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Protein Adsorption on Poly(*N*-isopropylacrylamide) Brushes: dependence on grafting density and chain collapse

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Abstract

The protein resistance of poly(*N*-isopropylacrylamide) brushes grafted from silicon wafers was investigated as a function of the chain molecular weight, grafting density, and temperature. Above the lower critical solution temperature (LCST) of 32°C, the collapse of the water swollen chains, determined by ellipsometry, depends on the grafting density and molecular weight. Ellipsometry, radio assay, and fluorescence imaging demonstrated that, below the lower critical solution temperature, the brushes repel protein as effectively as oligoethylene oxide terminated monolayers. Above 32°C, very low levels of protein adsorb on densely grafted brushes, and the amounts of adsorbed protein increase with decreasing brush grafting densities. Brushes that do not exhibit a collapse transition also bind protein, even though the chains remain extended above the LCST. These findings suggest possible mechanisms underlying protein interactions with end-grafted PNIPAM brushes.

Keywords

Poly(*N*-isopropylacrylamide); protein adsorption; smart materials; polymer brush

INTRODUCTION

Stimulus-responsive materials^{1, 2} such as the temperature-responsive polymer poly(*N*-isopropylacrylamide) (PNIPAM) are used in numerous applications, including biosensors,³ protein purification,^{4–11} drug delivery,^{12, 13} and tissue engineering.¹ PNIPAM, in particular, undergoes a solubility switch under mild conditions at its lower critical solution temperature (LCST) of 32 °C, such that the polymer swells in water below the LCST, but becomes less soluble and collapses above the LCST. With a transition near physiological temperature and at neural pH, PNIPAM has broad potential for applications in biotechnology and the clinic.

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Supporting Information Available: Supplemental Figures S1 of PNIPAM brush refractive index change and Figure S2 of protein adsorption measurement on PNIPAM and OEG samples using ellipsometry. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Cell-sheet engineering is a notable example.¹⁴ In this case, cells adhere to PNIPAM at 37°C, to form confluent cell sheets that can be detached intact, simply by decreasing the temperature, and without compromising the monolayer integrity.^{14–24} The assumption is that above the LCST, cells attach to protein adsorbed on the presumably collapsed, hydrophobic polymer. Below the LCST, the polymer is predicted to be more hydrophilic and to swell in water, causing protein lift-off and cell detachment.

Several applications are founded on the assumption that substantial changes in the interfacial properties of PNIPAM coatings associated with this collapse transition promote reversible protein adsorption. The collapsed chains presumably adsorb protein, whereas the water-swollen state below the LCST is assumed to repel protein. Nevertheless, an increasing number of reports suggest that PNIPAM brushes do not always exhibit a collapse transition above the LCST, and that proteins do not adsorb to all PNIPAM coatings. For example, PNIPAM spin-coated onto pre-conditioned silicon wafers did not adsorb human serum albumin, either above or below the LCST.²⁵ Cell attachment on PNIPAM hydrogels polymerized by electron beam irradiation on tissue culture polystyrene or on glass depends on the gel thickness, with thicker gels repelling cells above and below the LCST.^{26–28} Cell adhesion requires adsorbed protein. In the latter example, fibronectin adsorbed on thin (15–20nm) PNIPAM gels but not on thick (> 30nm) coatings, despite the 10 degree increase in the water contact angle on the dry, thick gels above the LCST.^{27, 28} On the other hand, plasma deposited PNIPAM coatings reversibly adsorb protein above the 32°C, regardless of film thickness.^{26, 29, 30}

Defining design rules for thermoresponsive coatings requires identifying key parameters governing the interfacial properties of PNIPAM and its interaction with proteins. This is challenging with some fabrication methods. However, chains grafted from immobilized initiators using atom transfer radical polymerization (ATRP) enable good control of grafting densities and molecular weights. This in turn facilitates investigations of the impact of these parameters on the thermally driven collapse transition, and on the mechanism and extent of protein adsorption. Prior studies with PNIPAM grafted from alkanethiols on gold showed that their temperature-dependent collapse transition depends on the polymer molecular weight and grafting density.^{31, 32} This intriguing finding suggested that some PNIPAM brushes might remain protein resistant above the LCST, and motivated the present investigation of the impact of grafting parameters on protein adsorption onto PNIPAM brushes.

Here, ATRP was used to generate controlled PNIPAM brushes. The results document the influence of brush architecture on i) the temperature-dependent collapse of PNIPAM brushes grafted from silicon wafers and ii) temperature-dependent protein adsorption onto those brushes. Ellipsometry, use of radio-isotope-labeled proteins, and fluorescence measurements in turn determined protein adsorption as a function of the brush architecture and temperature.

