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

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Abstract:

Templated surfaces can be used to create patterns of proteins for applications in cell biology, bio-sensors and tissue engineering. A diblock copolymer template, which contains a pair of hydrophobic blocks, has been developed. The template is created from well-ordered, non-equilibrium surface structures of poly(styrene-*b*-isoprene) (PS-*b*-PI) diblock copolymers, which are achieved in ultrathin films having a thickness less than one domain period. Adsorption and nanopatterning of bovine serum albumin (BSA) on these thin films were studied. After incubation of the copolymer templates in BSA solutions (500 µg/ml) for a period of one hour, BSA molecules formed either a striped or a dense, ring-like structure, closely resembling the underlying polymer templates. In this “hard-soft” PS-*b*-PI system, BSA molecules were preferentially adsorbed on the hard PS domains, rather than on the soft PI domains. SIMS and contact angle analysis revealed that with more PI localized at the free surface, fewer BSA molecules were adsorbed. SIMS analysis confirmed that BSA molecules were adsorbed selectively on the PS blocks. This is the first example of two *hydrophobic* blocks of a diblock copolymer being used as a protein patterning template. Previously reported diblock copolymer templates used hydrophilic and hydrophobic pairs. A potentially useful characteristic of this template is that it is effective at high protein solution concentrations (up to 1 mg/ml) and for long incubation times (up to two hours), which broadens its range of applicability in various uses.

Keywords: PS-*b*-PI, thin film; self-organization, template, BSA, nanopattern; diblock; protein

Introduction

Biomolecule patterning is of great interest both for fundamental research in cell biology¹ and for applications in biosensors and tissue engineering.^{2,3} 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,⁴ electron lithography,⁵ dip-pen lithography,⁶ and imprint lithography,⁷ 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.^{8,9} 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.¹⁰ These self-assembled patterns have been applied as nanolithographic masks and as templates for the synthesis of inorganic or organic structures.^{11,12} Extensive work has been done to control the self-assembly and orientation of block copolymer thin films.¹³⁻¹⁵ 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.*¹⁶⁻¹⁹ 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.*²⁰ 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^{21,22} have used polymer blend systems to study the selective protein adsorption on phase-separated polymer surfaces. Meanwhile, researchers^{23,24} 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.

Hydrophobic and hydrophilic polymers both adsorb proteins, but there is less protein adsorption on the hydrophilic polymer surface.²⁵ Hence, the differences in hydrophilicity have been used in previous reports to explain observed protein templating on hydrophobic/hydrophilic diblock copolymers.²⁰ (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.¹⁶⁻¹⁹ 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 PS-*b*-PI films without prior thermal or solvent annealing. Atomic force microscopy (AFM), Time-of-flight secondary ion mass spectroscopy (ToF-SIMS) and water contact angle analysis (WCAA) are complementarily used to confirm the protein preferential adsorption and nanopatterning on the diblock copolymer templates.

Methods

Film preparation

Two different polystyrene-block-polyisoprene (1,4 addition) (PS-*b*-PI) block copolymers (Polymer Source Inc., Montreal, Canada) with a number-average molecular weight, M_n , of 91 kg/mol were used: a *symmetric* PS(45)-*b*-PI(46) and an *asymmetric* PS(65)-*b*-PI(26) copolymer, where the numbers in parentheses denote the M_n of each block in units of kg/mol. Thin films were prepared by spin-coating toluene solutions onto (100) single-crystal silicon wafers with a spin rate of 2000 rpm. The film thicknesses were adjusted by changing the solution concentration over a range from 0.3 to 3 wt.%. Film thicknesses were measured with spectroscopic ellipsometry (J.A. Woollam Co., USA) over a wavelength range from 400 to 800 nm. A 189 nm

PS(45)-*b*-PI(46) film was annealed for 24 hr at 130 °C in vacuum and until reaching the equilibrium state. All other films were used without annealing. For comparison to the copolymer films, 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. Poly(1,4-isoprene) (PI) with a molecular weight of 100 kg/mol was purchased from Sigma-Aldrich. A 3.5 wt.% solution in toluene was spin-coated onto the substrate to deposit a 250 nm thick film.

