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Switchable surface coatings for control over protein adsorption

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ABSTRACT

Control over biomolecule interactions at interfaces is becoming an increasingly important goal for a range of scientific fields and is being intensively studied in areas of biotechnological, biomedical and materials science. Improvement in the control over materials and biomolecules is particularly important to applications such as arrays, biosensors, tissue engineering, drug delivery and 'lab on a chip' devices. Further development of these devices is expected to be achieved with thin coatings of stimuli responsive materials that can have their chemical properties 'switched' or tuned to stimulate a certain biological response such as adsorption/desorption of proteins. Switchable coatings show great potential for the realisation of spatial and temporal immobilisation of cells and biomolecules such as DNA and proteins.

This study focuses on protein adsorption onto coatings of the thermosensitive polymer poly(N-isopropylacrylamide) (pNIPAM) which can exhibit low and high protein adsorption properties based on its temperature dependent conformation. At temperatures above its lower critical solution temperature (LCST) pNIPAM polymer chains are collapsed and protein adsorbing whilst below the LCST they are hydrated and protein repellent.

Coatings of pNIPAM on silicon wafers were prepared by free radical polymerisation in the presence of surface bound polymerisable groups. Surface analysis and protein adsorption was carried out using X-ray photoelectron spectroscopy, time of flight secondary ion mass spectrometry and contact angle measurements.

This study is expected to aid the development of stimuli-responsive coatings for biochips and biodevices.

Keywords: Surface modification, protein adsorption, radical polymerisation, stimuli-responsive polymers, biochips

1 INTRODUCTION

Biochips, microarrays and microfluidic devices are becoming a central element of biomedical and biotechnological research. With biomolecules and cells at interfaces being fundamental to these devices much research has been directed to modification of surface chemistry which is known to be a determining factor in protein adsorption and cell attachment [1-3].

Commonly, chemical modification is employed and has focused on the generation of both low fouling chemistries such as poly(ethylene oxide) and bio-adhesive chemistries in order to control the spatial location of biomolecules and cells and to provide a biocompatible surface to allow optimal biological function [3-5]. However this remains a passive approach to direct the location of biomolecules and further control over adsorption and desorption is desired for advanced biomedical and biotechnological applications alike.

A key focus in the further development of advanced devices is the integration of smart, switchable or stimuli-responsive coatings to allow better control over biological elements coming in contact with the surface of engineered devices [6-8]. Here, the development of switchable surfaces and materials with properties that can present both resistant and adsorbent characteristics towards cells and biomolecules such as protein and DNA is desirable [9-12]. Switchable materials and systems can be triggered or controlled by a number of means some of which include temperature, light, solvent, pH, salt concentration and electric fields [13-18]. Some stimuli-responsive materials are sensitive to multiple triggers and others can be engineered to include components sensitive to multiple stimuli [14, 19, 20].

One such switchable material commonly studied is the thermosensitive stimuli responsive polymer poly (N-isopropylacrylamide) (pNIPAM). In water pNIPAM has a lower critical solution temperature of 32 °C which means that

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below 32 °C it is soluble and hydrated and at temperatures above 32 °C it is insoluble and has collapsed conformation [21, 22]. Attachment of pNIPAM to surfaces can be achieved by a number of methods with minimal impact on its phase transition behaviour. Among the pNIPAM coating techniques surface initiated radical polymerisation, plasma polymerisation and electron beam irradiation polymerisation are the most popular [23-25].
The nature of the phase transition of pNIPAM is such that at temperatures below the LCST it is low fouling and will resist the adsorption of proteins whilst at temperatures above the LCST pNIPAM is fouling or bio-adhesive [8]. This property as well as the sharp phase transition and close proximity of the LCST to physiological temperature make pNIPAM attractive for application in biomedical and biotechnological devices. One of the most popular examples of the application of stimuli responsive materials is the use of pNIPAM in cell sheet engineering [23, 26-28]. In this technique cells may be grown to confluency on pNIPAM surfaces at temperatures above the LCST and then released as an intact sheet by lowering the temperature below the LCST [12, 22, 26]. However protein adsorption, an important precursor to cell recognition, is less understood and requires further study along with the mechanism of switchable coatings [1, 29].
In this study we report the generation of stimuli responsive pNIPAM coatings able to resist the adsorption of proteins which alternately can be switched via the application of heat in order to induce the adsorption of proteins. We use highly sensitive surface analytical techniques to probe for the presence of protein at the interface and to investigate the switching mechanism and properties of pNIPAM coatings.
The development of switchable surface coatings and devices promise to provide a new degree of control with the ability to attract or repel biomolecules to and from a solid phase on demand. Secondary events such as cell attachment, proliferation and differentiation may also be controlled with the ability to manipulate biomolecules such as proteins at the solid/liquid interface.
Advancements in this area is expected to benefit both in *vitro* applications such as microarrays, biosensors and chromatography devices as well as in *vivo* applications, such as tissue engineering devices and stimuli responsive drug delivery [30-34].

