

Functionalization of Solid Surfaces with Thermoresponsive Protein-Resistant Films

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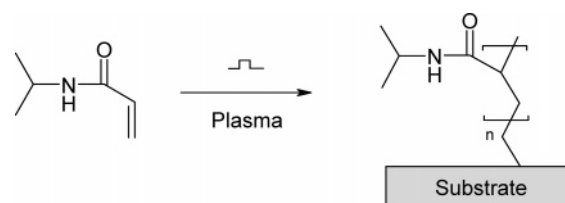
Pulsed plasma polymerization of *N*-isopropylacrylamide leads to the deposition of thermoresponsive films. The reversible (switching) behavior of these poly(*N*-isopropylacrylamide) surfaces has been exemplified by screening the adsorption of fibrinogen and fluorescein isothiocyanate labeled bovine serum albumin proteins by surface plasmon resonance (SPR) and fluorescence microscopy at low and elevated temperatures.

1. Introduction

One class of smart polymers is renowned for its ability to undergo a sharp structural transition in response to external stimuli^{1–4} (e.g., pH, temperature, exposure to ultraviolet light, electric charge, etc.). Poly(*N*-isopropylacrylamide) is considered as being a thermoresponsive example of such materials as a consequence of its lower critical solution temperature (31–32 °C⁵) lying between room temperature (~20 °C) and human body temperature (37 °C). At temperatures below the lower critical solution temperature, poly(*N*-isopropylacrylamide) forms an expanded structure in aqueous solution with a random coil configuration.⁶ This stems from hydrogen bond formation between the secondary amide hydrogen N–H and carbonyl C=O groups and surrounding water molecules.⁷ However, above the lower critical solution temperature, the polymer collapses into a globular structure,^{6,8} and hydrophobic interactions predominate, leading to polymer precipitation out of water. This remarkable hydration–dehydration switching phenomenon above the lower critical solution temperature is attributable to the accompanying entropy gain achieved during amide group dehydration. Effectively, at higher temperatures, the poly(*N*-isopropylacrylamide) surface becomes packed with propyl groups, i.e., alkane-like. Upon dropping below the lower critical solution temperature, the polymer surface switches back to being hydrophilic as the amide groups hydrate.^{9,10} Water contact angle values for a poly(*N*-isopropylacrylamide) surface below its lower critical solution temperature (20–25 °C) typically range between 45 and 55°, whereas the water contact angle value rises to approximately 90° at body temperature (37 °C, i.e., above the lower critical solution temperature), which is akin to an alkane-like surface.^{11,12} These thermoresponsive properties can strongly influence the polymer's affinity toward proteins and cells. Hence, poly(*N*-isopropylacrylamide) is a potential candidate for separation applications,¹³ controlled drug release,¹⁴ and tissue engineering.^{11,15–18} For instance, cell culture can be grown on a poly(*N*-isopropylacrylamide) surface at body temperature (37 °C) and subsequently “popped off” by allowing the substrate to rapidly hydrate (i.e., become protein resistant) by cooling to below the lower critical solution temperature (e.g., 20 °C, room temperature).^{19–21}

Various methods are documented for the preparation of poly(*N*-isopropylacrylamide) surfaces. These include electron beam

SCHEME 1: Pulsed Plasma Polymerization of *N*-Isopropylacrylamide.



grafting,^{11,22} plasma grafting,^{6,9} atom transfer free radical polymerization,²³ photoinitiated polymerization,^{24–26} surface grafting pre-formed poly(*N*-isopropylacrylamide) polymer,²⁷ and free radical polymerization using ammonium persulfate²⁸ and dialkylazene initiators.^{29–31}

Plasma polymerization is an attractive alternative because it potentially offers a single-step, substrate-independent route for making thermally responsive protein-resistant surfaces. Previously, continuous wave plasma polymerization of the *N*-isopropylacrylamide monomer³² has been reported to yield films which adsorb protein when heated to 37 °C; however, cooling back down to 20 °C showed that protein adsorption is not completely reversible.^{33,34} This lack of reversibility is most likely due to flaws in the macromolecular structure derived from the highly energetic nature of the continuous wave glow discharge employed in that particular study. During continuous wave plasma deposition, the surface is constantly being bombarded by damaging energetic species (e.g., photons, ions, and electrons). Furthermore, there is a constant surface plasma sheath potential which assists in bombardment.

