly thereafter, thus cell passaging or sub-culturing is needed to maintain the culture. In this work, cultures were passaged every 48 hours by a dilution factor of 1/6 or every 72 hours by a dilution factor of 1/7. This passage frequency is used to ensure cell confluence maintains at around 80% before passaging and the dilution factor (split-ratio) is selected to sustain the plating density in a fixed range in order to minimize its influence to adhered cell density. After the cells reached confluence, they were first rinsed with sterile PBS and then detached from the flask wall by incubating with 0.05% of trypsin-EDTA (purchased from Gibco) solution for 3-5 min. For seeding samples, culture flasks (75 cm² growth area) of 80% confluent CHO cells were trypsinized, washed and suspended in fresh media. The suspension of cells was then diluted with cell growth media to the desired cell concentration. Cell suspensions were then added to polystyrene Petri dishes (13 mm diameter) containing prepared FN
substrates on glass and incubated for one hour. For microscopy and imaging experiments, the cell plating density was 1000-1500 cells/mm².

6.2.4 Fixing and staining cells

The cell-seeded substrates were then washed with PBS twice and fixed with 4% paraformaldehyde in PBS for 20 min. After fixation, the samples were rinsed gently with PBS and permeabilized with 0.1% non-ionic surfactant (Triton X, Sigma) in PBS for 5 min. Then samples were thoroughly rinsed with PBS and stained. Samples were incubated with phalloidin (1:30, AlexaFluor phalloidin, Molecular Probes, Eugene, OR) for 30 min at room temperature to stain actins of cells. After rinsing with PBS, samples were subsequently incubated with Draq5 (BD Biosciences Limited) for 10 min at room temperature to stain nuclei of cells. Stained cells were mounted with a coverslip in mounting medium containing DAPI (Vectashield, Vector Laboratories, Inc., Burlingame, CA) to help to prevent the rapid loss of fluorescence during microscopic examination and sealed with nail varnish. Double-labelled samples were then ready for examining with confocal microscopy.

6.2.5 Confocal Microscopy

Imaging was conducted by using a Zeiss LSM 510 META laser scanning confocal microscope. For double stained cells, phalloidin (actin stain) was excited with the argon laser line at 488 nm, and Draq5 (nuclei counterstain) excited with a helium-neon laser line at 633 nm. The emission signals passed through 505-530 nm and 649-799 nm filters, respectively. All images were captured with a Plan-Apochromat 40×/1.4 oil and Plan-Apochromat 63×/1.4 oil DIC objective and collected in multichannel mode.

6.2.6 Image analysis

Cell morphology on each test substrate was quantified using image analysis software, Image J (version 1.42), from the United States National Institutes of Health. Image J automatically detects the cell outline and calculates parameters such as the quantity of cells, cell area, and coverage per unit area of the substrate. The dimensions were calibrated using a stage micrometer.
6.3 Results and discussion

6.3.1 FN nanopattern imaging and characterization

AFM images of the surface structures of PS, PS(45)-b-PI(46), and PS(65)-b-PI(26) films have been shown in Chapter 4. The surface structures of the PS(45)-b-PI(46), and PS(65)-b-PI(26) copolymer films with a thickness of ca. 18 nm are dot-like and stripe-like nanopatterns, respectively. The AFM images of FN surfaces after adsorption on PS and PS-b-PI substrates are shown in Fig 6.1. After comparing these images to those from the original surfaces, our preliminary interpretation is that FN molecules appear to cover fully the PS surface and create a dense and uniform carpet (Fig 6.1a and b).

On the stripe-like copolymer template obtained from PS(65)-b-PI(26), it appears that the FN molecules also formed a stripe-like nanopattern resembling their underlying copolymer structure (Fig 6.1 c and d). As was also found on the original copolymer surface, there is a strong contrast in the height and phase images. The FN pattern is very dense and the spacing of the pitch is only 10-15 nm. Although the protein molecules are aggregated densely, there is still a strong contrast in the height and phase images, which is because of a preferential adsorption of the FN rather than having a blanket coverage (comparing with Fig 6.1a and b). On the dot-like PS(45)-b-PI(46) copolymer surface, the FN forms a ring-like network structure (Fig 6.1e and f). This interpretation is explained by the selective adsorption of FN on PS domains and exclusion from the dot domains of PI. The feature size of this nanopattern is ca. 50 nm, which is larger than the size of the stripe-like pattern.
Figure 6.1. AFM (a) height and (b) phase images of a PS film after FN incubation for 1h; AFM (c) height and (d) phase images of a 17.8 nm PS(65)-b-PI(26) film after FN incubation; and AFM (e) height and (f) phase images of a 17.7 nm PS(45)-b-PI(46) film after FN incubation. Image sizes are 2 μm x 2 μm.

