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Protein Conformation Changes on Block Copolymer Surfaces Detected by Antibody-Functionalized AFM Tips

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Abstract

Conformational changes of fibronectin deposited on poly(methyl methacrylate) and poly(acrylic acid) block copolymers with identical chemical compositions were detected using an antibody-functionalized atomic force microscope (AFM) tip. Based on the antibody-protein adhesive force maps and phase imaging, it was found that the nanomorphology of the triblock copolymer is conducive to the exposure of the arginine-glycine-aspartic acid (RGD) groups in fibronectin. For the first time, X-ray photoelectron spectroscopy (XPS) was used to elucidate surface chemical composition and confirm AFM results. The findings demonstrate that block copolymer nanomorphology can be used to regulate protein conformation and potentially cellular response.

Keywords

block copolymers; atomic force microscopy; X-ray photoelectron spectroscopy; protein conformation

1. Introduction

Biomaterial surfaces are rapidly coated with a dynamic protein layer upon implantation into a living host. The interactions of this protein layer and cells with biomaterial surfaces are widely studied due to their implications to a wide range of applications such as the development of new materials for cell culture, dental and surgical implants [Garcia et al., 1999; Parker et al., 2002; Keselowsky et al., 2003; Matsushita et al., 2006; Klein et al., 2011] and biosensors [Bhushan et al., 2005, 2006, 2008; Scarpa et al., 2010; Zhao et al., 2010]. The deposited protein layer is composed of many proteins, including extracellular matrix (ECM) and serum proteins such as fibronectin, laminin and collagen, and will regulate the adhesion, proliferation and differentiation of cells on synthetic biomaterials. The behavior of these proteins, including their conformation, are known to be highly dependent on surface chemistry, topography and rigidity of the substrate, and for in vitro situations, the solvent used to disperse the proteins [Keselowsky et al., 2003; Michael et al., 2003; Baugh and Vogel, 2004]. These factors influence the conformation of the adsorbed proteins, thus

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affecting the cell behavior upon exposure to the biomaterial surface. Therefore, it is possible to control cellular adhesion and differentiation on a given polymer surface by controlling the nano-morphology, and hence, the protein conformation.

The optimization of biomaterials for cell adhesion and proliferation therefore involves designing surfaces that maintain the appropriate conformation of the adsorbed proteins. Nanomorphology is well known to influence the conformation of proteins adsorbed on a surface. Because of their ability to generate a wide diversity of nanomorphologies, biocompatible block copolymers represent a class of materials that can regulate protein absorption and conformation. Since the surface morphology of block copolymers can be modified using various polymer synthesis methods, they can be used to modulate the conformation of the proteins. The morphology of block copolymers is a function of the composition and the molecular weight of each individual block, as well as the spatial relationship of the blocks, i.e., A-B block copolymers (diblock) will have a different morphology from (A-B-A) block copolymers (triblock). This difference in morphology, based on the spatial relationship of the blocks, can affect the interfacial properties of these polymers. For block copolymers composed of poly(methyl methacrylate) (PMMA) / poly(acrylic acid) (PAA) and poly(methyl methacrylate) (PMMA) / poly(2-hydroxyethyl methacrylate) (PHEMA), it has been demonstrated that their adhesive interactions with proteins vary as a function of both the block composition and arrangement [Palacio et al., 2011]. They were able to show variation in the measured adhesive force between proteins and polymers with different block arrangement but identical chemical compositions.

Fibronectin (Fn) is a high molecular weight (450 kDa) dimeric ECM protein found in blood and other body fluids. It plays an important role in various cell functions such as adhesion, growth and differentiation both *in vitro* and *in vivo* [Garcia et al., 1999; Pankov and Yamada, 2002]. The ability of fibronectin to facilitate favorable cell-surface interactions is attributed to the presence of the cell-binding domain (CBD), which contains the Arginine-Glycine-Aspartic Acid (RGD) sequence. It has been proposed that the proper conformation of Fn on a surface causes the RGD sequence and the adjacent amino acid sequences to be exposed, which is necessary for the interactions of fibronectin with cells. Moreover, this sequence is also considered as an epitope or an antigenic determinant and its exposure while adsorbed to a surface ensures recognition and binding by antibodies [Dickinson et al., 1994; Kowalczyńska et al., 2005; Giambianco et al., 2011]. Due to its importance in regulating cell adhesion, fibronectin is a widely used protein model to evaluate the molecular level biocompatibility of biomaterial surfaces.

