Switchable coatings for biomedical applications

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ABSTRACT

The control over protein adsorption is of major importance for a variety of biomedical applications from diagnostic assays to tissue engineered medical devices. Most research has focused on the prevention of non-specific protein adsorption on solid substrates. Examples for surface modifications that significantly reduce protein adsorption include the grafting of polyacrylamide, poly (ethylene oxide) and polysaccharides.
Here, we describe a method for creating surfaces that prevent non-specific protein adsorption, which in addition can be transformed into surfaces showing high protein adsorption on demand.
Doped silicon wafers were used as substrate materials. Coatings were constructed by deposition of allylamine plasma polymer. The subsequent grafting of poly (ethylene oxide) aldehyde resulted in a surface with low protein fouling character. When the conductive silicon wafer was used as an electrode, the resulting field induced the adsorption of selected proteins.
Surface modifications were analysed by X-ray photoelectron spectroscopy and atomic force microscopy. The controlled adsorption of proteins was investigated using a colorimetric assay to test enzymatic activity. The method described here represents an effective tool for the control over protein adsorption and is expected to find use in a variety of biomedical applications particularly in the area of biochips.

Keywords: Surface modification, protein adsorption, plasma polymerisation

1 INTRODUCTION

Protein, DNA and cellular arrays are becoming extremely important for the areas of proteomics, genomics and molecular cell biology research as well as being of high interest in the development of the next generation of biotechnological devices such as 'lab on a chip', microreactor and protein purification devices. [1-6] These devices commonly consist of a micromachined, microfluidic or nanofabricated platform for the spatial localisation of biomolecules so that vast amounts of interactions may be screened simultaneously. [1-6] Platforms employed for the development of biochips and similar applications are commonly engineered using various substrates, which include metals (gold, platinum), silicon based materials (eg silicon, glass, and quartz) and a range of different polymers. [5, 6] Silicon and gold platforms are commonly applied to biochips and arrays due their conductivity. [3, 7]
A vital aspect of all biomedical devices is the control or prevention of biofouling. Here, chemical modification of surfaces has particularly concentrated on the control over protein and cell/biomaterial interactions and the generation of low-fouling surfaces.[8,9]
There exists a multitude of techniques for the generation of low-fouling surfaces including but not limited to solvent casting, self-assembling monolayers, plasma polymer deposition, and covalent immobilisation.[8, 10, 11]
Low-fouling surfaces are commonly investigated by their ability to prevent protein adsorption, however other research has involved adsorption of lipids and more complex solutions of biomolecules to mimic physiological conditions. [8, 9, 12] From these studies, it has been shown that initial fouling or conditioning of the film with certain biomolecules may facilitate the adhesion of other biomolecules. [8-10, 12]
The most promising results from low-fouling films to date have been from surface modification with poly (ethylene oxide) (PEO). High density PEO graft polymers have been shown to be highly successful in preventing biofouling due to the steric barrier that prevents proteins from contacting the underlying less hydrophilic surface.[8-10, 13]