2 EXPERIMENTAL

2.1 Silicon wafer preparation

Silicon wafers were diced to a size of 10 x 10 mm and ultrasonically cleaned in ultra pure MilliQ (MQ) grade water (18.2 MΩ). Following this, wafers were immersed in 7:3 (v/v) concentrated H_2SO_4 / 30% H_2O_2 (Piranha) solution for a minimum of 30 min, washed with copious amounts of MQ water and dried under a stream of nitrogen.

2.2 Silanisation

Wafers were immersed in a 5 % v/v solution of 3-trimethoxysilylpropyl methacrylate (γMPS, 98%, Aldrich, Sydney, Australia) in acetone for 2 h at room temperature. Samples were then washed with copious amounts of acetone and MQ water and dried under a gentle stream of nitrogen.

2.3 Poly (N-isopropylacrylamide) grafting

N-isopropylacrylamide (NIPAM, 97 %, Sigma, Sydney, Australia) was purified by recrystallisation from distilled n-hexane. Purified NIPAM was made to 7 % w/v solutions in MQ water along with 0.1 % w/v 4,4'-azobis(4-cyanopentanoic acid) (ACPA, 98%, Fluka). Functionalised wafers were immersed in NIPAM/ACPA solution and purged with nitrogen for a minimum of 20 min. Free radical polymerisation occurred in 15-20 min with stirring at 65 °C. Samples were then washed in a large excess of MQ water overnight at 20 °C. Prior to contact angle or XPS analysis samples were dried with a gentle stream on nitrogen

2.4 Protein adsorption experiments

The adsorption and desorption of protein at temperatures above and below the LCST was investigated using bovine serum albumin (BSA, Sigma, Sydney, Australia). Solutions of BSA were made to 1 mg/mL in 1x phosphate buffered saline (PBS, 137 mM NaCl, 27 mM KCl, 6.64 mM $Na_2HPO_4.H_2O$, 1.76 mM KH_2PO_4) at pH 7.4. Samples of Si-γMPS-pNIPAM were incubated in protein solutions under controlled temperatures to investigate their ability to both resist and promote the adsorption of protein in a simulated physiological environment. To investigate the protein resistive properties of formed pNIPAM coatings samples were incubated in protein solution at 20 ± 2 °C with gentle shaking for a period of 4 h. For investigation of protein adsorption at temperatures above the LCST Si-γMPS-pNIPAM samples were incubated at 37 ± 2 °C for 15 min with gentle shaking. Following incubation in protein solutions samples were washed in MQ water at 20 °C or 37 °C for resistive and adsorptive experiments respectively and allowed to dry. Incubation of Si-

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γMPS-pNIPAM in PBS without protein at 37 ± 2 °C for 4 h served as a negative control for protein adsorption experiments and was used to investigate the stability of coatings under these conditions. Samples were stored in a desiccator prior to analysis by ToF SIMS or XPS.

2.5 X-ray photoelectron spectroscopy analysis

X-ray photoelectron spectroscopy (XPS) analysis of surface modified samples was performed on an AXIS Ultra spectrometer (Kratos Analytical Ltd., England) equipped with a monochromatised Al Kα source. The pressure during analysis was typically 1×10^{-9} mbar. Elemental composition was obtained from survey spectra which were collected with a pass energy of 160 eV whilst high resolution spectra were collected with a pass energy of 20 eV. Binding energies for all spectra were referenced to the aliphatic hydrocarbon peak at 285.0 eV.

