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Relating material surface heterogeneity to protein adsorption: the effect of annealing of micro-contact-printed OTS patterns

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Abstract

We have investigated the influence of micrometer- and sub-micrometer-scale surface heterogeneities in patterned octadecyltrichlorosilane (OTS) films on human serum albumin (HSA) adsorption and its spatial distribution. 5- μm -wide OTS patterns were created on glass substrates by micro-contact printing and in some instances subsequent annealing was used to alter OTS molecule distribution within the patterns. Scanning force microscopy (SFM), advancing water contact angles and water vapor condensation figures were used to characterize the OTS films and to assess the nature of the heterogeneities within the various surface areas. High-resolution fluorescence microscopy was used to record images of fluorescently labeled albumin on OTS patterned films and fluorescence intensity was quantified and converted into the adsorbed amount. Adsorbed albumin was also characterized through SFM measurements. Combined SFM topography and lateral force microscopy (LFM) imaging revealed that micro-contact printing of OTS onto glass both replicated the stamp pattern and created small islands within the non-stamped regions between the patterns. The OTS coverage within stamped regions was not fully continuous but improved with subsequent annealing. Annealing also resulted in OTS island growth within the non-stamped regions and decreased average wettability on both the stamped and non-stamped areas. The extent of albumin adsorption was not proportional to OTS coverage, but correlated with the sub- μm distribution of OTS chains. We inferred that the surface distribution of ligands such as OTS on a sub- μm length scale determines the nature of albumin adsorption and its kinetics.

Keywords

Surface heterogeneity; micro-contact printing; force microscopy; albumin adsorption; fluorescence microscopy

1. INTRODUCTION

Adsorption of proteins on biomaterials and model surfaces has been studied extensively in the past using a number of analytical techniques. A majority of these techniques, including ellipsometry, total internal reflection spectroscopy, surface plasmon resonance and Fourier transform infrared attenuated internal reflection, provided an averaged measure of protein adsorption on presumably uniform surfaces because of their inherent inability to spatially resolve the buildup of protein films at a sub-micrometer scale [1–4]. The majority of experiments conducted on protein adsorption to date fail to address the effects that adsorbent surface heterogeneities have on sub-micrometer scale protein 2D distribution in the adsorbed layer. To address this shortfall in the study of protein adsorption, scanning force microscopy

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(SFM) techniques, which provide nanometer-scale resolution surface information, have been used to investigate protein distribution on chemically heterogeneous substrates [5–8]. However, in order to resolve individual proteins, SFM requires ultra-flat substrates and has been shown to disrupt protein layers [6], especially in contact mode scanning. Similarly, scanning near-field optical microscopy, although it appears to be a promising method to monitor spatial protein distribution in protein layers [9], may also cause protein film damage. As a result of all these limitations, little has been done to show how micrometer and sub-micrometer substrate heterogeneities affect spatial and temporal protein adsorption patterns.

In the present study, we have combined SFM with high-resolution fluorescence microscopy (hrFM) to gain new information about adsorbent material heterogeneity effects on protein adsorption. Although limited by optical diffraction, hrFM can be used to monitor spatial and temporal protein adsorption on biomaterial surfaces both qualitatively and quantitatively [10–13].

In the past, self-assembled monolayers (SAMs) of organosilanes and alkanethiols have been frequently used as model surfaces to study the nature of protein adsorption on solid surfaces. The degree of organic molecules coverage on these surfaces and thus the surface wettability can be precisely controlled through a number of methods, as described in the literature [14, 15]. The hydrophobicity of SAMs has been known to control the amount of protein a modified substrate will adsorb [15,16]. SAM substrates can be readily and reproducibly fabricated with single or multiple types of organic molecules. A variant of SAMs with spatially controlled hydrophobicity is the so-called ‘wettability gradient surface’ in which organic molecule coverage changes along one of the surface linear dimensions [17]. Using octadecyltrichlorosilane (OTS) wettability gradient surfaces, we have shown previously that albumin adsorption increases with increasing surface coverage of OTS up to a certain limit: any increase of OTS surface coverage above 42% neither affected albumin adsorbed amount nor its adsorption kinetics from a dilute albumin solution [17].

Micro-contact printing (μ CP) is another well-known method to spatially control proteins on surfaces through creating SAM patterns [18–20]. μ CP methods have been refined to enable the formation of well-defined sub-micrometer scale OTS features [21,22] and the physical structure and organization of stamped OTS films have also been characterized [23–27].

The aim of this study was to investigate how heterogeneities and micro-gradients in a patterned OTS film directed the spatial distribution of adsorbed albumin. We used hrFM to monitor the course of human serum albumin adsorption on patterned OTS films with distinct differences in surface heterogeneities. The hrFM protein adsorption data were then matched and statistically correlated with the SFM-detected spatial distributions of OTS heterogeneity on the adsorbent material. Adsorbed albumin layers were also characterized with SFM techniques to confirm the results observed with hrFM measurements.