To verify this interpretation of the AFM images, the surfaces of FN dense layers and FN on polymer templates were analyzed by ToF-SIMS. Their positive and negative ion spectra were collected. The positive spectra were used for analysis because of their discriminating characterization. Figure 6.2 is the typical positive spectra of pure FN film on Si substrate over the mass range $m/z = 0$-200 Da. FN has unique peaks corresponding to nitrogen-containing fragments at $m/z = 18, 30, 44, 60, 70$ and $86$ Da. These peaks are not found in the spectra of PS-PI hydrocarbon polymer
fragments and can be used to quantify the FN and to differentiate it from the underlying polymer components.

Figure 6.2. Positive ToF-SIMS spectra in the mass range m/z 0-200 Da for FN on Si substrate.

In the SIMS spectra, every peak presents a characteristic ion fragment dislodged from some special amino acid in the long FN chain. Table 6.1 shows the positive ion fragments of marked peaks on Fig 6.2.

Table 6.1. Unique peaks of amino acids of fibronectin and their characteristic fragments in ToF-SIMS spectra.

<table>
<thead>
<tr>
<th>Mass</th>
<th>Positive ion fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>NH₄</td>
</tr>
<tr>
<td>30</td>
<td>CH₄N</td>
</tr>
<tr>
<td>44</td>
<td>C₂H₄N</td>
</tr>
<tr>
<td>60</td>
<td>C₂H₄NO</td>
</tr>
<tr>
<td>70</td>
<td>C₄H₈N</td>
</tr>
<tr>
<td>86</td>
<td>C₂H₁₂N</td>
</tr>
</tbody>
</table>

Table 6.2 gives the summed RPI of the characteristic FN peaks on each polymer sample after protein adsorption. The summed RPI of FN on the PS substrates is 10⁹×10⁻³. The FN RPI number on PS(65)-b-PI(26) substrate is only 9×10⁻³ less than the number on PS. This is because the stripe-like PS(65)-b-PI(26) substrate was densely covered by FN molecules. Dot-like PS(45)-b-PI(46) substrate has a lower PS component on its surface. After FN adsorption, the RPI of FN is about 80×10⁻³ and it
is less than the number on stripe-like pattern. This means the coverage percentage of FN on PS(45)-b-PI(46) substrate is less than that on PS(65)-b-PI(26) substrate. This conclusion is consistent with the AFM results. The pure 350 nm thick PI sample also adsorbed a few FN molecules after only 10 min of incubation, but less than any thin copolymer samples. The RPI of FN on PI film after 30 min incubation is much higher than the number after 10 min incubation. The signal strength is also ca. $80 \times 10^{-3}$. It is speculated that the PI film is partly covered by FN molecules or the PI film changed its topography under aqueous atmosphere. This hypothesis will be further confirmed by using the SIMS mapping image.

**Table 6.2.** Relative peak intensities (RPI) ($\times 10^{-3}$) of characteristic fibronectin (FN) ion peaks on substrates. (the uncertainty is in the range of $0.2 \times 10^{-3}$-$3 \times 10^{-3}$)

<table>
<thead>
<tr>
<th>FN peaks (m/z)</th>
<th>FN on PS (1 h incubation)</th>
<th>FN on PS(65)-b-PI(26) 17.8 nm (1 h incubation)</th>
<th>FN on PS(45)-b-PI(46) 17.7 nm (1 h incubation)</th>
<th>FN on PI (30 min incubation)</th>
<th>FN on PI (10 min incubation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>6.9</td>
<td>6.7</td>
<td>5.6</td>
<td>3.9</td>
<td>0.9</td>
</tr>
<tr>
<td>30</td>
<td>29.5</td>
<td>29.6</td>
<td>20.3</td>
<td>17.3</td>
<td>6.1</td>
</tr>
<tr>
<td>44</td>
<td>19.9</td>
<td>15.2</td>
<td>12.9</td>
<td>21.3</td>
<td>5.8</td>
</tr>
<tr>
<td>60</td>
<td>14.1</td>
<td>14.0</td>
<td>13.5</td>
<td>7.9</td>
<td>0.6</td>
</tr>
<tr>
<td>70</td>
<td>23.9</td>
<td>26.8</td>
<td>21.9</td>
<td>20.4</td>
<td>3.5</td>
</tr>
<tr>
<td>86</td>
<td>14.7</td>
<td>8.4</td>
<td>7.6</td>
<td>9.7</td>
<td>1.4</td>
</tr>
<tr>
<td>Totals</td>
<td>109</td>
<td>100.7</td>
<td>81.8</td>
<td>80.5</td>
<td>18.3</td>
</tr>
</tbody>
</table>

SIMS mapping can show the component distribution with a resolution of 100 nm through the bright and dark areas in an image. The bright area shows the higher concentration of the desired component. Figure 6.3a, b, and c are the mappings of several FN ion fragments which were sputtered by the ToF-SIMS gun. Fig 6.3a is the map of the CH$_4$N fragment. It is a polygonal cellular structure and the area inside the polygon is much brighter than the rim area. Fig 6.3b and 6.3c are the maps of C$_2$H$_6$N and C$_4$H$_8$N fragments, respectively. These two maps also show the same polygon structure and component distribution. All these fragments come from fibronectin amino acid sequences. The bright and dark contrast means the FN molecules are concentrated on the inner areas of polygons. Fig 6.3d is an AFM phase image of PI film that has dewetted to a polygon structure after incubation in DI-water. In this
image, the rim of the polygon is composed of converged PI and the bright areas inside polygon are the exposed oxide layer of the Si wafer.

