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Imaging Hydrated Albumin on a Polystyrene−Poly(methyl methacrylate) Blend Surface with X-ray Spectromicroscopy

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Human serum albumin (HSA) adsorbed to thin films of phase-segregated polystyrene (PS)−poly(methyl methacrylate) (PMMA) was examined under hydrated and dry environments with scanning transmission X-ray microscopy (STXM). Quantitative mapping of the protein and polymer components at 30 nm spatial resolution was achieved using near-edge X-ray absorption fine structure (NEXAFS) spectral contrast at the C 1s edge. Under fully hydrated conditions (0.005 mg/mL HSA), adsorbed HSA thicknesses in excess of its crystallographic dimensions suggest bilayer adsorption to the polar PMMA regions. Upon washing, these loosely bound protein molecules adsorbed to PMMA were removed. Upon drying, the thickness of HSA on the nonpolar PS region decreased by ∼40%, indicative of conformational changes. It is suggested that this change occurs due to the free energy gain from the ability of the protein to unfold on the less crowded PS surface.

1. Introduction

Biomaterials are increasingly produced with improved biocompatibility, stealth properties, and responsiveness.[1] However, nonspecific protein adsorption, which can initiate biofouling, still greatly impacts the performance of biomedical devices.[2] The ultimate goal of biomaterials research is the development of materials with the ability to control and evoke the correct biological response upon implantation within the human body or in blood-contact applications extracorporeally.[3] In order to achieve this goal, in-depth understanding of the interactions between proteins and surfaces is required.

Analytical microscopies have emerged as a superior means for detecting surface composition in two dimensions, chemically distinct regions, and lateral spatial localization of protein preferential adsorption sites. Advances in characterization methods for studying proteins at interfaces have mostly yielded techniques with either enhanced chemical sensitivity or high spatial resolution. For example, X-ray photoelectron spectroscopy (XPS)[4,5] and time-of-flight secondary ion mass spectrometry (TOF-SIMS)[6,7] offer quantifiable chemical analysis with limited spatial resolution (below 10 μm), while atomic force microscopy (AFM)[8,9] and transmission electron microscopy (TEM)[10] can be used to image with angstrom level spatial resolution but suffer from a lack of chemical recognition. Synchrotron-based scanning transmission X-ray microscopy (STXM) obtained via near edge X-ray absorption fine structure (NEXAFS) spectroscopy offers both quantitative chemical characterization and good spatial resolution (∼30 nm).

The presence of a hydrated environment is imperative for establishing meaningful conditions for proteins adsorbed at surfaces, since water is essential for forming the native protein structure.[11,12] These analytical studies should be performed with residual water/buffer, since dehydration can significantly change protein conformation.[13] Even lyophilization or freeze-drying methods used in ultrahigh vacuum (UHV) experiments have been shown to cause often-irreversible conformational changes.[14,15]

Synchrotron-based STXM is used to study human serum albumin (HSA) adsorption to a model biomaterial consisting of a thin film of phase segregated polystyrene−poly(methyl methacrylate) (PS−PMMA) under fully hydrated conditions. STXM spectromicroscopy offers both quantitative chemical characterization and good spatial resolution (∼30 nm). More importantly, since STXM is a photon-in, photon-out technique, fully hydrated samples can be examined, which is a significant advantage compared to alternative He, vacuum, or UHV techniques.[16] To our knowledge, this is the first report of the use of STXM and a wet cell to chemically map unlabeled HSA adsorbed to a polymer surface under fully hydrated conditions.

2. Experimental Section

Spun-cast films of PS−PMMA (25−40 nm thick) were floated onto X-ray transparent (75 nm) silicon nitride windows (Norcada Inc., Edmonton, AB). For the wet cell, 2 μL of 0.005 mg/mL HSA in DDI water was added to the surface and immediately another silicon nitride window was placed on top. Epoxy was used to seal the

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four edges, leaving a hydrated sample between the two windows (Supporting Information Figure S1). For the washed sample, 2 μL of 0.005 mg/mL HSA in DDI water was added to the surface of a PS−PMMA film floated onto a silicon nitride window. After 20 min, the sample was diluted three times with 10 μL of DDI water. After washing, the surface was immediately covered with another silicon nitride window and sealed with epoxy along all four sides. For the dry sample, a drop of 0.005 mg/mL HSA was placed on the surface of PS−PMMA, and HSA was allowed to interact for 20 min, followed by rinsing with DDI water and then left to air-dry.