The adhesion of cells with fibronectin is mediated by the integrin group of cell-surface receptors. Integrins are known to anchor cells, support cell spreading, and trigger signals that can regulate cellular proliferation and differentiation. It has been shown that the conformation of fibronectin is sensitive to changes in the surface chemistry of the substrate where it is adsorbed. This leads to the modulation of the binding of fibronectin to integrins and its ability to facilitate cell adhesion [Keselowsky et al., 2003; Michael et al., 2003].

Fibronectin conformation has been examined by a variety of methods, including radioactive isotopes, ELISA and FRET [Garcia et al., 1999; Keselowsky et al., 2003; Baugh and Vogel,

2004; Kowalczyńska et al., 2005; Little et al., 2008]. In this study, the conformation of Fn will be investigated using atomic force microscopy (AFM). AFM is capable of studying specific molecular recognition events if the tip (usually made of Si or Si₃N₄) is modified (or functionalized) to chemically attach an antibody that would exhibit strong interactions with the sample surface containing the protein of interest. In addition to quantifying Fn conformation, maps of the proteins on the surface can potentially be generated. In many cases, the adhesive interaction is the unbinding force between an antibody and the epitope contained in the protein. A schematic is presented in Fig. 1 to highlight the difference in specific adhesion between the antibody (on the AFM tip) and the epitope (in the protein) and non-specific adhesion when the epitope is not in the desired conformation or is buried within the protein. The detection of strong adhesive forces corresponding to specific molecular recognition implies that the desired protein conformation exists on the surface [Hinterdorfer et al., 1996; Allen et al., 1997; Stevens et al., 2002; Kienberger et al., 2005; Lee et al., 2007].

X-ray photoelectron spectroscopy (XPS) has been used to elucidate the surface chemical composition of biomaterial surface with adsorbed proteins. It has the sensitivity to quantitatively detect the extent of adsorption as a function of the protein solution concentration [Browne et al., 2004]. XPS can also detect the covalent bonding of proteins to a polymer substrate by monitoring binding energy shifts [Nelson et al., 2010]. By monitoring changes in the intensity at multiple angles of incidence (angle-resolved experiment), the thickness of protein layers adsorbed on a substrate can be determined [Awwsiuk et al., 2010].

AFM was used in this study to investigate the adhesion between fibronectin (adsorbed on polymer) and an RGD epitope-specific antibody (attached to the tip) to model the effects of the polymer morphology and surface composition. XPS was used to elucidate chemical information on the protein adsorption to the polymer surface. The difference between triblock, diblock, and random PMMA/PAA copolymers is examined to illustrate that fibronectin-antibody adhesion, and hence, the fibronectin conformation is modulated not only by surface chemistry, but also by the specific nanomorphological variation induced by differences in the block arrangement of the copolymer components.

2. Experimental

2.1 Materials

Click coupling between PMMA and PAA homopolymers was undertaken to synthesize the PAA-b-PMMA-b-PAA (0.5/1/0.5 mol ratio) triblock copolymer [Schrick et al., 2010]. Films from the triblock copolymer and PMMA were created on silicon substrates using the drop casting method. The polymers were dissolved in tetrahydrofuran (THF) to attain a concentration of 10 mg/mL. Silicon wafers cut into 1 × 1 cm² squares were placed into a chamber kept at a temperature of 22 °C and a relative humidity (RH) ranging from 85–95%. Droplets of the polymer solution (approx. 50 µL) were cast onto the silicon surface while it is exposed to high relative humidity. The water droplets adsorbed on the silicon surface as a result of condensation served as templates for block copolymer film assembly. The evaporation of the volatile THF solvent led to the deposition of the polymer films on silicon.

It should be noted that pure PAA was not studied because it is water-soluble, making it unstable in AFM imaging experiments in liquid medium [Palacio et al., 2011].

Protein solutions containing either fibronectin or bovine serum albumin (BSA) (both from Sigma-Aldrich, St. Louis, MO) were added to the sample coupons with the polymer film and equilibrated for an hour. Afterwards, the sample coupons were rinsed with phosphate buffered saline (PBS, pH 7.4, from Invitrogen, Carlsbad, CA) followed by deionized water to remove any proteins that did not adsorb to the polymer surface.

2.2 Functionalized tip preparation

Silicon AFM probes on a 225 μm rectangular cantilever with a nominal stiffness of 0.1 N/m (Vista Probes, Phoenix, AZ) were functionalized to attach a fibronectin monoclonal antibody (MAB 88916, Millipore, Billerica, MA). Briefly, the AFM probes were treated with 3-aminopropyltrimethoxysilane (APDMES, Gelest, Morrisville, PA) to aminosilane the surface. Afterwards, the probes were immersed in a solution containing PBS, the coupling agent 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC, from Sigma-Aldrich, St. Louis, MO), and the antibody in order to attach the antibody to the AFM tip.

