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Immunological Identification of Fibrinogen in Dual-Component Protein Films by AFM Imaging

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Abstract

The success of long-term blood-contacting implanted devices is largely dependent upon the interaction of the blood components with the device biomaterial surface. The ability to study these interactions has been hindered by a lack of methods to measure single-molecule interactions in complex multi-protein environments similar to the environment found in-vivo. In this paper, we demonstrate the use of atomic force microscopy (AFM) in conjunction with gold nanolabels to detect the protein fibrinogen under aqueous conditions without the topographical clues usually necessary for high resolution visualization. BSA was patterned onto both muscovite mica and plasma-treated polydimethylsiloxane (PDMS) substrates and these test substrates were subsequently backfilled with fibrinogen to yield a featureless protein layer. The fibrinogen in this dual protein layer was detected using high resolution AFM imaging following infusion of anti-fibrinogen conjugated with nanogold particles. This AFM immuno-detection technique will potentially be applicable to complex multi-component protein films adsorbed on clinically-relevant polymers used in medical devices.

Keywords

AFM; fibrinogen; protein adsorption; immunochemistry; biomaterials; gold labeling; biocompatibility; polymers

1. INTRODUCTION

The adsorption of plasma proteins is one of the first events to occur following blood biomaterial contact and this adsorbed protein influences subsequent biological events including platelet adhesion and thrombogenesis on the biomaterial surface (Gorbet and Sefton, 2004). Fibrinogen is the third most abundant protein in plasma and plays a key role in the development of surface-induced thrombosis. Fibrinogen is a key structural glycoprotein in thrombogenesis; activation by thrombin cleaves fibrinogen to fibrin, which then assembles to form an insoluble fibrin clot (Hantgan, et al., 2001). Fibrinogen binding to the platelet integrin receptor $\alpha_{IIb}\beta_3$ links platelets together (platelet aggregation), while surface adsorbed fibrinogen serves as a ligand for

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subsequent platelet immobilization (platelet adhesion) (Farrell, et al., 1992, Plow and Shattil, 2001). Fibrinogen interaction with platelets can lead to platelet activation through outside-in signaling mediated by integrin binding (Calvete, 1994, Calvete, 1999). The end product of these processes is the formation of a platelet-fibrin plug, generally referred to as a thrombus, which is composed of a mixture of platelets and blood cells entrapped in a fibrin mesh. Embolization of a thrombus can lead to fatal strokes and other ischemic events and therefore remains a significant problem in the design of medical devices for blood-contacting applications. An improved understanding of the initial steps of the biological response to blood contacting materials can provide crucial information for the development of improved biomaterials for use in medical devices.

Protein adsorption is a complex phenomenon where both the amount and biological activity of adsorbed proteins are dependent on the properties of the substrate. Although the complex interactions between surface and proteins are not completely understood, it is believed that both short and long range forces including van der Waals interactions, electrostatic interactions, hydrogen bonding and particularly hydrophobic interactions are all important in protein adsorption. Furthermore, the properties of the surfaces including charge, chemistry, topography, and wetting behavior have all been suggested to play some role in protein adsorption (Horbett, 1993, Noh and Vogler, 2006). Improved understanding of plasma protein interactions with biomedically relevant surfaces can provide the necessary framework for understanding the cellular response and eventually designing improved biomaterials.

Protein adsorption in complex and competitive environments have been studied by a variety of techniques including ellipsometry (Lassen and Malmsten, 1996), surface plasma resonance (Green R, et al., 1999), quartz crystal microbalance (QCM) (Glasmaster, et al., 2002), tensiometry (Krishnan, et al., 2006), total internal reflectance fluorescence (TIRF) (Wertz and Santore, 2001), electrophoretic depletion measurements (Noh and Vogler, 2007) and radiolabeling (Tsai, et al., 1999) as well as spectroscopy methods including Fourier Transform Infrared Spectroscopy (FTIR) (Chittur, 1998), Electron Spectroscopy for Chemical Analysis (ESCA) (Vogler, et al., 1995) and Time of Flight Secondary Ion Mass Spectrometry (Wagner, et al., 2003, Wagner, et al., 2003) to name just a few. Although these techniques have provided a wealth of macroscopic and microscopic information describing adsorption behaviors of fibrinogen and other proteins, the nature of protein surface activity is still not completely understood. Moreover, most of the techniques used in detecting the proteins are indirect and measure average quantities of adsorption events. The advent of atomic force microscopy (AFM) has provided a new tool for the study of individual plasma proteins at molecular scale with ultrahigh resolution and under physiologically relevant aqueous conditions (Binnig, et al., 1986, Marchant, et al., 2002).

