Temperature dependent activity and structure of adsorbed proteins on plasma polymerized *N*-isopropyl acrylamide

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Thorough studies of protein interactions with stimulus responsive polymers are necessary to provide a better understanding of their applications in biosensors and biomaterials. In this study, protein behavior on a thermoresponsive polymer surface, plasma polymerized *N*-isopropyl acrylamide (ppNIPAM), is investigated using multiple characterization techniques above and below its lower critical solution temperature (LCST). Protein adsorption and binding affinity are probed using radiolabeled proteins. Protein activity is estimated by measuring the immunological activity of an antibody adsorbed onto ppNIPAM using surface plasmon resonance. Conformation/orientation of the proteins is probed by time-of-flight secondary ion mass spectrometry (TOF-SIMS) and principal component analysis (PCA) of the TOF-SIMS data. In this work, we find that at low protein solution concentrations, ppNIPAM-treated surfaces are low fouling below the LCST, but protein retentive above it. The protein adsorption isotherms demonstrate that apparent affinity between soluble protein molecules and the ppNIPAM surface are an order of magnitude lower at room temperature than at 37 °C. Although direct protein desorption is not observed in our study when the surface temperature drops below the LCST, the binding affinity of surface adsorbed protein with ppNIPAM is reduced, as judged by a detergent elution test. Furthermore, we demonstrated that proteins adsorbed onto ppNIPAM are functionally active, but the activity is better preserved at room temperature than 37 °C. The temperature dependent difference in protein activity as well as TOF-SIMS and PCA study suggest that proteins take different conformations/orientations after adsorption on ppNIPAM above and below the LCST. © *2006 American Vacuum Society.*
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I. INTRODUCTION

Smart polymers that respond to environmental stimulations such as *p*H,[1] temperature,[2] ionic strength,[3] electrical potential,[4] magnetic field,[5] light[6] and sound[7] have recently attracted much interest for applications in biosensors, biocatalysis, controlled release, tissue engineering, actuators and basic biological research.[8] Thermosensitive poly(*N*-isopropyl acrylamide) (pNIPAM) is among the most extensively studied due to its reversibility, fast response, and mild switching conditions (physiological temperature and neutral *p*H). In an aqueous environment, pNIPAM is capable of transition between collapsed and swollen state depending on the solution temperature. When it is immobilized on a substrate, the chain conformational change leads to transitions in surface wettability[9,10] and chemistry.[11] Since both factors affect protein behavior on a surface,[12–14] it is expected that the amount of adsorbed proteins as well as the affinity, activity and conformation/orientation of the adsorbed protein layer will vary as the polymer is cycled through its lower critical solution temperature (LCST).

In the literature, protein interactions with pNIPAM-immobilized surfaces have primarily been studied by monitoring the amount of adsorbed protein as a function of temperature.[15–17] The general observation is that a pNIPAM surface adsorbs significantly more protein above its LCST than below it. Protein binding strength and reversible desorption are also discussed in some studies.[18–20] However, the activity and conformation/orientation of proteins adsorbed onto pNIPAM has not been systematically explored. Thus, a thorough examination of protein interactions with pNIPAM (including all the points mentioned above) is necessary to further advance the application of thermoresponsive polymers in protein-based sensors and devices. In addition, many mammalian cells interact with surfaces through the extracellular matrix (ECM),[21] which is a network of proteins that provides a supporting structure for cell adhesion. Therefore, understanding how proteins behave is instructive for cell-

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based applications utilizing temperature sensitive surfaces. To address this need, this study examines protein interactions with pNIPAM using multiple, complementary analytical techniques.

The pNIPAM surface deposition technique used in this study is plasma polymerization (ppNIPAM).[22] This technique is a one-step, solvent free and vapor-phase method for producing conformal, sterile, tightly adhering and ultrathin coatings on a variety of substrates, including those used in this study. Previously, we demonstrated that the transition of ppNIPAM occurs at $31-32$ °C,[11] with the surface mechanical properties, wettability and chemistry all changing in this temperature range. Furthermore, ppNIPAM stimulates different cell responses depending on the surface temperature, a property that may be useful for applications such as cellomic chips.[23] Here, we characterize protein adsorption and binding strength to ppNIPAM using radiolabeled protein adsorption and detergent elution. To study protein activity on the thermoresponsive polymer, we test the immunological activity of an adsorbed antibody using surface plasmon resonance (SPR).[24] Additionally, quantifying protein activity provides an indirect measure of the protein conformation/orientation on the surface. A comparison of protein conformation/orientation versus temperature is performed by analysis of time-of-flight secondary ion mass spectrometry (TOF-SIMS) spectra using principal component analysis (PCA). Our multi-technique analysis facilitates understanding of protein interaction with thermosensitive surfaces, which will help to guide the use of ppNIPAM for protein and cell-based applications.

II. MATERIALS AND METHODS

A. Sample preparation

Silicon wafers were obtained from Silicon Valley Microelectronics (San Jose, CA) and diced into $1 \text{ cm} \times 1 \text{ cm}$ squares. Poly(ethylene terephthalate) (PET) sheets were purchased from Fisher Scientific (Houston, TX) and cut into $0.8 \text{ cm} \times 0.8 \text{ cm}$ squares. Glass substrates for SPR were purchased from Schott Glass Technologies (Duryea, PA). All samples were cleaned by sonication in methylene chloride (Fisher Scientific, Houston, TX), acetone (Fisher Scientific, Houston, TX), methanol (Fisher Scientific, Houston, TX) and $18 \text{ m}\Omega$ de-ionized water (Millipore, Billerica, MA) twice each for 10 min. Surfaces were dried by nitrogen purge before plasma deposition. Radiolabeled protein adsorption was carried out using bare PET or ppNIPAM coated PET. SPR was performed on gold-coated glass substrates with or without a plasma film. TOF-SIMS spectra were obtained on ppNIPAM-treated silicon chips. NIPAM plasma treated PET, gold and silicon substrates demonstrated similar chemical composition by ESCA analysis.[22] The protein adsorption experiments were performed in a water bath and the SPR measurements were taken in a homemade housing with temperature controller. The temperature variation in all the experiments was less than 1 °C.

B. Plasma deposition

Plasma polymerization of NIPAM (Sigma-Aldrich, Milwaukee, WI) was carried out in a custom-built reactor using the protocol described earlier.[22] In brief, the powered electrode is connected to a 13.56 MHz radio frequency power source and a manual impedance matching network. The deposition process included an 80 W methane plasma deposition, followed by NIPAM plasma deposition with stepwise decreasing powers from 80 to 1 W with a processing pressure of 140 mTorr. The ppNIPAM-grafted surfaces were rinsed three times with cold de-ionized water before use to remove uncross-linked molecules.

Radio frequency glow discharge fluoropolymer was generated using a similar reactor to the one for NIPAM deposition.[25] Perfluoropropylene (C_3F_6) (Matheson Gas Products, Newark, CA) was used as the precursor. During deposition, a pressure of 150 mTorr and a flow rate of 4 sccm were maintained under 20 W power for 3 min to obtain the fluoropolymer coating. Both sides of the samples were treated at the same time by hanging the samples in the plasma reactor and the surfaces were rinsed three times with ethanol and dried before use.