In this article, the much milder and controlled conditions associated with pulsed plasma deposition are shown to lead to reversible protein adsorption behavior with changing temperature for poly(*N*-isopropylacrylamide) films, Scheme 1. This surface functionalization technique entails modulating the electrical discharge on the ms-μs time scales in the presence of gaseous precursors containing polymerizable carbon–carbon double bonds.³⁵ Mechanistically, there are *two distinct reaction regimes* corresponding to the plasma duty cycle on- and off- periods.³⁶ Namely, there is monomer activation and the generation of reactive sites at the surface during each short (μs) burst of plasma (via UV irradiation, ion, or electron bombardment) followed by conventional polymerization during the much longer (ms) off-time (in the absence of any UV-, ion-, or electron-induced damage, and also no surface plasma sheath potential).

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Extremely high levels of structural retention of the precursor molecule can be achieved, and therefore specific functionalities can be incorporated onto the surface. Furthermore, by programming the pulse duty cycle, it is possible to control (i.e., tailor) the desired chemical group surface density (in contrast to conventional grafting techniques). Examples devised in the past include: anhydride,³⁶ epoxide,³⁷ carboxylic acid,³⁸ cyano,³⁹ hydroxyl,⁴⁰ furfuryl,⁴¹ and perfluoroalkyl groups.⁴² The pulsed plasma deposited poly(*N*-isopropylacrylamide) films in the present study have been characterized by water contact angle measurements and X-ray photoelectron and infrared spectroscopies. The reversible protein adsorption behavior of these surfaces has been screened by surface plasmon resonance (SPR) and fluorescence microscopy.

2. Experimental Section

N-Isopropylacrylamide (+97%, Aldrich) monomer was loaded into a sealable glass tube, followed by degassing using multiple freeze–pump–thaw cycles. Pulsed plasma polymerization was carried out in a cylindrical glass reactor (4.5 cm diameter, 460 cm³ volume, 2×10^{-3} mbar base pressure, and 1.4×10^{-9} mol s⁻¹ leak rate) surrounded by a copper coil (4 mm diameter, 10 turns, located 15 cm away from the precursor inlet) connected to a 13.56 MHz radio frequency (RF) power supply and an L–C matching network. The plasma chamber was located inside a temperature-controlled oven and a Faraday cage. A 30 L min⁻¹ rotary pump attached to a liquid nitrogen cold trap was used to evacuate the reactor, while the system pressure was monitored with a Pirani gauge. All fittings were grease-free. During pulsed plasma deposition, the RF power source was triggered by a signal generator, and the pulse shape monitored with an oscilloscope.

Prior to each deposition, the apparatus was scrubbed with detergent, rinsed in 2-propanol, and oven dried. Further cleaning entailed running a continuous wave air plasma at 0.2 mbar pressure and 40 W power lasting 30 min. Next, silicon wafers, gold chips, or poly(tetrafluoroethylene) strips (10 mm \times 15 mm) were inserted into the reactor and the reactor pumped down to base pressure. At this stage, a continuous flow of *N*-isopropylacrylamide vapor was introduced into the chamber at a pressure of 0.1 mbar and 40 °C temperature for 5 min prior to plasma ignition. The duty cycle corresponded to 35 W continuous wave bursts lasting between 60 and 100 μ s (t_{on}), followed by an off-period (t_{off}) fixed at 2 ms. Upon completion of deposition, the RF generator was switched off, and the monomer allowed to continue to purge through the system for a further 5 min prior to evacuating to base pressure and venting to atmosphere.

A spectrophotometer (nkd-6000, Aquila Instruments Ltd.) was used for plasma polymer film thickness measurements. The obtained transmittance–reflectance curves (350–1000 nm wavelength range) were fitted to a Cauchy material model using a modified Levenberg–Marquardt algorithm.⁴³

Contact angle analysis on the pulsed plasma deposited poly(*N*-isopropylacrylamide) films was carried out using a video capture system (ASE Products, model VCA2500XE). 2.0 μ L sessile droplets of deionized water were placed onto the polymer surface and contact angle measurements taken at 20 °C (room temperature) and also at 40 °C (above the lower critical solution temperature). The temperature of the heated substrate holder was monitored with a thermocouple.