MATERIALS AND METHODS

Chemicals

N-isopropylacrylamide (NIPAM), and 1,1,4,7,7-pentamethyldiethylenetriamine (PMDETA) were purchased from Acros. NIPAM monomer was re-crystallized from hexane. 2-[methoxy(polyethyleneoxy)propyl]-trimethoxysilane (OEG) was from Gelest Inc. CuBr, methanol and anhydrous toluene were purchased from Aldrich. The initiator, 11-(2-Bromo-2-methyl)-propionyl undecyl trichlorosilane, was synthesized as described.³³ Chicken egg white lysozyme and human serum albumin (HSA) were purchased from Sigma

Aldrich. All aqueous solutions were prepared with Milli-Q purified water (Millipore, Bedford, MA) with a resistivity of 18.2 M Ω •cm.

Surface-Initiated Polymerization of *N*-Isopropylacrylamide Brushes

The poly(*N*-isopropylacrylamide) (PNIPAM) brush was synthesized by surface-initiated atom transfer radical polymerization (ATRP) of *N*-isopropylacrylamide from initiator functionalized silicon substrates, according to published procedures.³¹ First, silicon wafers were cleaned in piranha solution (25% (v/v) hydrogen peroxide and 75% (v/v) sulfuric acid) at 60 °C for 1 hr. (*Warning: piranha solution can react strongly with organic compounds. It should be handled with extreme caution and it should not be stored in closed containers.*) The silicon wafers were then rinsed with deionized water and dried under a stream of nitrogen. Next, self-assembled monolayers of the initiator (SAM-Br) and 2-[methoxy(polyethyleneoxy)propyl]-trimethoxysilane (SAM-OEG) were formed on the substrates by immersing the clean silicon wafers in a toluene solution containing 2 mM mixtures of the initiator and OEG for 4 hr. Substrates with different SAM-Br surface densities were prepared by adjusting the molar percentages of SAM-Br and SAM-OEG in solutions used to assemble the monolayers. After cleaning and drying, the substrates then were placed in a reaction vessel and degassed with three freeze-pump-thaw cycles. NIPAM (3.955g, 35 mmol) was dissolved in a mixture of MeOH/H₂O (v/v: 7/3) and degassed with nitrogen for 1 hr.³¹ The catalyst, CuBr (50.75 mg, 0.35 mmol), and the ligand, PMDETA (227.92 μ L, 1.05 mmol), were then added to the monomer solution. This mixture was transferred, via a cannula, into the reaction vessel containing the silicon wafers functionalized with only SAM-Br or with a SAM-Br:SAM-OEG mixture. The polymerization reaction was at room temperature under a nitrogen atmosphere, for specified times. After the reaction, the vessels were disconnected from the nitrogen line and the substrates were rinsed with methanol, followed by sonication in methanol, ethanol, and then water before drying under a stream of nitrogen.

Ellipsometric Determination of PNIPAM Brush Thickness

The thickness of the PNIPAM brushes was measured with a Gaertner ellipsometer (Model L116C) and LGEMP software (Gaertner Scientific Corp, USA). The incident angle of 632.8nm light was 70° in all experiments. The refractive index and extinction coefficient of the clean silicon wafer were 3.85 and -0.02, respectively. The PNIPAM brush thickness on the silicon wafer was obtained by fitting the data to a single-layer box model. The native oxide thickness was subtracted to give the monolayer thickness. For thickness measurements in air, a refractive index of 1.46 was used for the organic layer. Three measurements were done at each of six different locations on each sample. A custom-built environmental cell was used to vary the temperature, in order to study the temperature response of the PNIPAM brushes in water. The temperature was increased at a rate of ~0.5°C/min. Given the rapid polymer response to temperature changes,^{34, 35} this should be sufficient to enable brush equilibration during the heating cycle. Thicknesses and refractive indices of the water swollen PNIPAM films were also determined by ellipsometry, as a function of temperature.

Contact Angle Measurement

The advancing water contact angle on the different dry PNIPAM brushes was measured with a Ramè-Hart goniometer (Model 100-00) in a temperature-controlled room ($\pm 0.2^\circ\text{C}$). The advancing static water contact angles were measured for each water droplet at room temperatures and at 37 °C. Measurements were taken from several different positions on each substrate, and the average of these values was determined.