2. EXPERIMENTAL

2.1. Micro-contact printing

Poly(dimethylsiloxane) (PDMS) stamps and stamp pads were fabricated by curing Sylgard-184 (Dow Corning) prepolymer in silicon molds at an elevated temperature. The patterned mold used in these experiments consisted of a $3 \times 3 \text{ mm}^2$ patterned area composed of $5 \mu\text{m}$ wide, 1540 nm high, 3 mm long mesas separated by $5 \mu\text{m}$ wide troughs (Mikromasch, Tallinn, Estonia). Planar silicon surfaces were used to fabricate featureless surface stamp pads. All molds and glass substrates used for OTS modification as well as glass surfaces used as controls were cleaned in a ‘piranha’ ($\text{H}_2\text{O}_2/\text{H}_2\text{SO}_4$, 1:2 (v/v)) mixture, rinsed several times with double-distilled deionized (DD) water, then dried with filtered nitrogen gas immediately prior to each

OTS reaction. A scanning force microscope (Explorer, Topometrix) was used to verify the faithful reproduction of the silicone mold topography on the PDMS stamps. OTS-patterned surfaces were created by a stamp pad method as described previously [21]. Briefly, 10 mM OTS in anhydrous n-hexane (Sigma) was applied to a PDMS stamp pad and allowed to evaporate and absorb into the pad. The stamp pad was then dried for 20 s with a stream of filtered nitrogen. A stamp was brought into contact with the stamp pad for 6 s using a stepping motor stage to enable the transfer of OTS molecules and a portion of any remaining solvent to the stamp. The stamp was finally brought into contact with a pre-cleaned glass coverslip (thickness #1) for 20 s.

Upon stamping, the OTS patterned substrates were either immediately rinsed with hexane to remove any non-reacted OTS and then sonicated in an ethanol/DD water (1:1 (v/v)) mixture or first annealed followed by hexane rinsing and ethanol/water sonication step. The annealing step consisted of placing the OTS-patterned substrate into a covered glass dish in an oven (VWR) for 20 s where the glass dish surface temperature was 122°C and the oven air temperature was 95°C. Positive controls of both non-annealed and annealed OTS films were produced using smooth, planar stamps. Clean glass surfaces served as negative controls.

2.2. OTS pattern characterization

2.2.1. Water contact angle—Advancing water contact angles were measured on the OTS-patterned substrates and on all control surfaces with a goniometer (Rame-Hart, A-100) equipped with a video camera (XC-73, Sony) and a data acquisition system (Power Macintosh 7100 with LG-3 frame grabber and Image software, Scion) using the sessile drop method. The OTS-patterned substrates were positioned with the OTS bands facing either in parallel or perpendicular direction with respect to the goniometer's optical axis. A 0.25 μ l droplet of deionized and double-distilled water ($\gamma = 72$ mN/m at 20°C) was carefully placed with a microsyringe (Hamilton) on the surface and immediately measured. The procedure was repeated at least 10 times on each sample.

The Cassie equation [28] was used to find the average wettability and fraction of OTS coverage for both the stamped and non-stamped bands of the patterned samples and for the controls:

$$\cos\theta_{adv} = f\cos\theta_1 + (1 - f)\cos\theta_2, \quad (1)$$

where θ_{adv} is the experimentally observed advancing water contact angle, f is a fraction of the OTS modified surface with characteristic contact angle, θ_1 , and θ_2 is the characteristic contact angle for an unmodified glass surface. The parameters required for solving the Cassie equation were obtained from the advancing water contact angles measured parallel ($\theta_{adv||}$) and perpendicular ($\theta_{adv\perp}$) to the patterned OTS bands. A water contact angle of $\theta_{adv} = 115^\circ$ was measured on a complete OTS monolayer and $\theta_{adv} = 0^\circ$ on a cleaned glass surface. The OTS coverage in the stamped bands (f_{OTS-st}) was calculated from:

$$f_{OTS-st} = \frac{\cos\theta_{adv||} - \cos(0)}{\cos(115) - \cos(0)}. \quad (2)$$

An advancing water contact angle on the non-stamped bands (θ_{advnst}) was computed from:

$$\cos\theta_{advnst} = \left(\frac{\cos\theta_{adv\perp} - f_{OTSsw} \times \cos\theta_{adv||}}{1 - f_{OTSsw}} \right), \quad (3)$$

where the fractional OTS band width, f_{OTSsw} , was found from AFM topographical images of the OTS patterns. The OTS coverage in the non-stamped bands ($f_{\text{OTS-nst}}$) was then found as:

$$f_{\text{OTS-nst}} = \frac{\cos\theta_{\text{advnst}} - \cos(0)}{\cos(115) - \cos(0)}. \quad (4)$$