2.6 Contact angle measurement

A custom built sessile drop apparatus with an Olympus SZ-PT microscope and lens system mated to a Sony CCD camera was use for temperature controlled analysis of static drop contact angle of Si-γMPS-pNIPAM surfaces. Temperature of the substrate during heating experiments was controlled by an AFM Heater Controller attachment (Nanoscope® Heater Controller HS-1, Digital Instruments, Veeco Metrology Group, USA) which was positioned below the sample. Samples were allowed to equilibrate on the heater for at least 10 min prior to measurement with the actual temperature at the surface estimated to be accurate to ± 2 °C. A 10 µL syringe (Hamilton, Reno, USA) was used to dispense droplets of MQ water of approximately 2-3 µL to substrates at 20 °C and 40 °C (according to the heater controller) for 3 separate pNIPAM coated samples. ImageJ software v1.34 was used to analyse droplet images.

2.7 Time of flight secondary ion mass spectrometry analysis

The ToF-SIMS analyses were performed with a PHI TRIFT II (model 2100) spectrometer (PHI Electronics Ltd, USA) equipped with ^{69}Ga liquid metal ion gun (LMIG). A 15 keV pulsed primary ion beam was used to desorb and ionize species from a sample surface. Pulsed, low energy electrons were used for charge compensation. Mass axis calibration was done with CH_3^+, $C_2H_5^+$ and $C_3H_7^+$ in positive mode and with CH^-, C_2H^- and Cl^- in negative mode of operation. A mass resolution m/Δm of ~ 4500 at nominal m/z = 27 amu ($C_2H_3^+$) was typically achieved.

In a ToF-SIMS instrument, the secondary ions are electrostatically extracted to mass a spectrometer and their mass to charge ratios (m/z), are determined by measuring the time it takes for the ions to come off the surface and hit the detector. This technique is "destructive" by nature, however, by applying an ion beam of low current it is possible to limit the probability of multiple ion impact and thus derive data from virtually an intact surface. In this study the primary ion fluxes used were between 3×10^{11} and 6×10^{11} ions cm^{-2}, meeting the static conditions regime [35]. This means that less than 0.1% of the surface atomic sites (1 in 1000) were struck by the primary ion beam in the time of the measurement. The spectra recorded under the 'static SIMS' conditions detailed information of the surface 1 to 2 monolayers, without significantly affecting their chemical integrity.

Samples of Si-γMPS-pNIPAM were characterised by ten positive and one negative mass spectra collected from different, not overlapping areas. A thick layer of BSA dried on a Si wafer was used as the protein control for comparison. Peak intensity normalisation to the total intensity of selected peaks was performed prior to data analysis. Spectra were subjected to further processing by *Analysis of Means* [36]. *Analysis of Means* yielded statistical variability within and between the samples based on a single variable (univariate) assessment.

3 RESULTS

Modified surfaces were characterised at each stage of modification using XPS and contact angle measurements. However, due to similarities between the spectra of proteins and pNIPAM the technique of XPS was not suitable to probe for small amounts of adsorbed protein. Instead, ToF SIMS which has higher surface sensitivity forms the basis for the investigation of protein adsorption to pNIPAM surfaces under different experimental conditions.

3.1 XPS analysis of modified substrates

The chemical composition of modified silicon substrates from XPS analysis is given in table 1. Following cleaning, bare silicon (Si) was shown to have 10.1 % carbon present from residual hydrocarbon contamination. Silicon and oxygen atomic percentages were 51.8 % and 38.1 % respectively indicating the presence of a silicon oxide surface. The oxide layer, containing silanol groups allowed the covalent immobilisation of alkoxy silanes to generate a functionalised surface bearing reactive methacrylate groups. After silanisation with 3-trimethoxysilylpropyl methacrylate, Si-γMPS

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samples show an increase of carbon to 26.7 % along with attenuation of oxygen and silicon to 36.8 % and 36.5 % respectively (Table 1). The methacrylate functionalised substrate was further modified by radical polymerisation of NIPAM to generate pNIPAM coatings. Reaction conditions were optimised so that the dry layer thickness of pNIPAM was sufficient to almost completely attenuate the signal from the underlying silicon substrate in XPS spectra. After polymerisation, XPS analysis of the Si-γMPS-pNIPAM grafted substrate showed carbon at 74.5 % along with oxygen at 12.7 % and the introduction of nitrogen at 12.6 % with the amount of each element present in excellent agreement with the expected atomic ratio of the polymer (Table 1). The observation of a small amount of silicon at 0.3% from the underlying substrate is indicative that the thickness pNIPAM coatings formed here are approximately equal to the XPS sample depth of 8-10 nm for polymers in the dry state. Analysis of Si-γMPS-pNIPAM following incubation in PBS at physiological temperature and pH for 4 h shows virtually no change in the N/C or O/C ratio indicating the coatings are stable under these conditions. A slight increase in silicon from 0.3 % to 0.9 % is noted however this is may be accounted for by variability in sample thickness.