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Grafting of low-fouling polymers with high density to substrates can be achieved by displaying a high number of functional groups on the substrate surface, which each act as potential attachment sites.[9, 14] Plasma polymer films can provide suitable functional coatings with high chemical resistance and good adherence.[15]
Plasma polymers have found many material and biomaterial applications owing to their ability to provide films with complete surface coverage (pinhole free), low roughness and controlled concentrations of functional groups.[15, 16] Plasma polymerisation also has the advantage of being a sterile solvent-free technique. [4, 16] Films of amine plasma polymers such as allylamine plasma polymers (ALAPP) are of significant value as coatings for biomedical devices due to the ease of subsequent modification. Studies into allylamine plasma polymers have found that allylamine is not as sensitive as other monomers towards fragmentation in the plasma, producing films with a high retention of the amine functionality within the deposited film.[16, 17]
Biochips containing protein, DNA or cell microarrays are exemplary applications that require spatially controlled attachment of biomolecules. This is currently, achieved using patterning of surface chemistry or robotic spotting methods.
Conventional patterning techniques include photolithography, electron beam irradiation and microcontact printing. [1, 2, 4] Methods for generation of sub micron patterned arrays have also included dip pen nanolithography (DPN), finely focused ion beam lithography (FFIB) and excimer laser ablation. [2, 17]
However further control over the adsorption of biomolecules is desirable and would be highly advantageous to biochips and other biomedical applications alike.
Methods to control the adsorption of biomolecules to surfaces via switching have the advantage of attracting and/or enriching biomolecules of interest on a solid phase as well as releasing them on demand. [4, 7] This also means that biomolecules may be delivered to a known location at a specific time, so that they may interact with attached cells or receptors. This gives a new dimension of control and has been demonstrated using electrostatic attraction and repulsion of charged species, thermoselective adsorption to switchable surface chemistries and ion exchange switching. [4,7,18, 19]
These mechanisms have exciting possibilities for in *vitro* applications such as microarrays, biosensors and biotechnological devices as well as applications in *vivo*, such as tissue engineered devices and stimuli responsive drug delivery. [14, 20, 21]

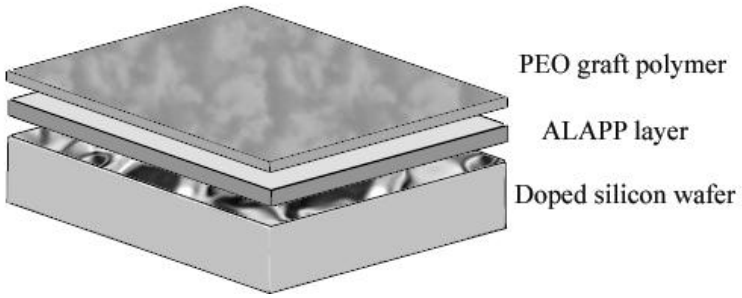

Fig. 1: Schematic representation of the surface modified silicon substrate (not to scale).

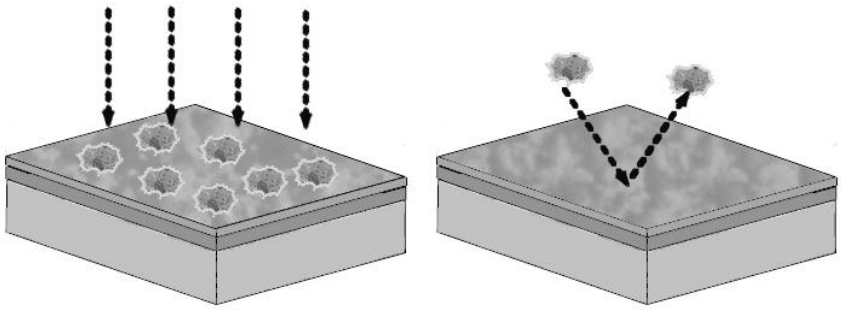

Fig. 2: Schematic representation of attraction and repulsion of enzymes. Left an electrostatic field draws proteins from solution to the surface based on their net charge at a given pH. Right: a repulsive bias assists in preventing protein adsorption at the surface.

In this paper, we describe a method for the adsorption of protein on demand to a low fouling surface. The concept for such a switchable device described in Figures 1 & 2, allows transformation of a surface with low fouling properties

under physiological conditions to a surface that can retain proteins by the application of an electrostatic bias. Proteins in solution of opposite net charge are attracted to the charged substrate based on their inherent net charge at a given pH.

2 EXPERIMENTAL

2.1 Silicon wafer preparation

For plasma polymerisation, silicon pieces were sonicated for 1 h in 2% v/v RBS (RBS 35, Pierce USA) solution followed by thorough washing with 10x 10 mL ultra pure (MQ) water (Labconco Water PRO PS, 18.2Ω). Silicon was dried with a stream of nitrogen and then UV treated in a custom-built UV cabinet polished side up for 1hr.