2.2.2. Scanning force microscopy—SFM cantilever tips (Veeco Metrology Group, ‘Ultrasharp’, nominal spring constant, $k = 0.5$ N/m, gold-coated S_3N_4 , V-shaped) were made hydrophobic using an adaptation of a previously described method [29]. Briefly, the probes were cleaned in an ice-chilled $\text{H}_2\text{O}_2/\text{H}_2\text{SO}_4$ (1:2 (v/v)) solution for 2 min, then serially rinsed with the following three solvents in the order DD water, ethanol and hexane. The chemical modification step was accomplished by placing the probes in a 10 mM OTS-hexane solution for 2 min followed by serial solvent rinsing in the order hexane, ethanol and DD water. The sessile drop method on the cantilever tip surface was used to verify the completion of the cleaning and OTS modification steps. The same SFM cantilever was used for all SFM measurements.

An SFM instrument (Explorer, Topometrix) was used to measure OTS and protein film topography using contact mode as well as to simultaneously map the friction forces using lateral force mode. Topometrix imaging software and SXM software (based on NIH Image software, S. Barrett, University of Liverpool) were subsequently used for image processing and to determine physical characteristics of the patterns such as film thickness, friction contrast and OTS island coverage within the non-stamped regions of the patterns. Best results were achieved by measuring OTS film topography and lateral force maps in air while adsorbed protein layers were measured in aqueous solution to avoid artifacts caused by drying the protein film.

2.2.3. Condensation figures—OTS-modified glass substrates were chilled and water vapor was quickly introduced to the substrate surfaces prior to taking bright field microscopy images using the microscope and CCD camera described in the FM measurements section below. Careful synchronization between water vapor introduction and image capture ensured imaging of surface-condensed water droplets within a fraction of a second of nucleation events.

2.3. Protein adsorption experiments

2.3.1. Protein labeling and purification—Human serum albumin (HSA; ICN, Fraction V, fatty acid free) was conjugated with Alexa Fluor 488 (af488, Molecular Probes) and the resulting labeled protein (af488-HSA) was isolated through gel-permeation chromatography and further purified through extensive dialysis. Thin layer chromatography was used to verify the separation of free dye from the labeled protein. UV-Vis spectrophotometry was used to determine the degree of labeling and protein concentration in the final af488-HSA solution [1]. The degree of HSA labeling was 0.83 mol af488 per mol HSA. The final af488-HSA solution was then diluted to a concentration of 5 $\mu\text{g}/\text{ml}$ in 10 mM phosphate-buffered saline (PBS, pH 7.4, ionic strength 0.165 M). Aliquots of the 5 $\mu\text{g}/\text{ml}$ af488-HSA stock solution were stored in small vials at -20°C and thawed immediately prior to each adsorption experiment. The same 5 $\mu\text{g}/\text{ml}$ af488-HSA stock solution was used for all adsorption experiments.

2.3.2. Flow cell configuration—The adsorption flow cell was designed to enable the flow of buffer and protein solutions across glass coverslip (thickness #1) surfaces while allowing real-time fluorescence imaging of protein adsorption on the OTS-modified glass coverslip surface facing the interior of the flow cell. The flow cell consisted of two aluminum blocks, which were used to clamp the OTS-modified glass coverslip and a glass slide together separated by a 305- μm -thick silicone rubber gasket. The flow cell was designed to have a microscope

objective accessible opening on the side of the OTS-modified coverslip. The flow cell was placed on the stage of an upright fluorescence microscope (Nikon Eclipse E400) and a syringe-pump was used to flow solutions through the flow cell and into a collection reservoir.

2.3.3. Adsorption experiment parameters—A vial of 5 $\mu\text{g/ml}$ af488-HSA solution was allowed to reach room temperature and was filtered with a 0.22 μm filter (Millipore) immediately before each adsorption experiment. The flow cell was initially filled with PBS and a dark count image was recorded of the sample surface. PBS in the inflow tubing was subsequently replaced with the af488-HSA solution immediately prior to beginning the adsorption experiment. Faster flow of protein solution and PBS were used during the first 30 s of adsorption and desorption processes to bring the solution to the observation area and thus limit the mixing and dilution effects. The flow was reduced to 0.1 ml/min thereafter. All experiments were conducted at room temperature.

2.3.4. Fluorescence imaging—Fluorescence images of adsorbed af488-HSA on the OTS-modified and clean coverslips were recorded using a 100 $\times$, NA = 1.25, oil immersion objective (Leitz) in the epifluorescence configuration. A 12-bit Pelletier-cooled CCD camera (MicroMax, Princeton Instruments) was used to obtain high-resolution images of the adsorbed af488-HSA layer. Neutral density filters were employed to attenuate the excitation light in order to minimize photobleaching of the labeled protein and an exposure time of 4 s was used to reduce the effects of fluctuations in the excitation light source (75 W Xenon lamp) intensity and position over short time periods. A set of excitation band-pass, dichroic mirror and emission band-pass filters (Omega) was used to separate the excitation and emission photons.