2.3 Atomic force microscopy

A Multimode AFM (Bruker, Santa Barbara, CA) equipped with a modified tip holder was used to perform the adhesion mapping in liquid medium [Bhushan et al., 2008]. A horizontal slot was carved out in the opening of a non-fluid Multimode tip holder in order to insert a glass slide. Prior to imaging, the PBS immersion medium was added to the sample surface and the AFM liquid cell.

Adhesive force mapping was conducted in force-volume mode using the protein-functionalized AFM tip. Using relative triggering, a 64×64 array of force-distance curves over a $10 \mu\text{m} \times 10 \mu\text{m}$ area was collected across the surface of a location of interest. For each force-distance curve, 128 sampling points were obtained. A custom program coded in Matlab was used to calculate the adhesive force [Palacio et al., 2011]. The adhesive force for each force distance curve was obtained by multiplying the maximum deflection of the cantilever in the retracted position with the cantilever spring constant.

Experiments were performed in tapping mode in order to obtain height and phase information with the sample immersed in PBS. A FORTA tip (AppNano, Santa Clara, CA) with a nominal spring constant of 3 N/m and a resonant frequency of 60–80 kHz was used. Scanning was performed on $10 \mu\text{m} \times 10 \mu\text{m}$ areas at a rate of 0.5 Hz along the fast scan axis.

2.4 X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) studies were performed on the block copolymers and PMMA with added fibronectin using a Kratos Axis Ultra spectrometer. Spectra were acquired using a monochromatic Al $K\alpha_{\text{mono}}$ X-ray radiation source operated at 12 kV and 10 mA in ultrahigh vacuum. Survey scans were performed in an energy range of 0–1400 eV at two incident angles, namely 90° and 30° . High-resolution spectra of C 1s, O 1s N 1s and

S 2s were taken on all the samples investigated at 90° and 30°. The resulting spectra were referenced to the C 1s (alkyl) peak at 285 eV [Moulder et al., 1992].

3. Results and Discussion

3.1 Atomic force microscopy

Morphology and adhesive force maps taken with the antibody-functionalized AFM tip while the samples are immersed in phosphate buffered saline (PBS, pH 7.4) are presented in Fig. 2. The PMMA surface (which is used as a reference surface) is smooth, and features are apparent only on the surfaces with added protein (fibronectin or BSA). Meanwhile, the triblock copolymer PAA-b-PMMA-b-PAA (where “b” stands for the block copolymer linkage) exhibits a pore morphology, which corresponds to cylinders in three dimensions [Palacio et al., 2011]. The diblock copolymer also exhibits a regular morphology, but with smaller features than the triblock copolymer. The random copolymer PMMA-co-PAA exhibits irregular surface features over the scan areas examined.

A close correspondence between the height and adhesive force is observed in the triblock and diblock copolymers without any added protein, where the recesses in the height image correspond to peaks in the adhesive force. These are attributed to the PAA-rich regions of the polymer, which are expected to be more adhesive. This implies that the PAA exhibits non-specific adhesion with the antibody on the AFM tip, such as hydrogen bonding and polar interactions. Upon addition of fibronectin to the triblock copolymer, the adhesive force map no longer traces the surface topography. Non-specific adhesive interactions with the polymer still exist. However, the tip now experiences specific antibody-antigen interactions with the exposed RGD epitopes of fibronectin, which is adsorbed throughout the triblock copolymer surface. The adhesive force map of the triblock copolymer with adsorbed BSA shows much fewer and fainter peaks, indicating that the antibody does not strongly interact with BSA, as expected. In contrast, the diblock and random copolymers exhibit fewer non-specific interactions with the antibody relative to the triblock copolymer. Even after the addition of fibronectin, the adhesive force maps do not appear to contain numerous peaks corresponding to antibody-antigen interactions.

The variation in the adhesive force between the antibody-functionalized tip and the polymer surfaces are shown in Fig. 3 and Table 1(a). It is observed that the PMMA and the triblock copolymer PAA-b-PMMA-b-PAA with added fibronectin have higher adhesive forces than the plain surface and the surface with adsorbed BSA. The average adhesion for the triblock copolymer is the highest among the polymers investigated. This implies that this polymer has the most adsorbed fibronectin with the RGD groups exposed on the surface. It also implies that the fibronectin adsorbed in the diblock and random copolymers do not possess the proper conformation, such that only non-specific adhesion is occurring. Both PMMA-b-PAA and PMMA-co-PAA (random) are less hydrophilic relative to the triblock copolymer, which could explain why fibronectin orients differently on these three surfaces [Palacio et al., 2011]. For comparison, the measured adhesive forces between an unmodified tip and the polymer surfaces with fibronectin are presented in Table 1(b). The range in the adhesive force is lower than the same surfaces with an antibody-functionalized tip (Table 1(a)), further confirming that the antibody-antigen unbinding was observed.

