

SURFACE FUNCTIONALIZATION OF POLYSTYRENE TO BIND WITH FMRF PEPTIDES FOR NOVEL BIOCOMPATIBILITY

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Abstract A generic method was described to change surface biocompatibility by introducing reactive functional groups onto surfaces of polymeric substrates and covalently binding them with biomolecules. A block copolymer with protected carboxylic acid functionality, poly(styrene-*b*-*tert*-butyl acrylate) (PS-*Pt*BA), was spin coated from solutions in toluene on a bioinert polystyrene (PS) substrate to form a bilayer structure: a surface layer of the poly(*tert*-butyl acrylate) (*Pt*BA) blocks that order at the air-polymer interface and a bottom layer of the PS blocks that entangle with the PS substrate. The thickness of the *Pt*BA layer and the area density of *tert*-butyl ester groups of *Pt*BA increased linearly with the concentration of the spin coating solution until a 2 nm saturated monolayer coverage of *Pt*BA was achieved at the concentration of 0.4% *W/W*. The protected carboxylic acid groups were generated by exposing the *tert*-butyl ester groups of *Pt*BA to trifluoroacetic acid (TFA) for bioconjugation with FMRF peptides *via* amide bonds. The yield of the bioconjugation reaction for the saturated surface was calculated to be 37.1% based on X-ray photoelectron spectroscopy (XPS) measurements. The success of each functionalization step was demonstrated and characterized by XPS and contact angle measurements. This polymer functionalization/modification concept can be virtually applied to any polymeric substrate by choosing appropriate functional block copolymers and biomolecules to attain novel biocompatibility.

Keywords: Surface functionalization; Biocompatibility; Bioconjugation reaction.

INTRODUCTION

The application of a polymeric material depends on both its bulk and surface properties that are very difficult to be controlled through a single industrial processing operation. Therefore, polymers with satisfying bulk properties often need to be functionalized/modified by post-processings to obtain desirable surface properties, such as biocompatibility, adhesion, wear resistance and hydrophilicity/hydrophobicity^[1, 2].

The most versatile approach of surface functionalization is to generate particular functional groups on polymeric surfaces. These functional groups can provide desirable surface properties either by themselves or by molecules covalently bound with them. For instance, most polymeric materials, even the so-called biomaterials, have limited applications in the biological field due to the lack of reactivity, hydrophilicity, or adequate interactions with cells^[3]. Although polymers with reactive functional groups can covalently bind with biologically active moieties to expand their applications^[3–7], most polymers do not have the necessary functionality in reality. It is therefore crucial to functionalize a polymeric surface by a simple yet generic method and subsequently attach biomolecules to attain novel bioproperties.

A number of techniques have been successfully developed to generate functional groups on polymeric

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surfaces in both industry and academia, such as plasma treatment, chemical reaction, corona discharge, flame treatment, UV irradiation and laser etching^[8, 9]. These methods are brute-force in nature and do not provide precise functionalization results, such as the exact nature of the functional groups produced or depth of the surface functionalization. The surfaces fabricated by these methods are thermodynamically unstable and can only keep the generated properties for a short period of time^[1].

Block copolymers have been employed as a physical means to lower the surface tension of a polymeric substrate^[10]. Recent research by our own and other groups has shown that block copolymers with functionality can also be used as delivery vehicles to carry functional groups to polymeric surfaces^[11, 12]. These block copolymers basically share two common features: one block of the block copolymer is usually identical in composition to the substrate and anchors the block copolymer onto the substrate; the second block comprises a lower surface tension polymer with functional groups and forms a surface layer at the air-polymer interface *via* preferential surface segregation^[12]. Once adsorbed on the substrate, the block of lower surface tension delivers its functional groups to the substrate surface to bind with other molecules and satisfy different applications^[11, 13].

Block copolymers with functional groups can be added onto polymeric surfaces by a number of strategies, such as segregation from the bulk phase^[14], adsorption from supercritical fluids^[8] and spin coating^[15]. Segregation from bulk phase during melt processing requires a long time for the block copolymer to diffuse to the surface due to the high viscosity of the polymeric matrix. In addition, block copolymers can form micelles because of the inherent incompatibility of the two copolymer blocks^[14]. These intrinsic disadvantages make this method impractical. Although adsorption from supercritical fluids works well in coating a block copolymer onto a polymeric substrate^[11], we choose fabricating the surface for the current study by the spin coating method since it is very straightforward to be executed and yet allows precise control of coating results. The thickness of the block copolymer film and the area density of the functional groups can be quantitatively controlled by changing the rotation speed and the concentration of the block copolymer solution used for spin coating^[16, 17].