In Chapter 3, we already concluded that the PI film thickness and incubation time both affected the feature size and the velocity of dewetting. The initial PI film thickness in Fig 6.3d is only 50 nm, but the thickness of the PI film used for the SIMS maps is 350 nm. Hence, the size of the polygonal structures in the SIMS maps is noticeably larger than the size of the structure in the AFM image. The SIMS mapping explains that the PI film dewetted and formed the polygonal structure when incubating in FN solution for 30 min. The FN molecules are aggregated on the Si wafer, and less FN is adsorbed on the PI polygonal boundaries. Although the RPI of FN adsorbed on PI after 30 min of incubation is much higher, the mapping results indicate that most FN molecules were adsorbed on the exposed Si wafer. These SIMS maps further confirm that PI is a highly protein-resistant material.

Figure 6.3. (a), (b), (c) are ToF-SIMS maps of FN fragments on a PI film (350 nm thick) after incubation in FN solution for 30 min; image sizes are 150 µm × 150 µm. (d) is the AFM phase image of a PI film (50 nm thick) after DI-water soaking for 15 min; image size is 50 µm × 50 µm.
Further analysis is conducted in order to identify the domains on which the FN is adsorbed. The relative intensities of characteristic PS and PI ion peaks after FN adsorption on PS, PI and PS-b-PI copolymer films were collected. The ion peak at m/z = 68 u has been removed from consideration because FN also provides strong intensities at this value from a hydrogen-carbon fragment. After FN adsorption, this will affect the PI’s analysis by SIMS.

Table 6.3 presents a comparison of the summed RPI of the PI and PS characteristic peaks before and after FN adsorption. The idea behind this analysis is that, preferential protein adsorption will cause a decrease in the yield of the polymer domains, as protein will cover the domain surface. The yield from the other block, however, should not change if there is no adsorption on it. The magnitude of the drop in the summed RPI for a particular polymer block is assumed to be proportional to the fraction of its surface covered by adsorbed protein. For the PI homopolymer film, the RPI of the characteristic PI peaks decrease by only about 25×10⁻³ after the FN adsorption, which is a relatively small change. For the samples in the left three columns in Table 6.3, all RPIs of characteristic PI peaks decrease or increase only slightly after the FN adsorption. These negligible changes of RPI value imply that very few PI domains are covered by FN molecules.

**Table 6.3.** Relative intensity changes of PS and PI ion peaks after FN adsorption.

<table>
<thead>
<tr>
<th></th>
<th>PS</th>
<th>PS(65)-b-PI(26)</th>
<th>PS(45)-b-PI(26)</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>17.8 nm</td>
<td>17.7 nm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly(isoprene) peaks ($\times10^{-3}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before FN Ads.</td>
<td>9.0</td>
<td>17.6</td>
<td>85.0</td>
<td>201.9</td>
</tr>
<tr>
<td>After FN Ads.</td>
<td>11.8</td>
<td>28.7</td>
<td>81.9</td>
<td>176.2</td>
</tr>
<tr>
<td>Difference</td>
<td>2.8</td>
<td>11.1</td>
<td>-3.1</td>
<td>-25.7</td>
</tr>
<tr>
<td>Poly(styrene) peaks ($\times10^{-3}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before FN Ads.</td>
<td>182.8</td>
<td>122.0</td>
<td>76.5</td>
<td>37.1</td>
</tr>
<tr>
<td>After FN Ads.</td>
<td>56.0</td>
<td>32.9</td>
<td>29.3</td>
<td>29.8</td>
</tr>
<tr>
<td>Difference</td>
<td>-126.8</td>
<td>-89.1</td>
<td>-47.2</td>
<td>-7.3</td>
</tr>
</tbody>
</table>
On the contrary, the RPIs of PS after FN adsorption decrease greatly for every sample which comprises PS components. After FN adsorption, the PS's RPI for the PS homopolymer is decreased by $126.8 \times 10^{-3}$. For the 17.7 nm PS(45)-b-PI(46) film and the 17.8 nm PS(65)-b-PI(26) film, the RPI values for PS is both ca. $30 \times 10^{-3}$ after FN adsorption and decrease by $47.2 \times 10^{-3}$ and $89.1 \times 10^{-3}$, respectively. The amount of FN adsorption on the PS block, as gauged by the RPI decrease, is found to be proportional to the fraction of PS blocks at the polymer film surface.