Incubation of polymer films in BSA solution

BSA ($\geq 98\%$, 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 $\mu\text{g/ml}$ in a phosphate buffer (PB) solution, pH=7.2) for 1 h. 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.

Thin Film and Surface Analysis

An atomic force microscope (NTEGRA, NT-MDT, Moscow, Russia) was used in an intermittent-contact mode to determine the topography of the templates before and after BSA incubation. Silicon cantilevers (NT-MDT, Moscow, Russia) with a spring constant of *ca.* 5 N/m and a resonant frequency of 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.

Spectroscopic ellipsometry (J.A. Woollam Co., USA) was performed on the copolymer templates in air before and after incubation in the protein solutions. The data were fitted with a slab model to obtain an estimate of the average thickness of the adsorbed protein layer.

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 $100\ \mu\text{m} \times 100\ \mu\text{m}$ area at a resolution of 64×64 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 $m/z = 0\text{-}500$ mass spectra.

The quantitative analysis of PS, PI and protein components on the surface is analyzed through the relative peak intensity (RPI) method.^{26,27} The RPI was calculated by

$$RPI = \frac{I_{indv}}{I_{total}} \quad (1)$$

where I_{indv} is the intensity of an individual peak of interest in the ToF-SIMS spectrum, and I_{total} is the intensity of all ion peaks in the same spectrum over the entire mass range (*i.e.* the total yield).

WCAA used the sessile drop method (Easy Drop, Krüss GmbH, Germany). 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.

Results and Discussion

Preparation of Copolymer Templates

The contact angle of water on PS films was found to be $95 \pm 0.7^\circ$, whereas it had only a slightly higher value of $101 \pm 1.2^\circ$ 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 ,²⁸ and hence PS molecules are in a glassy state at a room temperature of 20°C . The T_g of PI is -65°C ,²⁸ 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.

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.²⁹⁻³² PI creates a wetting layer at the air interface, and etching has been used to reveal the sub-surface phase structure. More recently, solvent-annealing and the control of the solvent evaporation rate have been found to create ordered structures in a thin film of a *similar* diblock copolymer, polystyrene-*b*-polybutadiene (PS-*b*-PB).³³⁻³⁵ Our approach is simpler: templates are created from the *non-equilibrium* in-frozen structure of the copolymer thin films after spin-casting.

Figure 1 presents the AFM phase images of the surfaces of PS(45)-*b*-PI(46) (symmetric copolymer) ultrathin films with increasing thickness. During the AFM imaging process, the “soft” PI domains will dissipate more energy than the “hard” PS when in contact with the AFM tip, because PI is viscous.³⁶ Hence PI domains are

presented as the darker areas (more dissipative) in the phase image.³⁷ The soft PI can be indented more by the AFM tip in comparison to the hard PS. Therefore, PI is represented as the darker area (lower height) in the height image.

Figure 1. Phase images of PS(45)-*b*-PI(46) as-spun films on silicon wafers with increasing thickness of (a) 12 nm, (b) 16 nm, (c) 19 nm (d) 60 nm. Image sizes are $2\ \mu\text{m} \times 2\ \mu\text{m}$. (e) and (f) are proposed diagrams of PS(45)-*b*-PI(46) as-spun thin films in a cross-sectional view. Diagram (e) corresponds to (a) a 12 nm thick film, and (f) is for (c) a 19 nm thick film.

There is a progression in Figure 1 from a stripe-like pattern (12 nm thickness), to a dot-like pattern (16 and 19 nm) to a “patchwork quilt” pattern (60 nm) in which

PI domains form a pattern of dots and short ribbons. The striped surface structure in Fig 1a is consistent with a perpendicular lamellar structure, which consists of lamellae arranged laterally across the cross section area of the film, as shown schematically in Fig 1e. This morphology has been noted in previous theoretical and experimental studies^{38,39} 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 (Fig 1b). At a thickness of 19 nm, almost all of the PI surface domains form uniform dots surrounded by the PS matrix (Fig 1c). Drawing on the structures reported for PS-*b*-PLMA films, which was called a hybrid structure,³⁹ we propose that the PS surface shown here in Fig 1c 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 1f.