X-Ray Photoelectron Spectroscopy (XPS)

The surface chemical composition was determined by XPS, using a Kratos Axis ULTRA spectrometer with monochromatic Al K α radiation at 1486.6 eV (225W, 40 eV pass energy) at a pressure of 10⁻⁹ Torr. The samples were loaded on a rectangular metal support, using double-sided adhesive tape. The C 1s hydrocarbon peak at 285.0 eV was used as the reference peak for all binding energy measurements. Surface chemical compositions were obtained from the integrated area under the peaks in the XPS spectra, normalized with the appropriate sensitivity factors.

Ellipsometric Determination of Adsorbed Protein on PNIPAM Brushes

The proteins investigated were chicken egg white lysozyme (Sigma Aldrich - L6876) and human serum albumin (HSA, Sigma Aldrich - A9511). Lysozyme is small and positively charged at neutral pH (pI = 10.9), while HSA is a larger, negatively charged protein (pI = 4.4), and the predominant globular protein in serum. The proteins were dissolved in phosphate buffered saline (pH 7.0, ionic strength 0.1).

The instrument used for these studies is a Horiba Jobin–Yvon MM-16 spectroscopic phase modulated ellipsometer. The light sources are halogen and LED lamps with the wavelength range 430–850 nm. Measurements were performed near the Brewster angle at an incident angle of 70°. All measurements were obtained over the entire spectral range.

The samples were contained in a home built cell used for *in situ* studies of protein adsorption at solid/liquid interfaces. The incident light enters the liquid via a thin glass window, reflects from the solid/liquid interface, and is detected after passing through another thin glass window. The temperature is regulated by a thermostatic water bath that circulates water at the base of the cell. The lid consists of poly(tetrafluoroethylene).

The adsorbed amount of protein Γ (mg/m²) was determined from:³⁶

$$\Gamma = \frac{h(n - n_0)}{10(dn/dc)}$$

where h is the thickness (in Å) of the layer (box) in which the protein is adsorbed, n_0 is the refractive index of the medium in the thin layer before protein insertion, n is the refractive index of the medium with adsorbed protein, and dn/dc is the refractive index increment of the protein (mL/g). In the case of protein insertion into the brush, h is the brush thickness, n_0 is the refractive index of the brush without protein and n is the refraction of the brush with adsorbed protein. Both for the lysozyme and HSA, dn/dc is about 0.19 mL/g.³⁷

A 1 mg/mL or 3.5 mg/mL solution of lysozyme was injected into the cell containing the PNIPAM brush. The PNIPAM samples were immersed into the protein solution, order to avoid flushing, which could disturb the brush and prevent weak protein adsorption. The PNIPAM brushes were first incubated with the protein solution at a given temperature (25°C or 37°C) for up to 5 hr to equilibrate the system, and then the solution was heated to 37°C or cooled to 25°C, respectively. The protein absorption onto the PNIPAM brushes was monitored as a function of the temperature change.

Radio-assays of Adsorbed Protein

To detect protein levels below the detection limit of ellipsometry, BSA adsorption on PNIPAM brushes was quantified with 125-iodine-labeled protein. BSA was iodinated with Iodobeads (Pierce) and carrier free Na¹²⁵I (Perkin Elmer). The labeled protein was

desalted with a PD-10 column (GE Healthcare Bioscience) to remove unbound ^{125}I , and the determined specific activity of labeled protein was 25–30 cpm/ng.

Replicate ($N = 4$) samples were affixed with SecureSeal™ Imaging Spacers (Grace Biolabs) with a 13mm circular cutout. This constrained the sample within a well-defined area. The sample was then overlaid with 200 μL of labeled BSA (1mg/ml). The incubation was carried out for 2hr at 23°C (room temperature) or at 37°C in an incubator. After the incubation, the solution was gently aspirated, and the samples were rinsed ten times by displacement with 100 μL buffer that was maintained at the incubation temperature. That is, for samples heated to 37°C, the buffer and sample were maintained at 37°C during all rinsing steps, to prevent brush reswelling. Substrates were placed in vials containing 5ml of scintillation cocktail and the counts were recorded with an LS 6500 scintillation counter (Beckman Instruments) with specified settings for 125-iodine detection. Control measurements were with oligoethylene oxide terminated silane monolayers. Each set of measurements was repeated to insure reproducibility.

Patterned PNIPAM Brush Formation

To visualize temperature-dependent differences in protein adsorption on the PNIPAM brushes above and below the LCST, patterns of the initiator were microcontact printed on clean glass slides. For comparison, we similarly patterned OEG-SAMs. A second, positive control used octadecyl trimethoxy silane (OTS) patterns with the non-patterned region backfilled with OEG-SAM. Stamps were fabricated by casting a mixture of poly(dimethyl siloxane) (PDMS) prepolymer and curing agent (v/v: 10/1) onto the silicon master that was photolithographically patterned. The PDMS was cured overnight at 65°C.