C. Protein labeling and adsorption

^{125}I-labeled human fibrinogen (Enzyme Research Laboratories, South Bend, IN), bovine serum albumin (BSA) (Sigma-Aldrich, Milwaukee, WI) and polyclonal anti-horse ferritin antibody (anti-Fe Sigma-Aldrich, Milwaukee, WI) were prepared using the iodine monochloride (ICl) technique.[26] Briefly, equal molar ratios of ICl (Sigma-Aldrich, Milwaukee, WI) and fibrinogen or anti-ferritin antibody were mixed in the presence of 1 mCi of ^{125}I (Amersham Biosciences, Piscataway, NJ). For albumin, twofold molar excess of ICl over albumin was used in the mixture. Iodinated protein was separated from unreacted iodine by liquid chromatography (Bio-Rad Laboratories, Hercules, CA) at room temperature using citrate-phosphate buffered saline containing sodium azide (CPBSz, pH = 7.4).

Protein adsorption was performed using ^{125}I labeled proteins for 2 h at either room temperature (23 °C) or 37 °C using citrate-phosphate buffered saline containing sodium iodide and sodium azide (CPBSzI, pH = 7.4).[27,28] Radiolabeled proteins were added to unlabeled protein solutions to obtain a specific activity of 5 cpm/ng and protein concentrations two times as high as the desired final concentration (2 × solutions). One milliliter 2× radiolabeled protein solution was added to a sample immersed in 1 ml of buffer to achieve a final 1× concentration. After a 2 hour adsorption, the reaction was terminated by dilution displacement of the protein solution with CPBSzI buffer. The radioactivity was measured by a gamma radiation counter (Model 1185, TM Analytic, Elk Grove, IL). Amounts of adsorbed proteins were calculated from the retained radioactivity (corrected for background), the specific activity of the protein solution, and the surface area of the sample.

Fibrinogen adsorption as a function of temperature cycles was studied in two ways: (1) samples were incubated at room temperatures for 2 h, after which the temperature was raised to 37 °C and samples were allowed to soak for another 2 h; or (2) samples were incubated at 37 °C for 2 h, rinsed, and then incubated in buffer at room temperature for two hours. All samples were rinsed and counted afterwards to calculate the amount of adsorbed protein.

The protein adsorption isotherms were obtained by exposing samples to protein solutions of a series of concentrations. The amount of adsorbed protein (Γ) at each solution concentration (C) was calculated and fit to the Langmuir equation [Eq. (1)] using Microcal^{TM} Origin Software (Microcal Software Inc., Northampton, MA)

$$\Gamma = \Gamma_{max}kC/(1 + kC), \tag{1}$$

where Γ_{max} is the monolayer adsorption capacity and k is the Langmuir constant or association constant, which is a measure of the apparent affinity of the protein with the surface. All the data points were repeated with five replicates.

D. Protein elution by detergent

Elution tests were performed on samples with adsorbed radiolabeled proteins to compare protein-binding affinity on ppNIPAM as a function of temperature. Samples were first incubated in radioactive protein solution at 37 °C for 2 h at solution concentrations of 0.1, 0.05, and 0.03 mg/ml for BSA, anti-Fe and fibrinogen, respectively, as described in Sec. II C. After rinsing, half of the samples were directly counted to calculate the amount of protein on the surface without elution (R_0). The other half of the samples were transferred to a 2 ml solution of sodium dodecyl sulfate (SDS; Bio-Rad, Hercules, CA) /de-ionized water of various concentrations and soaked for two additional hours at either room temperature or 37 °C. Then, sample radioactivity was measured with a γ counter, and the amount of protein remaining on the surfaces following elution was calculated (R_f). Elutability of adsorbed protein was calculated from $100 \times (R_0 - R_f)/R_0$. Five replicates were used for each data point.

E. Surface plasmon resonance (SPR)

SPR spectroscopy was used to characterize the antibody activity at different temperatures after adsorption to ppNIPAM surfaces. SPR was performed with an instrument developed at the University of Washington.[29] Generic SPR chips were prepared by coating a glass substrate with an adhesion-promoting chromium layer (2 nm) and a surface-plasmon-active gold layer (50 nm) using electron beam evaporation under vacuum. To perform SPR experiments, a plasma-deposited or bare gold chip was attached to the base of the prism, and optical contact was established using a refractive index matching fluid (Cargille Laboratories, Cedar Grove, NJ). A dual-channel Teflon flow cell containing two independent parallel flow channels with small chambers was used to contain a liquid sample during the experiments. A

peristaltic pump (Ismatec, Glattbrugg, Switzerland) was used to deliver a liquid sample to the two chambers of the flow cell. The resonant wavelength was determined by means of an optical spectrograph (Ocean Optics, Dunedin, FL). To account for the effect of temperature on the signal shift, one channel in the dual channel system was used for protein flow, while the second was used as the reference channel with only buffer flowing in parallel. The wavelength shift from the protein adsorption was obtained by subtracting the signal from the reference channel from the protein flow channel. For experiments above the LCST, a warm air blower with temperature controller (Omega Engineering, Stamford, CT) and a custom-built plastic cage were used to heat and keep the SPR stage and all the solutions at 37 °C.

The immunological activity of antiferritin was determined as follows. The SPR signal was first stabilized in CPBSz solution. Then, an antiferritin solution (500 μg/ml at room temperature and 50 μg/ml at 37 °C) (Sigma-Aldrich, Milwaukee, WI) was flowed into the SPR cell for 20 min, followed by flushing with CPBSz solution for 10 min to remove reversibly bound antibodies. Afterwards, 1 mg/ml bovine serum albumin (BSA) in CPBSz was injected to block nonspecific binding sites on the surface. Next, 50 μg/ml ferritin (Sigma-Aldrich, Milwaukee, WI) in CPSBz solution was flowed over the sample for 15 min, followed by flushing with CPBSz. The antigen to antibody molar ratio was calculated from the SPR wavelength shift to estimate the antibody activity on the surface.[30] Nonspecific reaction was checked by replacing anti-ferritin antibody with a nonspecific antibody, goat IgG (Sigma-Aldrich, Milwaukee, WI) and repeating the above steps. To determine the statistical significance of differences observed between the samples, two-tailed student *t*-tests were performed, and each condition was repeated with five replicates.

F. Sample preparation for TOF-SIMS analysis

The ppNIPAM coated silicon chips were first incubated in antiferritin at room temperature or 37 °C for 2 h. As differing surface coverage would impact data analysis, the concentrations determined from the protein adsorption and SPR studies were used to obtain similar surface coverage of proteins. After rinsing with CPBSz buffer, they were further dip rinsed three times in pure water to remove buffer ions from the surfaces, as the presence of salts on the surface induces a matrix effect, cationizing the substrate and affecting the yield of ions from the surface.[31] The samples were then soaked in 0.1 wt% trehalose (Sigma-Aldrich, Milwaukee, WI) solution for 0.5 h at the same temperature as used for protein adsorption, followed by spin drying using a spin caster (Nuclear Corporation of America, Deville, NJ) at 4000 rpm for 20 s. The treatment with trehalose has been shown to protect the protein conformation in the high vacuum environment during TOF-SIMS analysis.[32]

TABLE I. Molecular weight, solution concentration and adsorption of bovine serum albumin (BSA), anti-ferritin antibody (Anti-Fe) and fibrinogen (Fg) on ppNIPAM coated PET and bare PET at both room temperature and 37 °C. The ppNIPAM surfaces are observed to be low fouling at room temperature but protein retentive at 37 °C. However, protein adsorption is high on PET at both temperatures.