Table 1: XPS data. Atomic concentrations of elements present on substrate and modified substrates in percentage.

	C%	N%	O%	Si%	N/C	O/C
Si	**10.1**	**0.0**	**38.1**	**51.8**	**0.00**	**3.77**
Si-γMPS	**26.7**	**0.0**	**36.8**	**36.5**	**0.00**	**1.37**
Si-γMPS-pNIPAM	**74.5**	**12.7**	**12.6**	**0.3**	**0.17**	**0.17**
Si-γMPS-pNIPAM (PBS stability test)	**74.3**	**12.8**	**12.0**	**0.9**	**0.17**	**0.16**

High resolution C1s spectra provided further insight into the chemical nature of surface coatings following chemical modification. Figure 1A shows the typical C1s spectra of Si-γMPS substrates with fitted curve components attributed to aliphatic hydrocarbon (C1) at 285.0 eV, ether groups (C2) at approximately 286.2 eV and ester groups (C3) at approximately 289.1 eV. The high resolution C1s spectra of Si-γMPS-pNIPAM in figure 1B shows aliphatic hydrocarbon (C1) at 285.0 eV along with amine groups (C2) at 286.2 eV and the introduction of amide functionality at approximately 287.8 eV. The quantification of components C1 – C3 for Si-γMPS-pNIPAM represents the % area under the fitted curves and can be used as a comparative ratio of functional species present in the coating. Components C1, C2 and C3 correspond to areas 65.3 %, 17.3 % and 17.4 % which is in excellent agreement with the theoretical ratio of the chemical structure of pNIPAM (Fig. 1B inset).

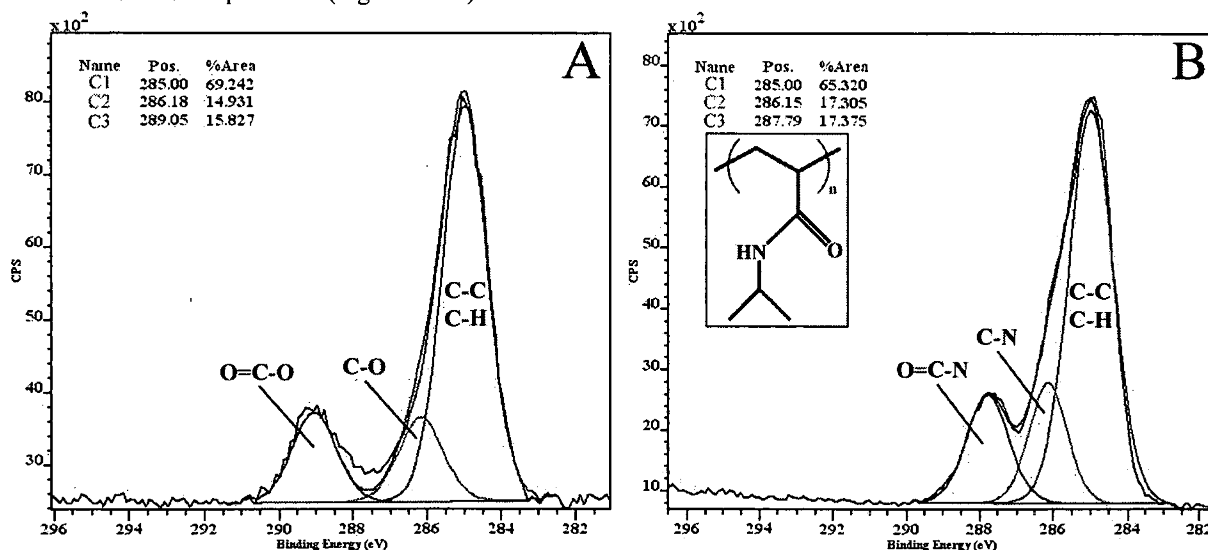

Fig.1: High resolution C1s spectra of Si-γMPS (A) and Si-γMPS-pNIPAM (B) substrates. Included in (B) is the structure of pNIPAM.