The complementary use of AFM and SIMS reveals that the FN can selectively adsorb on PS domains rather than on the PI domains on copolymer substrates. The two-dimensional PS-b-PI templates can be used to form stable, well-organized FN nanopatterns. All FN nanopattern results tell a similar story with the BSA adsorption results. Taken together, the results indicate that the PS-b-PI hard-soft templates can selectively adsorb a variety of proteins (not only BSA, as shown in Chapter 4). This nanopatterning method can be used in all kinds of bioadhesive materials. In the following work, fabricated FN layers on two control surfaces (a glass coverslip or on flat, PS homopolymer film) and on nanopatterned surfaces (both stripe-like and ring-like patterns) were prepared to explore the influence of nanopattern shape and the spacing of FN-ligand on cell adhesion functions. This topic is considered next.

### 6.3.2 Cell adhesion analysis by confocal microscopy

CHO cells following one hour of incubation on the control and on the nanopatterned FN on glass substrates was examined by confocal laser scanning microscopy. In Figure 6.4, the double stained CHO cells shows different adhesion behaviours depending on the substrate. The red areas are DRAQ5-stained CHO cell nuclei and the green areas are phalloidin-stained CHO cell actin. It is evident that cell adhesion density on the ring-like FN nanopattern is much higher than on the other three FN substrates. Meanwhile, the cell spreading on this ring-like pattern is better than others.
Figure 6.4. Confocal microscopy images with nucleus and actin staining that show CHO cell adhesion on different FN substrates: (a) ring-like FN nanopattern on PS(45)-b-PI(46) with ca. 50 nm domain spacing, (b) stripe-like FN nanopattern on PS(65)-b-PI(26) with ca. 12 nm domain spacing, (c) homogeneous FN surface on glass as a control surface, and (d) a FN surface on pure PS as a control surface. The red scale bar in each image is 10 μm.

The confocal images in Fig. 6.4 are representative of what was found in many observations. In each experiment, five confocal images were obtained from each of four different samples of the same type of template. The cell number density of attached cells and the percentage area of coverage by the spreading cells were determined from the images using Image J software. The entire experiment was repeated a second time, so that in total 40 images from eight samples of the same template type were analysed. The mean CHO cell densities are presented for each template type in Fig. 6.5, and the error bars represent the standard deviations obtained from analysis of the 40 images. The adhesion cell densities on the two control FN surfaces (FN on glass and FN on PS) and on the stripe-like FN surface are in the range between 400 and 500 cells/mm². On the other hand, the cell density on the ring-like
FN pattern obtained on the PS(45-b-PI(46) template is ca. 750 cells/mm², which is 50% more than obtained for the other three samples. In addition to the different CHO cell numbers, the fraction of the surface covered by the cells is also different. The cell coverage on the ring-like FN surface is 65±22%, which means that in a unit image area, the majority of the FN surface is covered by attached cells. This number is double the percentage of other three FN surfaces. This result indicates that the ring-like FN nanopattern surface with a 50 nm domain spacing can increase the cell adhesion in the initial adhesion stage (i.e., after one hour of incubation). Meanwhile, pure PS surface without FN treatment was incubated in CHO cell suspension as well. There are almost no or very few cells adhered on the PS substrate within one hour. Then the result of cells on PS surface is insignificant for decent comparison.

**Figure 6.5.** (a) CHO cell densities on four different substrates with pre-adsorbed FN. (b) Area percentage covered by CHO cells after adhesion and spreading for 1 hr on the four FN-coated substrates.
The actin cytoskeleton structure is difficult to observe in the double stained images presented in Fig 6.4. However, Figure 6.6 shows the cell images with actin staining on the four FN substrates.

Figure 6.6. Confocal images with actin staining to show CHO cells on different substrates: (a) a ring-like FN nanopattern on PS(45)-b-PI(46) with ca. 50 nm domain spacing, (b) a stripe-like FN nanopattern on PS(65)-b-PI(26) with ca. 12 nm domain spacing, (c) a homogeneous FN surface on glass, and (d) a FN surface on PS. The red scale bar is 20 μm. The blue arrows indicate cells that are not well spread.

It is apparent that the cells on the ring-like FN surface have attached and spread on the surface after only one hour of seeding. Almost all of the cells in the image are seen to be well spread. On the other three FN surfaces, however, some cells (identified by the blue arrow) finished the attachment but have not begun to spread.
The adhesion cell shape and size are also different. There is a pronounced difference in cell shape, with the cells forming a more rounded phenotype on the ring-like FN surface and a more elongated phenotype on the other three FN substrates. Meanwhile, the single cell spreading size on the ring-like FN is larger than the spreading cell on the other FN surfaces.

Large bundles or fibres of actin are observed on both the control and the nanopatterned FN surfaces. We assume they link focal adhesions where the cell binds to the substrates. The development of actin fibres in CHO cells on ring-like FN substrates is very apparent. The spreading cells form abundant and highly-aligned actin fibres. The actin fibres in a single cell are in multiple directions. On other three FN substrates, there is less stress fibre formation, and most of the fibres are distributed in the cell periphery.