In this paper, we illustrate a generic approach to chemically modify polymeric surfaces that is applicable for almost all polymeric substrates. A polystyrene (PS) surface was first spin coated with poly(styrene-*b*-*tert*-butyl acrylate) (PS-*Pt*BA) dissolved in toluene. Spin coating formed a surface layer of the *Pt*BA blocks ordering at the air-polymer interface and a bottom layer of the PS blocks entangling with the PS substrate. The surface layer were further functionalized with carboxylic acid groups by hydrolysis of the *tert*-butyl ester groups on *Pt*BA blocks, accomplished by exposing the ester groups to trifluoroacetic acid (TFA)^[18]. The surface carboxylic acid groups produced in this fashion render the surface hydrophilic and also provide reactive sites for subsequent bioconjugation reaction with FMRF neuropeptides to change the biocompatibility of hydrophobic and bioinert PS. In a similar way, the modified surface can also obtain other biological properties by simply reacting carboxylic acid groups with corresponding biomolecules such as cell adhesion ligands, proteins, and growth factors^[6, 18]. The functionalizations and reactions studied in this work were characterized by contact angle and angle-dependent XPS measurements.

EXPERIMENTAL

Materials

Polystyrene (M_n : 145×10^3 , $M_w/M_n = 1.07$) was purchased from Seebio Biotechnology Inc. Trifluoroacetic acid was purchased from Accela ChemBio Inc. Ethylene glycol (analytical pure) and acetone were purchased from Jingke Company. FMRF was purchased from GI Biochem Ltd. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS) were purchased from Fisher Scientific China. Poly(styrene-*b*-*tert*-butyl acrylate) (M_n : 6.4×10^3 – 7×10^3 , $M_w/M_n = 1.33$) was purchased from Polymer Source.

Sample Preparation and Characterization

Silicon wafers (1 cm by 1 cm) were cleaned in piranha solution (3:1 *V/V* H₂SO₄:H₂O₂) for at least half an hour and then were washed extensively with deionized water and acetone. After being dried with nitrogen, the wafers

were spin coated by a model TC100A spin coater (Siyouyen Electronics Technology Co., Ltd) with a 0.77% *W/W* solution of PS in toluene at 2500 r/min for 40 s to produce a 40 nm thick PS substrate.

A layer of block copolymer PS-*Pt*BA was subsequently spin coated onto the PS substrate at 2500 r/min for 30 s to generate a bilayer structure of *Pt*BA-PS/PS. To study the relationship between the *Pt*BA layer thickness and the concentration of the spin coating solution, we prepared the PS-*Pt*BA films by varying the concentration of PS-*Pt*BA solutions in toluene from 0.1% to 0.6% *W/W*. The resultant surfaces were then annealed at 110°C for 5 h and then cooled to room temperature in a vacuum oven to ensure the uniformity of the surface films.

For surface bioconjugation with FMRF peptides, the *Pt*BA blocks of *Pt*BA-PS/PS on silicon wafers were first reacted with TFA into poly(acrylic acid) (PAA) to form a structure of PAA-PS/PS. After *Pt*BA was hydrolyzed into PAA, the bioconjugation reaction of surface-bound PAA and FMRF peptides was made in an aqueous solution since both PAA and peptides are soluble in water. The carboxylic acid groups on PAA were first activated by EDC and sulfo-NHS for 15 min, rinsed by deionized water to wash away the excess EDC and sulfo-NHS, and finally immersed in a 5 mg/mL FMRF solution in deionized water overnight to complete the bioconjugation reaction. The resultant samples were cleaned by deionized water and dried with nitrogen for XPS characterization. The bioconjugated sample is referred as FMRF-PAA-PS/PS in this paper.

Measurements

Ethylene glycol contact angle measurements were carried out at room temperature with a model JC2000C1 contact angle goniometer (Zhongchen Digital Technic Apparatus Co., Ltd). The contact angles were recorded immediately after dispensing 10 μ L ethylene glycol with a pipet onto the surface. The reported values were average values of measurements at three different locations for each sample.