2.2 Plasma polymerisation

Plasma polymerisation experiments were carried out in a custom-built reactor as described elsewhere. [22] Briefly, the cylindrical reactor chamber is defined by a height of 35 cm and a diameter of 17 cm. Samples were placed on a circular electrode with a diameter of 9.5 cm, approximately 12.5 cm below a U shaped electrode. Allylamine (Aldrich 98% purity) was used as monomer. Parameters for the deposition of the allylamine plasma polymer (ALAPP) were a frequency of 200 KHz, a power of 20 W, an initial monomer pressure of 0.333 mbar and a deposition time of 25 s.

2.3 Poly (ethylene oxide) grafting

Grafting of methoxy poly (ethylene oxide) aldehyde (PEO, Shearwater Polymers Inc.) with an average molecular weight of 5000 to freshly deposited ALAPP films was carried out by reductive amination using $NaCNBH_3$ (Sigma, 90%) as the reducing agent. Grafting was performed under 'cloud point' conditions at 60°C in 0.1 M sodium phosphate buffer containing 11% w/v K_2SO_4. The reaction mixture contained 3 mg/mL $NaCNBH_3$ and 2.5 mg/mL of the PEO. The reaction time was 16 h. Afterwards samples were rinsed repeatedly with MQ water and finally allowed to stand in MQ water for at least 2 h.

2.4 Adsorption to different surface chemistries

Samples of PEO-ALAPP and ALAPP on silicon were incubated overnight at 4 ^{o}C in 0.1 or 0.5 mg/mL PBS solutions of horseradish peroxidase (HRP, Sigma) or FITC conjugated horse radish peroxidase (FITC-HRP, Sigma) in sealed glass containers.

2.5 Electrostatic attraction of enzymes

Using a custom-made Teflon cell, horseradish peroxidase (HRP, Sigma was attracted to PEO modified silicon wafer surfaces. Solutions of HRP were made to 0.5 mg/mL in 1x, 0.5x and 0.1x PBS (1x PBS (4g NaCl, 0.1g KCl, 0.705g Na_2HPO_4, 0.12g KH_2PO_4 in 1L MQ water buffered to pH 7.4 and then diluted to 0.1 mg/mL protein.

The experimental set up included PEO samples being clamped against a Teflon cell with a 6mm O-ring to provide a seal. This formed a reproducible surface area exposed to protein solutions.

Following application of a ±1 V potential between the doped silicon substrate and a platinum counter electrode, 1 mL of PBS followed by 1 mL of 0.1 mg/mL enzyme solution was pipetted into the cell. Attraction or repulsion experiments were performed for periods of 10 and 15min followed by washing with 8x 2 mL of PBS with bias still present. Samples were then removed from the cell and dried with a stream of nitrogen followed by immersion in 3,3', 5,5'-tetramethylbenzidine (TMB, liquid substrate system for ELISA, Sigma).

2.6 X-ray photoelectron spectroscopy analysis

X-ray photoelectron spectroscopy (XPS) analysis of surface modified samples was performed on an AXIS HSi spectrometer (Kratos Analytical Ltd, GB), equipped with a monochromatised Al Kα source. The pressure during analysis was typically 5 x 10^{-8} mbar. The elemental composition of samples was obtained from survey spectra, collected at a pass energy of 320 eV. High-resolution spectra were collected at a pass energy of 40 eV. Binding energies were referenced to the aliphatic carbon peak at 285.0 eV.

2.7 Atomic force microscopy analysis

Atomic force microscopy (AFM) was performed using a Multi Mode, Nanoscope IV Digital Instruments microscope (Veeco Corp. Santa Barbara, USA) in both tapping and contact mode in air. Ultra-Sharp (NT-MDT, Moscow, Russia) and FESP (Digital Instruments, Veeco, USA) silicon cantilevers with 150-350 and 40-70 KHz resonance frequencies

respectively were used for tapping mode and silicon nitride tips (DI Instrument) with a nominal spring constant of 0.58 – 5.5 Nm^{-1} were used for contact mode.