While immobilized ECM proteins can be used to control the initial adhesion of cells on a substrate, most adsorbed ECM surfaces are unstable in the presence of cells and thus are only transiently defined. Cells can remodel surfaces on which they are attached over a time period as short as two hours. In our experiment, the cell interaction with the FN surface is only monitored for the first one hour. Compared with other FN substrates, the ring-like pattern not only increases the cell adhesion quantity but also the percentage of coverage by the cells. Focal adhesions and related structures are major cellular sites responsible for cell-ECM attachment and adhesion-mediated signalling. The well-organized actin cytoskeleton reflects the strong cell interaction with substrates. It is apparent that the ring-like FN-ligand pattern with around 50 nm spacing can positively affect the cell attachment and spreading.

6.3.3 Influence of FN nanopatterns on cell adhesion

In Fig. 6.5, the cell density and area percentage of coverage on the ring-like FN surface have large standard deviations. This means that the spread of values results from the cell adhesion behaviour being non-uniform on this substrate. Examples of optical images of cells on the ring-like FN surface, presented in Fig 6.7, indeed show that the density and spread shape of adhered cells are not uniform across a patterned substrate. In some regions, the cell density is high, but in other regions, fewer cells are adsorbed on the substrates.
The non-uniformity demonstrated in Fig 6.7 implies either the underlying FN chemical or geometric property affects cell adhesion behaviour. Additional experiments explored the heterogeneity of the substrates and correlated it with cell adhesion.

Figure 6.7. Optical images of CHO cells after 1 h adhesion on the ring-like FN substrates for different areas on the same sample. All images are at the same magnification. White scale bar size is 40 μm.

Before cell culture, several FN-coated glass coverslips were divided into nine sub-areas and the relative hydrophilicity of every sub-area was determined by water contact angle analysis. Here, the water contact angle is not of direct interest, but rather it is useful to determine it in order to correlate with the FN structures, as determined by AFM. Measurements of cell densities were collected from two different samples in which a copolymer film on a glass substrate had been templated with FN.

The mean cell densities were determined in each of the nine different sub-areas through analysis of several images obtained from that sub-area. Owing to small differences in how the surfaces were rinsed with buffer solution and DI water, the distribution of water contact angles on the two surfaces differ. Furthermore, there is variability in the water contact angle across each substrate, which results in a spread
of contact angles. Figure 6 shows values of the mean cell density in a sub-area as a function of the mean contact angle in its sub-area. The error bars on the densities represent the standard deviation of the measurements from the images in a sub-area corresponding to the particular water contact angle.

There is a clear trend showing that more cells adhere to the areas with a larger water contact angle. The maximum mean cell density on the ring-like FN pattern on PS(45)-b-PI(46), which is $680\pm103$ mm$^{-2}$, is found in a sub-area with a high water contact angle (65°). The sub-area with the lowest water contact angle (21°) has a mean cell density of only $118\pm29$ mm$^{-2}$.

For the FN surfaces on the PS(65)-b-PI(26)) template, data points were likewise obtained from two samples. The peak mean cell density on this stripe-like FN substrate is $470\pm65$ mm$^{-2}$ and was found in a sub-area with a high water contact angle, whereas the area with a low water contact angle (42°) has a mean cell density of only $70\pm13$ mm$^{-2}$.

![Figure 6.8](image)

**Figure 6.8.** Adhered cell density as a function of the water contact angle on pre-adsorbed FN surfaces. Black square points are cells on a FN+PS(45)-b-PI(46) template; and red round points are cells on a FN+PS(65)-b-PI(26) template.

FN is hydrophilic, and the PS-PI copolymer is hydrophobic (with a water contact angle of 110°). The variability in the water contact angles of the patterned FN surfaces might therefore be expected to be explained by variability in the patterns.
Figure 6.9. AFM phase images showing different sizes of FN ring structures on a PS(45)-b-PI(46) template. All images are 2 μm × 2 μm in area.

Although the FN on the same PS(45)-b-PI(46) template forms ring-like patterns in every sub-area, the ring sizes are variable across the surface. Examples of
the FN ring structures are presented in Fig 6.9. It is clear that the FN ring sizes are very different. Some ring sizes roughly match the size of their underlying copolymer template in the first several images in Fig 6.9. But in the latter images in Fig 6.9, the FN ring sizes are gradually increased. When the FN ring size is much bigger, the underlying copolymer dot-like structure is clearly apparent in the AFM images, especially in the lower right image.

In Chapter 4, BSA molecules were adsorbed on PS-b-PI templates and formed very stable ring-like structure. There was not any ring size variability across the sample surface, and the ring size matched the copolymer template in all areas. We speculate that the much bigger size of the FN molecules prevents them from adapting to structures with fine features. Obtaining more uniform templates is an objective of future research.