AFM has been used extensively to obtain nanometer scale images of biosystems including proteins, lipid membranes, DNA and cells, generally utilizing ultrasmooth model surfaces such as muscovite mica, highly oriented pyrolytic graphite or self assembled monolayers (Argaman, et al., 1997, Radmacher, 1997, Reviakine and Brisson, 2000, Reviakine, et al., 2000, Marchant, et al., 2002, Osada, et al., 2003, Touhami, et al., 2003, Hussain, et al., 2005, Toscano and Santore, 2006). The low surface roughness of these model surfaces is ideal to characterize nanoscale protein features, including specific domains and conformational changes in these proteins upon adsorption. However AFM experiments are still somewhat limited in scope, particularly in complicated multi-protein adsorption experiments, because the proteins can be difficult to distinguish based on topography alone (Truong, et al., 1998). Perhaps more importantly, clinically used biomaterials have rough topographies that often dwarf the dimensions of the proteins of interest making detection of specific proteins on these surfaces even more difficult. Holland and Marchant successfully used phase imaging for detecting fibrinogen on two clinically relevant biomaterials: poly(dimethylsiloxane) (PDMS) and low

density polyethylene (LDPE) (Holland and Marchant, 2000). While fibrinogen could occasionally be observed in the phase images when present at submonolayer concentrations, the surface often proved too rough to clearly distinguish the adsorbed protein. Figure 1 illustrates an example of fibrinogen adsorbed onto a commonly used biomedical polymer, poly (urethane urea) and imaged by tapping mode AFM. Neither height nor phase images reveal any details sufficient to identify protein on this material. There remains a need to develop new techniques that do not rely heavily on topography to recognize specific proteins.

Alternative non-conventional AFM techniques have been developed for detection of specific proteins without dependence on topography. The force spectroscopy techniques of AFM utilize molecular recognition events to detect artificially patterned areas, red blood cells and antibody-antigen interactions (Hinterdorfer, et al., 1996, Radmacher, 1997). Our group has previously used an AFM probe modified with polyclonal antibodies to detect fibrinogen in a dual-protein environment. The dual protein layer containing bovine serum albumin (BSA) and fibrinogen dual protein layer was formed using microcontact printing techniques and adhesion mode AFM was used to detect the fibrinogen using the antibody-modified probe (Agnihotri and Siedlecki, 2005). A limitation of this method was the amount of time required for generation of an adhesion map (~60 minutes). This limitation is particularly important when studying the post adsorptive transitions in the fibrinogen structure on different substrates (Balasubramanian, et al., 1999, Agnihotri and Siedlecki, 2004). Moreover, this technique yields relatively low resolution images when used with standard AFM equipment and relies on complex statistical analysis to generate maps of protein distribution. Nanogold particles, commonly used in electron microscopy, (Hainfeld and Powell, 1997, Yang, et al., 2000, Montesano-Roditis, et al., 2001) have been used in conjunction with AFM. Mikoshiba used 10 nm gold particles to visualize the inositol 1,4,5-trisphosphate receptor using AFM and Transmission Electron Microscopy (TEM) (Wakako, et al., 2006). Hussain and Siedlecki used nanogold particles to directly visualize individual ligand-receptor interactions in physiologically relevant environment where the nanogold labels were conjugated to an RGD peptide and the presence of a peptide ligand bound to integrin receptors could be detected by the differences in the mechanical properties measured by AFM phase imaging (Hussain, et al., 2005). Similar gold bead labeling techniques were used by Putnam et al. to label cells (Putnam, et al., 1993) and Eppell et al. to perform correlative microscopy studies on platelets (Eppell, et al., 1995). These types of labels were used for protein analysis by Lin et al. to map the heparin binding site on fibronectin (Lin, et al., 2000) and by Raghavachari and Marchant to examine the exposure of functional domains in von Willebrand Factor following conformational changes induced by fluid shear (Raghavachari, et al., 2000). However, in each of these protein labeling studies, the protein was adsorbed in submonolayer amounts to visualize the binding events.