The PI component has a strong interaction with the Si substrate and a lower surface free energy compared with the PS component.²⁹ Therefore, we expect that in thicker films the PI will wet the Si substrate and also create a wetting layer at the free surface to minimize the total interfacial free energy when in an equilibrium state. Knoll et al.⁴⁰ reported a 10 nm thick wetting layer of the lower-surface-energy poly(butadiene) (PB) phase in an *equilibrated* triblock PS-PB-PS thin film. Their work also warns against mis-interpretation of AFM images. They found that AFM imaging was sensitive to the lamellar structure lying *beneath* the wetting layer. The images presented in Figure 1 are from films that have been frozen in non-equilibrium states in the spin-casting process. SIMS analysis, presented later, indicates that both PS and PI are present at the film surfaces in the expected ratio. There is no evidence for a wetting layer. Subsurface structures were not investigated further in our work, as our emphasis is on the creation of templates for protein adsorption, in which the surface structure is the relevant factor.

Theoretically, the *asymmetric* PS(65)-*b*-PI(26) copolymer is expected to create a cylindrical structure in the bulk.⁴¹ Structures will be different in thin films because of the confinement by the substrate and free surface. At a thickness of 12 nm (Fig. 2a), the morphology appears to consist of PI spheres dispersed in a PS matrix, which is consistent with a cubic structure. There is less PI component residing at the free surface compared with the 12 nm thick PS(45)-*b*-PI(46) film surface (Fig. 1a). 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. 2c). With the film thickness continuing to increase, the lengths of the stripes decrease. At a thickness of 39 nm (Fig. 2d), the morphology becomes a complex structure of spheres and short ribbons.

Figure 2. Phase images of PS(65)-*b*-PI(26) as-spun film surfaces on silicon wafers with increasing film thickness: (a) 12 nm, (b) 19 nm, (c) 25 nm (d) 39nm. Image sizes are $2\ \mu\text{m} \times 2\ \mu\text{m}$.

One interpretation of this structure 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 (which is about 60 nm according to a previous report⁴²). Previous theoretical⁴³ and experimental⁴⁴ study of the parallel and vertical morphologies of cylindrical domains likewise reported such a thickness dependence. 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.²⁹⁻³¹ The results in Fig. 1 and Fig. 2 show that the surface structure of non-equilibrium 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 .

BSA Adsorption on the Copolymer Templates

From the survey just presented, two nanostructures were chosen for subsequent application as protein templates: a PI dot pattern (as in Fig. 1c) and a striped pattern (as in Fig. 2b). 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. 3a 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 3d and 3e) 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 a single layer of BSA molecules with a diameter of 4 nm.⁴⁵ The pitch of the profile after BSA adsorption matches that of the original copolymer surface.

Figure 3. 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 $\mu\text{m} \times 2 \mu\text{m}$.

We next consider a 17.6 nm film of the asymmetric PS(65)-*b*-PI(26) copolymer, which presents a striped pattern (Figure 4a and 4b). (The small dot-like domains that appear dark in the Figure 4b image are presumed to be defects in the structure consisting of PI blocks.) After BSA adsorption, the images indicate that the BSA molecules adsorb in a striped pattern resembling the underlying polymer structure (as viewed in Figure 4d and 4e). 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 preferentially 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. 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 $2\mu\text{m} \times 2\mu\text{m}$.

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 height and phase images (Figure S1) are provided in the Supporting Information. The images are quite similar to those of the adsorbed BSA layers presented in Fig. 3 and Fig. 4. The well-ordered protein nanopatterns maintain stability independent on the circumstance of protein solution concentration and incubation time. In previous reports on other templates,¹⁶⁻¹⁹ much shorter times (20 – 60 s) and lower protein concentrations (4-20 $\mu\text{g/ml}$) were used. Thus, the PS-PI templates have a different range of process parameters over which they have been shown to be applicable.