Chemical characterization by X-ray photoelectron spectroscopy (XPS) was undertaken using a VG ESCALAB II electron spectrometer equipped with a non-monochromated Mg K $\alpha_{1,2}$ X-ray source (1253.6 eV) and a concentric hemispherical

analyzer. Photoemitted electrons were collected at a takeoff angle of 30° from the substrate normal, with electron detection in the constant analyzer energy mode (CAE, pass energy = 20 eV). The XPS spectra were referenced to the C(1s) peak at 285.0 eV and fitted with a linear background and equal full-width-at-half-maximum (fwhm) Gaussian components⁴⁴ using Marquardt minimization computer software. Instrument sensitivity multiplication factors were taken as C(1s): O(1s): N(1s): Si(2p) = 1.00: 0.36: 0.57: 1.02.

Surface infrared spectroscopy was performed on gold substrates using an FTIR spectrometer (Perkin-Elmer, model Spectrum One) equipped with a liquid nitrogen cooled MCT detector operating at 4 cm⁻¹ resolution over the 700–4000 cm⁻¹ range. The instrument was fitted with a reflection–absorption spectroscopy accessory (Specac) and a KRS-5 p-polarizer with the reflection angle set to 84°.

For surface plasmon resonance (SPR) studies, 20 nm thick poly(*N*-isopropylacrylamide) films were pulse plasma deposited onto a gold sensor chip (Biacore). Protein adsorption at 25 °C and 36 °C was monitored using an SPR biosensor system (Biacore 1000 upgrade). For each flow cell, the cross sectional area is 0.05 \times 0.5 mm and the length is 2.4 mm, giving a volume of about 60 nL; and the total sensor chip area in each flow cell is 1.2 mm². The surface plasmon resonance protocol for measuring protein adsorption entailed first cleaning the surface by flowing a 40 mM sodium dodecyl sulfate (+99% Sigma) in phosphate buffered saline solution over the surface for 3 min, followed by flushing with phosphate buffered saline (pH 7.4) for 10 min. At this stage, the absence of detergent associating with the poly(*N*-isopropylacrylamide) surfaces⁴⁵ was confirmed by XPS. Next, protein solution (1 mg mL⁻¹ in phosphate buffered saline, pH 7.4) was passed over the surface for 30 min. Finally, phosphate buffered saline was purged through the system for 10 min in order to remove loosely bound protein. Throughout, the flow rate was kept constant at 10 μ L min⁻¹ and identical baselines reestablished before each SPR measurement. The proteins screened were fibrinogen (from bovine plasma, Sigma, pI = 5.8) and lysozyme (egg white, Sigma, pI = 11.0).

Finally, a patterned thermally responsive protein-resistant surface was prepared by embedding cut 10 mm \times 15 mm pieces of poly(tetrafluoroethylene) (PTFE, 0.25 mm thick, Goodfellow) with nickel grids (200 mesh, 28 μ m bar size, 97 μ m hole size, Agar Scientific) using a force of 40 kN (an approximate pressure of 400 MPa) for 10 s. With the embossed grid in place, *N*-isopropylacrylamide pulsed plasma deposition was carried out onto this surface as detailed above. The nickel grid was then lifted off the PTFE substrate to leave behind a well-defined array of plasma polymer. These substrates were used for fluorescence microscopy in conjunction with fluorescein isothiocyanate-labeled bovine serum albumin (FITC–BSA, Fluka, 0.5 mg mL⁻¹ in phosphate buffered saline). Fluorescein isothiocyanate has an excitation wavelength of 495 nm and emits at 520 nm.⁴⁶ Each coated PTFE substrate was immersed in fluorescein isothiocyanate labeled bovine serum albumin solution at either 20 °C or 40 °C for 30 min, then rinsed in phosphate buffered saline at the same respective incubation temperature, and subsequently imaged with a home-built fluorescence microscope. The fluorescence microscope system comprised an inverted microscope (Olympus IX-71) fitted with a UV–vis mercury lamp for excitation. Light was transmitted through a band-pass filter (UG-1) and reflected off a dichroic mirror (Comar Instruments, 475-BK shortwave reflecting) onto the sample surface via a 10 \times lens (UPLFL Olympus). The fluorescence