2.4. Fluorescence image processing and analysis

Each fluorescence image was corrected for long-term variations in the excitation light intensity due to Xenon arc-lamp aging and changes in the microscope optical alignment, unevenness of the excitation illumination, and for fluorescence background originating from solution fluorescence, scattered light and electronic CCD readout noise according to the following equation:

$$I_{\text{Corr}}[x_i y_j] = k_{\text{LTVar}} \times \left\{ \left[\frac{(I_{\text{Meas}}[x_i y_j] - I_{\text{Dark}}[x_i y_j])}{(I_{\text{FlatF}}[x_i y_j] - I_{\text{Dark}}[x_i y_j])} \times k_{\text{FlatF}} \right] - k_{\text{Backgr}} \right\}, \quad (5)$$

where $I_{\text{Corr}}[x_i y_j]$ is the fluorescence image with the intensity corrected at any given pixel position, $I_{\text{Meas}}[x_i y_j]$ is the recorded fluorescence image, $I_{\text{Dark}}[x_i y_j]$ is the recorded dark count image, $I_{\text{FlatF}}[x_i y_j]$ is the recorded flat-field fluorescence image k_{LTVar} is the correction factor for long-term variations in excitation light normalized to the intensity of the background fluorescence caused by the af488-HSA solution in the flow cell under steady-state adsorption conditions, k_{FlatF} is the average pixel intensity of the flat-field image and k_{Backgr} is the average pixel intensity of the background protein solution fluorescence in the flow cell for the given experiment. Each flat-field fluorescence image was recorded using either a free af488 in PBS (for adsorption times less than 60 s) or a densely adsorbed layer of af488-HSA on a uniform OTS-treated glass coverslip (for adsorption times longer than 60 s), each taken immediately before the given experiment.

2.4.1. Conversion of fluorescence intensity into adsorbed protein surface density

—In fluorescence imaging of protein adsorption, the fluorescence intensity corrected at any given pixel position, $I_{\text{Corr}}[x_i y_j]$, is composed of the in-focus fluorescence emission from the adsorbed layer (I_{Ads}) and the out-of-focus fluorescence emanating from the protein solution in the flow cell (I_{Sol}). Since each of the two fluorescence contributions to the total fluorescence

intensity can be separately determined in experiments and the concentration of the af488-HSA solution, c_{Sol} , is known, the surface concentration of surface bound af488-HSA, Γ_{HSA} , can, in principle, be calculated by finding the optical relationship between the two fluorescence components, I_{Ads} and I_{Sol} . The conversion from I_{Ads} and I_{Sol} to Γ_{HSA} used in this study paralleled the quantification of protein adsorbed amount in total internal fluorescence microscopy [30,31] and will not be described in detail here (data not shown). The error in the measurement of the adsorbed HSA surface density was up to $\pm 3.5\%$ (estimated from the standard deviations of protein fluorescence intensity measured within OTS bands). A linear relationship between the measured fluorescence intensity and the adsorbed HSA surface density was assumed. This assumption was based on the previous quantification of fluorescein-labeled HSA adsorption using radiography and mass-transfer limited adsorption process [17, 32]. Alexa Fluor 488 dye is both brighter and more photostable than fluorescein; consequently, its very low photobleaching and relatively large Stokes shift (21 nm), together with the low degree of protein labeling, ensured that the self-quenching and photobleaching of af488-HSA conjugate in the surface layer was minimal.

2.4.2. Protein adsorption for SFM measurements—OTS-patterned glass substrates and substrates with control surfaces were placed in an adsorption chamber containing PBS, which was then exchanged with filtered 5 $\mu\text{g}/\text{ml}$ af488-HSA solution. The samples were simultaneously incubated in af488-HSA for 90 min at room temperature then rinsed with and stored in PBS until analyzed.

3. RESULTS

3.1. OTS pattern characterization

3.1.1. SFM topographical imaging of OTS patterns and controls—SFM images of the topography of various OTS patterned surfaces used in this study provided local information about OTS film thickness and distribution. Figure 1A and 1B displays the faithfulness of the reproduction of the OTS stamped pattern on the glass surfaces. Stamped bands in the non-annealed patterns (Fig. 1A) were $5.37 \pm 0.38 \mu\text{m}$ wide, while the annealing step increased average stamped band width to $5.92 \pm 0.3 \mu\text{m}$ (Fig. 1B). The average height of OTS patterns found in the stamped regions corresponded to a slightly less than reported monolayer thickness of 2.5 nm [23, 24], thus indicating that the stamped pattern might have been either compressed by the scanning SFM tip, or contained less than complete monolayer OTS coverage in these regions. Similarly to what was reported by others [23], the island formation within the non-stamped regions was also apparent and was found to increase with stamp contact time. Our initial hypothesis was that vapor phase transfer of OTS molecules from the stamp to the neighboring glass surface was responsible for the island formation. The contact time of 20 s was, therefore, selected to minimize island formation in non-stamped regions (Fig. 1). However, we also found that even with 20 s of stamp contact time a substantial quantity of non-reacted OTS remained in the stamped regions and that these molecules could: (a) either be quickly removed by solvent rinsing to produce a sharp step-function type of OTS pattern, or (b) be allowed to spread and react with the neighboring regions in a short annealing step. Using this second approach, we have combined μCP with a vapor diffusion-controlled silanization method [33], a combination that proved valuable in producing OTS films containing unique patterns of surface heterogeneities.