To explain the apparent templating, the BSA adsorption on the PS-*b*-PI surfaces can be compared to what is found for the two homopolymers, PS and PI. The surface morphologies of PS and PI, after BSA incubation and rinsing, are shown in Figure 5. As viewed in Fig 5a, the PS surface is densely coated by BSA molecules, which form a “carpet” of densely-packed BSA particles. 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 literature⁴⁶ 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.⁴⁵ 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 isolated white spots are observed in the topographic AFM 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 is required to reach a firm conclusion.

Taken together, the AFM images in Figures 3 through 5 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.

Figure 5. AFM height images of (a) PS and (b) PI films after BSA incubation for 1h; image sizes are $1\ \mu\text{m} \times 1\ \mu\text{m}$.

Surface Analysis to Verify Preferential Adsorption

ToF-SIMS was applied to the basic materials (PS, PI, and BSA) and to the PS-*b*-PI copolymer surfaces. Experiments were performed on the exact same samples used for the AFM analysis already presented. Although both positive and negative ion spectra were collected, the negative spectra have fewer peaks and are quite similar when comparing the PS and PI mass spectra. Therefore, the positive ion spectra were chosen to analyze the surfaces.

Figure 6 shows the low-mass-range spectra for PS and PI homopolymers and the pure BSA film on a Si substrate. These so-called “fingerprint” spectra contain peak intensity patterns which are dependent on the material type. The PS homopolymer has characteristic peaks at $m/z = 63, 89, 91, 103, 115, 117, 128, 165, 178$ and 193 u, 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 u, which are significantly more intense than the peaks of PS. Meanwhile, BSA has unique peaks corresponding to nitrogen-containing fragments at $m/z = 18, 30, 44, 60, 70$ and 86 u, none of which are found in the spectra of PS-PI hydrocarbon polymer fragments.

Figure 6. Positive ToF-SIMS spectra in the mass range m/z 0-200 u for (a) PS on Si substrate, (b) PI on Si substrate, (c) BSA on Si substrate.

Table 1 gives the *summed* RPI, which is defined as the total of the individual RPIs for each of the characteristic “fingerprint” peaks (as identified in Fig. 6) for each of the copolymer samples. The 18.2 nm thick PS(45)-*b*-PI(46) copolymer sample gives a PI relative intensity of 89.8×10^{-3} , whereas the 17.6 nm thick PS(65)-*b*-PI(26) sample has a PI relative intensity of only 18.4×10^{-3} . On the contrary, the PS(45)-*b*-PI(46) sample gives a PS relative intensity of 76.5×10^{-3} but PS(65)-*b*-PI(26) sample has a PS relative intensity of 122×10^{-3} . It is clear that the PS(65)-*b*-PI(26) sample has a greater fraction of PS blocks and a lower fraction of PI blocks on the surface

compared with the PS(45)-*b*-PI(46) sample. 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 non-equilibrium structures of the spin-cast copolymer films.

Additionally, the summed RPI of PI peaks of the PS(45)-*b*-PI(46) sample with a 189 nm thickness is similar to that of the PI homopolymer. As reported elsewhere⁴⁷, 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. In AFM images, this PS-*b*-PI sample showed that only one block segregated on the top surface (not shown). Considering the SIMS data, we conclude that only PI occupies the surface of the thick copolymer film.

Table 1. Relative peak intensities (RPI) of characteristic PS and PI ion peaks

	PI	PS(45)- <i>b</i> - PI(46) 189nm	PS(45)- <i>b</i> - PI(46) 18.2nm	PS(65)- <i>b</i> - PI(26) 17.6nm	PS
Summed RPI of PI peaks ($\times 10^{-3}$)	196.8 $\pm$ 5.6	192.0 $\pm$ 2.8	89.8 $\pm$ 6.6	18.4 $\pm$ 1.2	9.3 $\pm$ 0.5
Summed RPI of PS peaks ($\times 10^{-3}$)	40.5 $\pm$ 3.5	30.8 $\pm$ 2.9	76.5 $\pm$ 1.9	122.0 $\pm$ 1.4	182.8 $\pm$ 3.8

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. A table (Table S1) of the RPI of characteristic BSA ion peaks (from the nitrogen fractions) after 1h BSA adsorption is given in the Supporting Information. Figure 7 shows 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), 189 nm PS(45)-*b*-PI(46)) and compared to the two homopolymers).