		BSA	Anti-Fe	Fg
M.W. (kDa)		66	150	340
Concentration (mg/ml)		0.1	0.05	0.03
Adsorption	RT	17±9	18±4	20±7
on ppNIPAM (ng/cm^2)	37 °C	190±18	104±8	168±18
Adsorption	RT	265±17	146±4	424±38
on PET (ng/cm^2)	37 °C	188±22	133±10	278±18

G. Time-of-flight secondary ion mass spectrometry (TOF-SIMS) and principal component analysis (PCA)

TOF-SIMS data acquisition was performed using a PHI Model 7200 Physical Electronics instrument (PHI, Eden Prairie, MN) equipped with an 8 keV Cs$^+$ primary ion source, a reflectron time-of-flight mass analyzer, chevron-type multichannel plate (MCP), a time-to-digital converter, and a pulsed flood gun for charge neutralization. Positive secondary ion mass spectra were collected over a mass range from $m/z = 0 - 400$ and analyzed with PHI TOFPAK software. Negative ion TOF-SIMS spectra were not considered in this study due to their low information content for proteins.[33] The area of analysis for each spectrum was 100 μm $\times$ 100 μm, and the total ion dose used to acquire each spectrum was less than 2×10^{12} ions/cm^2. The mass resolution of the secondary ion peaks in the positive spectra was typically between 4000 and 6000. The ion beam was moved to a different spot on the sample for each spectrum. Positive spectra were calibrated using CH_3^+, $C_2H_3^+$, $C_3H_5^+$, $C_7H_7^+$ peaks before further analysis. Five samples were prepared for each sample type, with four to five spectra acquired on each sample.

TOF-SIMS spectra from proteins adsorbed onto ppNIPAM at different temperatures (room temperature or 37 °C) were compared using principal component analysis (PCA), which determines the linear combination of peaks that describe the majority of variation in a dataset (the PCs). Detailed discussion of PCA can be found in the work by Jackson[34] or Wold.[35] Briefly, all spectra were mean centered prior to PCA analysis. A limited peak set containing only molecular fragments previously identified with amino acids was generated from representative spectra of all sample types.[33] The peak areas for each spectrum were then normalized to the intensity of the sum of the selected peaks to account for fluctuations in secondary ion yield between different spectra. PCA was then used to analyze the positive TOF-SIMS spectra using the PLS Toolbox v. 2.0 (Eigenvector Research, Manson, WA) for MATLAB (the MathWorks, Inc., Natick, MA). From PCA, an output of both "scores" and "loadings" plots were obtained to compare the main differences of the samples and contribution of the chemistries to the difference. Although data were obtained up to m/z 400, only those masses in the range from $m/z = 0$ to 180 are shown in the loadings plot, as few peaks of interest were observed beyond this range in this study.

III. RESULTS AND DISCUSSION

A. Protein adsorption

To test the temperature response of the ppNIPAM films to protein adsorption, we quantitatively measured adsorption of ^{125}I-labeled bovine serum albumin (BSA), anti-horse ferritin antibody (Anti-Fe) and fibrinogen (Fg) of fixed concentrations at both room temperature (below the LCST) and 37 °C (above the LCST) during a 2 hour time period. The solution concentrations of BSA, anti-ferritin antibody and fibrinogen were 0.1, 0.05, and 0.03 mg/ml, respectively. These proteins and concentrations are commonly used in our laboratory to test the fouling properties of various surfaces.[36,37] These three proteins are representative of important components in blood plasma and they were selected for their distinct molecular weights (Table I) and shapes (globular and fibril). As summarized in Table I, less than 25 ng/cm^2 of BSA, IgG or fibrinogen adsorb on ppNIPAM surface at room temperature. At 37 °C, the amount of protein adsorption is observed to increase by an order of magnitude, which is a considerably larger increase than one would expect from a pure increase of temperature-dependent endothermic adsorption.[38] In addition, this drastic increase in protein adsorption as a function of temperature is only observed on the thermoresponsive ppNIPAM surfaces, not on the control PET substrates. Furthermore, the amount of protein adsorbed onto ppNIPAM at 37 °C is approximately equal to or slightly lower than that on the control PET, suggesting near monolayer protein coverage on ppNIPAM at 37 °C.

Having first determined the adsorption of protein on ppNIPAM films as a function of temperature, we next consider adsorption as a function of protein concentration. Isotherms resulting from adsorption of BSA, anti-Fe and Fg are presented in Fig. 1. As the protein concentrations span a wide range, the x axis is plotted on a log scale. Each isotherm shows a rapid increase in protein adsorption with the solution concentration before they eventually plateau. Thus, the experimental data are fitted to the Langmuir model,[39] shown as solid lines in Fig. 1. The corresponding monolayer adsorption capacity (Γ_{max}) and association constant (k) values from each isotherm are summarized in Table II.
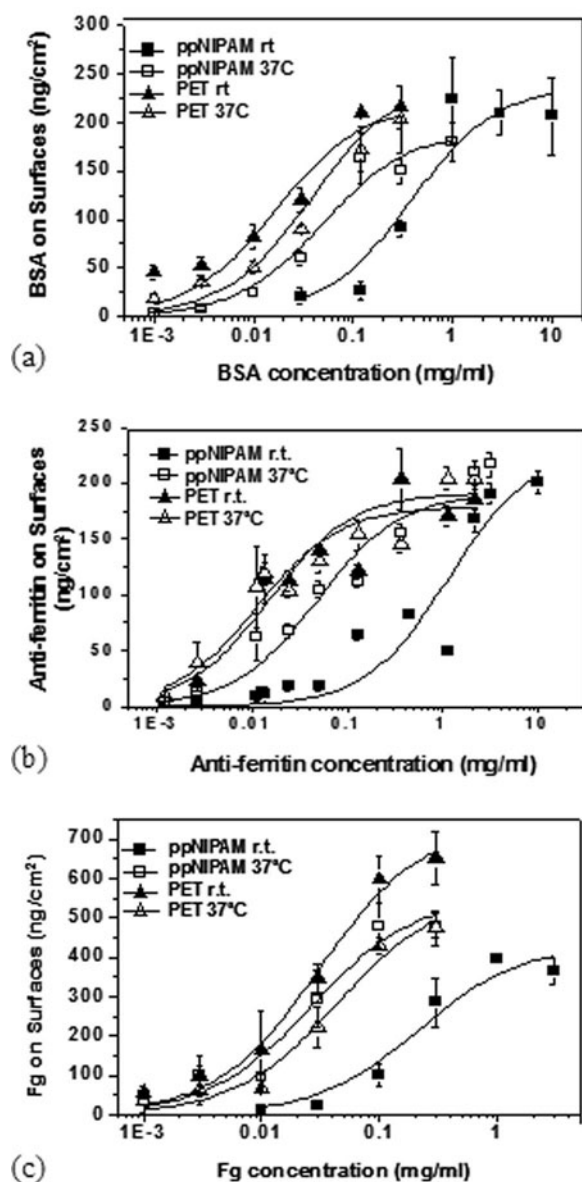
FIG. 1. Isotherms of bovine serum albumin (BSA) (a), anti-ferritin antibody (b) and fibrinogen (Fg) (c) adsorption on ppNIPAM (squares) and PET (triangles) at both room temperature (solid symbols) and 37 °C (open symbols). The data are fit to the Langmuir model, which is shown as the solid lines. Note that on PET, the adsorption isotherms for the three proteins at both temperatures are identical. In comparison, on ppNIPAM-treated PET, the adsorption isotherms at room temperature are shifted up by an order of magnitude compared with the isotherms at 37 °C.