2.8 Detection of adsorbed enzymes

A Molecular Devices νmax Kinetic Plate Reader was used to detect converted TMB substrate solution by reading the absorbance at 650 nm (A_{650}). Softmax® PRO software was used to collect plate reader data with a typical plate experiment containing a blank as well as the final washes plus sample solutions. A HRP enzyme standard curve with a range of 0.1 –10 ng was obtained by pipetting (in quadruplicate) 100 uL dilutions to the wells of a 96-well plate and incubating overnight. It was assumed that all of the protein adsorbed to the plate surface. The solution was then aspirated from the wells and TMB substrate solution added. The standard curve could be applied to experiments with assay incubation ranges of 2-180 minutes.

3 RESULTS

3.1 Plasma polymerisation of allylamine

ALAPP films were investigated by XPS with respect to elemental composition (Table 1). Due to the lack of silicon from the substrate surface, it was concluded that films generated were thicker than the sampling depth of the XPS (~10 nm). XPS results also show the presence of 9.0 % oxygen in the deposited film, which is most likely due to a small amount of oxygen present in the plasma chamber during treatment as well as oxygen uptake of the deposited film after removal from the plasma chamber. The latter is known to occur due to the presence of highly reactive species still present in the film upon exposure to air. [16]

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

	C1s	N1s	O1s	F1s	Si2p	O/C	N/C
Si	12.9	0.0	34.2	2.1	50.8	2.66	0.00
Si-ALAPP	80.0	11.0	9.0	0.0	0.0	0.11	0.14
Si-ALAPP-PEO	73.1	5.7	21.0	0.0	0.3	0.29	0.08

The high amount of nitrogen (11.0%) detected after plasma polymerisation allows the conclusion that a high number of nitrogen containing groups have been incorporated in the plasma polymer film, including amine functional groups, which can be used for further chemical modification.

The high resolution C1s spectrum of ALAPP shows the typical profile of a plasma polymer with a broad peak due to the variety of functional groups present (Fig. 3). From lower to higher binding energy we see the aliphatic carbon peak referenced to 285.0 eV followed by a peak associated with ether and amine groups providing further support that ALAPP films retain a high degree of their amine functionality. Then we have a peak associated with carbonyl and amide groups and finally a small peak attributed to the presence of ester groups.

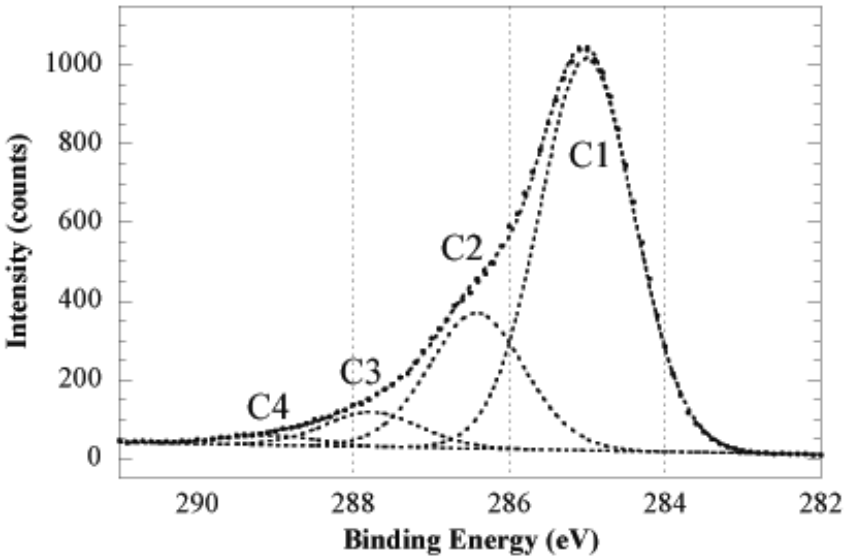

Fig. 3: The figure represents the high resolution C1s spectrum of a fresh ALAPP coating. The spectrum is fitted with 4 components; Component C1 at 285.0 eV represents C-C & C-H, C2 at 286.4 eV represents the C-O & C-N, C3 at 287.8 eV represents C=O & N-C=O peak and finally C4 at 289.1 eV represents O-C=O.