In this work we describe a novel application of nanogold labels conjugated to antibodies against human fibrinogen to determine the distribution of fibrinogen molecules in a dual protein layer that fully covers the surface. This study was carried out initially on the model substrate mica and then extended to a model polymer substrate, glow discharge plasma-treated poly (dimethylsiloxane) (PDMS). Furthermore, this technique can potentially be extended for detection of proteins on complex polymers used in biomedical applications.

2. MATERIALS AND METHODS

2.1 General

Phosphate buffered saline (PBS, 0.01 M sodium phosphate buffer, 150 mM NaCl, pH 7.4, Sigma Inc.) was prepared using filtered water from a Millipore Simplicity 185 system which utilizes two ultraviolet filters (185 and 254 nm) to reduce carbon contaminants. Human fibrinogen (90% clottable) and polyclonal rabbit anti-human fibrinogen (90% IgG) were used as received from Calbiochem, La Jolla, CA. Anti-fibrinogen antibody was dissolved in

phosphate buffer to yield a concentration of 1 mg/ml and aliquots were stored at -20°C until use. Bovine serum albumin (BSA) was obtained from Sigma Chemicals Co, St. Louis, MO.

2.2 Preparation of Nanogold conjugates

Sulfo-N-hydroxy-Succinimido Nanogold (Nanoprobes Inc., Yaphank, NY) are functionalized gold particles 1.4 nm in diameter. These functionalized particles have reactivity to primary amines and can be covalently linked to any protein. The Nanogold reagent was dissolved in 200 μ l of deionized water. Polyclonal rabbit anti-human fibrinogen was conjugated to Nanogold using the protocol for labeling proteins with molecular weight greater than 15,000 Da specified by the company. The anti-fibrinogen solution (1mg/ml) was reacted with the activated Nanogold solution at pH 7.4 for 1 hour at room temperature while rotating on a hematology mixer to ensure optimal conjugation. The reaction mixture was concentrated to a volume of 10 μ l using a centricon centrifugal filter (Millipore Inc.) with a 30,000 molecular weight cut-off. The retinate was brought to a volume of 100 μ l before purification by High Performance Liquid Chromatography (HPLC, Superose 200 column, Beckman Coulter Inc., Fullerton, CA). Conjugated nanogold was collected and stored at 4°C until use.

2.3 Dual protein layer formation using micro-contact printing

Proteins were patterned on mica and PDMS substrates using microcontact printing as described previously (Agnihotri and Siedlecki, 2005). Briefly, a 15 mm diameter poly(dimethylsiloxane) (PDMS, Sylgard 184, Dow Corning) rubber stamp having ~700 nm diameter holes was prepared by replication molding of a photoresist pattern prepared using optical lithography techniques (Milner, et al., 2006). The PDMS stamp was incubated in 1 or 5 mg/ml bovine serum albumin (BSA) for 1 hour; the stamp was then rinsed in PBS for 5 minutes and dried with nitrogen. Freshly cleaved, hydrophilic, muscovite mica (Ted Pella Inc., CA) or glow discharged plasma-cleaned PDMS was used as the substrate for stamping experiments. The PDMS substrate was rendered hydrophilic (water contact angle $\sim 15^\circ$) by glow-discharge plasma cleaning in an ambient environment for 45 minutes at 100 W power. The protocol for sample preparation and labeling is shown schematically in figure 2. A BSA-linked PDMS stamp was placed onto the mica or PDMS substrates under a 5 mg weight for 30 seconds to ensure proper stamping of proteins onto the substrate (Fig. 2a). The stamp was carefully peeled off and the substrate surface was immediately imaged using tapping mode AFM under PBS. The sample was transferred to an external fluid cell similar in design and dimension to the AFM fluid cell used for imaging (Fig. 2b). Human fibrinogen solution (1 mg/ml) was delivered through the fluid cell at a rate of 1 ml/hour for 1 hour to allow fibrinogen to adsorb in the protein-free holes created by printing (Fig. 2c). After the sample was rinsed with buffer solution in the external fluid cell for 5 minutes, Nanogold conjugated anti-fibrinogen (1:5 dilution) was delivered to the fluid cell at a rate of 1 ml/hr for 1 hour (Fig. 2d). The sample was again rinsed with PBS before being moved to the AFM imaging stage for analysis.