Silicon (111) wafers and glass substrates were cleaned in piranha solution for 1 hr. After rinsing with excess ultra pure water (18.2 M $\Omega\cdot\text{cm}$), substrates were dried with a stream of nitrogen. Silicon wafers were used for reference thickness measurements of patterned PNIPAM brushes. Glass slides were used for obtaining fluorescence microscopy images of PNIPAM patterns, after incubation with fluorescein isothiocyanate (FITC) labeled albumin. The image was obtained with a Zeiss Axiovert 200 M fluorescence microscope (Thornwood, NY).

The PDMS stamp was inked with a 10 mM solution of the initiator in hexane and dried with a stream of nitrogen for 3 seconds. The inked PDMS was brought contact with a clean silicon wafer (or glass) for 10 seconds. Subsequently, the initiator-printed surface was washed with ethanol and dried with nitrogen. For backfilled samples, the unprinted regions were coated with OEG silane by immersing the substrates in a 2 mM toluene solution of OEG silane for 1 hr at room temperature. Finally, surfaces were washed with toluene, ethanol, and then deionized water, before drying with nitrogen. Patterned PNIPAM brushes were grown from patterns of SAM-Br by following the same procedure as the non-patterned PNIPAM brushes, except that the polymerization took place for 2 hr.

Statistical Analyses

The student's t-test was used for pairwise comparisons of data.³⁸ The difference between two measurements was considered to be statistically significant, if $p < 0.05$.

RESULTS

Initiator SAMs and PNIPAM Brush Synthesis

Self-assembled monolayers of the initiator (SAM-Br) and OEG (SAM-OEG) were first characterized by ellipsometry. The measured values for the SAM-Br and SAM-OEG

monolayer are $18 \pm 2 \text{ \AA}$ and $12 \pm 1 \text{ \AA}$ respectively, and compare well with prior studies.^{39, 40}

To control the initiator coverage, SAMs were prepared from mixtures of initiator silane and OEG silane. The surface composition of the mixed initiator:OEG monolayers was determined by XPS (Figure 1). Table 1 shows the surface densities of the initiator ($\#/\text{nm}^2$) determined for the mixed-SAMs, as a function of the solution composition of the mixture from which the monolayers were assembled. The decreasing amount of initiator on mixed SAMs with decreasing percentage of initiator in solution demonstrates the ability to control the initiator surface density. The SAM-Br surface coverage does not depend linearly on the mole percent of the initiator in the initiator/OEG solutions. This may reflect differences in their relative diffusivity and reactivity.

PNIPAM brushes were then synthesized from surfaces that were functionalized with SAM-Br or mixed SAM-Br:SAM-OEG. A comparison of XPS spectra before and after polymerization in Figure 1 showed that the nitrogen peak appears and the bromide peak disappears after polymerization, which indicates that PNIPAM has been grafted on surface. Table 2 summarized the thicknesses of dry and water swollen PNIPAM brushes. The wet brush thicknesses were determined at 23°C and 37°C. The swelling ratio, which is the ratio of the hydrated PNIPAM thickness to dry PNIPAM brush thickness at 23°C, increases with decreasing grafting density (Table 2). This trend might be expected since the chains in more densely packed brushes are already highly stretched and should swell less upon full hydration. The decrease in refractive index (Figure S1a) with decreasing initiator density also agrees with the expected increase in the water content of the more dilute chains. In addition, from the estimated initiator densities and dry film thicknesses, the molecular

weight of the grafted PNIPAM was estimated with the formula $\sigma = \frac{h\rho N_A}{M_n}$. Here, σ is the chain density, determined from the surface density of initiator and chain initiation efficiency, h is the measured brush thickness ($\text{\AA}$), ρ is the density of dry NIPAM, N_A is Avogadro's number, and M_n is the number average molecular weight of the chains. The surface coverage σ and M_n (Table 3) were estimated from XPS data, the maximum packing density of 2.1 bromine initiator/ nm^2 , a monomer density of 0.95 mg/cm^3 , and a chain initiation efficiency of $\sim 10\%$, reported for polystyrene.^{41–43}

To determine whether the grafted polymers are in the brush regime, we calculated the distance between grafting sites s relative to the Flory radius $R_F = L \cdot n^{3/5}$,⁴⁴ assuming that water at 23°C is a good solvent for PNIPAM. Here L is the estimated monomer size ($\sim 3 \text{ \AA}$), and n is the polymerization degree. If $s \ll 2R_F$, then the chains form stretched brushes.⁴⁴ The values of R_F and distances between grafting sites $s = \sigma^{-1/2}$ are in Table 3. Based on these calculations, all PNIPAM preparations used here produced brushes.