Atomic force microscopy of ALAPP shows that the formed films are quite smooth and free of pinholes (Fig. 4). They typically exhibit features with heights of less than 1 nm to yield a surface roughness RMS of 0.3 nm ± 0.03. For the purpose of comparison, AFM of the polished silicon substrate is provided presenting a smooth surface (RMS 0.2 nm) as the platform for plasma modification (Fig. 4).

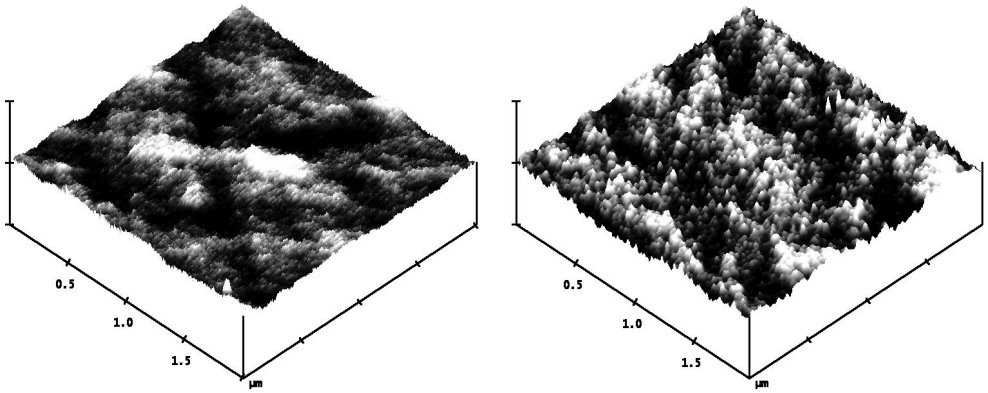

Fig. 4: 2 x 2 µm AFM images of silicon substrate (left) and substrate modified with ALAPP (right). Z scale for images is 5 nm.

3.2 PEO grafting on ALAPP coated substrate

Atomic concentration data of the Si-ALAPP-PEO surface show an increase in the O/C ratio and a decrease in the N/C ratio indicating an increase in ether groups due to the successful grafting of the PEO layer (Table 1). This observation is reiterated by the presence of an intense peak at approximately 286.6 eV due to C-O-C groups of the PEO in the C1s spectra (Fig. 5). The fact that the C-O-C peak is visible in addition to the peaks associated with the plasma polymer suggests a PEO coating thickness of less than 10 nm in the dry state.