In Figure 7, 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, there is evidence that the thick (189 nm) PS(45)-*b*-PI(46) copolymer adsorbs nearly as little BSA as the PI homopolymer film. 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. Also in Figure 7, 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.

Figure 7. The dependence of the BSA's RPI *after* BSA adsorption on the PI's RPI *before* BSA adsorption over one hour 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 2 gives a comparison of the summed RPI of the PI and PS characteristic peaks *before* and *after* BSA adsorption. (Note that some strong peaks for PS are not unique for the homopolymer but are also found as very weak peaks for PI, and *vice versa* for some strong PI peaks.) 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 189 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 30×10^{-3} after the BSA

adsorption, which is a relatively small change. (We estimate the uncertainty to be less than 5×10^{-3} in the RPI in Table 2.) For the samples in the right three columns of Table 2, 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 2. After BSA adsorption, the PS's RPI for the PS homopolymer is decreased by 126.8×10^{-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 35×10^{-3} and 80.9×10^{-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 2. RPI changes of characteristic PS and PI ion peaks after 1 h of BSA adsorption

	PI	PS(45)- <i>b</i> -PI(46) 189 nm	PS(45)- <i>b</i> -PI(46) 18.2 nm	PS(65)- <i>b</i> -PI(26) 17.6 nm	PS
Poly(isoprene) peaks ($\times 10^{-3}$)					
Before BSA Ads.	184.1	161.6	85.0	17.6	9.0
After BSA Ads.	153.8	133.4	98.5	22.9	11.8
Difference	- 30.3	- 28.2	13.5	5.3	2.8
Poly(styrene) peaks ($\times 10^{-3}$)					
Before BSA Ads.	40.5	30.8	76.5	122.0	182.8
After BSA Ads.	27.1	26.3	41.5	41.1	56.0
Difference	- 13.4	- 4.5	- 35.0	- 80.9	-126.8

The data in Table 2 are presented graphically in Figure 8. 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 (9×10^{-3}); likewise, the RPI of the PS has been corrected by subtracting the RPI for PI (40.5×10^{-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 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.

Figure 8. The correlation between the RPI of PS (filled symbols) and PI (open symbols) *after* BSA adsorption and the RPI of the same polymers *before* BSA adsorption. The solid line has a gradient of 1. The best-fit for the PS data (dashed line) has a gradient of 0.14 ± 0.07 .

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 3 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 ($\approx 101^\circ$) 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 3. Water contact angles of polymer and adsorbed BSA layers and angle differences before and after BSA adsorption

	PS	PS(65)- <i>b</i> -PI(26) 17.6 nm	PS(45)- <i>b</i> -PI(46) 18.2 nm	PS(45)- <i>b</i> -PI(46) 189 nm	PI
WCA of initial polymers ($^\circ$)	95.0 $\pm$ 0.7	96.9 $\pm$ 0.9	99.8 $\pm$ 1.0	101.1 $\pm$ 1.2	101.5 $\pm$ 1.2
WCA after BSA 1h ads. ($^\circ$)	67.0 $\pm$ 1.5	68.0 $\pm$ 1.7	79.3 $\pm$ 1.4	93.8 $\pm$ 1.9	99.5 $\pm$ 1.5
WCA differences before and after BSA ads. ($^\circ$)	-29	-28.9	-20.5	-7.3	-2

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.^{48,49} 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.

Summary and Conclusions

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. A regular ordering of the block copolymer over microscopic distances was achieved by controlling the diblock ratio and the film thickness. 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. 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. Both dotted and striped templates were used to nanopattern BSA molecules at relatively long incubation times (2 hr.) 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 relatively broad window of applicability for the templating process. We note, however, that BSA concentration in blood is greater than 1 mg/ml, and so further experiments are required to determine the feasibility of templating from undiluted blood.

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Supporting Information Available

Additional SIMS data and AFM images of copolymer templates after incubating at longer times and higher concentrations can be found in the Supporting Information.