All data were collected at the Advanced Light Source using the polymer STXM on beamline 5.3.2.[17] Monochromated soft X-rays from the bend magnet beamline are focused by a zone plate (Centre for X-ray Optics, 25 nm outer zone, 240 μm diameter) to a spot size of 30 nm, and the sample is (x,y) raster scanned through the zone plate focus while synchronously recording the transmitted X-rays using a phosphor (P31) to convert them to visible light pulses, which are converted to an electrical pulse by a photomultiplier tube (Hamamatsu) and counted. Typically, the spectral information which is the basis of the chemical analysis is collected as sequences of 25−100 images (stacks), which are aligned postacquisition (if needed) and converted from transmission to optical density scale using the incident flux spectrum (I_0) recorded through the wet cell or Si_3N_4 window (dry sample), off the polymer film. For studies of wet samples sandwiched between two Si_3N_4 windows, two stacks were recorded simultaneously with one stack located on the PS−PMMA film and protein solution (or water, in the washed case) and another stack in a region where there is protein solution (or water, for the washed cell) but no polymer. By setting the latter stack as I_0, the spectral contribution of the free HSA molecules in solution is removed. Thus, for the wet cells, the reported HSA thicknesses arise only from protein adsorbed on the polymer surface. The spectrum at each pixel of the image sequence is then fitted with reference spectra by means of singular value decomposition (SVD).[18,19] Since the reference spectra are set to the intensity response of 1 nm thickness of standard density of each material, the array of pixel-by-pixel fit coefficients for each chemical component (PS, PMMA, HSA) corresponds to a quantitative component map. The analysis is verified by applying a threshold mask to each component map to isolate pixels corresponding to the PS, PMMA, or interdomainal regions (Figure 1). The interdomainal region is the boundary between PS and PMMA domains. Previously, atomic force microscopy (AFM) revealed the presence of small microdomains[20] composed of the opposite polymer found within the discrete domains of PS and PMMA; however, many of these small domains are below the spatial resolution of STXM. Next, the average NEXAFS spectrum in each region was extracted and fitted with a linear combination of the PS, PMMA, and HSA reference spectra.[21] Several stacks were quantitatively analyzed to determine the uncertainty as the standard deviation (±5%) of the replicates of the fitting procedure. This uncertainty does not include any consideration of systematic errors nor does it represent the homogeneity of protein adsorption within each chemically specific region of the surface.

3. Results and Discussion

HSA (0.005 mg/mL) was adsorbed to a thin film of PS−PMMA sandwiched between two X-ray transparent silicon nitride windows

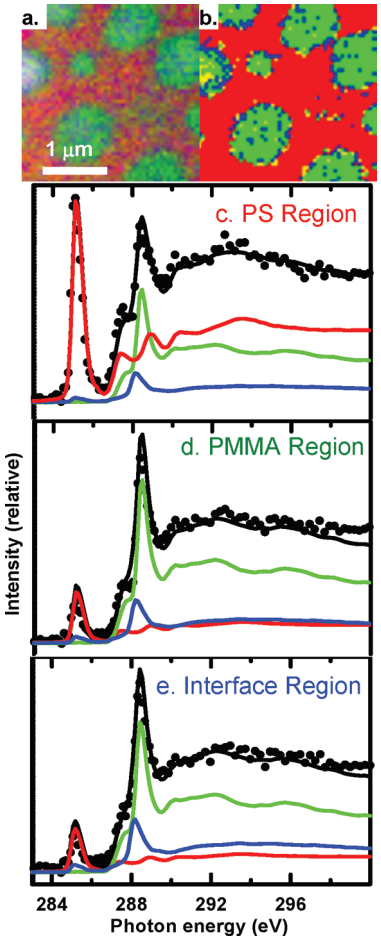

Figure 1. (a) STXM color coded composite map (red = PS, green = PMMA, blue = HSA). (b) Mask used to extract spectra of specific regions. Red denotes PS-rich regions; green denotes PMMA-rich regions, defined by threshold masking the PS and PMMA component maps with the blue being the remaining pixels which define areas with intermediate compositions representing the interdomainal regions between the PS-rich and PMMA-rich domains. (c) Curve fit of the average C 1s spectrum of the PS-rich region (data, dots; fit, black line; components, colored lines). (d) Curve fit of the average C 1s spectrum of the PMMA-rich region (same color coding). (e) Curve fit of the average C 1s spectrum of the interdomainal region (same color coding).