2.4 AFM imaging

All images were acquired in tapping mode (intermittent contact mode) with a Nanoscope IIIa Multimode[®] AFM (Digital Instruments, CA) under both ambient and aqueous PBS buffer using short thick cantilever silicon nitride probes (NP – S; spring constant ~ 0.6 N/m², Digital Instruments, CA). Topographic images and phase images were captured at 512×512 pixels resolution with scan size of 5×5 μ m². All imaging was carried out at a scan rate of 1 Hz with a resonant frequency of ~ 8 kHz. The tapping force can be controlled by the r_{sp} value, which is the ratio of set-point amplitude to free amplitude of oscillation. The AFM probe free amplitude of oscillation and r_{sp} values were fixed at 20 nm and 0.75, respectively, for all images. AFM images were flattened using a first order line fit and low pass filtered to remove high frequency

noise spikes from the images. Quantitative analysis of the dual protein layer thickness was made using the cross-section tool provided with the instrument software.

3. RESULTS AND DISCUSSION

3.1 Dual protein layer formation on mica

Mica is frequently used as a substrate for high resolution AFM imaging because it is quite easy to produce clean, hydrophilic ultrasmooth surfaces by simply cleaving with adhesive tape. Figure 3 shows height and phase images of BSA micro-contact printed onto a freshly cleaved muscovite mica substrate and imaged under both ambient conditions and aqueous buffer conditions by tapping mode AFM. The topography of the patterned surface imaged under ambient conditions is illustrated in figure 3a. The dark regions are mica substrate with diameter of ~800 nm, surrounded by BSA protein layer. Section analysis (not shown) calculates the height of the BSA pattern to be ~2.5 nm, approximately equal to a BSA monolayer (Green R. J., et al., 1997). The pattern can also be observed in the phase image (Fig. 3b) which detects the mechanical property differences between the protein and the substrate.

Given the large differences in size of BSA (4 nm × 8 nm) and fibrinogen (8 nm × 45 nm) (Green R, et al., 1997), there is only one plausible scenario in which a uniform 2 - protein layer can be created using a monolayer of BSA and fibrinogen. After backfilling the BSA pattern with fibrinogen, if all BSA molecules are adsorbed in an end-on configuration and all fibrinogen molecules are adsorbed in a side-on configuration, it would produce an ~8 nm thick uniform film. Considering the difficulties in achieving a completely uniform dual protein layer when the proteins are segregated as they are here, the PDMS stamp was incubated with higher BSA concentration (5 mg/ml) to create a multi-layer of BSA onto mica. When imaged in buffer, the holes in the patterned surface appear slightly enlarged which can be attributed to small AFM scanner drift which is common in AFM experiments in aqueous conditions (Agnihotri and Siedlecki, 2005). Both the height and phase images (Fig. 3c and 3d) illustrate that there is no protein in the patterned holes. Section analysis in figure 3e revealed that the BSA height was ~12.5 nm, which is approximately 5 times the height of a single monolayer of BSA (2.5 nm on mica surface).