The association constants of proteins with ppNIPAM at room temperature are observed to be an order of magnitude lower than those at 37 °C or on the control surfaces. This result indicates that ppNIPAM-treated surfaces have a much weaker apparent affinity with soluble proteins at room temperature. We consider the increase in protein association with a decrease in surface wettability a significant result. (In a previous publication, we demonstrated that ppNIPAM surfaces are slightly more hydrophobic at 37 °C.[11]) Furthermore, it has been noted widely that increased surface hydrophobicity promotes stronger protein adsorption from solution.[40–43] Thus it is expected as ppNIPAM surface wettability drops above the LCST, the apparent affinity of soluble protein with the surface increases.[44]

Also of note, we find that although the Γ_{max} values on PET and ppNIPAM are relatively similar for BSA and anti-ferritin antibody isotherms, those for fibrinogen adsorption vary with respect to surface and temperature. At 37 °C, the Γ_{max} values for fibrinogen on both PET and ppNIPAM surfaces are about 550 ng/cm^2. However, the Γ_{max} for fibrinogen at room temperature is more than 700 ng/cm^2 on PET and less than 450 ng/cm^2 on ppNIPAM. The observation of the different Γ_{max} behavior for BSA and fibrinogen is consistent with previous findings by Tanaka *et al.*,[45] who observed that BSA reaches a relatively constant Γ_{max} on surfaces of different wettabilities, but Γ_{max} for fibrinogen is strongly dependent on the surface chemistry. The variation of Γ_{max} of fibrinogen may be due to different faces of the fibrinogen molecule (which has a high aspect ratio) being presented at different temperatures and on different surfaces.

From these results, we conclude ppNIPAM has a much higher apparent affinity with proteins in the solution above its LCST than below it. However, static protein adsorption itself does not reveal the state of the adsorbed proteins. We will address this topic in the rest of the article with other techniques.

B. Protein desorption and elution

To test whether proteins adsorbed to PET and ppNIPAM are reversibly or irreversibly bound, we performed temperature cycles between room temperature and 37 °C on two sets of samples. Set 1 consisted of ppNIPAM-treated surfaces incubated with ^{125}I-labeled proteins at room temperature, and subsequently heated to 37 °C in the same protein solution.

TABLE II. Monolayer adsorption capacity (Γ_{max}) and association constant (k) obtained by fitting the isotherms in Fig. 2 to the Langmuir equation. The association constants for protein adsorption on PET at the two temperatures are similar, but the constants for adsorption on ppNIPAM at room temperature are an order of magnitude lower than the ones at 37 °C.

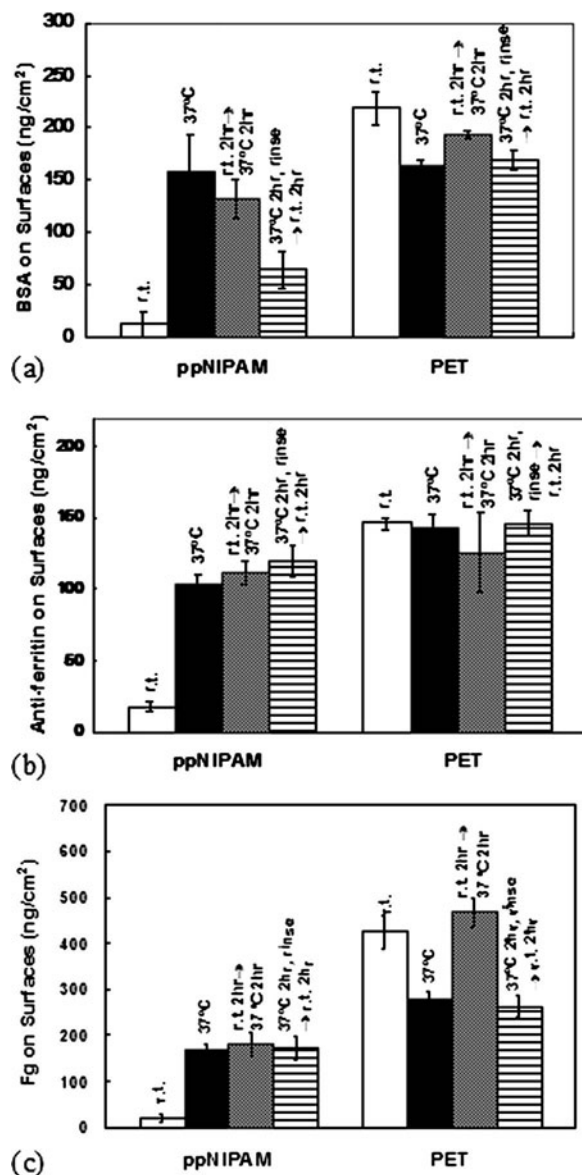|	Γ_{max} (ng/cm^2)		k (ml/mg)	
	RT	37 °C	RT	37 °C
BSA on ppNIPAM	238±28	188±22	3±1	18±6
BSA on PET	218±21	243±26	63±23	32±10
Anti-Fe on ppNIPAM	228±34	211±14	1±0	12±3
Anti-Fe on PET	192±24	182±12	67±40	90±27
Fg on ppNIPAM	432±66	553±54	5±1	38±13
Fg on PET	736±41	565±49	33±6	23±6

FIG. 2. Bovine serum albumin (BSA) (a), anti-ferritin antibody (b) and fibrinogen (Fg) (c) adsorption through temperature cycles on ppNIPAM and control PET using ^{125}I-labeled protein. Irreversible fibrinogen and anti-ferritin antibody adsorption are observed on ppNIPAM, while BSA adsorption is partially reversible on ppNIPAM. The error bars represent the standard deviation from five replicates.

Set 2 consisted of ppNIPAM-treated surfaces incubated with ^{125}I-labeled at 37 °C, after which they were transferred to buffer at room temperature. (Note: the rationale behind changing from protein solution to buffer in the second set of samples was to reduce secondary adsorption from the solution after protein desorption at room temperature.) Following the change in temperature, both sets were incubated for an additional 2 h.

The results of protein adsorption through temperature cycles are presented in Fig. 2. From Set 1, we find that while ppNIPAM-treated surfaces are initially low fouling at room temperature (<25 ng/cm^2 for the three proteins on ppNIPAM), they become protein retentive with incubation at

37 °C. From Set 2, we find that the fibrinogen and anti-ferritin antibody adsorbed onto the fouling ppNIPAM surface (originally incubated at 37 °C) does not detach spontaneously from ppNIPAM as a result of the temperature drop, while BSA partially detaches from ppNIPAM when temperature drops. Thus fibrinogen and antibody adsorption onto ppNIPAM are irreversible over the time frame of several hours. These results on ppNIPAM are consistent with observations of irreversible protein adsorption on nonthermoresponsive polymers such as polyethylene, Teflon and Silastic.[28]

The seemingly conflicting observations between lower apparent affinity of soluble proteins and irreversible adsorption of some proteins could be explained by either of two hypotheses: (1) the polymer surface conformation is "frozen" in the hydrophobic state by adsorbed proteins at 37 °C, from which it cannot recover to the swollen state after returning below the LCST; or (2) the decrease in the binding strength of surface adsorbed proteins to ppNIPAM upon temperature drop may not be sufficient to overcome other interactions present, and allow protein detachment. To test these hypotheses, protein elution with detergent was performed at both room temperature and 37 °C to determine protein-binding affinity to ppNIPAM at the two temperatures.[27,28] If the first hypothesis is correct, proteins will elute similarly at the two temperatures. If the second hypothesis is correct, more protein will be eluted at room temperature than at 37 °C.