(Supporting Information Figure S1). We have previously characterized HSA adsorption to PS−PMMA at this concentration in the dry state.[21] Figure 2a−c displays the hydrated component maps of PS, PMMA, and HSA, with Figure 2d showing the color-coded rescaled overlap for the hydrated sample in red, green, and blue, respectively. The bright white pixels in Figure 2c correspond to areas high in HSA. There is a marked correlation between the locations of protein adsorption and the PMMA domains. Upon close inspection, a greater intensity of protein signal exists in the interdomainal region between PS and PMMA. As observed before for both STXM and X-ray photoemission electron microscopy (X-PEEM) studies of HSA/PS−PMMA[20−23] or HSA/PS-polylactide (PLA)[24] samples prepared by air-drying, the interdomainal region between the PS and

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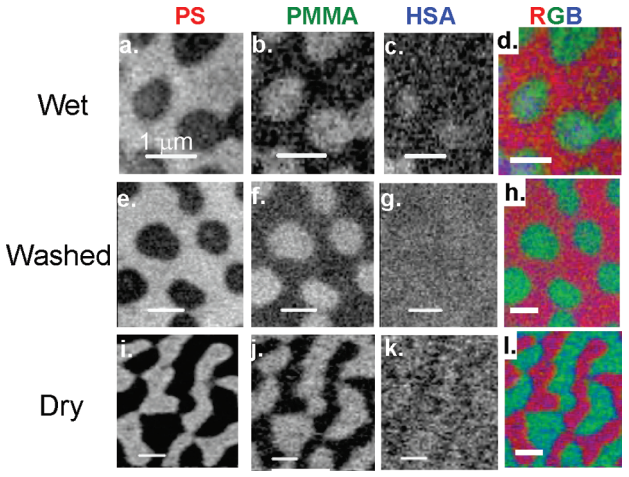

Figure 2. (top) Component maps derived from C 1s STXM image sequences. Fully hydrated wet cell of HSA adsorbed to a PS−PMMA thin film: (a) PS, (b) PMMA, (c) HSA, and (d) rescaled color composite map. (center) Component maps from a fully hydrated sample, but washed repeatedly with DDI water prior to sealing the wet cell: (e) PS, (f) PMMA, (g) HSA, and (h) rescaled color composite map. (bottom) Component maps from HSA adsorbed to PS−PMMA, washed with DDI water after 20 min exposure and air-dried: (i) PS, (j) PMMA, (k) HSA, and (l) rescaled color composite map. In each case, the color coding for the composite maps is: red, PS; green, PMMA; blue, HSA. HSA = 0.005 mg/mL.

PMMA (or PLA) domains is the preferred location for protein adsorption, and it is thus most likely the site of lowest free energy on the surface (although kinetic factors are also known to be involved in this system[22]).

Figure 2i−k shows comparative micrographs of the dry system. The rescaled color-coded map of the dry (Figure 2l) and wet (Figure 2d) sample reveals a much "pinker" map in the case of the dry sample, indicative of more protein on the PS region. Similar images were obtained for a fully hydrated sample washed with DDI water (Figure 2e−h), with a much "pinker" PS region compared to the hydrated unwashed system (Figure 2a−d), suggesting that a greater amount of protein is found on the PS matrix relative to the PMMA domains after washing.

Quantitative data (Table 1) show that under dehydrated conditions, protein adsorption follows the order: interdomainal > PS > PMMA, similar to previously published X-PEEM results for the same concentration of HSA adsorbed to PS−PMMA.[20] X-PEEM is a surface sensitive UHV technique which probes the top 10 nm of the surface, while STXM samples the amount of adsorbed protein against the entire film thickness. Hence, the percentages of adsorbed HSA are much higher in the X-PEEM data compared to the STXM data. More importantly, the percentages from the X-PEEM and STXM measurements cannot be compared directly due to the different sampling thicknesses; however, the relative amounts (ratios) of HSA adsorbed to the three chemically distinct surface chemistries (PS domains, PMMA-domains, and the interdomainal region) can be compared. These ratios are listed in Table 1 with the value for the amount on PMMA set to 1.0. The relative amounts of HSA on the three different regions of the surface are quite similar for the STXM and X-PEEM data on the dried film but very different for the hydrated sample, where there is much more HSA on the PMMA domains.

Quantitative results from the hydrated wet sample reveal apparent adsorbed protein thicknesses in excess of 10 nm on PMMA and interdomainal regions. Since HSA has crystallographic dimensions of 8 nm × 8 nm × 3 nm,[25] the STXM results suggest an end-on adsorbed orientation. However, both surface plasmon resonance (SPR)[26] and neutron reflection[27] studies of hydrated HSA and bovine serum albumin (BSA) adsorption to PS and silica, respectively, suggested a side-on adsorption orientation. At the low concentration used in these experiments, it is unlikely that HSA is sterically forced into an end-on adsorption orientation.[28] The STXM result of 10 nm HSA thickness on PMMA and at the interdomainal region may thus indicate a bilayer of HSA with side-on orientation in these regions.