A comparison of the distance between grafting sites relative to the protein dimensions also determined the feasibility of primary protein adsorption by penetrating the brush to adsorb on the underlying substrate.⁴⁵ The dimensions of lysozyme are $45 \times 35 \times 35 \text{ \AA}$ and those of HSA are $140 \times 40 \times 40 \text{ \AA}$, such that, even at the lowest grafting density, the chain density and molecular weight should be sufficient to repress primary adsorption.⁴⁵ Proteins could still penetrate the brush (ternary adsorption), but they would not likely adsorb to the underlying surface.

Temperature-Dependence of Advancing Water Contact Angles

The dry PNIPAM brushes were characterized with advancing water contact angle measurements at 37°C and at 23°C (Figure 2). Advancing water contact angles generally tracked with the extent of the brush collapse above 32°C. The difference in contact angles

above and below the LCST was greatest with the PNIPAM brush at the highest grafting density, and then decreased somewhat with decreasing grafting density. However, no change was observed with the most dilute brush ($0.08/\text{nm}^2$) or with the lower molecular weight PNIPAM synthesized at the intermediate initiator density ($0.09/\text{nm}^2$; Table 3).

Temperature-Dependence of Hydrated PNIPAM Brush Thickness

Figure 3 shows the measured thickness of the water swollen PNIPAM brushes at increasing temperatures. Two observations can be made. First, changes in the hydrated thicknesses similarly depended on the chain grafting density, when PNIPAM was synthesized under otherwise identical conditions. Different from the swelling ratios at 23°C , chains at the higher grafting densities exhibited the greatest decrease in brush thickness above 32°C . The thickness changes of the two most densely packed brushes were similar, and refractive index changes (Figure S1b) suggest a decrease in the water content of the brushes. There was no apparent change in the thickness of swollen chains grafted from the least dense initiator monolayers ($0.08/\text{nm}^2$). The initial thickness of PNIPAM at the intermediate grafting density ($0.09/\text{nm}^2$) was less than brushes grafted from initiator densities of $0.11/\text{nm}^2$ and $0.21/\text{nm}^2$, as expected, and the brush similarly collapsed above the LCST. PNIPAM grafted from SAM-Br at $0.09/\text{nm}^2$ appears to lie at a crossover between two regimes: namely, higher molecular weight chains ($\sim 85\text{kDa}$) collapsed above the LCST, but the thinner monolayers ($\sim 48\text{kDa}$) remained swollen above 32°C . This agrees with a prior report, in which the collapse of PNIPAM grafted from alkanethiols on gold depends on the grafting density and on the polymer molecular weight.³¹ Second, for the dense PNIPAM brush, the chain collapse was not abrupt, but exhibited a more gradual decrease over the temperature range of $\sim 26^\circ\text{C}$ to 32°C .

Ellipsometry Measurements of Adsorbed Protein on PNIPAM Brushes

Next, protein adsorption measurements determined whether the grafting density and molecular weight dependence of the chain collapse generate corresponding differences in protein adsorption. Control measurements with 100 mole% SAM-OEGs first confirmed that the underlying OEG monolayer is protein resistant. The SAM-OEG regions beneath the brushes should therefore repel the adsorption of both protein and PNIPAM. A total of 14 measurements were then carried out with PNIPAM brushes.

Figure 4 shows the difference in the ellipsometric angles ψ and Δ at 546 nm before and after incubation with the protein solution. The difference is maximal in this wavelength region. In all cases, there was no significant change in the ψ and Δ spectra before and after incubation with 1mg/mL lysozyme solution. Contrary to expectations,^{8, 10, 30} there was no detectable protein adsorption on any of the PNIPAM brushes studied at either 25°C or 37°C . Even with a higher lysozyme concentration (3.5 mg/mL), there were no significant changes in Ψ and Δ . Identical results were obtained with a 1 mg/mL solution of HSA. Two measurements with PNIPAM brushes grafted from dense initiator monolayers ($0.11/\text{nm}^2$) were carried out at initial temperatures of 25°C and at 37°C . In both cases, the thicknesses, refractive indices, and percentages of solvent in the brush were similar to the polymers in the absence of protein.

Simulations determined the instrument sensitivity to protein adsorption. Changes in ψ and Δ spectra were simulated for adsorbed protein amounts of 0, 0.5, 1.5, 2.5 and 3.5 mg/m^2 in the PNIPAM brush (Figure S2a). Spectral shifts at $\Gamma = 0.5\text{ mg/m}^2$ were barely detectable. However, the spectra are particularly sensitive to protein at $\Gamma > 1\text{ mg/m}^2$. At 1 mg/m^2 , ψ and Δ shift by, respectively 0.269° and 1.016° at 546 nm. These differences could be easily detected by ellipsometry. Comparison of these simulations with experimental data in Figure 4 indicates that any adsorbed protein is below 1 mg/m^2 , and supports the experimental

finding of negligible protein adsorption on both swollen and collapsed PNIPAM brushes, under the experimental conditions investigated.