In the elution test, samples incubated in protein solution at 37 °C for 2 h were immediately transferred to SDS solutions of varying concentrations at room temperature or 37 °C. After incubating for an additional 2 h and rinsing with CPBS, the sample radioactivity was counted to calculate the protein elutability. Figure 3 compares the elutability of three proteins (BSA, anti-ferritin antibody and fibrinogen) from ppNIPAM and PET at room temperature and 37 °C. As observed in the figures, the elutability from ppNIPAM is higher at room temperature than at 37 °C, while temperature has an opposite effect on anti-ferritin antibody elution from PET. As temperature has a positive effect on SDS efficiency,[46] the different elutability from PET possibly reflects the temperature influence on the detergent. Thus, more protein is eluted from ppNIPAM at room temperature than at 37 °C and this difference should be more dramatic when the detergent efficiency is taken into account. The higher elutability of these three proteins at room temperature is consistent with the hypothesis that protein affinity to ppNIPAM decreases after the surfaces are cooled from 37 °C to room temperature, although instantaneous desorption may not occur. It also suggests that ppNIPAM is capable of the phase transition after protein adsorption.

This decreased binding strength between surface-bound proteins and ppNIPAM upon temperature drop is consistent with the observation of soluble protein adsorption (Fig. 1). However, there is no common observation about protein desorption from thermosensitive polymers. In contrast to our observations of irreversible adsorption of fibrinogen and antibody, smaller proteins such as albumin,[47,48] lactalbumin
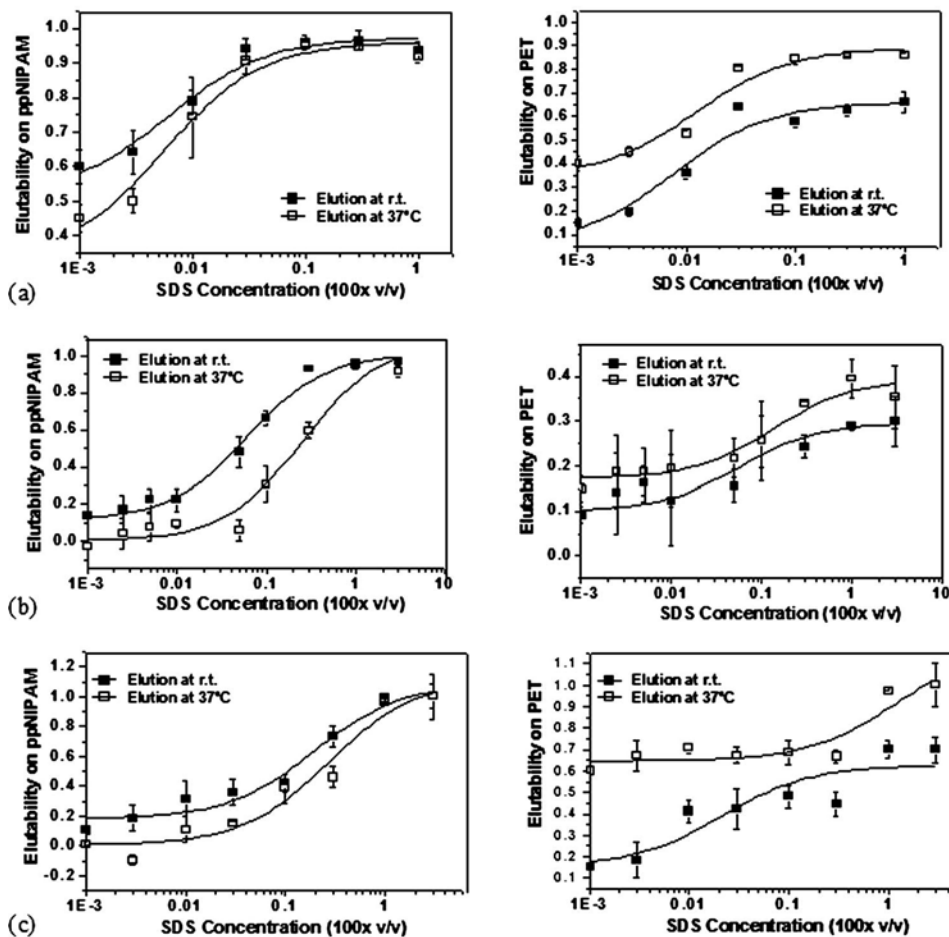
FIG. 3. Elution of bovine serum albumin (BSA) (a), anti-ferritin antibody (b) and fibrinogen (c) from ppNIPAM and PET at room temperature (solid squares) and 37 °C (open squares). The elutability is higher when proteins are eluted from ppNIPAM at room temperature, indicating proteins bind to ppNIPAM surfaces more tightly at 37 °C. The error bars represent the standard deviation from five replicates.

hydrolysate.[49] and myoglobin[50] have all been observed to desorb instantaneously from thermoresponsive surfaces upon temperature drop. This difference is likely due to changes in protein structure and/or conformation after contacting the surface at the elevated temperature. In a review on protein adsorption,[51] Norde notes that proteins change structure to expose the interior hydrophobic amino acid residues upon encountering a hydrophobic surface. Furthermore, the more flexible a protein is, the more capable it is of undergoing extensive conformation changes.[52] Thus, for a large, easily unfolding protein like fibrinogen, adsorption onto collapsed ppNIPAM may induce unfolding at multiple locations. As a result, some of the conformation changes are not able to fully recover when the polymer rehydrates below its LCST and the protein continues to bind on the surface with a reduced binding strength. This scenario is strongly supported by the elution tests presented above. Smaller proteins like myoglobin, on the other hand, may experience less unfolding per molecule during adsorption to the surface. Thus they are easier to refold and desorb once the surface transition to the more hydrophilic state has occurred. The idea of different levels of conformational changes among different proteins is also in line with recent studies of the rate constants of protein

unfolding after adsorption. In these studies, fibrinogen is found to have a higher unfolding rate compared to albumin and immunoglubulin.[53,54] Extensive relaxation and spreading of fibrinogen after adsorption can possibly result in its resistance to detergent elution.

The second possible explanation for the loss of reversibility involves the influence of the substrate and immobilization technique. For example, Yoshioka *et al.* found that IgG reversibly adsorbed onto a pNIPAM oligomer that was immobilized onto a silica gel surface,[55] while Taniguchi *et al.* reported irreversible binding of IgG on poly(styrene-*N*-isopropyl acrylamide) core-shell latex particles.[19] Thus, it would not be surprising if the irreversibility observed in our experiment was due to a variation in the chemistry of ppNIPAM from the conventionally synthesized thermoresponsive polymer. In fact, we have demonstrated in a previous article that plasma polymerized NIPAM is cross-linked on the surface.[11] The cross-linked polymer may influence protein-binding reversibility in a different way than the linear polymer used by Yoshioka *et al.*[55]

Another factor that may contribute to adsorption reversibility is length of time used for the incubation process. Since protein conformation slowly changes on the substrate as the

incubation time is increased, the time-dependent denaturation level will determine the ease of protein desorption afterwards.[20]

It is important to note that in contrast to our findings that large proteins adsorb irreversibly to ppNIPAM-treated surfaces, large proteins have been observed to detach from ppNIPAM-treated substrates upon temperature drop when an active force is exerted. Specifically, when cells cultured on ppNIPAM are brought to room temperature, the cells will reversibly detach from the surface, along with the majority of the extracellular matrix (ECM) proteins such as collagen, laminin and fibronectin.[56,57] The reversible cell interaction with ppNIPAM through the ECM proteins is more evidence that binding affinity between proteins adsorbed onto ppNIPAM is dependent on whether solution temperature is above or below the LCST.[58]

From the elution test, we conclude that the binding strength between the surface-bound protein molecules and ppNIPAM weakens below its LCST. Whether protein adsorption is reversible may depend on other factors such as the size and flexibility of the protein molecules, the substrate, and the adsorption procedures.