3.2 Fibrinogen adsorption

The sample was placed in an external fluid cell and fibrinogen was adsorbed onto the stamped BSA layer to form a uniform protein layer. We investigated various concentrations of fibrinogen (250 µg/ml, 500 µg/ml and 1 mg/ml) and adsorption times (30 minutes and 1 hour) to determine parameters for reproducibly creating a smooth protein layer in which the protein patterns are not visible. This is important because the objective of the technique is to detect proteins without having topographical clues about the distribution of the different protein types. Figure 4 shows representative height and phase images obtained under aqueous buffer following fibrinogen adsorption for 1 hour from a 1 mg/ml solution. We found this time and concentration combination suitable for reproducibly generating smooth protein layers and this protocol was used in all subsequent images. The distribution of the two protein types are not distinguishable in either the height or the phase image thereby demonstrating the formation of a uniform layer with fibrinogen presumably located within the holes produced by micro-contact printing and the BSA surrounding these fibrinogen regions. This situation can be considered analogous to imaging of proteins on polymer biomaterial samples where individual proteins cannot be identified due to the density of the adsorbed proteins and the inherent roughness of the underlying substrate.

3.3 Nanogold labeling of fibrinogen

The Nanogold conjugate is functionalized with sulfo-N-hydroxysuccinimide ester having reactivity towards primary amines and therefore it can be covalently coupled to the antibodies against human fibrinogen by a simple protocol. In this study, we utilize the mechanical properties of the gold label to visualize binding of the antibody to the protein antigen of interest directly by AFM tapping mode phase imaging. The patterned protein substrates were washed thoroughly prior to addition of the nanogold conjugate to ensure that there is no free fibrinogen in solution that might react with the antibody and subsequently undergo nonspecific adsorption to the surface. Height and phase images shown in figure 5 illustrate that the protein patterns are visualized by tapping mode AFM after addition of conjugate. The nanogold-conjugated antibody binds to the surface-adsorbed fibrinogen and is identified from the phase angle shift induced by the presence of the hard nanogold particles. The data shows that the region containing fibrinogen and conjugated nanogold seems to present a lower phase angle than the BSA coated region. Generally, regions of increased hardness or modulus appear as bright spots in the phase image. However, it has been shown that phase images can be sensitive to the operating parameters including r_{sp} and the free amplitude of oscillation, particularly in a heterogeneous system having different compliances (Bar, et al., 1997). Phase images can even undergo contrast reversals as imaging parameters are changed, making it difficult to assign features in height and phase images to different components based on the direction of phase shift alone (Pickering and Vancso, 1998).

Section analysis performed on the topography image (Fig. 5c) showed a small decrease in the apparent height following conjugate binding although the roughness of the sample makes it difficult to measure the exact height difference. As the nanogold conjugates should only interact with fibrinogen on the sample, an increase in height in the fibrinogen regions on the sample is expected, which is contrary to what we observed. However, we have previously observed a “shadowing effect” in height images, it is simply an image artifact that arises from differences in the interactions of the AFM tip with the underlying substrate that changes the apparent height of the sample (see for example fig. 5 in (Agnihotri, et al., 2006)).

To confirm that this apparent loss of height in the fibrinogen regions was not a result of desorption of the fibrinogen during labeling, a dual protein layer was again prepared, washed with protein-free PBS for 1 hour at 1 ml/hour flow rate and imaged by tapping mode AFM. Height and phase images from these experiments are shown in figures 6a and 6b respectively. No patterns were observed in either the height or phase images, demonstrating that there is no detectable desorption of fibrinogen from the sample surface.

A second concern was that the anti-fibrinogen antibody was binding to the fibrinogen in the patterned regions and was somehow in itself responsible for the height and phase contrast. This would be expected to yield an increased height image, but nonetheless we tested the effects of adding unconjugated anti-fibrinogen antibodies to the images. Figures 6c and 6d show height and phase images of the dual protein layer after anti-fibrinogen antibody incubation. Again, tapping mode imaging did not show evidence of the patterns thereby demonstrating that the contrast seen in figure 5 arises from the conjugated anti-fibrinogen and is not an artifact of the labeling procedures.