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3.2 Contact angle measurement

To investigate the switching of formed pNIPAM coatings analysis of contact angle was used to study surface wettability at temperatures above and below the phase transition temperature. Changes in wettability of up to 140 degrees have been reported for pNIPAM coatings on substrates with engineered topography however for flat substrates the difference is much less pronounced [37, 38]. At 20 °C the average contact angle of Si-γMPS-pNIPAM samples was 55 ± 4 degrees whilst at 40 °C the samples were more hydrophobic in nature with a contact angle of 85 ± 4 degrees. The observed contact angle difference of 30 degrees indicates that the properties of formed pNIPAM coatings may be transformed to a more hydrophobic state with a relatively small heat stimulus.

3.3 Time of Flight Secondary Ion Mass Spectrometry analysis of adsorbed protein

XPS analysis of small amounts of protein deposited onto pNIPAM modified substrates is hampered by spectral similarities between proteins and polyamides as this technique analyses for elemental compositions, which are similar for proteins and pNIPAM. For this reason many studies have analysed protein solutions after they have come in contact with pNIPAM surfaces to investigate a loss or gain in protein from adsorption to or desorption from the surface and thus make assumptions on the amount of protein remaining at the interface [17, 30]. Surface analysis with ToF SIMS however can provide actual evidence for minute amounts of protein remaining at the interface. Figure 2 shows survey spectra from positive ion static SIMS (SSIMS) for Si-γMPS-pNIPAM samples before and after exposure to protein solutions at different temperatures along with BSA as a control. Figure 2A shows the typical fragmentation pattern of BSA, the protein control whilst figure 2B represents the pNIPAM control. As can be seen, the mass spectra of BSA and pNIPAM differ substantially; though many of the same peaks are evident, unique peaks also exist and moreover the intensity patterns differ substantially (Fig. 2A, 2B). Comparison of the survey spectra of pNIPAM prior to BSA exposure (Fig. 2B) with the spectra of pNIPAM samples after BSA exposure at 20 °C (Fig. 2C) and 37 °C (Fig. 2D) shows no obvious difference in fragmentation peaks and indicates that more detailed analysis is required.

By using the intensities of selected mass fragments that are unique to either the polymer or protein ToF SIMS is excellent technique to probe for the presence of adsorbed protein in amounts much less than monolayer.

This is apparent in figure 3 which shows the high mass resolution spectra of the m/z 58 amu region. Here two peaks are present at 58.0293 amu and 58.0656 amu representing C2H4NO+ and C3H8N+ fragments respectively. The C3H8N+ mass peak features in both the BSA (Fig. 3A) and pNIPAM (Fig. 3B) spectra however the less intense C2H4NO+ peak is characteristic of BSA and can therefore be used to probe for adsorbed protein. Figures 3C and 3D show predominantly C3H8N+ however figure 3D appears to have a small contribution from C2H4NO+.

The results in figure 3 were critically evaluated using statistical data analysis. Figure 4a shows the mean normalised intensities of the C2H4NO+ and C3H8N+ peaks and their associated confidence intervals. The intensities of the fragments vary allowing differentiation between different samples. Peaks attributed to C2H4NO+ from amino acids asparagine and glutamine present in BSA and C3H8N+ arising from the N-isopropyl groups of pNIPAM distinguish BSA and pNIPAM controls respectively (Fig. 4A). With pNIPAM + BSA at 20 °C the intensity for C2H4NO+ and C3H8N+ peaks is at the same intensity as that of the pNIPAM control indicating that BSA, containing the C2H4NO fragment, has not adsorbed under these conditions (Fig. 4A). For pNIPAM + BSA at 37 °C the intensity of the C2H4NO+ fragment matches that of the BSA control whilst the C3H8N+ fragment intensity from the polymer has been slightly attenuated (Fig. 4A). This indicates that a small amount of protein is present on the pNIPAM coating.