AFM phase images were taken on all polymers with added fibronectin to corroborate the results of the adhesive force mapping. Phase mapping is capable of detecting local variations in composition, adhesion, viscoelasticity, as well as material stiffness [Bhushan, 2010, 2011]. Phase images for the polymer surfaces with added fibronectin are shown in Fig. 4. As all images were taken in liquid medium (PBS), phase contrast due to adhesion is not a contributing factor. Tip-surface adhesive forces originate from meniscus formation at the interface, which is absent when the interface is immersed in liquid. In addition, the tip used for phase imaging was not functionalized (i.e., no attached antibodies), so specific interactions between the tip and adsorbed proteins are not present. Therefore, any observed phase contrast due to composition variation is due to differences in the viscoelasticity and material stiffness. A qualitative interpretation can be made on the surface composition based on the distribution of the phase as shown in the histogram. For PMMA, the histogram shows only one peak, corresponding to a one-component system. The random PMMA-co-PAA has two peaks, corresponding to the two block copolymer components. In both cases, the adsorbed protein does not appear as a distinct peak. However, for the triblock and diblock copolymers, additional higher phase signals are observed, which is attributed to the presence of a softer material (in this case, fibronectin). For the triblock case, there are actually three higher phase signals, indicating that different conformations of fibronectin are present on the polymer surface. The absence of these higher phase peaks on PMMA and on the random copolymer could mean that fibronectin may not be adsorbing at the desired conformation on those surfaces as a consequence of denaturation. By correlating the data from the adhesion and phase imaging studies, it is seen that higher adhesive force and the presence of additional phase modes correspond to the desired surface exposure of the RGD groups of fibronectin. This shows how the nanomorphology and block arrangement of block copolymers can affect protein conformation.

3.2 X-ray photoelectron spectroscopy

The XPS survey spectra of the polymer surfaces with added fibronectin at an incident angle of 90° are shown in Fig. 5. (Data for the incident angle of 30° are not presented, as the peak locations are identical.) Aside from peaks corresponding to the silicon substrate, peaks corresponding to C 1s, O 1s and N 1s photoelectrons are observed. The C and O peaks originate from either the polymer or protein, while the N peaks indicate presence of the protein. Sulfur is another element attributable to the presence of proteins. While sulfur peaks are not easily observable in the survey spectra, they are present in the high-resolution spectra, as discussed below.

High-resolution spectra at an incident angle of 30° corresponding to C 1s, O 1s, N 1s and S 2s peaks are shown in Fig. 6, and a tabulation of the peak assignments is presented in Table 2. It should be noted that spectra taken at the incident angle of 30° has the advantage of a shallower sampling area, thereby eliminating or minimizing the effect of the underlying substrate. In all the polymers studied, the C 1s spectra have three prominent peaks; one mode corresponds to the aliphatic environment (C-C or C-H), another mode corresponds to either C-O or C-N, and another mode due to the presence of the carbonyl group (C=O). The O 1s spectra have three major peaks, due to the C-O-C, O-C, and O=C bonding modes. The N 1s spectra have one peak, which is due to the N-H and N-C bonding environments. The S

2s spectra also have one peak, which is attributed to the S-H and S-C bonds present in the protein. The S spectra exhibit more noise and lower intensity, signifying its low surface concentration. In general, the peak locations are consistent with that observed from previous studies [Ton-That et al., 2001; Wagner et al., 2003; Nelson et al., 2010].

The surface elemental concentrations of nitrogen, oxygen and sulfur are reported as ratios with respect to carbon. Results for both the 90° and 30° incident angles are shown in Table 3. The effect of varying the incident angle led to a decrease in the O/C ratio, but mixed results for N/C and S/C. As mentioned above, the use a non-90° angle enables the sampling of a shallower sample thickness. For oxygen, the decrease in the O/C ratio from the 90° to the 30° experiment means that more of the protein is being analyzed (instead of the block copolymer surface), because amino acids contain more carbon than oxygen. For nitrogen, the N/C ratio did not change for PMMA, triblock and diblock copolymers, and a slight change on the random copolymer. This also indicates that the protein is the main contributor to the observed signals, and not the block copolymer. The mixed results for sulfur, where the S/C ratio increased for PMMA, decreased for the triblock and diblock copolymers and remained constant for the random copolymer, could be due to the signal noise arising from the low concentration of sulfur-containing groups on the surface. An important point to emphasize is that this variation in the elemental ratios for the three block copolymers means that fibronectin is adsorbed differently in these three surfaces, in spite of their identical chemical compositions. This finding is consistent with the AFM adhesion and phase data discussed earlier.