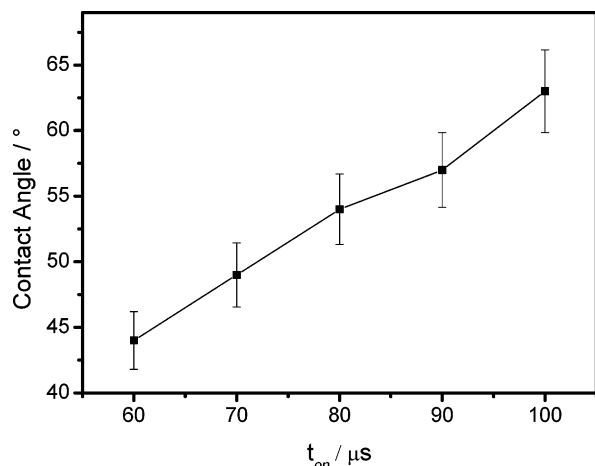


Figure 1. Variation of water contact angle with varying duty cycle on period (t_{on}) for pulsed plasma deposited poly(*N*-isopropylacrylamide) surfaces at 20 °C ($P_p = 35$ W, $t_{off} = 2$ ms). (The films washed off at shorter duty cycles.)

TABLE 1: XPS Elemental Analysis for Plasma-Deposited Poly(*N*-isopropylacrylamide)^a

conditions	stoichiometry $\pm 1\%$			
	%C	%O	%N	%Si
monomer	74	13	13	0
pulsed	78	10	12	0
continuous wave	75	12	13	0

^a ($P_p = 35$ W (CW and pulsed); $t_{on} = 60 \mu s$ and $t_{off} = 2$ ms (pulsed)).

(650 × 500 pixel) image was passed through a 520 nm cutoff filter (Comar Instruments) prior to detection using a CCD camera (HX-916, Starlight-Xpress). The images were corrected for background fluorescence from plasma-deposited poly(*N*-isopropylacrylamide) on PTFE in order to accurately determine the level of fluorescence arising from the fluorescein isothiocyanate labeled bovine serum albumin.

3. Results

The surface hydrophilicity of the poly(*N*-isopropylacrylamide) films could be tailored by varying the pulsed plasma duty cycle (i.e., shorter duty cycles improve structural retention), Figure 1. A duty cycle corresponding to $P_p = 35$ W, $t_{on} = 60 \mu s$, and $t_{off} = 2$ ms yielded the lowest water contact angle value of 44° while maintaining covalent attachment of the poly(*N*-isopropylacrylamide) film to the substrate. When the temperature of this coating was raised to 40 °C, the water contact angle increased to 81°. This compares favorably with previously reported water contact angle values of 42° and 90° at 20 °C and 36 °C, respectively, for conventional solution phase synthesized poly(*N*-isopropylacrylamide) coatings.¹²

The absence of any Si(2p) XPS signal from the underlying silicon wafer substrate verified homogeneous film thicknesses greater than 2–5 nm, Table 1. The XPS C(1s) envelope can be fitted to four different carbon environments associated with the precursor: hydrocarbon ($C_{x}H_y = 285.0$ eV), carbon adjacent to a carbonyl group ($C-C=O = 285.7$ eV), carbon attached to nitrogen ($C-N = 285.9$ eV), and carbon singly bonded to nitrogen and doubly bonded to oxygen ($N-C=O = 288.1$ eV),⁴⁷ Figure 2. In the case of pulsed plasma deposition, the distribution of peaks representing the different carbon environments correlates well with the expected theoretical fit. Whereas continuous wave plasma polymerized *N*-isopropylacrylamide reveals much greater structural disruption, there are also additional compo-

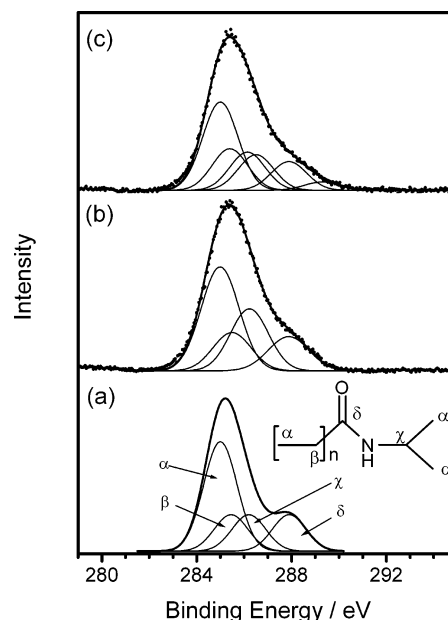


Figure 2. XPS C(1s) envelope of (a) theoretically predicted conventional poly(*N*-isopropylacrylamide); (b) pulsed plasma ($P_p = 35$ W, $t_{on} = 60 \mu s$, $t_{off} = 2$ ms) polymerized *N*-isopropylacrylamide; and (c) 35 W continuous wave plasma polymerized *N*-isopropylacrylamide.