Figure 4B shows the intensity ratio of C3H8N+/C2H4NO+ for samples in figure 4A and highlights the similarity of pNIPAM and pNIPAM + BSA at 20 °C indicating a lack of adsorbed protein. Also apparent is that the ratio of C3H8N+/C2H4NO+ for pNIPAM + BSA at 37 °C is vastly different from the 20 °C sample and similar to the BSA sample ratio indicating adsorption of BSA has occurred (Fig. 4B). This result shows that by controlling the temperature to below the LCST the pNIPAM coatings in their hydrated form are able to screen the surface and resist adsorption of BSA, a particularly sticky plasma protein. Alternately, by switching the pNIPAM coatings with heat, small amounts of protein were able to be adsorbed and retained at the interface.

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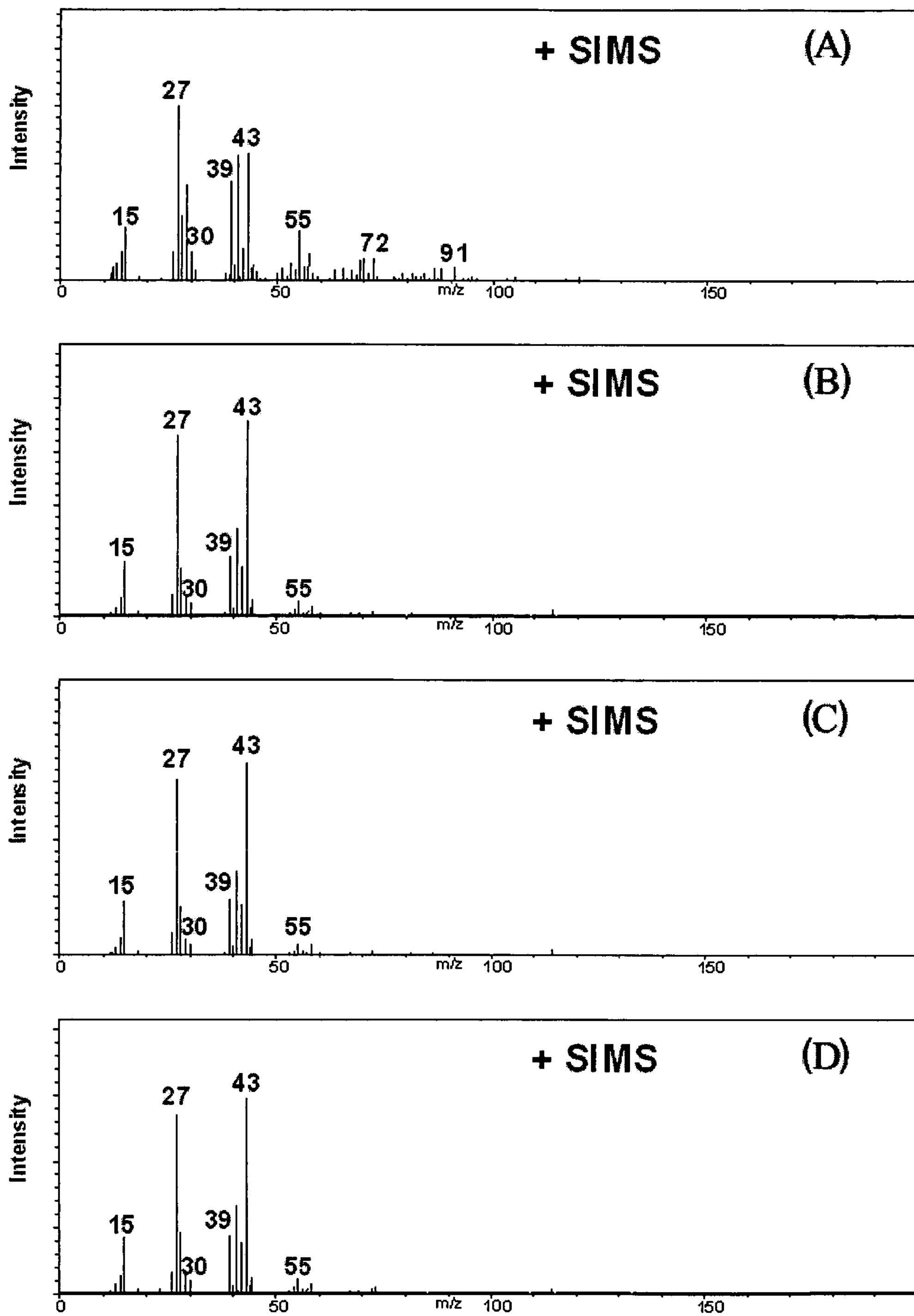