In a hydrated environment, these results indicate that protein adsorption occurs preferentially on the hydrophobic polar domain (PMMA). Molecular dynamics (MD) simulations of a model protein (cytocrome c) adsorbed to alkane or hydroxyl-terminated self-assembled monolayers (SAMs) under hydrated conditions indicated this protein adopted a 9−10% larger conformation on polar compared to nonpolar surfaces.[29] Furthermore, an ∼25% increase in the adsorption of egg lysozyme protein on very polar ($-CF_3$ and $-OPh$) surfaces compared to nonpolar ($-CH_3$) or slightly polar ($-CN$) surfaces was observed under hydrated conditions using attenuated total reflection Fourier transform infrared (ATR/FTIR) spectroscopy.[30] The preference of lysozyme for polar surfaces was attributed to an interaction between the surface and polar protein side chains. A similar preference of HSA is apparent in our hydrated experiments.

Evidently, a large proportion of the < 10 nm thick adsorbed HSA layer on the PMMA and interdomainal regions of the unwashed hydrated system is due to loosely bound protein molecules. Upon washing with 30 μL of DDI water (∼10-fold dilution), the HSA thickness decreases by almost 50% on the PMMA and interdomainal regions. It seems likely that more loosely bound HSA molecules are removed with the vigorous washing/drying process used for the completely dry STXM and X-PEEM samples (∼100× dilution). Bilayers of loosely bound protein molecules are not found on the nonpolar PS region. With detected HSA thicknesses of ∼3−4 nm, adsorption clearly occurs as a side-on monolayer.

Under dry conditions, more HSA is adsorbed to the PS region than PMMA, resulting in a more "pink" color of the PS region in the qualitative analysis. However, the thickness of HSA decreases by > 40% when comparing the washed system with the dry, indicative of conformational changes. FTIR spectroscopy in the amide I region has shown that structural distortions result from protein dehydration.[31] In fact, it was found that the β-sheet content increased by 16% and the α-helix decreased by 28% upon lyophilization.[32] Although these experiments refer to freeze-dried proteins, it is possible that similar structural rearrangements occur from air-drying adsorbed proteins. Moreover, the extent of surface coverage (sparse/crowded) could affect the degree of conformational change (unfolding). With respect to the present work, the protein molecules adsorbed to the PS region are more likely to spread out in order to minimize the configurational free

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Table 1. Average Thickness of HSA (nm/Pixel and %) on the PS, PMMA, and PS/PMMA Interdomainal Regions (0.005 mg/mL HSA Solution; Uncertainty, 5%)

		hydrated (STXM)		washed (STXM)		dry (STXM)		dry (X-PEEM)
region	component	nm/pixel	%	nm/pixel	%	nm/pixel	%	%
PS	PS	19.5	50	16.7	51	13.9	60	65
	PMMA	14.5	37	11.5	35	6.6	28	14
	HSA	4.7	12	4.6	14	2.7	12	21
PMMA	PS	7.5	15	7.1	18	1.9	8	23
	PMMA	31.9	64	27.4	69	19.4	82	64
	HSA	10.6	21	5.4	14	2.3	10	13
interdomainal region	PS	4.3	10	9.2	26	4.6	18	38
	PMMA	27.6	66	19.5	55	16.1	63	37
	HSA	10.2	24	6.7	19	4.8	19	25
HSA ratios (PS/PMMA/interface)		0.4/1.0/1.0		1.1/1.0/1.2		1.2/1.0/1.9		1.6/1.0/1.9

energy following adsorption,[33] resulting in decreased protein thickness. Thus, we conclude that structural rearrangement and removal of loosely bound protein molecules accounts for the thickness variations between fully hydrated and completely dried layers of HSA adsorbed on PS−PMMA thin films.

By examining HSA adsorption under hydrated conditions with varying protein concentration, significant changes in adsorbed HSA thicknesses were observed, especially between the washed and unwashed system. This qualitative insight into the strength of HSA adsorption to inhomogeneous surfaces, especially in the unwashed case, gives valuable insight into in vivo adsorption conditions where the concentration of hydrated protein is considerably higher.

Conventionally, techniques used to probe protein adsorption with respect to protein site selectivity have been challenged by a lack of spatial resolution and the necessity for dry, UHV conditions. STXM is emerging as a powerful probe sensitive to spatial and chemical variability and capable of detecting minute changes in protein thickness and site preference under hydrated conditions. The latter is a key requirement for understanding protein surface interactions under real in vivo conditions.

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4. Conclusions

In summary, HSA adsorption to PS−PMMA thin films under hydrated and dried conditions revealed a preference for hydrophobic polar PMMA and hydrophobic nonpolar PS regions, respectively. The difference in preferential adsorption regions likely arises from the adsorption of bilayers on the hydrated PMMA domains. With washing, these bilayers are removed from the PMMA regions. Upon drying, conformational changes are detected on the PS region, with the detected HSA thickness decreasing by ~40%.

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Supporting Information Available: Wet cell schematic. This material is available free of charge via the Internet at http://pubs.acs.org.