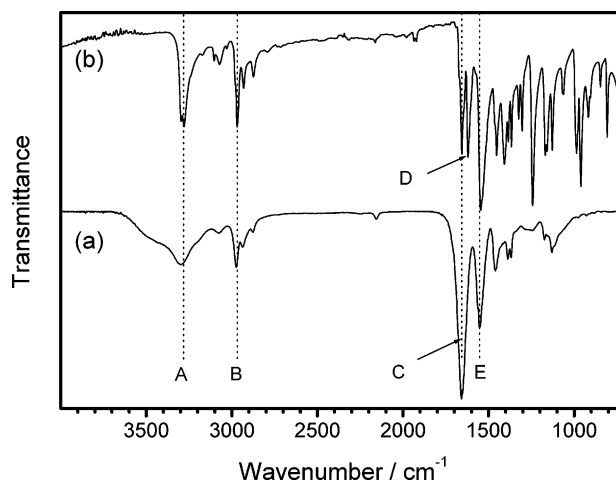


Figure 3. FTIR spectra of (a) pulsed plasma polymerized *N*-isopropylacrylamide ($P_p = 35$ W, $t_{on} = 60 \mu s$, $t_{off} = 2$ ms), and (b) *N*-isopropylacrylamide monomer.

nents due to $C-O$ at 286.5 eV and $O-C=O$ at 289.3 eV which represent functionalities stemming from extensive monomer fragmentation and rearrangement within this more energetic electrical discharge.

Structural retention for the pulsed plasma deposited poly(*N*-isopropylacrylamide) layers was also authenticated by infrared spectroscopy, Figure 3. Characteristic absorption bands include the N-H stretch at 3300 cm^{-1} (A), the amide I stretch at 1660 cm^{-1} (C), and the amide II stretch at 1540 cm^{-1} (E). Absence of the C=C stretch at 1630 cm^{-1} (D) corroborates *N*-isopropylacrylamide monomer polymerization. These pulsed plasma deposited poly(*N*-isopropylacrylamide) films were also found to form stable coatings on other substrates (polyethylene, cotton, polystyrene microspheres, glass, and steel).

Surface plasmon resonance analysis of a negatively charged protein (fibrinogen) exposed to 25 nm thick pulsed plasma deposited poly(*N*-isopropylacrylamide) films at pH 7.4 showed a significant increase in adsorption when the substrate temperature was raised from 25 °C (below the lower critical solution