Simulated ellipsometry data for protein adsorption onto the OEG monolayer at 0, 0.5, 1.5, and 2.5 mg/m² (Figure S2b) also confirmed that such measurements would be sensitive to protein adsorption, such that 0.1 mg/m² would generate detectable shifts in ψ and Δ of, respectively, 0.011° and 0.567° at 546 nm (Figure S2b). At 0.1 mg/m², the change in ψ is insignificant, while the change in Δ could be detected easily. Comparison with ellipsometry data indicates that any adsorbed protein on OEG must be $\Gamma < 0.1 \text{ mg/m}^2$.

¹²⁵I]-BSA Adsorption on PNIPAM Brushes

More sensitive radioisotope assays revealed subtle differences in the low protein adsorption on the different brushes. Results are summarized in Figure 5. At 23°C, the amount of adsorbed BSA on the PNIPAM brushes is comparable to the OEG control. Although the adsorbed amount of $20 \pm 3 \text{ ng/cm}^2$ on OEG-terminated SAMs is slightly higher than reported by Yu et al.,⁴⁶ it is comparable to similar reports of protein adsorption on ethylene oxide coated surfaces.^{47, 48}

At higher grafting density (0.11 chain/nm^2), the large decrease in the brush thickness at 37°C did not coincide with a corresponding increase in adsorbed protein. The slight measured increase was comparable to that on OEG-terminated films at 37°C and may reflect general, temperature-dependent changes in protein and surface solvation. Conversely, the amount of adsorbed BSA on chains at $0.08/\text{nm}^2$, which did not collapse at elevated temperatures, increased from $22 \pm 5 \text{ ng/cm}^2$ to $55 \pm 7 \text{ ng/cm}^2$. This is below the detection limit of ellipsometry. Interestingly, at the intermediate grafting density ($0.09/\text{nm}^2$), there was no statically significant difference in BSA adsorption on either high or low molecular weight chains, despite differences in the collapse behavior of the different brushes. In both cases, adsorbed BSA was greater than on OEG-terminated monolayers at 37°C, but less than on the more dilute brushes ($0.08/\text{nm}^2$). These findings indicate that protein adsorption on PNIPAM brushes above the LCST is low in all cases. However, observed differences in the amounts of bound protein correspond to decreasing chain density. Adsorption appears to be governed by the capacity of proteins to penetrate the brush and by temperature-dependent changes in segment-solute (protein) interactions, rather than on the chain configuration.

Protein Adsorption on Patterned PNIPAM Brushes

We further visualized the adsorption of fluorescently-tagged BSA onto patterned PNIPAM, above and below the LCST. Patterned PNIPAM brushes do not have the identical grafting density as non-patterned PNIPAM brushes, due to the difference in the SAM-Br preparation methods. Nevertheless, our measurement shows that the PNIPAM brush prepared on the microcontact-printed SAM-Br is around 30nm.

Images of printed OEG-SAM at 23°C and 37°C, are shown for comparison (Figure 6a,b). The black regions are the patterned OEG and the fluorescence is from protein adsorbed on the uncoated glass. The resulting images obtained with the PNIPAM brush are consistent with the ellipsometry studies of protein adsorption; namely, the polymer patches appear black in the fluorescence images (Figures 6c, d) at both 23°C and 37°C. The background fluorescence shows adsorbed protein on the unmodified glass slide, whereas there is no detectable fluorescence on the PNIPAM pattern at either temperature, similar to patterned SAM-OEG on glass (cf Figures 6a,b). If the regions surrounding the SAM-Br pattern were instead backfilled with SAM-OEG, prior to polymerization, then there little detectable fluorescence on the patterns and no contrast between the PNIPAM pattern and SAM-OEG background (not shown). However, fluorescence images obtained with the positive control,

comprising printed SAM-OTS with unprinted regions backfilled with SAM-OEG, show clear fluorescence on the OTS patterns, but no protein in SAM-OEG regions (Figures 6e, f). These results validate the ellipsometry data, and show that the PNIPAM brushes repressed protein adsorption, both above and below the LCST.