Finally, control experiments were carried out to verify that there is no desorption or migration of BSA onto the regions where fibrinogen is expected to adsorb. A multilayer of BSA was stamped on mica and was monitored for 3 hours in a hydrated fluid cell. Figure 7 shows tapping mode images taken every hour and clearly shows an absence of marked migration of the BSA off the protein layer or into the holes, thereby demonstrating the stability of the BSA multilayer on mica substrate.

3.4 Nanogold labeling of protein patterns on PDMS

The efficacy of the nanogold technique was tested on a clinically relevant polymer, PDMS. PDMS is a relatively smooth homopolymer generally lacking any significant topographical features which might cause difficulty in producing/imaging of protein patterns. Moreover, glow discharge plasma cleaning can change the PDMS surface from hydrophobic to hydrophilic, which has been shown to be essential for proper stamping of proteins (Thibault, et al., 2005). The PDMS stamp was incubated with 0.1 and 1mg/ml of BSA for 1 hour (similar conditions for data shown in figs. 3a and 3b) and then stamped onto hydrophilic PDMS substrate. Tapping mode images in ambient conditions were not able to detect any pattern, most likely due to the inherent roughness of the polymer surfaces. Only upon increasing the concentration to 5mg/ml could a pattern be detected, as shown in figure 8. The dark regions are bare PDMS surface while the protein surrounding the regions is BSA. Fibrinogen was backfilled (1 mg/ml for 1 hour) to form a dual protein layer on PDMS. Figure 9 shows a distinct decrease in both the height (a) and the phase signal (b) between the BSA pattern and adsorbing fibrinogen after delivering fibrinogen (1 mg/ml) for 0.5 hour. After 1 hour incubation of fibrinogen, the two proteins were indistinguishable from one another in the phase image thereby forming a uniform protein layer on PDMS substrate (Figs. 9c and 9d).

The binary protein layer was incubated with conjugated nanogold solution in a manner similar to that for the mica substrate described earlier. The antibody binds to the fibrinogen in the pattern and is again identified using the phase lag produced by the hard gold labels with respect to the surrounding protein. In figure 10, the dark holes represent fibrinogen adsorbed on the PDMS substrate whereas the brighter regions represent the patterned BSA protein layer.

AFM methods typically utilize sub-monolayer distributions of a single protein type from dilute solutions onto model material surfaces. This situation lies far from the actual physiologic reality where all of the nearly 500 proteins present in plasma have the potential to adsorb onto rough polymeric biomaterial surfaces, and AFM identification of protein types at the molecular level is nearly impossible in such a system. We previously described an adhesion mapping method for protein identification in a 2-component protein film that was based on measuring the interaction forces between antibodies coupled to the end of an AFM probe and adsorbed proteins on the surface (Agnihotri and Siedlecki, 2005). Force curves obtained from the non-specific interactions between antibodies and BSA were distinctly different from the specific antibody-fibrinogen interactions and could be used to identify fibrinogen on mica substrate. While this adhesion mapping technique allowed for measurement of the distribution of one specific type of protein, the new method utilizing a gold-labeled antibody described in this work offers numerous advantages over the adhesion force technique used previously; data can be acquired at much higher resolution (512×512 pixels vs 32×32 pixels) and much faster acquisition times (≤ 8 minutes vs ~ 60 minutes for the adhesion technique) using standard AFM equipment. It is a direct imaging method and therefore it does not require the same complex statistical analyses that were necessary to interpret AFM force measurement images. Furthermore, using this technique in combination with various monoclonal antibodies could offer unique opportunities to address how the distribution of functionally active protein molecules affects subsequent biological functions, moving the field closer to single molecule immunochemistry techniques and providing much needed information on structure/function relationships in single proteins, and contributing to our knowledge of protein-surface interactions that are important in the biological response to implanted biomaterials.