C. Antibody activity

Surface wettability is also known to alter the activity of the adsorbed protein layer via induced changes in conformation and/or orientation.[30] To determine the effect of ppNIPAM surface wettability on protein functionality, we tested the antibody immunological activity after adsorption on ppNIPAM at different temperatures using SPR. First, an antibody and its corresponding antigen were flowed across a ppNIPAM-treated chip. Next, the specific activity was determined by calculating the antigen/antibody binding ratio. Curve B in Fig. 4(a) presents a typical SPR sensorgram of the ppNIPAM-treated gold substrate exposed to sequential injections of antiferritin and ferritin at 37 °C. With the injection of 50 μg/ml antiferritin, a wavelength shift of ~17 nm is observed. This result is comparable to results reported in the literature with the same antibody and suggesting near monolayer coverage.[32] After blocking nonspecific binding sites on the surface, the immunological reactivity of the adsorbed antiferritin layer is then measured. With the injection of 50 μg/ml ferritin at 37 °C, the second increase observed in the SPR curve (~18 nm) is due to the specific antigen/antibody interaction.

In Sec. III A, we demonstrated that the apparent affinity of proteins with ppNIPAM varies with respect to temperature. It is also known that variations in protein surface coverage directly impact the activity of a protein on the surface.[59] Therefore, to make a direct comparison of antibody activity at room temperature versus 37 °C, we must first determine what concentration of antiferritin solution will give similar surface coverage at both temperatures. To make this determination, a series of experiments using different concentrations of antiferritin were done at room temperature. From this series, the concentration that gave a signal shift most similar to that at 37 °C was selected.[59] Curve A in Fig.
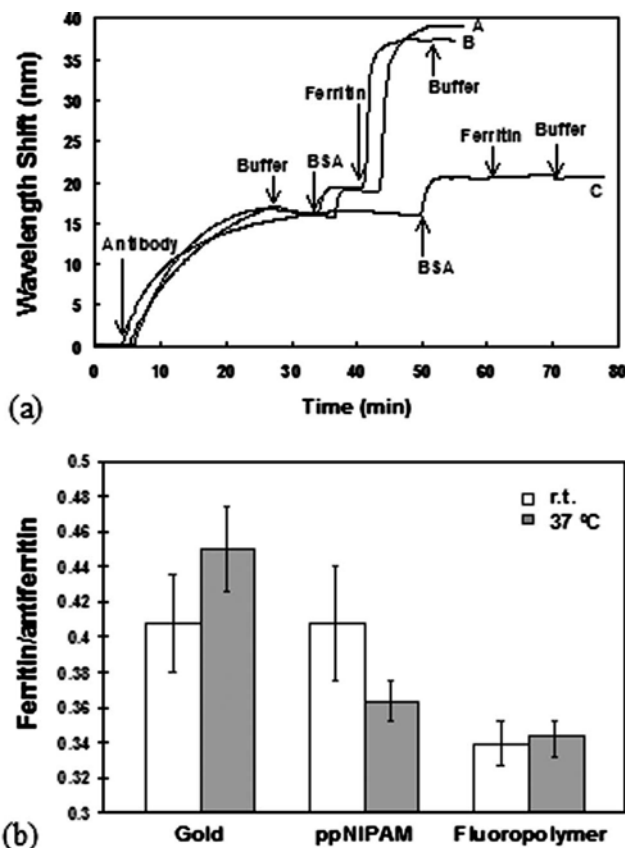

FIG. 4. (a) Typical SPR response curves for specific (curves A and B) or nonspecific (curve C) antibody adsorption and followed by antigen response to adsorbed antibody on ppNIPAM. Curve A shows the wave number shift of 500 μg/ml antiferritin injection followed by 50 μg/ml ferritin injection to ppNIPAM-treated gold substrate at room temperature. Curve B represents a typical SPR sensorgram of the same substrate exposed to sequential injections of 50 μg/ml antiferritin and 50 μg/ml ferritin at 37 °C. With similar antiferritin adsorption in curves A and B, more ferritin adsorbs to antiferritin at room temperature, indicating a higher retention of immunological activity after adsorption at this temperature. When 50 μg/ml nonspecific antibody (goat IgG) is first adsorbed onto the substrate (curve C), no obvious SPR response is observed upon flow of the ferritin solution over the goat IgG monolayer, indicating the ferritin adsorption in curves A and B are specific. (b) Antigen/antibody binding ratio as a function of surfaces and temperatures. The ratios on ppNIPAM fall between the hydrophilic gold and hydrophobic fluoropolymer, with a higher binding ratio at room temperature. The error bars represent the standard deviation from five replicates.

4(a) presents the typical SPR sensorgram of the ppNIPAM-treated gold substrate when exposed to 500 μg/ml of antiferritin followed by 50 μg/ml ferritin at room temperature. It is observed that antiferritin adsorbed onto ppNIPAM at room temperature is more active for ferritin binding than at 37 °C.

The specificity of this reaction is assessed by replacing the specific antigen/antibody reaction (antiferritin/ferritin) with that of a nonspecific reaction (goat IgG/ferritin). Injection of 50 μg/ml of goat IgG on the ppNIPAM surface yields an increase in the SPR curve similar to adsorption of antiferritin (compare Fig. 4, curve C to B). However, the increase of the SPR response upon flow of the ferritin solution over the goat IgG monolayer cannot be readily distin-

guished from that of background noise. Thus the increase after ferritin injection in curve A and curve B in Fig. 4 was the result of specific binding between the antibody and the antigen. Although the difference in wavelength shift between curve A and B is not large, it is reproducible ($n=5$) and is therefore a measurable result.

From the wavelength shift of the SPR signal, the antigen/antibody molar ratio can now be calculated. As a first order approximation, the wavelength shift in SPR response is linearly proportional to the average thickness of the adsorbed protein film. Furthermore, when it is assumed that the proteins under study have the same density,[60] the wavelength shift in SPR response is also linearly proportional to the amount of adsorbed proteins per unit area (g/cm^2).[60] Therefore, from the wavelength shift near the plateau induced by their respective adsorptions, the mass ratio of adsorbed antigen and antibody can be calculated. To obtain the molar ratio of adsorbed antigen to antibody, the mass ratio is divided by the molecular weight ratio.