Migration of non-reacted OTS from the stamped regions could also occur by processes other than vapor phase transfer. For example, surface-diffusion could be responsible for the somewhat increased width of the stamped bands in annealed samples ($5.37 \pm 0.38 \mu\text{m}$ for non-annealed pattern width vs. $5.92 \pm 0.3 \mu\text{m}$ for annealed pattern) (Fig. 1). In some instances, the edges of the stamped bands in the annealed pattern are more than a monolayer thick, indicating

that some piling up of the OTS molecules at the edges has occurred during the annealing step (Fig. 1B). Height profiles of the two patterns also demonstrate the existence of narrow OTS concentration gradients between stamped and non-stamped regions. A number of large OTS islands in the non-stamped bands in non-annealed samples (Fig. 1A) could also have been formed by the ejection of small amounts of OTS during the stamping process.

3.1.2. Water contact angle measurements—Figure 2 shows the top view of the water sessile drop edges on the OTS pattern. Water protrudes into the more hydrophilic bands and recedes from the more hydrophobic regions creating an undulating pattern of the drop edge perpendicular to the orientation of the OTS bands (Fig. 2A). The water drop edges positioned parallel to the OTS bands stop on the boundary of a hydrophobic band forming a straight drop edge (Fig. 2B).

Table 1 lists the measured water contact angles for the various areas on the OTS pattern and on controls as well as the calculated average OTS coverage for each area. Contact angles observed parallel to the stamped bands were used to calculate OTS coverage within these regions according to equation (2). The measurements taken both parallel and perpendicular to the stamped bands were required to calculate these contact angles and the average OTS coverage within the non-stamped bands using equation (4). Due to the annealing process, the average OTS coverage increased from 43.2% to 75.9% in the stamped regions and from 14.4 % to 24.9% within the non-stamped bands. There was good agreement between calculated OTS coverage for the stamped OTS areas and the OTS controls both before and after annealing treatment indicating that the methodology developed to compute each OTS coverage (i.e., use of equations (2)–(4)) was reliable.

3.1.3. Condensation figures—Water vapor condensation figures have been demonstrated as a convenient method to visualize local differences in substrate wettability [34]. Images of water vapor condensation figures on non-annealed and annealed stamped patterns revealed the nature of heterogeneity within the OTS films (Fig. 3). Water droplets, seen under bright field illumination as darker spots in Fig. 3, readily nucleated on cooled OTS patterned surfaces and initially began to grow on both the non-stamped and stamped bands of the non-annealed OTS pattern (Fig. 3A). The most visible difference between non-annealed and annealed stamped regions is that fewer droplets nucleated on the stamped OTS regions in the annealed sample. Assuming that the defects in the OTS-stamped regions served as droplet nucleation centers, we inferred that the OTS-stamped regions contained a smaller number of defects after the annealing process, while the non-stamped regions in both the non-annealed and annealed samples contained, overall, a larger number of nucleation sites.

3.1.4. Lateral force microscopy imaging of OTS patterns and controls—Lateral force microscopy (LFM) has been used in the past to visualize the extent of surface chemical heterogeneity that often eludes mere SFM topography imaging [5]. As evidenced in Fig. 4, LFM provided higher resolution images of the OTS patterns and associated microstructures than those found in SFM topography images (Fig. 1). The brighter regions in the LFM images in Fig. 4 corresponded to areas of higher OTS coverage. A collection of closely packed, nearly equiaxed islands was observed in the OTS-stamped regions in the non-annealed sample indicating that the OTS reaction with glass during stamping proceeds through parallel nucleation and growth of many islands. A smaller number of similar size isolated islands was also found in the non-stamped regions. The annealing step appears to have coalesced the OTS island boundaries within the stamped regions but it also greatly increased the number of small OTS islands in the non-stamped bands (Fig. 4B). Interestingly, these islands did not grow in size upon annealing but remained similar in size to the islands found before the annealing process. A distinct, smaller population of larger OTS islands was found on both samples in the region between stamped and non-stamped bands. We conclude that the annealing process

affected the extent of surface micro-heterogeneities on the stamped and non-stamped regions differently: it improved the uniformity of stamped OTS regions, while at the same time it decreased the uniformity in the non-stamped regions (Fig. 4A).