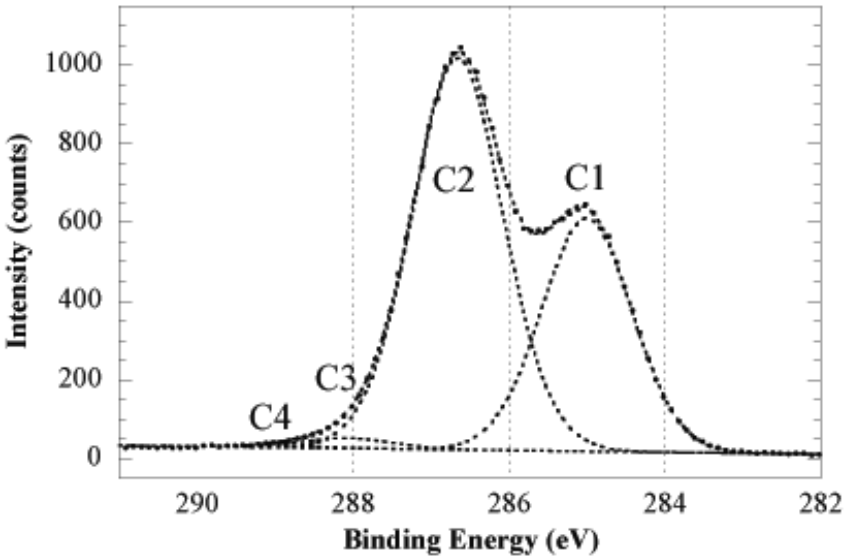

Fig. 5: The modification of the Si-ALAPP coating with PEO shows a significant increase in the C2 peak at 286.6 eV attributed to the covalently attached PEO graft polymer.

The area under the deconvoluted components of Si-ALAPP and Si-ALAPP-PEO modified substrates is summarised in table 2. Most notable is the rise in component C2 of approximately 38 % due to the presence of the ether groups in the grafted PEO chains.

Table 2: Curve quantification shows the percentage area under fit curves.

	Si-ALAPP	Si-ALAPP-PEO
C1	68.35	36.34
C2	23.84	61.75
C3	6.12	1.60
C4	1.69	0.32

Analysis of PEO surface topography by AFM showed smooth films with roughness slightly higher than that of the plasma polymer with an RMS of 0.51 nm ± 0.09 (Fig. 6). The presence of PEO surface structures and aggregates was noted in some samples and is thought to be indicative of a high-density graft. [13]

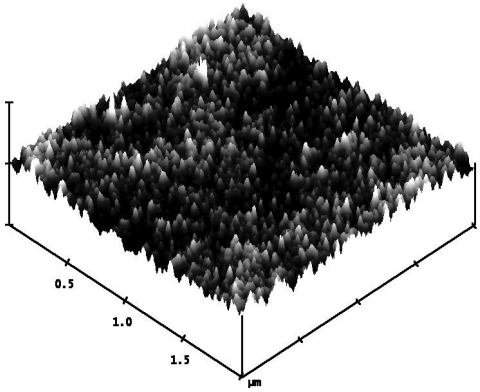

Fig. 6: 2 x 2 µm AFM image of dry PEO showing the smooth surface of formed films. Z scale is 5 nm.

3.3 Adsorption of enzymes

An enzyme was used as a model compound to investigate the amount of protein that could be adsorbed to a specific surface area. An initial study into the low fouling ability of PEO was carried out by immersing PEO and ALAPP samples in a solution of HRP followed by exposure to substrate solution and subsequent colorimetric analysis. With HRP enzyme, it was shown that adsorption occurred preferentially to ALAPP in comparison to PEO surfaces. It was detected however that some protein adsorption was occurring on PEO samples. However this was thought to be due to immersing the whole wafer in enzyme solution including areas not covered by PEO followed by substrate solution thus allowing enzyme adsorption to the bare silicon to contribute to the signal.

A standard curve was generated using a 96 well plate with known amounts of protein adsorbed overnight. The A_{650} was taken at a variety of intervals so that the standard curve could be applied to experiments where A_{650} had been analysed at a number of times. A working range of 0.1-10 ng was identified from standard curves for up to 10 minutes. Following this a range of 0.1-5 ng was identified for up to 3 hours although most experiments were less than 1 hour incubation. In order to employ the standard curve we required control over the surface area exposed to enzyme solution.

To investigate this further, we used a Teflon cell, which allowed us to expose a defined surface area for adsorption experiments. It also allowed application of an electrical bias to the unmodified back of the silicon wafer. PEO exposed to HRP without a bias showed negligible amounts present with $A_{650} = 0.042$ compared to a control value of 0.035 from substrate solution at the same time. Results for electrostatic attraction and repulsion experiments to enrich or minimise the amount of protein at the surface can be seen in Figure 7 below.