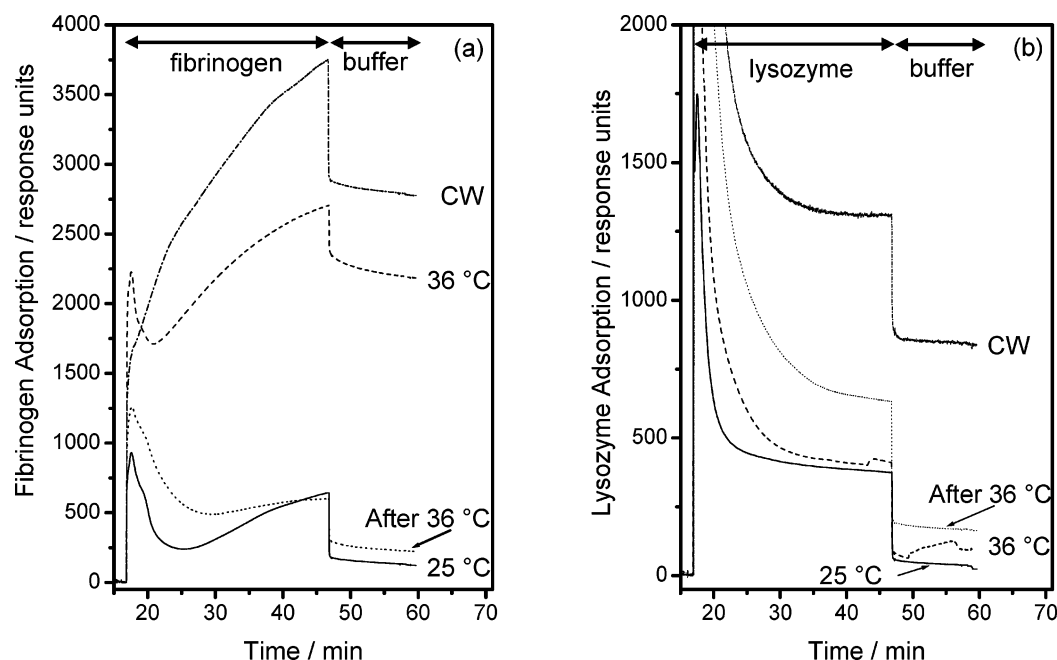


Figure 4. SPR of protein adsorption onto pulsed plasma polymerized *N*-isopropylacrylamide films at different temperatures, ($P_p = 35$ W, $t_{on} = 60$ μ s, $t_{off} = 2$ ms): (a) fibrinogen and (b) lysozyme. Readings have been taken at 25 °C, 25 °C after heating to 36 °C ('After 36 °C'), and 36 °C. The corresponding measurement for a 35 W continuous wave plasma deposited poly(*N*-isopropylacrylamide) film at 25 °C is also shown as a comparison ('CW') (± 100 response units).

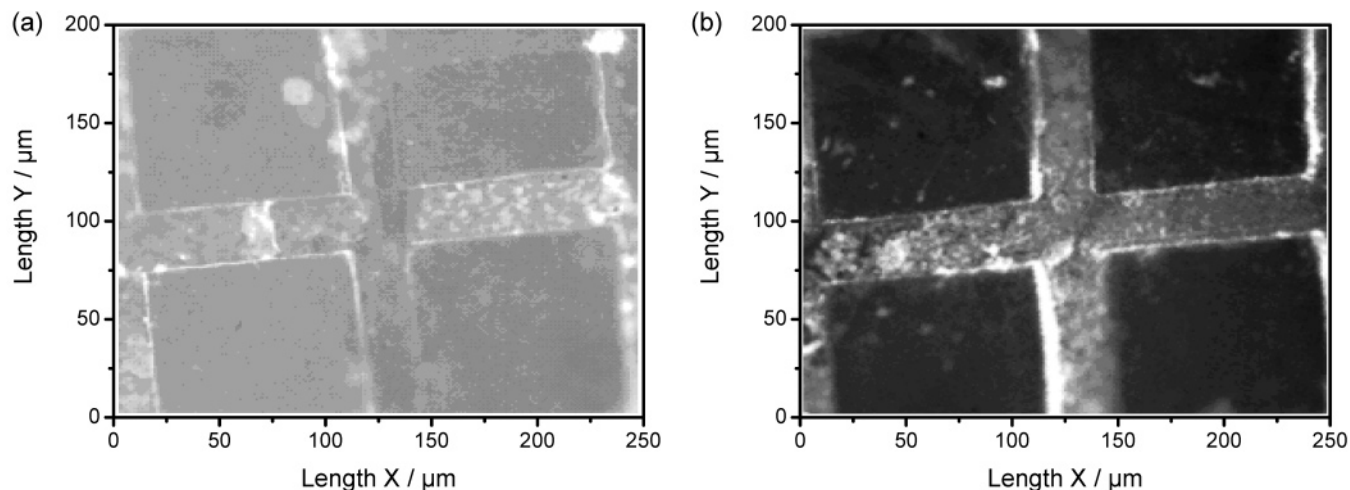


Figure 5. Fluorescence micrograph of pulsed plasma polymerized *N*-isopropylacrylamide array following immersion in fluorescein isothiocyanate labeled bovine serum albumin/phosphate buffered solution at: (a) 40 °C and (b) 20 °C ($P_p = 35$ W, $t_{on} = 60$ μ s and $t_{off} = 2$ ms).

temperature) to 36 °C (above the lower critical solution temperature), Figure 4. Adsorption of fibrinogen was found to be reversible by cycling the substrate temperature. Surface restructuring is a plausible explanation for the nonmonotonic adsorption during protein adsorption. Lysozyme adsorption (a positively charged protein)⁴⁸ was not as greatly affected, with only a marginal enhancement detected at the higher temperature. In marked contrast, adsorption of both proteins onto the continuous wave plasma polymerized *N*-isopropylacrylamide surfaces was found to be the greatest, owing to the high level of structural disruption in the film structure, Figure 4.