Figure 4(b) shows the calculated molar ratio of ferritin/antiferritin adsorption on ppNIPAM compared to results obtained on gold and a plasma polymerized fluoropolymer. These two surfaces were chosen for comparison to ppNIPAM-treated surfaces as they represent extreme cases of hydrophilic and hydrophobic surfaces. It has previously been demonstrated that gold is an excellent substrate for enzyme immobilization. In fact, the specific catalytic activity of enzymes adsorbed onto gold nano-particles is comparable to the free enzyme in solution.[61–63] In comparison, fluoropolymers are highly hydrophobic, and generally promote changes in protein structure and loss of protein activity.[52,59] Therefore, it is not surprising to see in Fig. 4(b) that antiferritin adsorbed onto gold retains significantly more functionality than that observed on the fluoropolymer ($p<0.05$, two-tail t test). The activity of antibodies adsorbed onto ppNIPAM falls between these two samples, with a better ($p=0.08$, two-tail t test) preservation of antibody activity at room temperature than at 37 °C. The activity of antibodies on ppNIPAM at room temperature is comparable to that on gold, while at 37 °C, the difference between the two is significant ($p<0.05$, two-tail t test).

A closer inspection of Fig. 4(b) reveals that when different surfaces are compared at the same temperature, decrease of protein functionality closely follows the decrease of surface wettability. The gold used in our study has immeasurable contact angles (~ 0 °) due to rapid spreading of water droplet on it. The plasma-deposited fluoropolymer is highly hydrophobic at both room temperature and 37 °C. (contact angles >90 °). In contrast, the surface energy of the ppNIPAM film is found to vary with temperature (contact angles 34 ° and 40 ° at room temperature and 37 °C, respectively).[11] It is interesting that the relatively small change in water contact angle above and below the ppNIPAM LCST leads to a remarkably large change in protein and cell interaction behavior.

The correlation between surface wettability and protein activity at the same temperature agrees with the theory that protein denaturation increases with surface hydrophobicity.[64–67] Additionally, surface wettability and chemistry can also affect protein orientation, due to the different ratios of hydrophilic and hydrophobic amino acids comprising the Fab and Fc fragments in the antibody.[59] In turn, this could affect antibody functionality. When antibody activity is compared on the same surfaces for different temperatures, opposite effects are observed for gold and ppNIPAM. The difference in antiferritin activity on gold at the two temperatures may arise from different binding constant between antiferritin antibody and the antigen. On ppNIPAM, the change in surface wettability and chemistry is the dominant factor, i.e., the lower antibody activity at 37 °C may arise from unfolding of the antibody and/or unfavorable protein orientation for antigen binding encountering different surface wettability and chemistry. Conformation/orientation difference resulting from the change of ppNIPAM surface wettability should be even more obvious when the temperature dependent binding affinity is taken into consideration.

D. Protein conformation/orientation

In the previous section, we observed using SPR that the activity of proteins adsorbed onto ppNIPAM-treated surfaces varies with temperature. In this section, we use principal components analysis (PCA) of positive ion TOF-SIMS data obtained from an adsorbed protein layer on ppNIPAM-treated surfaces to determine whether this effect is due to changes in the proteins' conformation and/or orientation. To reduce alteration of the protein structure in the TOF-SIMS high vacuum environment, we protected the protein films with trehalose. Previously, we demonstrated that using this technique, we were able to preserve the structure of antiferritin adsorbed onto gold.[68]

To perform this analysis, we first adsorbed antiferritin on to ppNIPAM-treated surfaces at room temperature or 37 °C in concentrations that would result in similar surface coverages (Sec. III C). Next, a thin layer of trehalose was added to protect the protein film in the ultrahigh vacuum environment. Following acquisition of positive ion TOF-SIMS spectra, PCA was used to aid in the interpretation of spectra by identifying related variables and focusing on the differences between spectra.[68]

Figure 5(a) shows a scores plot of principal component 1 (PC1), which captures 47.6% of the variance in the data, versus the spectrum number. Examination of Fig. 5(a) shows that antiferritin adsorbed onto pNIPAM at the two temperatures is distinctly grouped into two clusters: room temperature incubated samples mainly cluster above the x axis and 37 °C incubated samples mainly cluster below the x axis.

To appreciate why the two groups are distinguished from each other, it is necessary to consider the loadings from PC 1 in Fig. 5(b). The positive peaks in the PC1 loadings plot (those above the origin) correspond to samples with positive scores (protein adsorbed onto ppNIPAM at room temperature). Conversely, the negative peaks in the PC1 loadings plot (those below the origin) correspond to samples with negative scores (protein adsorbed onto ppNIPAM at 37 °C).
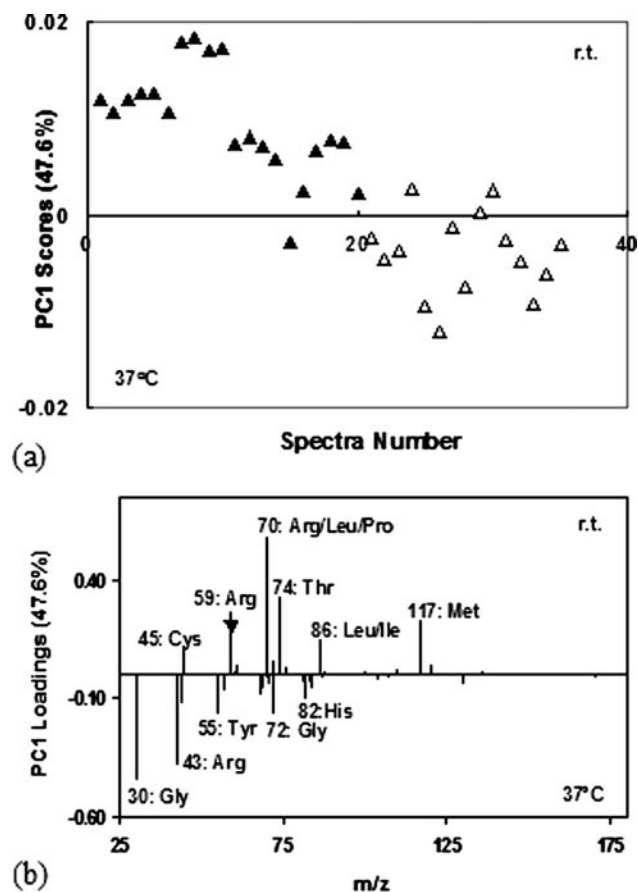
FIG. 5. Results from PCA analysis of positive ion TOF-SIMS data illustrating antiferritin conformation/orientation differences on ppNIPAM at room temperature and 37 °C. In the scores plot (a), spectra from protein adsorption at the room temperature (solid triangles) are separated from those at 37 °C (empty triangles). The differences are due to different protein conformation/orientation as reflected by the loadings of the different amino acid fragments in the loadings plot (b).

Upon inspection of the scores and loadings for these samples, we find that of the limited peak set of those molecular fragments previously identified with amino acids,[33] a number of amino acid fragments (e.g., arginine, cysteine, glutamine, leucine/isoleucine, methionine, and phenylalanine) appear to load positively with the samples adsorbed onto the room temperature surface. In comparison, amino acids such as alanine, asparagine, glycine, histidine, serine, proline, tryptophan and tyrosine appear to correlate with protein adsorbed onto the ppNIPAM at 37 °C. However, the assignment of a few of these amino acids (e.g., serine, threonine, and valine) is uncertain, as fragments of individual amino acids are found to load both positively and negatively (e.g., valine, asparagines, L-serine, and tryptophan). In some cases, all of the fragments load exclusively with either the room temperature samples (positive loadings) or 37 °C (negative loadings). For example, all fragments of arginine (C_4H_8N, $C_5H_5N_3$, $C_5H_8N_3$, and $C_4H_{10}N_3$) load positively (m/z 70.0657, 59.0483, 110.0718, and 100.08747, respectively). In Table III, each of the amino acids that load positively, negatively, or inconclusively is presented.