XPS spectra were recorded with a PHI 5500 spectrometer with Al $K\alpha$ monochromator radiation source (1486.6 eV). The test chamber pressure was maintained below 1.33×10^{-6} Pa during spectral acquisition. A low energy electron flood gun was used as required to neutralize surface charging. Binding energies were calibrated with respect to the aliphatic C1s peak at 285 eV. High-resolution spectra were acquired with a pass energy of 23.5 eV and a binding energy resolution of 0.05 eV. The take-off angle was defined as the angle between the surface and the photoelectron detector and set to 45° for all of the measurements. Atomic concentrations were calculated by normalizing peak areas with the elemental sensitivity factor data provided in the XPS database.

RESULTS AND DISCUSSION

The objective of this study is to functionalize surfaces from a molecular point view of to provide desirable bioproperties without changing the bulk properties. The basis of the approach is the fact that block copolymers with one low surface tension block are known to spontaneously self-assemble at the surface of a homopolymer matrix of higher surface tension^[19, 20]. As an illustration of this concept, a PS substrate with poor biocompatibility was modified with a functional PS-*Pt*BA block copolymer and then decorated with FMRF peptides. The system was chosen for several reasons: we have shown previously that the hydrophobic *tert*-butyl ester groups on *Pt*BA are readily hydrolyzed to hydrophilic carboxylic acid groups by exposure to ultraviolet and photo acid generator or TFA^[12]; *Pt*BA is biocompatible, known to be tolerated by biomolecules that might be immobilized onto it^[21]; and finally, the surface tension of *Pt*BA (30.4 mN/m) is lower than that of PS (40 mN/m) so that the *Pt*BA blocks segregate to the air-polymer interface after surface functionalization^[12, 22]. Therefore, spin-coated films of PS-*Pt*BA block copolymer on PS substrate are expected to self-organize into a bilayer structure depicted schematically in Fig. 1. The PS blocks of PS-*Pt*BA entangle with the PS molecules of the substrate because it has a higher surface tension and they are chemically identical, while the *Pt*BA blocks segregate to the top of the modified surface with its *tert*-butyl groups for further modification/functionalization.

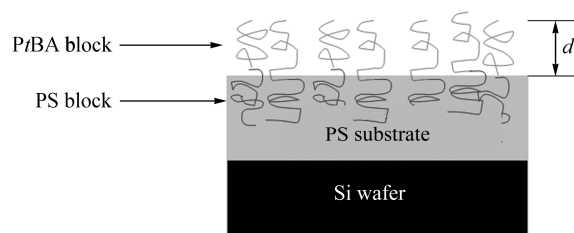


Fig. 1 Schematic picture of PS-PtBA block copolymer coated PS substrate

XPS measurements were employed to confirm the successful coating of PS-PtBA onto the PS substrate. An XPS spectrum of PS and a XPS spectrum of PS modified with 0.4% *W/W* solution of PS-PtBA in toluene (PtBA-PS/PS) are shown in Fig. 2 (spectra a and d, respectively). The XPS spectrum of the PS substrate exhibits only a single peak centered at 285 eV corresponding to the unshifted C_{1s} carbon-carbon bonds in PS. The C_{1s} spectrum of the block copolymer coated PS features signals that correlate with different chemical shifts in binding energies associated with the carbon atoms in PtBA. The signals are divided into three C_{1s} peaks, which are assigned to the carbonyl carbon ($\text{COO}-\text{C}$) in PtBA at 289.4 eV, the ester carbon ($\text{COO}-\text{C}$) in PtBA at 287.4 eV, and the rest carbons in PtBA as well as all of the carbons in PS at 285 eV.

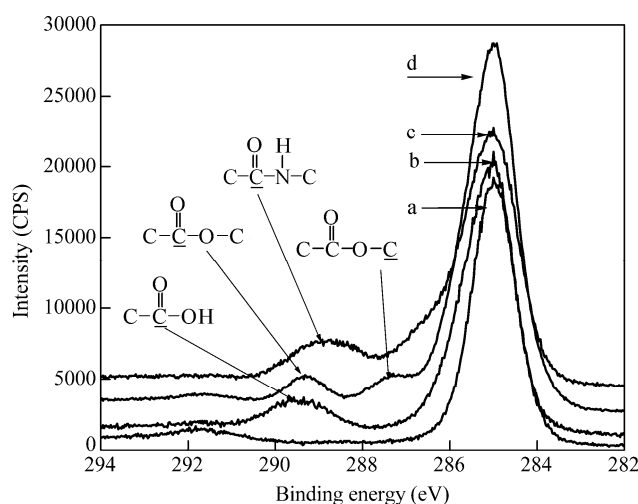


Fig. 2 High resolution C_{1s} spectra of (a) PS surface, (b) PAA-PS/PS surface, (c) FMRP-PAA-PS/PS surface and (d) PtBA-PS/PS surface