This information is available free of charge via the Internet at <http://pubs.acs.org>

References

- (1) Suh, K. Y.; Seong, J.; Khademhosseini, A.; Laibinis, P. E.; Langer, R. *Biomaterials* **2004**, *25*, 557-563.
- (2) Veiseh, M.; Zareie, M. H.; Zhang, M. Q. *Langmuir* **2002**, *18*, 6671-6678.
- (3) Lin, C. C.; Co, C. C.; Ho, C. C. *Biomaterials* **2005**, *26*, 3655-3662.
- (4) He, W.; Halberstadt, C. R.; Gonsalves, K. E. *Biomaterials* **2004**, *25*, 2055-2063.
- (5) Chang, Y.; Ahn, Y. S.; Hahn, H. T.; Chen, Y. *Langmuir* **2007**, *23*, 4112-4114.
- (6) Lee, K. B.; Park, S. J.; Mirkin, C. A.; Smith, J. C.; Mrksich, M. *Science* **2002**, *295*, 1702-1705.
- (7) Hoff, J. D.; Cheng, L. J.; Meyhofer, E.; Guo, L. J.; Hunt, A. J. *Nano Lett.* **2004**, *4*, 853-857.
- (8) Senaratne, W.; Andruzzi, L.; Ober, C. K. *Biomacromolecules* **2005**, *6*, 2427-2448.
- (9) Voros, J.; Blattler, T.; Textor, M. *MRS Bulletin* **2005**, *30*, 202-206.
- (10) Fasolka, M. J.; Mayes, A. M. *Annu. Rev. Mater. Res.* **2001**, *31*, 323-355.
- (11) Black, C. T.; Guarini, K. W.; Milkove, K. R.; Baker, S. M.; Russell, T. P.; Tuominen, M. T. *Appl. Phys. Lett.* **2001**, *79*, 409-411.
- (12) Morkved, T. L.; Wiltzius, P.; Jaeger, H. M.; Grier, D. G.; Witten, T. A. *Appl. Phys. Lett.* **1994**, *64*, 422-424.
- (13) Kim, S. H.; Misner, M. J.; Xu, T.; Kimura, M.; Russell, T. P. *Adv. Mater.* **2004**, *16*, 226-231.

- (14) Thurn-Albrecht, T.; Schotter, J.; Kastle, C. A.; Emley, N.; Shibauchi, T.; Krusin-Elbaum, L.; Guarini, K.; Black, C. T.; Tuominen, M. T.; Russell, T. P. *Science* **2000**, *290*, 2126-2129.
- (15) Kim, S. O.; Solak, H. H.; Stoykovich, M. P.; Ferrier, N. J.; de Pablo, J. J.; Nealey, P. F. *Nature* **2003**, *424*, 411-414.
- (16) Kumar, N.; Parajuli, O.; Hahm, J. *J. Phys. Chem. B* **2007**, *111*, 4581-4587.
- (17) Kumar, N.; Hahm, J. *Langmuir* **2005**, *21*, 6652-6655.
- (18) Kumar, N.; Parajuli, O.; Dorfman, A.; Kipp, D.; Hahm, J. *Langmuir* **2007**, *23*, 7416-7422.
- (19) Kumar, N.; Parajuli, O.; Gupta, A.; Hahm, J. *Langmuir* **2008**, *24*, 2688-2694.
- (20) Lau, K. H. A.; Bang, J.; Kim, D. H.; Knoll, W. *Adv. Funct. Mater.* **2008**, *18*, 3148-3157.
- (21) Morin, C.; Hitchcock, A. R.; Cornelius, R. M.; Brash, J. L.; Urquhart, S. G.; Scholl, A.; Doran, A. *J. Electron. Spectrosc. Relat. Phenom.* **2004**, *137-140*, 785-794.
- (22) Li, L.; Hitchcock, A. P.; Robar, N.; Cornelius, R.; Brash, J. L.; Scholl, A.; Doran, A. *J. Phys. Chem. B* **2006**, *110*, 16763-16773.
- (23) Neto, C. *Phys. Chem. Chem. Phys.* **2007**, *9*, 149-155.
- (24) Cai, Y.; Newby, B. M. *Z. Langmuir* **2008**, *24*, 5202-5208.
- (25) Koutsopoulos, S.; Patzsch, K.; Bosker, W. T. E.; Norde, W. *Langmuir* **2007**, *23*, 2000-2006.
- (26) Shimizu, K.; Abel, M-L.; Watts, J. F. *J. Adhes.* **2008**, *84*, 725-741.