The average FN ring diameters were calculated using the Nova software of the AFM (NT-MDT). The ring diameters in Fig 6.9 are 45, 55, 63, 86, 102, 125, 160 and 180 nm. The correlation of FN surface water contact angle with the FN ring size is shown in Fig 6.10. It indicates that the water contact angle and the ring diameter of FN surface have a positive linear relationship except for the 45 nm point. This linear trend is reasonable because the bigger that the FN ring size is, then the more that the copolymer components are exposed to the air interface. The hydrophobicity of the copolymer then makes a greater contribution.
So far, it is apparent that there is a correlation between the cell adhesion density and the water contact angle. In turn, the water contact angle depends on the FN ring diameter. Combining these results, it can be concluded that the cell adhesion correlates with the FN ring diameter. In Fig 6.11, the graph on the left one shows that the cell density correlates with the FN ring diameter. The areas with water contact angles lower than 45° are all ascribed to full coverage and then the ring size is essentially 0 nm. This graph shows there is a higher cell density when there is a ring size greater than about 50 nm. The cell density does not vary with FN ring-size above 50 nm.

The FN coverage area fraction of the images in Fig 6.9 was calculated using Image J software. The areas with water contact angle lower than 45° were ascribed to 100% FN coverage. The graph on the right in Fig 6.11 indicates that there is a higher cell density when the FN coverage is less than ca. 85%. Blanket coverage by FN leads to lower cell density.

![Figure 6.11. The relationships of (a) cell density with FN ring diameter, and (b) cell density with the area coverage by FN on a PS(45)-b-PI(46) template.](image)

Fabricated nanopatterns of biological molecules, such as adhesive peptides, are widely used to induce specific cellular responses dependent on cell adhesion. Maheshwari et al. 36 functionalized star-shaped polymers with RGD-containing peptide over a non-adhesive background to achieve a controlled surface density and local spatial distribution of the peptide. When the RGD peptide was presented in clusters of at least five peptides per star, but not in the case of a random single RGD peptide per star, cells developed well-formed actin stress fibres and mature FAs.
Other research\textsuperscript{37} indicates that higher ligand clusters and higher ligand densities reinforce the cell adhesion. These studies lead to the hypothesis that cell spreading might be dependent on critical densities of submicron integrin clusters to begin the recruitment of FA and cytoskeleton proteins. Such a local integrin density could be critical for the initiation of a mature and stable FA assembly.

In our study, although we do not know the local concentration of FN after being adsorbed, the FN solution concentrations are the same for every polymer substrate. The FN molecules are randomly distributed on glass and PS surfaces. But on the PS(45)-b-PI(46) copolymer template, the FN forms ring-like nanopatterns with over 50 nm spacing. The spacing induces more FN localisation on the PS network area, and the FN density on these areas is increased. This high local density of adhesive FN-ligands perhaps enhances the integrin clustering and the focal adhesion. The cells after 1 h seeding on the ring-like pattern spread very well and form abundant actin stress fibres. The stripe-like FN nanopattern with a smaller spacing cannot prominently improve the FN density in local areas. The cell adhesion behaviour on this substrate is similar to the adhesion on homogeneous substrates.

In our work, we did not study the cell adhesion behaviour on different topography. But this kind of work has been done by others. For example, Lim et al.\textsuperscript{38} studied the cell response to the polymer-demixed nanoisland topography with varying heights. They concluded that cell adhesion and proliferation were greater with decreasing island height. Bettinger et al.\textsuperscript{21} concluded that the synthetically nanofabricated topography can influence cell morphology, alignment, adhesion, migration and cytoskeleton organization. One interesting result is that nanogratings generally appear to enhance the cell adhesion in various cell-biomaterial geometries, while nanopots and nanopits generally reduce initial cell attachment.

In our work, we found that the ring-like FN nanopattern adheres more cells compared with the stripe-like nanopattern. But the reason for the increases in the cell density and focal adhesion is attributed to the domain size rather than the pattern shape. If the spacing size of the stripe-like pattern can be increased to more than 50 nm, we speculate that this template will increase the cell adhesion too. This needs more experiments in the future to confirm. Commonly, researchers used dot-like patterns to study the cell adhesion behaviour. For example: Lehnert et al.\textsuperscript{23} have
studied the cell behaviour on dot-like micropatterned substrata. They found the spacing between dots affect the cell spread shape and the extent of cell spreading is directly correlated to the total substratum coverage with ECM. Arnold et al.\textsuperscript{27} have studied the cell function on a hexagonally packed nanodot template with single integrin adhered on each dot. They found that a separation of $\geq 73$ nm between the adhesive dots result in limited cell attachment and spreading. It is concluded from other person’s work that the ECM domain size and domain spacing can influence the cell adhesion behaviour.

As a final note, we expect there to be negligible amounts of organic solvent (toluene) trapped in the glassy polymer phase. Any solvent that is trapped, however, would be released very slowly from the glass, because of the low molecular mobility. Furthermore, the solubility of toluene in water is very low. For these reasons, we do not expect there to be significant contamination of the cells by solvent in the polymer films. A study of the toxicological effects of the solvent is beyond the scope of this thesis.