Fig. 2: Positive SSIMS spectra for: (A) BSA; (B) pNIPAM; (C) pNIPAM + BSA (20 °C); (D) pNIPAM + BSA (37 °C)

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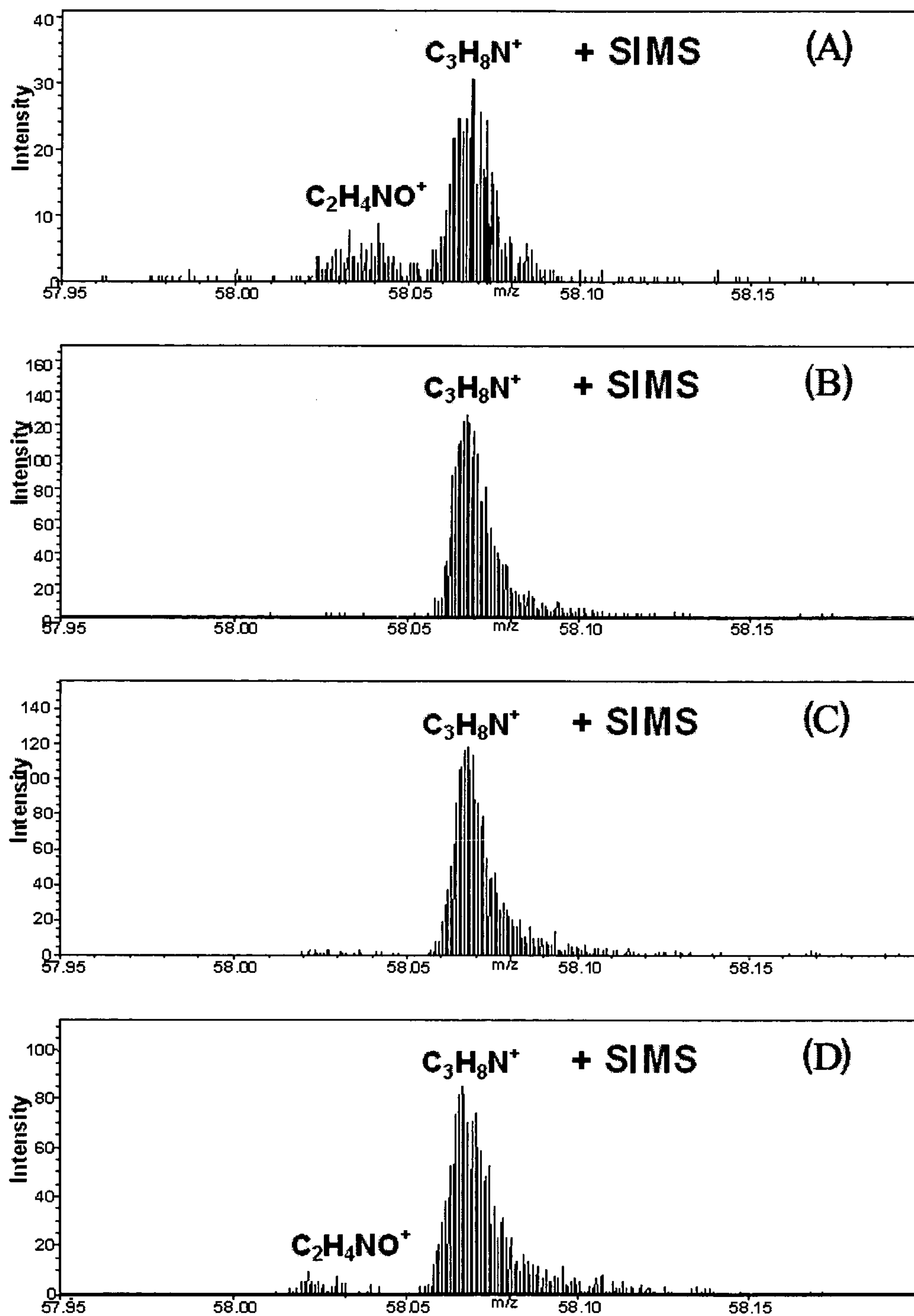

Fig. 3: High resolution positive SSIMS spectra for: (A) BSA; (B) pNIPAM; (C) pNIPAM + BSA (20 °C); (D) pNIPAM + BSA (37 °C)