The effect of concentration of the block copolymer solution employed for spin coating on functionalization results was characterized by contact angle measurements under ambient conditions as shown in Fig. 3. Ethylene glycol was used as the test liquid for this system because of the large ethylene glycol contact angle difference between PtBA (69°) and PS (55°). By contrast, water contact angles of PtBA (90°) and PS (85°) are close to each other according to our measurements. The contact angle on functionalized surfaces increased significantly between concentrations of 0.1% and 0.4% *W/W* and asymptotically approached a value of 69° , the value for contact angle of pure PtBA, signaling that the PS was fully covered by a saturated PtBA monolayer at the concentration of 0.4% *W/W*. The appearance of a saturated PtBA layer on the surface verified that the PtBA blocks ordered at the air-polymer interface and the bilayer structure was produced as illustrated in Fig. 1 after spin coating. The contact angle dropped when the copolymer concentration exceeded 0.4% *W/W*, suggesting that a partial multilayer of PS-PtBA was formed on PS substrate. In this case, the surface was covered by a mixture of PtBA and PS blocks. This phenomenon related to surface saturation was also observed in other similar systems^[12, 23].

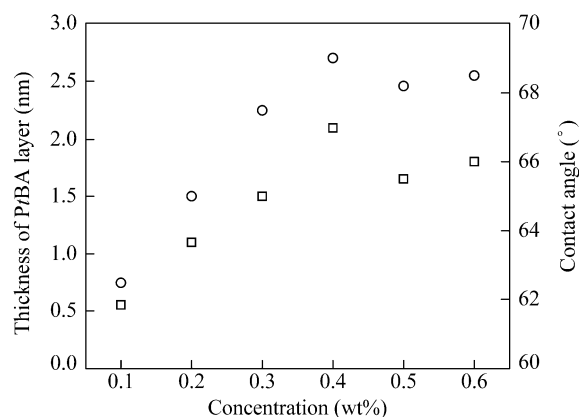


Fig. 3 Ethylene glycol contact angle (○) and thickness of PtBA layer (□) as a function of PS-PtBA concentration

An equation for the bilayer structure was then used to estimate the thickness of the PtBA layer based on XPS measurements. The derivation and validation of the equation were presented in our previous publications^[8, 11]. Briefly, the thickness of the PtBA layer can be calculated by using the following relationship:

$$\ln \left(\frac{1 - 7 \frac{I(C_{\text{carbonyl}})}{I(C_{\text{total}})}}{1 + 7 \frac{I(C_{\text{carbonyl}})}{I(C_{\text{total}})} \left(\frac{N_s}{N_{\text{tBA}}} - 1 \right)} \right) = \frac{-d}{\lambda \sin \theta} \quad (1)$$

where

$$\frac{N_s}{N_{\text{tBA}}} = \frac{M(\text{tBA})}{M(\text{styrene})} \times \frac{\rho(\text{PS})}{\rho(\text{PtBA})} \times \frac{8}{7} = \frac{128}{104} \times \frac{1.05}{1} \times \frac{8}{7} = 1.477$$

Here I is the signal from a particular atomic species and directly measured by XPS, θ is the take-off angle, λ is the mean free path of electrons with a value of 3.1 nm^[24], d is the thickness of the PtBA surface layer, N_{tBA} is the total carbon atomic density in the PtBA layer, and N_s is the total carbon atomic density in the underlying PS layer. The only unknown in Eq. (1) is the thickness of PtBA layer d that can be calculated with the experimental data of I .