4. CONCLUSIONS

Gold nanoprobe labels were conjugated to an anti-fibrinogen polyclonal antibody to produce a marker that identified fibrinogen distributed in 2-component protein films by AFM phase imaging. A patterned sample consisting of circular regions of fibrinogen dispersed in BSA was

prepared by micro-contact printing to test the specificity of the label. The data showed that the gold-conjugated labels identify the regions of the sample corresponding to fibrinogen with little-to-no nonspecific labeling seen. This labeling technique offers advantages over other single molecule AFM identification technique that are based on adhesion force measurements, including improved resolution and faster acquisition times, and could potentially be used to study competitive protein adsorption, to address conformation/functional changes in proteins on biomaterial surfaces and to study the composition and makeup of the adsorbed proteins layer in retrieved biomedical implants.

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Figure 1. AFM images of poly (urethane urea) (PUU) sample after incubation with phosphate buffer for 1 hour and fibrinogen (500 $\mu\text{g/ml}$) for 15 minutes: Height (a) and phase (b) images do not identify fibrinogen due to the inherent roughness of the biomaterial polymer surface.

Figure 2.

Schematic representation showing the procedure for preparation of patterned samples and application of the nanogold conjugate. A microtextured PDMS stamp is inked with BSA and placed into contact with mica or glow-discharge plasma-cleaned PDMS substrates (a) yielding a BSA pattern (b). Fibrinogen is added to produce a topographically uniform dual protein layer of BSA and human fibrinogen (c), which is then labeled by a gold-conjugated antibody and imaged by tapping mode AFM (d).

Figure 3.

AFM tapping mode images of a BSA pattern micro-contact printed onto mica: Height (a) and phase (b) images of a monolayer of BSA under ambient conditions. The circular holes seen in the images in ambient condition have a diameter of ~800 nm with the height of the protein layer ~2.5 nm; height (c) and phase (d) images of multilayer of stamped BSA in phosphate buffer saline. The BSA pattern is clearly visible. Enlargement of the pattern (diameter ~ 1100 nm) is from AFM imaging drift common in buffer conditions; section analysis (e) shows the height of the BSA multilayer to be ~ 12.5 nm.

Figure 4.

AFM tapping mode images of BSA-patterned mica substrate after human fibrinogen adsorption (1 mg/ml delivered through the fluid cell at the rate of 1 ml/hr for 1 hour): Height (a) and phase (b) images show that the micro-contact printed pattern is not visible after backfilling of the holes with fibrinogen, demonstrating the formation of a uniform layer of the two proteins.

Figure 5. Tapping mode images of patterned dual protein layer following Nanogold-antibody incubation (delivered to the fluid cell at a rate of 1 ml/hr for 1 hour): Height image (a) and phase image (b). The dark regions indicate the location of the conjugated Nanogold binding to fibrinogen while the bright regions indicate lack of binding to BSA.

Figure 6.

AFM images of control experiments: Height (a) and phase (b) images after washing of the patterned dual protein layer with PBS buffer for 1 hour. Height (c) and phase images (d) of the patterned dual protein layer following labeling with unconjugated anti-fibrinogen. Tapping mode imaging reveals that the pattern was not detected in these experiments, demonstrating that the phase differences seen previously arise from binding of the nanogold-antibody conjugates.

Height images

Figure 7. Sequential AFM tapping mode images of stamped multilayer of BSA on a mica substrate under PBS: Immediately after stamping on mica substrate (a), and after 1 hour (b), 2 hours (c) and 3 hours (d) of hydration. Images show that there is no migration of BSA from the patterns.

Figure 8. Tapping mode AFM images: BSA pattern stamped onto PDMS substrate imaged by height (a) and phase (b). The circular patterns (diameter ~800 nm) are PDMS substrate and the protein surrounding the holes is BSA.

Figure 9.

Height (a) and phase (b) images following fibrinogen adsorption for ½ hour demonstrate the filling of the circular pattern with fibrinogen. After 1 hour of fibrinogen adsorption, the pattern is still faintly seen in the height image (c) but undetectable in the phase image (d), demonstrating the formation of a uniform layer of two proteins.

Figure 10. Height (a) and phase (b) images after incubation with conjugated nanogold illustrate the fibrinogen pattern. The dotted squares in the figure highlight dark regions showing fibrinogen and the bright regions corresponding to BSA.