\textbf{6.4 Conclusion}

In this chapter, FN nanopatterns were created by the adsorption on well-organized copolymer thin film templates. Protein preferentially adsorbs on PS blocks rather than on PI blocks, which was confirmed through the complementary use of AFM and ToF-SIMS. Preferential adsorption induces FN patterns resembling the underlying block copolymer surface morphology. The use of self-assembled PS-b-PI has been found to provide a precise, two-dimensional template for the nanopatterning of a variety of proteins.

The ring-like FN nanopattern on PS(45)-b-PI(46) substrate increases the CHO cell’s adhesion compared with the cells on homogeneous FN surfaces and patterns on PS(65)-b-PI(26). The adhered cell density and coverage area fraction on the ring-like FN surface is higher than on the other three substrates. The ring-like pattern also develops more actin stress fibres, cell spreading, and focal adhesion. Cell adhesion is high when the FN ring size is $> 50$ nm and when the surface coverage of FN is $< \text{ca. 85\%}$. The increasing cell adhesion on ring-like nanopattern is attributed to a high local FN density on the ring areas. It is proposed that a high FN-ligand density increases the
integrin clustering and develops stable focal adhesion. From a practical standpoint, the FN templating method provides a simple way to increase cell adhesion to surfaces for applications in tissue engineering.
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Chapter 7

Summary and future work

7.1 Summary

This work employed a hydrophobic copolymer pair (polystyrene-block-polyisoprene, PS-b-PI) as templates to nanopattern proteins through selective adsorption of proteins on one polymer domain. Cell adhesion behaviors on the nanopatterned ECM protein (i.e. fibronectin, FN) were further investigated.

Under the atmospheric conditions where the protein adsorption operated, PS is in its glassy state with its $T_g$ of 100 °C, while PI is in its rubbery state with a $T_g$ of -65 °C. This PS-b-PI copolymer is termed as a hard-soft copolymer. The ultra low glass transition temperature gave the PI polymer chain a high mobility at room temperature. Hence, the PI film is able to dewet during incubation under the water. The dewetting process for this non-solvent dewetting is quite similar with the process of conventional thermo-dewetting. The film undergoes the growth of holes, coalescence of holes, formation of a polygon cellular structure, and droplet formation after the breakup of the PI cellular feature. Whereas, the PS film with a thickness of more than 10 nm was stable under the aqueous condition.

Two-dimensional PS-b-PI copolymer thin film templates were prepared by spin-coating. The morphologies of the spin-coated thin films are not in an equilibrium state, but are stable for months. The PS-b-PI copolymer films form different surface morphologies with varying parameters, such as block weight ratios, film thickness and annealing condition. Under the confinement between substrate-polymer and polymer-air interfaces, several well-organized surface structures were observed in the ultrathin films ($h<L$). For example, PI forms dots dispersed in a PS network matrix on a 18 nm PS(45)-b-PI(46) thin film surface, while a stripe-like structure is formed on a 18 nm
PS(65)-b-PI(26) thin film surface. These surface structures were examined by atomic force microscopy (AFM), and the surface chemical components were confirmed by secondary ion mass spectroscopy (SIMS).

After incubation of the dot-like and stripe-like copolymer templates in the bovine serum albumin (BSA) protein solution with a high concentration (0.5 mg/ml) for 1 h, the BSA formed similar ring-like and stripe-like structures resembling the underlying copolymer structures. The SIMS and water contact angle analysis confirmed that most BSA molecules were localized on the PS domains rather than on PI domains. More BSA will adsorb on the film if there are more PS components on the free surface of the film. The preferential BSA adsorption on PS domains rather than on PI induces a protein pattern on the nanoscale. The selective adsorption might be explained by the high mobility of the rubbery PI blocks at room temperature which can resist the adsorption of protein molecules.

Binary and ternary polymer blends of PS, PI and PS-b-PI were also prepared by spin-coating and studied for their ability to pattern proteins. Different from the two-dimensional structure by microphase separation of copolymer, the PS, PI blends form three-dimensional topographies via macrophase separation. With increasing PI mass fraction ($\phi_{PI}$) in the blends, the structures change from depressed PI circular holes to elevated PS circular islands. These feature sizes are much bigger than the sizes of copolymer templates. In the PI/PS(45)-b-PI(46) binary system, the films undergo microphase separation and form smooth surfaces with varying weight ratios. With increasing $\phi_{PI}$, the PS domain changes from a ring-like network to cylinders and then to spherical dots. In the PS/PI/PS(45)-b-PI(46) ternary system, the film formation mechanism is more complicated. The macrophase and microphase separation will compete to control the film formation when the polymer mass ratios are adjusted. This process formed more complex structures compared with the binary system.