4. Conclusions

Among the biomaterials of interest in protein and cellular adhesion studies, block copolymers offer a unique advantage as they can produce numerous nanomorphologies using the same starting materials. In this study, atomic force microscopy and X-ray photoelectron spectroscopy were used for the first time to demonstrate that the nanomorphology of block copolymer surfaces could be tuned to control the conformation of the adsorbed protein. In addition, it was found that the effect of nanomorphology on protein adsorption behavior is independent of the chemical composition of the polymer substrate, as seen in the results for triblock, diblock and random copolymers of PMMA and PAA. Beyond confirming the importance of nanoscale chemistry and morphology on adsorbed protein conformation, these findings are significant, as it allows one to exploit the numerous possible nanoscale surface morphologies available within a given block copolymer system to design novel biomaterials with optimized protein conformation and cellular adsorption characteristics.

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Fig. 1. Schematic illustrating the effect of epitope exposure (or non-exposure) to the measured adhesion with the antibody-functionalized AFM tip.

Fig. 2.

AFM height and adhesive force maps of the interactions between the antibody-functionalized tip and the polymer surfaces with adsorbed fibronectin or BSA with the bare surface shown for reference.

Fig. 3. Bar chart showing the variation in the measured adhesive force between the antibody-functionalized tip and the polymer surfaces investigated.

Fig. 4.

Phase images taken on the polymer surfaces with added fibronectin, along with corresponding the frequency distribution of the measured phase angle variation.

Fig. 5.
XPS survey spectra for the polymer surfaces with added fibronectin.

Fig. 6. High-resolution C 1s, O 1s, N 1s, and S 2s XPS spectra for the polymer surfaces with added fibronectin.

Table 1

Measured adhesive forces with antibody-functionalized and unmodified tips

Polymer/Protein	Adhesive force (pN)
(a) Antibody-functionalized tip	
PMMA	620 ± 40
PMMA + Fibronectin	720 ± 100
PMMA + BSA	670 ± 50
PAA-b-PMMA-b-PAA	680 ± 90
PAA-b-PMMA-b-PAA + Fibronectin	980 ± 150
PAA-b-PMMA-b-PAA + BSA	620 ± 80
PMMA-b-PAA	710 ± 40
PMMA-b-PAA + Fibronectin	780 ± 100
PMMA-b-PAA + BSA	810 ± 80
PMMA-co-PAA(Random)	670 ± 130
PMMA-co-PAA(Random) + Fibronectin	610 ± 50
PMMA-co-PAA(Random) + BSA	740 ± 80
(b) Unmodified tip	
PMMA + Fibronectin	660 ± 80
PAA-b-PMMA-b-PAA + Fibronectin	600 ± 30
PMMA-b-PAA + Fibronectin	690 ± 40
PMMA-co-PAA(Random)+ Fibronectin	520 ± 50

Table 2

Binding energies (in eV) of the XPS peaks

	C 1s		
	CH_x	C-O/C-N	C=O
PMMA	285	286.5	288.8
PAA-b-PMMA-b-PAA	285	285.8	288.5
PMMA-b-PAA	285	286.2	289
PMMA-co-PAA (Random)	285	286	288.5

	O 1s		
	O=C	O-C	C-O-C
PMMA	-	532.4	534.4
PAA-b-PMMA-b-PAA	-	532.5	-
PMMA-b-PAA	531.2	532.4	533.4
PMMA-co-PAA (Random)	531.2	532.7	-

	N 1s
	N-C/N-H
PMMA	400.5
PAA-b-PMMA-b-PAA	400.2
PMMA-b-PAA	400.2
PMMA-co-PAA (Random)	400.4

	S 2s
	S-C/S-H
PMMA	228.5
PAA-b-PMMA-b-PAA	228.5
PMMA-b-PAA	226.5
PMMA-co-PAA (Random)	228.5

Table 3

Elemental concentration ratios at 90° and 30° incident angles

	O/C		N/C		S/C	
	90°	30°	90°	30°	90°	30°
PMMA	0.35	0.33	0.10	0.10	0.002	0.003
PAA-b-PMMA-b-PAA	0.48	0.27	0.05	0.05	0.006	0.003
PMMA-b-PAA	0.40	0.36	0.03	0.03	0.004	0.0001
PMMA-co-PAA (Random)	0.92	0.57	0.12	0.11	0.005	0.005