3.2. Albumin adsorption

Monitoring protein adsorption as a function of time through high-resolution fluorescence imaging provided information about how the adsorption process was affected by the micro-heterogeneities in the OTS layer. Figure 5 displays six snapshots of af488-HSA accumulation on the annealed and non-annealed OTS patterns at six different adsorption times, 1.5, 4, 10, 15, 30 and 90 min. Based on the hrFM images, we found that the adsorption of af488-HSA proceeded at a faster rate and reached steady state sooner on the OTS stamped regions than on the non-stamped regions for both the annealed and non-annealed OTS patterns. In the case of the non-annealed pattern (Fig. 5A), af488-HSA adsorbed at a higher surface density on the less wettable OTS-stamped areas: Γ_{HSA} was $0.188 \mu\text{g}/\text{cm}^2$ on the areas with 43.3% average OTS coverage compared with $\Gamma_{\text{HSA}} = 0.165 \mu\text{g}/\text{cm}^2$ on the non-stamped regions where average OTS coverage was 14.4%. Somewhat surprisingly, in the case of the annealed OTS pattern (Fig. 5B), protein adsorption was greater on the non-stamped regions than on the stamped regions, as indicated by the fluorescence image of the af488-HSA adsorbed layer at 90 min of adsorption. A higher surface density of af488-HSA, $\Gamma_{\text{HSA}} = 0.220 \mu\text{g}/\text{cm}^2$, was found on the less wettable, non-stamped regions, where average OTS coverage was 24.9%, than on the stamped bands where Γ_{HSA} was $0.179 \mu\text{g}/\text{cm}^2$ (average OTS coverage 75.9%). The adsorption contrast between stamped and non-stamped bands for the annealed OTS pattern switched at about 12 minutes into the adsorption process.

Calculated af488-HSA coverage densities at 90 min of adsorption agree well with data published in the literature [17,32,35]. af488-HSA adsorption on the control OTS surfaces (i.e., on uniformly annealed and non-annealed OTS films on glass) were similar to those for the respective stamped areas of the OTS patterned surfaces, while the adsorbed amount of af488-HSA on the clean glass surface reached an average density of only $0.057 \mu\text{g}/\text{cm}^2$ after 90 min of adsorption. Although each sample was subjected to 90 min of desorption by PBS buffer, only a small fraction of bound af488-HSA desorbed. The average desorbed amount for the OTS-modified substrates was 4% of the total adsorbed amount and was never greater than 6% for any of the samples. The clean glass substrate experienced a 10% reduction in protein coverage after 90 min of PBS desorption.

3.3. Characterization of af488-HSA adsorption with SFM techniques

Topographical characteristics of all the samples were altered by af488-HSA adsorption. Figure 6 shows that af488-HSA adsorption on the non-annealed pattern increased the average height difference between the stamped and non-stamped bands from 1.65 ± 0.10 to 2.04 ± 0.15 nm, while on the annealed patterned surface the average height difference between stamped and non-stamped bands decreased from 1.84 ± 0.10 to 1.22 ± 0.20 nm after protein adsorption (Fig. 6A). The observed changes in the average height difference between stamped and non-stamped bands in the non-annealed and annealed patterns support the fluorescence data that protein preferentially adsorbed on the stamped areas in the non-annealed pattern and adsorbed in greater amounts on non-stamped areas in the annealed pattern. Interestingly, the adsorption process decreased the friction difference between the stamped and non-stamped bands in the non-annealed pattern indicating protein coverage in both areas, whereas little change in friction differences, if any, between the stamped and non-stamped bands was caused by HSA adsorption on the annealed pattern (Fig. 6B).

4. DISCUSSION

Based on OTS glass chemistry, we have fabricated and characterized model surface patterns designed to come in contact with complex biomacromolecules. Although the OTS patterns on these substrates appeared macroscopically homogeneous, they were found to contain chemical heterogeneities on a μm and sub- μm scale in both stamped and non-stamped regions. The OTS stamped regions and defects therein, concentration gradients between stamped and non-stamped bands, and island formation in non-stamped regions all provide interesting features to facilitate the investigation of the effects of surface micro-heterogeneity on protein adsorption.

Macroscopic techniques, such as advancing water contact angles, are typically used to characterize surface wettability; but they sample large areas and provide only an average measure of surface coverage of hydrophobic moieties (Fig. 2). Similarly, water condensation images (Fig. 3), while convenient as a means to estimate the number of defects in the OTS patterns, are not only indirect but also difficult to standardize. Almost all OTS surface features, however, could be imaged on a sub- μm scale using SFM. In particular, LFM has provided the highest resolution contrast in imaging OTS stamped regions, concentration gradients between stamped and non-stamped bands, defects in stamped bands and island formation in non-stamped regions (Fig. 4).