Compared to PS, PI has a lower surface free energy. The annealing treatment makes PI migrate to the free surface of the film, although both components stay on the free surface on the as-spun state. However, in the polymer blend system, there is always a thin PI layer aggregation on a thick film surface even without the annealing treatment. This is because of a lack of the covalent bond between PI and PS chains in a blend. This inhibits the application of these structures with a bigger feature size for
protein patterning. When the film thickness was decreased to less than 15 nm, some PS components were exposed at the free surface with some striking morphologies with several hundred nanometer spacing. These chemically heterogeneous substrates can be used to form complex protein nanopatterns.

Another protein, fibronectin, which has a bigger size and is an extracellular matrix (ECM) protein, was also used to adsorb on the copolymer templates and form nanopatterns. FN is a typical protein used in cell adhesion and tissue engineering. After incubation, FN molecules also preferentially adsorb on PS blocks rather than on PI blocks, which was confirmed through the complementary use of AFM and ToF-SIMS. This result implies that the selective protein adsorption by this hard-soft PS-b-PI template is general, and could lead to applications in the bioengineering or biomedical fields.

The FN nanopatterns were then used to investigate the influence of the two-dimensional ECM ligand shape or spacing on the cell behavior. After CHO cell incubation for one hour, the adhered cell density and coverage area fraction on ring-like FN on a PS(45)-b-PI(46) substrate is greater than those on homogeneous FN surfaces or patterns on PS(65)-b-PI(26). The ring-like pattern not only increases the cell quantity but also develops more actin stress fibers. After analyzing the cell adhesion behavior with the FN ring spacing and coverage, it was found that the cell adhesion density is high when the FN ring size is bigger than about 50 nm and the surface coverage less than about 85%. The increased cell adhesion on ring-like FN nanopatterns is attributed to the high local FN density on the ring area. The high FN-ligand density increases the integrin clustering, reorganizes the actin fibers, and develops stable focal adhesion.

The highly-organized PS/PI polymer templates offer a versatile and simple bottom-up approach for the nanopatterning of proteins. The templates were created from the non-equilibrium structure and did not require annealing. This means that the templates can be deposited on fragile, heat-sensitive substrates. Comparing with previous protein patterning techniques on hydrophobic-hydrophilic systems, this system can afford a long deposition time and a high incubation concentration without destroying the protein pattern. Hence, the template is more suitable for bioengineering.
applications. For example in this work, the FN nanopattern provides a simple method to increase cell adhesion for applications in tissue engineering.

7.2 Future work

In Chapter 5, a series of PS/PI films were created with different two-dimensional morphologies spin-cast from PI and PS(45)-b-PI(46) binary blends and three-dimensional topographies from binary blends of PS and PI and ternary blends of PS, PI and PS(45)-b-PI(46). The feature sizes of the film structures are distributed from several tens of nanometers to several micrometers. The variety of the polymer film structure and dimensional size broadens the feasibility of the pattern for protein or other biomaterial templates. However, almost all films with a thickness more than 15 nm have an ultra thin PI layer (<10 nm) localized on the free surfaces. This eliminates the chemical heterogeneity of the polymer surface and prevents it from being used to pattern protein. Then, how to expose both components on the free surface is the question we need to resolve.

The periodic nanostructures of block copolymers are widely used as masks for nanolithography. For example, Park et al. etched the film and transferred the patterns to an underlying substrate. The film was used as a mask by removing the minority block (in this case by selective ozonation, which attacks the double bonds of polyisoprene and polybutadiene) and then used a non-selective physical etch (reactive ion etching) to transfer the pattern of holes or cylinders to the underlying semiconductor substrate. Similarly, poly(styrene-b-methyl methacrylate) was also employed for nanolithographic applications, since the nanodomains of PMMA will degrade by radiation and then can be removed with acetic acid to create a mask. This technique can be used in the future work to etch off the PI layer on the free surface. UV ozonation is one method to cleave the double bond of the PI chain with a low intensity and slow etching speed. The key technique is to control the process conditions to etch off the surface PI layer but avoid etching off all PI components in the film. Another choice is to use a reactive ion etching technique to remove the thin PI layer and expose PS domains to the free surface. Once the chemically heterogeneous polymer substrates are prepared, the templates can be used to pattern the protein molecules with desired domain shape, size and spacing.
Previous research\textsuperscript{3} used micropatterned ECM geometry (ECM dots with a defined separation size) to study cell behaviors. They found that a cell adapts its shape to the ECM pattern. The ability of a cell to spread and to migrate is also influenced by the ECM dots spacing. In the future work, after patterning the ECM protein on the micro-size templates after etching off a surface PI layer, the protein substrates can also be used to explore their effects on cell adhesion and function. The influencing mechanisms can be explored as well. In addition, the substrate topography at the micro- and nanoscale can also control cell function.\textsuperscript{4} Then the films of polymer blends with three-dimensional topography can be incubated in cell suspensions to explore the cell behavior.

In the future work, more cell lines can be added to investigate the cell function on our protein templates. An understanding of the profound mechanisms of cell behaviors on these templates can broaden their applications in tissue engineering.
References