- (27) Rattana, A.; Hermes, J. D.; Abel, M-L.; Watts, J. F. *Int. J. Adhes. Adhes.* **2002**, 22, 205-218.
- (28) Brandrup, J.; Immergut, E. H.; Grulke, E. A., Eds., *Polymer Handbook, 4th ed.*; John Wiley & Sons: Chichester, 1999, Ch. 5
- (29) Harrison, C.; Park, M.; Chaikin P. M.; Register R. A.; Adamson D. H.; Yao, N. *Polymer* **1998**, 39, 2733-2744.
- (30) Harrison, C.; Park, M.; Chaikin P. M.; Register R. A.; Adamson D. H. *J. Vac. Sci. Technol. B* **1998**, 16, 544-552.
- (31) Park, M.; Harrison, C.; Chaikin P. M.; Register R. A.; Adamson D. H. *Science* **1997**, 276, 1401-1404.
- (32) Mykhaylyk, T. A.; Mykhaylyk, O. O.; Collins, S.; Hamley, I. W.; *Macromolecules* **2004**, 37, 3369-3377.
- (33) Huang, H.; Hu, Z.; Chen, Y.; Zhang, F.; Gong, Y.; He, T. *Macromolecules* **2004**, 37, 6523-6530.
- (34) Huang, H.; Zhang, F.; Hu, Z.; Du, B.; He, T.; Lee, F. K.; Wang, Y.; Tsui, O. K. *C. Macromolecules* **2003**, 36, 4084-4092.
- (35) Turturro, A.; Gattiglia, E.; Vacca, P.; Viola, G. T. *Polymer* **1995**, 36, 3987-3996.
- (36) Anczykowski, B.; Gotsmann, B.; Fuchs, H.; Cleveland, J. P.; Elings, V. B. *Appl. Surf. Sci.* **1999**, 140, 376-382.
- (37) Lei, C.; Ouzineb, K.; Dupont, O.; Keddie, J. L. *J. Coll. Interf. Sci.* **2007**, 307, 56-63.

- (38) Matsen, M. W. *J. Chem. Phys.* **1997**, *106*, 7781-7791.
- (39) Fasolka, M. J.; Banerjee, P.; Mayes, A. M. *Macromolecules* **2000**, *33*, 5702-5712.
- (40) Knoll, A.; Magerle, R.; Krausch, G. *Macromolecules* **2001**, *34*, 4159-4165.
- (41) Bates, F. S.; Fredrickson, G. H. *Phys. Today* **1999**, *52*(2), 32-39.
- (42) Park, J.; Ha, J. S.; Ryu, J.; Chang, T. *Jpn. J. Appl. Phys.* **2004**, *43*, 762-766.
- (43) Suh, K. Y.; Kim, Y. S.; Lee, H. H. *J. Chem. Phys.* **1998**, *108*, 1253-1256.
- (44) Knoll, A.; Horvat, A.; Krausch, G.; Sevink, G. J. A.; Zvelindovsky, A. V.; Magerle, R. *Phys. Rev. Lett.* **2002**, *89*, 035501-1-4.
- (45) McClellan, S. J.; Franses, E. I. *Coll. Surf. B* **2003**, *28*, 63-75.
- (46) McClellan, S. J.; Franses, E. I. *Coll. Surf. A* **2005**, *260*, 265-275.
- (47) Hasegawa, H.; Hashimoto, T. *Macromolecules* **1985**, *18*, 589-590.
- (48) Ji, J.; Feng, L. X.; Qiu, Y. X.; Yu, X. J. *Polymer* **2000**, *41*, 3713-3718.
- (49) Otsuka, H.; Nagasaki, Y.; Kataoka, K. *Langmuir* **2004**, *20*, 11285-11287.

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