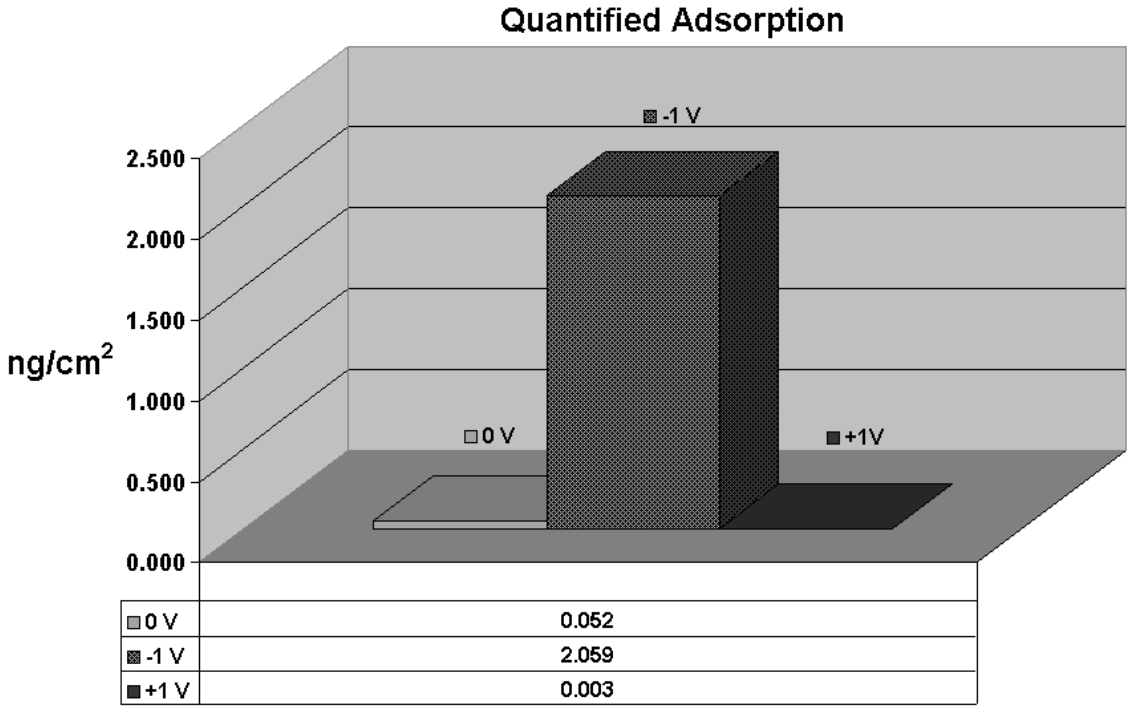

Fig. 7: Amount of HRP adsorbed to PEO surfaces exposed to protein solution in controlled surface area experiments.

The isoelectric point of HRP isozyme C is approximately 8.5 therefore its net charge in pH 7.4 buffer solution is positive. [23] Upon application of a negative bias to the substrate we were able to attract significant amounts of protein and overcome the low fouling nature of the PEO surface. Upon application of the opposite bias to the substrate we were able to assist the prevention of enzyme adsorption to the PEO coated surface.

The ability to switch surfaces from protein repellent to protein adsorbent is expected to be of value to a range of biomedical applications.

4 CONCLUSION

The successful implementation of "lab on a chip" approaches relies, at least in part, on the precise control over chip material surface properties. The advent of methods for control over the interactions of biomolecules with surfaces of engineered materials will be paramount for progress towards the goal of sensitive, cost-effective high-throughput devices.

In this study we were able to demonstrate for the first time the ability to induce adsorption of proteins to a low fouling surface by the application of an electrostatic field under physiological conditions.

The preliminary work carried out here may be valuable to future generations of functional biotechnological and biomedical devices since many of these devices will require switchable surface properties.

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