One aim of this study was to relate these small-scale surface heterogeneities with albumin adsorption. High-resolution fluorescence microscopy was selected as a method to simultaneously monitor protein adsorption kinetics on OTS patterns, as well as to monitor protein spatial-temporal distributions on a μm and sub- μm scale. But even in hrFM experiments, the adsorbed protein fluorescence is observed from many surface sites and is summed up to a composite fluorescence intensity measured at each pixel of the image recording device (Fig. 5). Hence, the analysis of the effects of surface micro-heterogeneity on protein adsorption is limited by the optical resolution of the hrFM. Protein adsorption kinetics can provide some estimate of the energy distribution of binding sites available to the molecule being adsorbed. Namely, higher affinity surface sites are expected to fill up with bound proteins faster than lower affinity sites, although albumin adsorption was probably initially transport-limited due to relatively low protein solution concentration. As a result, a cluster of high-affinity sites will lead to a faster increase of fluorescence intensity and, conversely, clusters of low-affinity binding sites will bind proteins more slowly.

Although it has been shown in the past that HSA preferentially adsorbs on non-polar, hydrophobic surfaces [36–38], there is also strong evidence that HSA will bind more readily and strongly to binding sites containing both hydrophobic and hydrophilic moieties and boundaries than either to hydrophobic solids or to surfaces with densely-packed hydrophobic molecules. Namely, it has been shown that the wetting transition zone of a carefully prepared wettability gradient surface will adsorb more HSA than more hydrophobic portions of the gradient [17,39]. It has also been shown that HSA molecules will preferentially adsorb on defect boundaries in organosilane films created on hydrophilic substrates [7,8]. Golander *et al.* postulated that HSA preferentially adsorbed on areas of intermediate organosilane coverage because of the amphiphilic and deformable nature of the HSA molecule [39]. Several studies have also shown that organosilane films of intermediate coverage irreversibly bind HSA with a higher affinity than more hydrophobic or hydrophilic areas indicating more favorable interactions between the alkyl chains and the HSA molecules [38–40]. In fact, it has been shown that an HSA molecule possesses several fatty-acid-binding sites, which can interact with alkyl chains [41]. Petrash *et al.* further postulated that HSA preferentially binds to substrates containing less densely packed alkanesilanes and at film boundaries because the chains in these

areas will have more freedom of movement which will enable an increased possibility for optimal interactions with HSA alkyl binding sites [40].

In the present study we observed the highest HSA adsorption on areas containing intermediate average OTS coverages of 24.9% (on non-stamped annealed bands, $\Gamma_{\text{HSA}} = 0.220 \mu\text{g}/\text{cm}^2$) and 43.3% (on stamped non-annealed band, $\Gamma_{\text{HSA}} = 0.188 \mu\text{g}/\text{cm}^2$) which corresponds well to previous findings [17]. The correlation between intermediate OTS coverage amounts and greater maximal HSA adsorption found in this study helps support evidence found by others [40] that greater adsorbed amounts may be due to surfaces where OTS molecules are less densely packed. The LFM maps (Fig. 4), however, provide more compelling evidence that increased HSA adsorption is not merely due to an average OTS packing, but rather to the surface heterogeneities present in the form of local OTS density distribution. In particular, HSA consistently adsorbed more readily on the non-stamped bands of the annealed patterns than on the stamped bands, despite the fact that the stamped bands had an average OTS surface coverage nearly three times greater than the non-stamped bands. We conclude, therefore, that the surface micro-heterogeneities in the OTS films prepared in this study have a significant impact on protein adsorption and that areas of intermediate coverage facilitate increased HSA adsorption when compared to densely packed hydrophobic organosilane films. Three different effects of local OTS concentration are quite interesting: (1) within the stamped OTS bands in the non-annealed samples one finds areas of lower fluorescence intensity, i.e., local areas of low HSA adsorption (Fig. 5A), (2) HSA adsorption on the OTS concentration gradients in the annealed OTS patterns increases faster than the adsorption on the neighboring stamped and non-stamped regions (Fig. 5B) and (3) within the non-stamped regions in the annealed samples one also finds darker spots, indicating local areas of low HSA adsorption (Fig. 5B). While the darker spots within the OTS-stamped regions (case 1) correspond to the level of af488-HSA adsorption which is comparable to that found on the non-stamped areas, similar darker spots within the non-stamped regions (case 3) seem to correspond to the af488-HSA adsorption on larger OTS islands and at the adsorption level found on the stamped areas.

It has been shown by others that albumins denature to a greater extent on more hydrophobic surfaces [6,42] and that initial binding rate increases with the ability of HSA to denature upon adsorption [43]. We postulate that strong hydrophobic interactions between a more densely-packed OTS region cause HSA molecules to denature upon adsorption in such a way that multiple surface binding sites are occupied. Because of the rapid depletion of these sites, one would expect to see albumin adsorption reach its steady state more quickly but at a lower adsorption level. We also postulate that areas containing mixed hydrophobic and hydrophilic sites bind protein at greater surface densities but more slowly due to the time required to find the best match between HSA alkyl binding sites and underlying substrate OTS chains. At very low OTS coverage, the OTS molecules are so distantly spaced that the number of OTS-HSA interactions is reduced so that adsorbed amount decreases with decreasing OTS coverage, i.e., with decreasing surface hydrophobicity.