The ability to control the thickness of the PtBA layer is also illustrated in Fig. 3 to compare with ethylene glycol contact angle data, where the XPS-estimated PtBA layer thickness is shown as a function of spin coating solution concentration of PS-PtBA. The dependence was linear as predicted by theory^[16] up to the concentration of 0.4% W/W . This linearity illustrates that it is straightforward to control the thickness of the functional PtBA layer on the surface. Both the XPS-estimated PtBA layer thickness and ethylene glycol contact angle data indicated that PtBA surface composition increased linearly with concentration until a saturated PtBA layer was formed at the concentration of 0.4% W/W in Fig. 3. Neither the bilayer model nor the XPS-estimated thickness is appropriate above the linear range where multilayer structures are formed as indicated by contact angle measurements. While the thickness data are not accurate when the concentration exceeds 0.4% W/W , they do reflect the change of surface structure. The XPS-estimated thickness of the saturated PtBA layer is about 2 nm in Fig. 3, close to the unperturbed radius of gyration of the PtBA block, estimated to be 2.1 nm^[25], but much smaller than the extended chain length of 13.74 nm^[11]. The block copolymer brushes produced by spin coating therefore behave as wet brushes with chains assuming nearly unperturbed dimensions. Within the linear range, the bilayer model is valid and the area density of *tert*-butyl groups on the surface can be easily calculated from the PtBA layer thickness as follows:

$$m_A = d\rho_P / M_U \quad (2)$$

wherein, m_A is the molar area density of the *tert*-butyl group, ρ_P is the density of PtBA, d is the thickness of the PtBA layer, and M_U is the molecular weight of the repeat unit of the PtBA block.

The next step of our strategy for surface functionalization is to expose the surface-bound PtBA to TFA. The aim of this step is to deprotect the ester groups on PtBA to produce reactive carboxylic acid groups for further bioconjugation reaction. PS substrate spin coated with 0.4% *W/W* PS-PtBA in toluene was chosen for the hydrolysis reaction to ensure the modified surface was fully covered with a pure 2 nm PtBA layer indicated by contact angle and XPS measurements. The success of TFA-induced deprotection and the time to complete the hydrolysis reaction were studied by monitoring the ratio of $\underline{\text{COOH}}/\underline{\text{C}}_{\text{total}}$ from the XPS spectra. As the reaction proceeded, the tertiary carbon peak of $\text{COO}-\underline{\text{C}}$ with a binding energy of 287.4 eV disappeared, while a new $\underline{\text{COOH}}$ peak occurred at 289.6 eV. The ratio of $\underline{\text{COOH}}/\underline{\text{C}}_{\text{total}}$ kept increasing until it reached a stable value that indicates all of the *tert*-butyl groups were transformed to carboxylic acid groups. The ratio of $\underline{\text{COOH}}/\underline{\text{C}}_{\text{total}}$ as a function of reaction time is shown in Fig. 4. The results show that the time required to hydrolyze all of the *tert*-butyl groups into carboxylic acid groups is 15 min. A high resolution C_{1s} spectrum after the hydrolysis reaction is shown in Fig. 2. The surface covered by carboxylic acid groups is hydrophilic and reactive whereas the original PS surface is hydrophobic and inert.

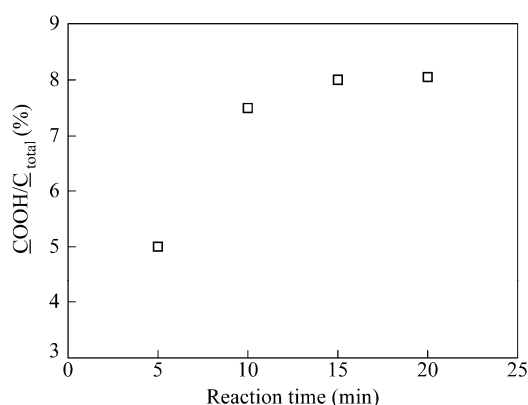


Fig. 4 The ratio of $\underline{\text{COOH}}/\underline{\text{C}}_{\text{total}}$ as a function of hydrolysis time

The final functionalization step is to link surface carboxylic acid groups with biomolecules to show the binding ability of the modified surface. A specific example of FMRF peptides binding to carboxylic acid groups in an aqueous solution was used to illustrate the new applications and properties of the functionalized surface. The most common linker for amino group and carboxylic acid group conjugation under this condition is EDC. EDC first reacts with a carboxylic acid group to form an active ester (*o*-acylisourea) leaving group, and then this active ester reacts with a terminal amino group on FMRF peptide. However, the reactive ester is subject to rapid hydrolysis in aqueous solutions, having a rate constant measured in seconds^[26]. The unstable esters can therefore hydrolyze before the peptides in the solution diffuse to them on the functionalized surface. To increase the reaction yield, sulfo-NHS was used to react with the EDC-activated unstable ester to produce a water-soluble sulfo-NHS ester that is stable in aqueous solutions for several hours^[27]. The sulfo-NHS esters ultimately reacted with the amino groups on peptides to form amide bonds.