DISCUSSION

Prior findings by us and by others^{31, 49} showed that the thermally driven collapse of PNIPAM brushes depends on the grafting density and molecular weight. These results confirm prior findings,^{31, 49} but also show that this transition (or its absence) does not correlate with protein adsorption, under the conditions investigated. These results instead support an emerging view that the reversible, temperature-dependent adsorption and lift-off of proteins and cells from PNIPAM coatings depend on the film architecture.^{11, 25, 50}

With grafted polymers, the chain densities and molecular weights (inferred from thicknesses) affect protein adsorption modes, which may include primary adsorption to the underlying surface, secondary adsorption at the outer edge of the brush, or ternary adsorption, involving brush penetration.⁴⁵ In this study, the high grafting densities of the brushes would prevent primary adsorption. The most densely packed brushes also appear to suppress secondary and ternary adsorption. At lower grafting densities where BSA could penetrate the brush (ternary adsorption), adsorption increases above the LCST. This suggests the formation of more extensive, attractive protein-segment interactions, which would support increased adsorption.

These results and postulated adsorption mechanisms are consistent with reported cell attachment studies. Other studies did not focus on the impact of chain density or MW on the polymer collapse, as this study does. However, enhanced cell attachment on dilute (low grafting density) or thin, e.g. low molecular weight brushes above the LCST is consistent with greater ternary or even primary protein adsorption.⁴⁵

These studies used a model protein BSA to probe qualitative changes in the interfacial properties of PNIPAM brushes. Altered surface properties, reflected in altered BSA adsorption would similarly impact the adsorption of other proteins, including extracellular matrix proteins that support cell adhesion. Changes in PNIPAM surface properties will necessarily affect all protein adsorption, although the adsorbed amounts of different proteins will differ quantitatively, on account of the variety of shapes, sizes, charge, and melting temperatures which also contribute to protein-surface interactions.

The low protein adsorption on dense brushes differs from the increased protein adsorption on thick films reported by Yu et al.⁴⁶ However, these results agree with other reports showing negligible protein deposition and cell attachment on dense, high molecular weight PNIPAM brushes⁵⁰. The reason for the discrepancy with Yu et al.⁴⁶ is presently unclear, but direct comparisons are hindered by the absence of molecular weight or grafting density information in the latter report.

Brushes with high PNIPAM grafting densities and molecular weights do not support reversible cell attachment.^{50–52} The cell and protein resistance of dense, high molecular weight brushes above the LCST suggest that, despite the difference in water contact angles on dry PNIPAM brushes above the LCST, in water, the collapsed brush presents a hydrophilic, repulsive outer layer.

This outer, hydrated region was proposed previously to account for the unexpected resistance of some PNIPAM films to cell attachment above the LCST.^{27, 50} This structure is analogous in some ways to a predicted vertical phase separation, in which more dilute,

hydrated chains extend beyond a denser, collapsed core.^{22, 53–55} The increased refractive index (Figure S1a) indicates a less polar core, as does steroid and peptide partitioning into PNIPAM coated chromatographic packing materials, above the LCST.^{1, 5, 10, 11, 22, 52, 56–58} Force measurements^{31, 32} and neutron reflectivity⁵⁵ also support the existence of hydrated outer chains. Here, the resistance to protein adsorption is also consistent with the solution stability of PNIPAM particles above the cloud point that is attributed to sterically stabilized polymer aggregates, or mesoglobules.⁵⁹ Although the structure and hydration of the outer edge of the dense PNIPAM brushes would be difficult to characterize, the present findings indicate that this region remains hydrophilic and does not present hydrophobic, protein adhesive segments. Instead, greater protein adsorption occurs on more dilute brushes that allow partial penetration and more extensive protein-segment interactions.

CONCLUSIONS

These results demonstrate the ability to control the collapse of PNIPAM brushes above the lower critical solution temperature of 32°C. They also provide a plausible mechanistic understanding of protein interactions with end-grafted PNIPAM films. Adsorbed protein levels were below the detection limit of ellipsometry, and required the use of isotope labeled protein for quantification. Differences in the low levels of adsorbed protein depended on the grafting density and, in part, on molecular weight. Dense brushes prevent protein penetration, and protein also does not adsorb at the outer edge of dense brushes. However, proteins adsorb to more dilute brushes that allow proteins to penetrate the layer and interact more extensively with the grafted chains. These findings suggest that protein adsorption above the LCST is determined by the capacity of proteins to penetrate PNIPAM brush layers and interact extensively with the chains, rather than on the collapsed configuration of chains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. XPS spectra of a self-assembled monolayer of initiator silane on a silicon wafer before (a) and after (b) the polymerization of PNIPAM from the monolayer.

Figure 2.