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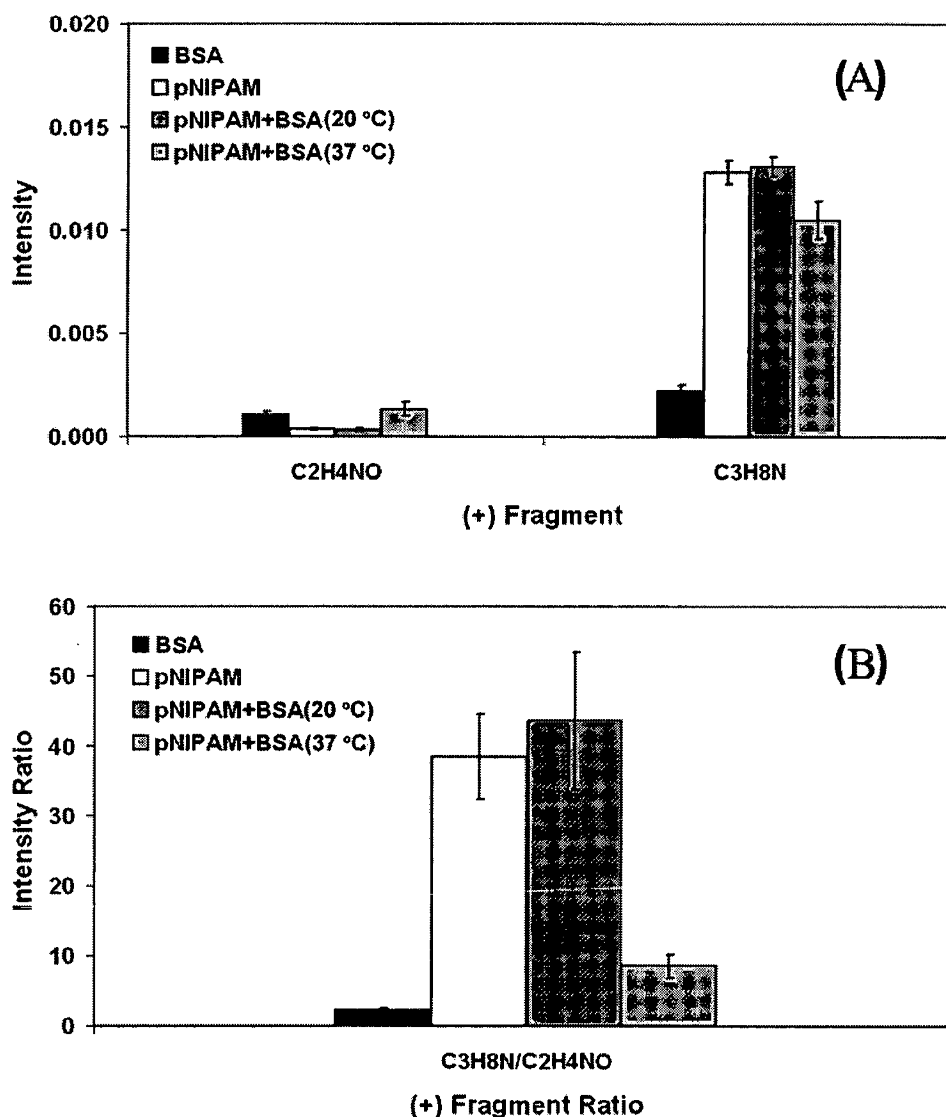

Fig. 4: C2H4NO+ and C3H8N+ intensities (A) and their ratios (B) for BSA, pNIPAM, pNIPAM + BSA 20 °C and pNIPAM + BSA 37 °C (analysis of means P = 95 %, t-distribution).

4 CONCLUSION

In this study we have demonstrated control over protein adsorption through manipulation of the surface properties of silicon substrates modified with thin coatings of thermosensitive poly(N-isopropylacrylamide). Control over the surface properties was achieved through manipulation of temperature above and below the LCST of the polymer coating which in turn allowed the repulsion or attraction of protein at the solid phase as evidenced by ToF SIMS spectra.

This study also demonstrates that the sensitivity of surface analytical techniques is an important factor for consideration when investigating small amounts of adsorbed biomolecules on biomaterial surfaces. Studies into enhancing protein adsorption on pNIPAM coatings and investigation of the reversibility of switching protein adsorption and desorption is the subject of further work.

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