Fluorescence microscopy of poly(*N*-isopropylacrylamide) arrays deposited onto a PTFE chip (97 μ m squares separated by 28 μ m) at 40 °C, following exposure to fluorescein isothiocyanate labeled bovine serum albumin, indicated a similar degree of fluorescence across the whole surface, Figure 5. Cooling to room temperature (20 °C) produced a marked contrast in fluorescence between the poly(*N*-isopropylacryl-

amide) squares and PTFE bars, Figure 5. The adsorption of protein at 40 °C was found to be reversible on the pulsed plasma polymer functionalized pixels, while the affinity for protein adsorption by the PTFE grid did not vary noticeably with temperature.

4. Discussion

Fibrinogen is a large plasma protein which interacts with platelets during blood clotting; as such, it is a good example of a "sticky protein".⁴⁹ Albumin is the most abundant plasma protein (at a level of 40 mg mL⁻¹ in the blood).⁵⁰ It is a large "soft" globular protein that when adsorbed results in surface passivation.⁵¹ The fully reversible adsorption of these two proteins with temperature in the present study is a marked improvement on previously reported continuous-wave plasma deposition of poly(*N*-isopropylacrylamide).³³ This can be attributed to a significant reduction in cross-linking during pulsed

plasma polymerization, which provides an increased degree of freedom for the polymer chains (i.e., permits hydration–dehydration changes in chain conformation to occur more readily during temperature cycling). The transition from a hydrophilic, protein-resistant surface to a more hydrophobic, protein-adsorbant surface is governed by the polymer chains' ability to reorganize their structure from an extended chain to a tight coil. The amount of cross-linking influences this phase transition; a highly cross-linked surface-bound polymer will not be able to change conformation as easily as a less cross-linked polymer. A balance must be drawn because if there are too few cross-links, the plasma deposited poly(*N*-isopropylacrylamide) film becomes susceptible to dissolution in aqueous media (as seen in Figure 1 at very short duty cycles).

The marked differences in the SPR kinetics between the adsorption of fibrinogen versus lysozyme have been previously observed for studies relating to alkanethiol-gold based SAMs,⁵² and are understood to stem from electrostatic effects. The small change in adsorption of lysozyme onto the pulsed plasma deposited *N*-isopropylacrylamide surfaces upon increasing the temperature above the lower critical solution temperature may be due to charge repulsion, since the measured XPS surface composition of the plasma polymer reveals a loss of oxygen compared to the expected theoretical stoichiometry, Table 1. Thus, a relative enhancement of nitrogen containing functionalities relative to oxygen may result in a net positive charge on the plasma polymer surface, and so increased repulsion toward positively charged lysozyme above the lower critical solution temperature.

The mechanism of protein resistance for solid surfaces is currently not fully understood. It is believed that the interaction of water with the surface plays an important role, causing steric repulsion,⁵³ and hence exclusion of the proteins from the surface–water interface. The packing and orientation of molecules may also influence the protein resistance of a surface.⁵⁴ It has been suggested that the surface molecules need to satisfy a set of four criteria in order to be protein resistant.⁵⁵ These molecular characteristics are: (i) they contain polar functional groups, (ii) they incorporate hydrogen bond accepting groups, (iii) they do not contain hydrogen bond donating groups, and (iv) they have no net charge. The pulsed plasma deposited poly(*N*-isopropylacrylamide) films in the present study appear to meet three of these when below their lower critical solution temperature, but contradict the requirement for the surface not to contain hydrogen bond donating groups.

Potential applications for the pulsed plasma surface functionalization of solid substrates with thermally responsive protein-resistant coatings include tissue engineering, where cells can be cultured at body temperature, preceding their removal by simply cooling the substrate to room temperature,^{19–21,56} and controlled release of water-soluble moieties.

5. Conclusions

Thermally responsive (switching) poly(*N*-isopropylacrylamide) films can be deposited by pulsed plasma polymerization. This is a viable substrate-independent, single-step process for controlling protein adsorption at solid surfaces.

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