5. CONCLUSIONS

In this study we have shown that high-resolution fluorescence microscopy is a viable technique to monitor protein adsorption and protein temporal-spatial changes down to a sub- μm length and a second time scale. By correlating hrFM data with OTS pattern properties, such as SFM-determined lateral force and topography, we have shown that heterogeneities in OTS films existing on the μm and sub- μm length scale have a significant effect on albumin adsorption kinetics, adsorbed amount, and its surface distribution. It is probable that the local OTS density distribution associated with heterogeneities found in the patterns created in this study and not mere hydrophobicity effects provides a more accurate model to predict the nature and extent of HSA adsorption on various areas on these substrates.

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(A)

(B)

Figure 1.

(A) SFM image of non-annealed OTS pattern topography. Brighter bands represent the stamped areas of the substrates (marked by an 'S') while non-stamped areas are darker (marked by an 'N'). An average height profile is presented in the panel above the image below. (B) SFM image of annealed OTS pattern topography. Brighter bands represent the stamped areas of the substrates (marked by an 'S') while non-stamped areas are darker (marked by an 'N'). An average height profile is presented in the panel above the image below.

Figure 2.

Top view of typical water-drop edges on OTS patterned substrates. (A) Water-drop edge positioned perpendicular to OTS bands reveals alternating hydrophobic and hydrophilic areas with a periodicity of approx. $10\ \mu\text{m}$. (B) Water-drop edges positioned parallel to the OTS bands stop on one of the hydrophobic bands forming a straight drop edge.

Figure 3. Water vapor condensation figures (water droplets appear as darker areas): S, stamped region; N, non-stamped region. (A) Non-annealed OTS pattern, (B) annealed OTS pattern. The lower contrast of the condensation image in the non-annealed pattern (A) is due to a lower density of nucleated droplets on the stamped OTS regions *versus* the non-stamped regions.

Figure 4. Lateral force microscopy images of OTS patterns: S, stamped, N, non-stamped regions. (A) Non-annealed pattern: the stamped areas are composed of many small islands and some islands exist in the non-stamped areas. (B) Annealed pattern: the stamped areas appear more continuous while considerable island formation has occurred within the non-stamped areas.

Figure 5.

Time-course fluorescence images of af488-HSA adsorption on OTS patterns. All images have the same intensity scale, i.e., the intensity is proportional to af488-HSA adsorbed amount. Lower left section of each image was processed (bas-relief) to enhance the details of the af488-HSA distribution. (A) Non-annealed OTS pattern, as evidenced by changes in fluorescence intensity af488-HSA initially adsorbed more quickly on the OTS-stamped regions. Heterogeneities in the stamped OTS regions result in spatial irregularities in protein adsorption: darker spots within the OTS-stamped regions correspond to the level of af488-HSA adsorption, comparable to that found on the non-stamped areas. (B) Annealed OTS pattern: af488-HSA adsorbs faster on the OTS-stamped region edges and reaches steady-state fluorescence on the OTS-stamped areas within minutes. Adsorption proceeded more slowly on the non-stamped regions but the adsorbed amount eventually exceeded that in the OTS-stamped regions: the reversal in the band brightness was seen starting at 12 min of adsorption time. Darker spots within the non-stamped regions correspond to the af488-HSA adsorption on larger OTS islands, which was found to be comparable to the adsorbed amount found on the stamped areas.

Figure 6.

Average SFM parameters before and after HSA adsorption. (A) Average height differences between stamped and non-stamped bands before and after protein adsorption. (B) Average friction force difference between stamped and non-stamped bands before and after protein adsorption. The friction force is not calibrated and is presented in AFM photodetector current units (nA). Error bars are ± 1 standard deviation.

Table 1

Advancing water contact angles ± 1 standard deviation on stamped and non-stamped regions of the patterned substrates and on controls, and calculated OTS coverage within each specified area

	Non-annealed		Annealed		Non-annealed (Control)	Annealed		Glass
	Stamped	Non-stamped	Stamped	Non-stamped		Control		
Contact angle (°)	67.4 ± 2.1	37.3 ^a	94.6 ± 2.3	49.8 ^a	67.5 ± 1.5	94.2 ± 1.9	0 ^b	
OTS coverage (%)	43.3	14.4	75.9	24.9	43.4	75.5	0	

^aContact angle values calculated from experimental data using the Cassie equation (equation (1)).

^bGlass coverslips completely wetted and so water contact angles could not be measured.