Unfortunately, the tertiary structure of antiferritin is incompletely defined, and no comparison between the amino acid fragments detected and their relative position within the protein (e.g., external versus internal, or at either terminus) may be made. Therefore, it is not possible to determine whether it is the protein's orientation (i.e., antibody binding pocket faced up versus faced down) or its conformation (i.e., native versus denatured) that is responsible for the reduced binding affinity observed by SPR using these data. However, it is interesting to note that each of the sulfur-containing amino acid fragments (e.g., C_2H_6NS from cysteine at m/z 76.0267; C_2H_5S and C_5H_9OS from methionine at m/z 61.0116 and 117.0247, respectively) load positively with the protein film adsorbed at room temperature. It is possible that this indicates that sulfur-containing residues (such as those that are found in the terminal ends of antibodies) are facing upward from the substrate upon adsorption to ppNIPAM-treated surfaces at room temperature. In any case, the fact that the amino acid fragmentation patterns in the loadings plot differ between proteins adsorbed above and below the LCST suggests that different regions of the proteins are exposed after adsorption at the two temperatures.

While these results suggest that the conformation/orientation of proteins adsorbed onto ppNIPAM-treated surfaces is altered in a temperature-dependent manner, they are preliminary. To confirm this hypothesis, it would be advantageous to study an adsorbed layer of a protein of known three-dimensional (3D) structure. In this way, knowledge of the relative abundance of amino acids (from the primary structure) and their location within the protein (from the tertiary and quaternary structures) could be related to PCA results obtained from the adsorbed films. Furthermore, it may be that while the protein film adsorbed at 37 °C is partially altered, this surface induced change in protein structure is not sufficient to completely denature the protein layer. Therefore, it would be advantageous to study the structure of a more completely denatured protein film adsorbed at a much higher temperature (e.g., >70 °C). If the conformation/orientation of proteins adsorbed onto ppNIPAM-treated surfaces is truly altered in a temperature-dependent manner, it would be expected that the structure of the film adsorbed at the highest temperature (70 °C) would be more denatured than that of the film adsorbed at the intermediate temperature (37 °C). In addition, by comparing the protein structure at different temperatures on different substrates, such as gold and fluoropolymer, we may better correlate the SPR results with the protein conformation and further appreciate the relationship between protein 3D structure and its activity.

Still, the results from PCA analysis of the TOF-SIMS do suggest that different regions of the proteins are exposed after adsorption at the two temperatures. When taken in conjunction with the protein activity results from SPR, we conclude that proteins adsorbed onto ppNIPAM-treated surfaces at room temperature have a different conformation/orientation from those adsorbed at 37 °C, and these protein films on the ppNIPAM surfaces at room temperature and 37 °C have different biological recognizabilities.

TABLE III. Assignments of amino acid fragments in the loadings plot in Fig. 5(b).

PC1 (47.6%)	Detected *m/z*	Theoretical *m/z*	Molec Frag	Amino Acid	Abbreviation	Alternate Identification
Positively Loaded Protein Fragments (assoc with RT)
0.577	70.067	70.066	C4H8N	ARGININE	Arg, R	LEUCINE, PROLINE
0.175	59.050	59.048	CH5N3	ARGININE	Arg, R	
0.020	110.075	110.072	C5H8N3	ARGININE	Arg, R	HISTIDINE
0.006	100.089	100.087	C4H10N3	ARGININE	Arg, R	
0.006	88.043	88.040	C3H6NO2	ASPARAGINE	Asn, N	ASPARTIC ACID
0.026	76.027	76.022	C2H6NS	CYSTEINE	Cys, C	
0.000	84.045	84.045	C4H6NO	GLUTAMINE	Glu, Q	
0.147	86.100	86.097	C5H12N	ISOLEUCINE	Ile, I	LEUCINE
0.013	60.049	60.045	C2H6NO	L-SERINE	Ser, S	
0.228	117.025	117.037	C5H9OS	METHIONINE	Met, M	
0.038	61.012	61.011	C2H5S	METHIONINE	Met, M	
0.035	120.084	120.081	C8H10N	PHENYLALANINE	Phe, F	
0.329	74.063	74.061	C3H8NO	THREONINE	Thr, T	
0.001	159.129	159.092	C10H11N2	TRYPTOPHAN	Trp,W	
0.053	72.083	72.081	C4H10N	VALINE	Val,V	
Negatively Loaded Protein Fragments (assoc with 37 °C)
PC1 (47.6%)	Detected *m/z*	Theoretical *m/z*	Molec Frag	Amino Acid	Abbreviation	Alternate Identification
−0.121	44.051	44.050	C2H6N	ALANINE	Ala, A	CYSTEINE
−0.005	87.058	87.056	C3H7N2O	ASPARAGINE	Asn, N	GLYCINE
−0.007	70.031	70.029	C3H4NO	ASPARAGINE	Asn, N	
−0.167	72.046	72.044	C3H6NO	GLYCINE	Gly, G	
−0.440	30.035	30.034	CH4N	GLYCINE	Gly, G	
−0.024	81.035	81.045	C4H5N2	HISTIDINE	His, H	
−0.099	82.067	82.053	C4H6N2	HISTIDINE	His, H	
−0.034	71.014	71.013	C3H3O2	L-SERINE	Ser, S	
−0.049	84.084	84.081	C5H10N	LYSINE	Lys, K	
−0.058	57.058	57.058	C3H7N	LYSINE	Lys, K	
−0.079	68.051	68.050	C4H6N	PROLINE	Pro, P	
−0.056	69.035	69.034	C4H5O	THREONINE	Thr, T	
−0.011	170.067	170.061	C11H8NO	TRYPTOPHAN	Trp, W	
−0.041	130.067	130.066	C9H8N	TRYPTOPHAN	Trp, W	
−0.003	107.052	107.050	C7H7O	TYROSINE	Tyr, Y	
−0.163	55.019	55.018	C3H3O	TYROSINE	Tyr, Y	
−0.030	83.051	83.047	C5H7O	VALINE	Val, V	

IV. CONCLUSIONS

In this work, we study protein adsorption and biological recognizability on a plasma polymerized NIPAM film above and below its LCST. Static adsorption studies using low protein concentrations demonstrate that ppNIPAM-treated surfaces are low fouling at room temperature, but protein retentive at 37 °C. Adsorption isotherms at the two temperatures indicate that the apparent affinity of soluble proteins to ppNIPAM at room temperature is approximately an order of magnitude lower than at 37 °C. That room temperature ppNIPAM-treated surfaces have a weaker affinity for proteins is further confirmed by our findings that they have increased detergent elutability of surface adsorbed proteins. However, direct desorption of proteins from ppNIPAM depends on other factors as well, including the adsorption time and the flexibility of the protein itself. Although we find that proteins adsorbed onto ppNIPAM-treated surfaces are functionally active at both room temperature and 37 °C, temperature appears to have an opposite effect on protein activity on ppNIPAM and gold substrates. From results of PCA analysis of positive ion TOF-SIMS data and SPR, we hypothesize that antibody activity is reduced on ppNIPAM above its LCST due to surface-induced changes in the proteins' conformation/orientation. To determine whether it is protein conformation or orientation that is at work, further study of the adsorption of a protein of known sequence and crystallography is necessary.

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