Advancing water contact angle on dry PNIPAM brushes at 23°C and 37°C. The numbers below the graph indicate the estimated chain grafting densities and corresponding molecular weights. Here * indicates a significant difference between the values measured at 23°C and 37°C for each sample ($p < 0.001$), and ** indicates a significant difference between the water contact angle at 37°C on the low density brush (0.08 chains/nm² at 44kD or 0.09 chains/nm² at 48kD) relative to the other samples ($p < 0.01$).

Figure 3.

Measured changes in the ellipsometric thickness of PNIPAM brushes in pure water as a function of temperature. The curves correspond to different brush preparations: black squares ($0.08/\text{nm}^2$, 44kDa); open squares ($0.09/\text{nm}^2$, 48kDa); open circles ($0.09/\text{nm}^2$, 85kDa); black circles ($0.11/\text{nm}^2$, 126kDa); open triangles ($0.21/\text{nm}^2$, 101kDa).

Figure 4.

Difference in ellipsometric angles ψ (a) and Δ (b) determined with incident light at 546 nm, before and after immersion in a 1 mg/mL lysozyme solution. The dashed lines correspond to the simulated values for the adsorbed amount of 1 mg/m² on PNIPAM. The dotted line indicates the simulated shift in Δ for 0.1 mg/m² adsorbed protein on the OEG monolayer.

Figure 5. ^{125}I -BSA adsorption on PNIPAM brushes at 23°C and at 37°C. The polymer grafting density and molecular weight are indicated below the bars. Adsorption on the OEG-terminated alkylsilane monolayer is shown for comparison. Statistically significant differences ($p < 0.05$) are indicated.

Figure 6.

Fluorescence images of adsorbed FITC-BSA (1mg/mL in 0.01M PBS buffer) on different micropatterned surfaces at two different temperatures. (a) Microcontact-printed OEG silane (dark zigzag region) on a clean glass substrate at 37 °C and (b) at 23°C; (c) Microcontact-printed PNIPAM brush (dark zigzag region) on a clean glass substrate at 37 °C and (d) at 23°C; (e) Microcontact-printed OTS on clean glass substrate (bright zigzag), which was backfilled with OEG silane (dark region) at 37 °C and (f) at 23°C.

Surface Chemical Composition of Self-assembled Silane Monolayers on Silicon Wafers, Determined by XPS. The Initiator Coverage was Estimated from the Br/Si Ratios.

Table 1

Mole% of SAM-Br in solution of SAM-Br and SAM-OEG	O	C	Si	Br	Br/Si	SAM-Br Coverage	σ (#/nm ²)
100%	22.37	30.52	44.6	2.51	0.056	100%	2.1
80%	26.24	20.93	51.19	1.64	0.032	57.1%	1.1
50%	26.14	20.28	52.22	1.35	0.0259	46.3%	0.9
20%	26.28	23.46	49.21	1.06	0.0215	38.4%	0.8

Table 2

Determined Thicknesses of Dry and Water Swollen PNIPAM Brushes, after Subtracting the Alkane Monolayer Thickness.

Mole% Initiator in the mixture of OEG and initiator (polymerization time)	Initiator monolayer (Å)	Dry film thickness (Å) at 23°C	Wet film thickness (Å) at 23°C	Wet film thickness (Å) at 37°C	Swelling ratio at 23°C
100% (2 hr)	18 ± 1	707 ± 4	1043 ± 4	876 ± 16	1.5
100% (10 min)	18 ± 1	352 ± 2	668 ± 9	519 ± 3	1.9
80% (10 min)	16 ± 1	243 ± 1	652 ± 3	516 ± 3	2.7
50% (10 min)	16 ± 1	134 ± 1	594 ± 11	484 ± 3	4.4
50% (4min)	16 ± 1	76 ± 5	490 ± 20	491 ± 7	6.5
20% (10 min)	12 ± 1	62 ± 2	490 ± 40	488 ± 8	7.9

Table 3

Summary of Poly(*N*-isopropylacrylamide) Brush Parameters.

Mole% Initiator in mixture of OEG and initiator (reaction time)	Estimated grafting density (#/nm ²)	Estimated Molecular Weight (g/mol)	Degree of polymerization	R _F (Å)	s (Å)	s/(2R _F)	Configuration
100% (2hr)	0.21	202,000	1789	268	22	0.04	brush
100% (10min)	0.21	101,000	891	176	22	0.06	brush
80% (10min)	0.11	126,000	1118	202	30	0.07	brush
50% (10min)	0.09	85,000	753	159	33	0.10	brush
50% (4min)	0.09	48,000	427	113	33	0.15	brush
20% (10min)	0.08	44,000	392	107	35	0.16	brush