After the bioconjugation reaction, high resolution of C_{1s} spectra in Fig. 2 show the energy shift due to transformation of $\underline{\text{COOH}}$ groups (peak centered at 289.6 eV) to $\underline{\text{CONH}}$ groups (peak centered at 288.9 eV). The XPS measurements therefore confirmed that the FMRF peptides were conjugated onto the surface *via* amide bonds. XPS spectra of PS, PtBA-PS/PS, PAA-PS/PS, and FMRF-PAA-PS/PS are all shown in Fig. 2 to illustrate the change of surface chemistry due to the functionalization steps. FT-IR measurements were also used to confirm the success of functionalization steps (data not shown for brevity). Only XPS data were presented in

this paper since XPS is an instrument that characterizes the change of surface chemistry more accurately and precisely. The yield of the bioconjugation reaction was calculated as follows: XPS data showed $\underline{\text{COOH}}/\underline{\text{C}}_{\text{total}}$ ratio of PAA surface was 7% and the $\underline{\text{CONH}}/\underline{\text{C}}_{\text{total}}$ ratio of FMRF conjugated surface was 10.4%. There are four $\underline{\text{CONH}}$ groups introduced onto the surface when one activated carboxylic acid group conjugates with one terminal amino group on FMRF. Wherein, three of them come from FMRF and one is formed by the conjugation reaction. The yield of bioconjugation reaction was then calculated as: $10.4\%/4/7\% = 37.1\%$. This value of yield was confirmed by the comparison of experimental and theoretical $\underline{\text{N}}_{\text{is}}/\underline{\text{CONH}}$ ratios. The bioconjugation reaction introduces seven nitrogens in a FMRF onto the surface at the same time of introducing four $\underline{\text{CONH}}$. The theoretical $\underline{\text{N}}_{\text{is}}/\underline{\text{CONH}}$ ratio was then $7/4 = 1.75$. XPS data showed the atomic concentration of C was 68.86% and that of N was 12.31% after the bioconjugation reaction. Since only 10.4% of carbon was in the form of $\underline{\text{CONH}}$, the experimental ratio of $\underline{\text{N}}_{\text{is}}/\underline{\text{CONH}}$ was then $12.31\%/(68.86\% \times 10.4\%) = 1.71$. The theoretical value is close to the experimental one, which indicates the value of yield is reliable. The area density of conjugated peptides, m_p , can be calculated based on equation (2) as follows with the knowledge that the area density of *tert*-butyl groups was equal to that of carboxylic acid groups and only 37.1% carboxylic acid groups reacted with peptides:

$$m_p = 37.1\% \times m_A = 37.1\% \times d\rho_p/M_U = 37.1\% \times 2 \text{ nm} \times 1 \text{ g/cm}^3/(104 \text{ g/mol}) = 7 \times 10^{-6} \text{ mol/m}^2$$

A bioinert polymer was modified with a functional block copolymer and then attained a novel biocompatibility by means of covalent attachment of FMRF peptides. Biologists can attach other desired biomolecules by following the same procedure to achieve required surface biocompatibility.

CONCLUSIONS

Spin-coated films of functional block copolymers constitute an effective means to quantitatively control the thickness and area density of functional groups on polymeric substrates. Polymers with reactive functional groups can be used as templates for bioconjugation with biomolecules to obtain novel biocompatibility. In the example described, a PS-*Pt*BA copolymer self-assembled at the surface of a PS substrate and formed a surface layer of *Pt*BA by spin coating method. The thickness of the *Pt*BA layer is linearly dependent on the concentration of the solution used for spin coating until a saturated monolayer of *Pt*BA was formed on the surface at the concentration of 0.4% *W/W*. The *tert*-butyl groups on *Pt*BA blocks were converted to reactive carboxylic acid groups by exposure to TFA for conjugation with biomolecules. This presented example illustrates how a bioinert PS surface can ultimately have biomolecules on the surface to change its biocompatibility through our generic functionalization process. The success of surface functionalization was demonstrated and characterized by XPS and contact angle measurements. A material of arbitrary chemistry can be functionalized by varying the functional block copolymer to obtain desirable reactive groups on the surface for binding with required biomolecules to achieve necessary biocompatibility.

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