Mater. Res. Soc. Symp. Proc. Vol. 1354 © 2011 Materials Research Society
DOI: 10.1557/opl.2011.1382

Universal Biomolecule Binding Interlayers Created by Energetic Ion Bombardment

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ABSTRACT

The ability to strongly attach biomolecules such as enzymes and antibodies to surfaces underpins a host of technologies that are rapidly growing in utility and importance. Such technologies include biosensors for medical and environmental applications and protein or antibody diagnostic arrays for early disease detection. Emerging new applications include continuous flow reactors for enzymatic chemical, textile or biofuels processing and implantable biomaterials that interact with their host via an interfacial layer of active biomolecules. In many of these applications it is desirable to maintain physical properties of an underlying material whilst engineering a surface suitable for attachment of proteins or peptide constructs. Nanoscale polymeric interlayers are attractive for this purpose.

We have developed interlayers[1] that form the basis of a new biomolecule binding technology with significant advantages over other currently available methods. The interlayers, created by the ion implantation of polymer like surfaces, achieve covalent immobilization on immersion of the surface in protein solution. The interlayers can be created on any underlying material and ion stitched into its surface. The covalent immobilization of biomolecules from solution is achieved through the action of highly reactive free radicals in the interlayer.

In this paper, we present characterisation of the structure and properties of the interlayers and describe a detailed kinetic model for the covalent attachment of protein molecules directly from solution.

INTRODUCTION

Environmental biosensors[2], antibody microarrays for early and precise disease diagnosis [3]and bio-mimetic surface coatings for medical implants[4, 5] require functional[4, 6] immobilised proteins, such as enzymes and antibodies. The capability to robustly immobilise all proteins expressed in a cell would enable “reverse phase” microarrays [7]. The biological responses to implanted biomedical devices could potentially be controlled with a conformal coverage of bioactive proteins or peptide segments [4, 8-10]. Simple techniques to achieve strong and functional binding[9] that perform well across a wide range of proteins and on a wide range of surfaces are required to facilitate the development of medical and sensing technologies that call for immobilised biomolecules with high functionality.

The *conformation* or shape of a biomolecule is the configuration in space that minimises the energy of the protein and its immediate environment. The *native conformation* is typically required to enable the protein molecule’s biological function. When attempting to immobilise

functional protein molecules we must therefore consider the effects of a proximate surface on the molecule's conformation. Figure 1 depicts schematically a protein molecule as a chain of covalently linked amino acids (a) that adopts configuration (b) to minimise free energy in solution. It results in predominantly hydrophilic amino acid residues (shown in white) on the surface of the molecule exposed to solution and hydrophobic residues (shown in black) in its centre. This energy minimising structure is known as the protein molecule's *native conformation*. In the presence of a hydrophobic surface there are other configurations that allow for energy minimisation of the system. These are conformations (c) in which the protein exposes predominantly hydrophobic residues to the surface while exposing predominantly hydrophilic residues to the surrounding water molecules. In the presence of a hydrophilic surface there are no significant energetic advantages to be gained from conformational changes but there are also no forces for adhesion. Strongly hydrophilic surfaces therefore to not adhere protein molecules from solution. An ideal immobilisation surface should be hydrophilic to avoid destruction of protein native conformations and must therefore rely on forces other than physical interactions to immobilise protein molecules. Covalent bonding has been demonstrated as a way to achieve strong immobilisation. In previously reported covalent coupling approaches, specially synthesised linker molecules (d) that have reactive end groups to form bonds with specific groups on amino acid side chains of the protein molecule and with reactive sites on the surface

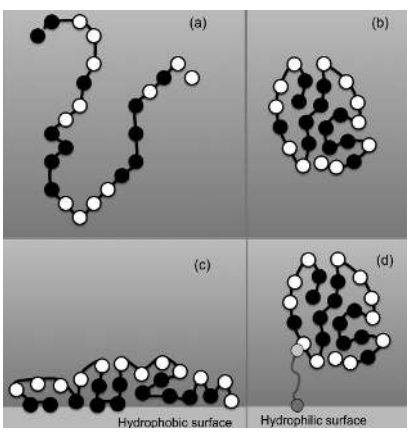

Figure 1: A protein, made up of a sequence of amino acids (a), covalently linked by amide bonds, adopts a configuration in solution (b), that minimises the Gibbs free energy of the system including the surrounding solvent. In the presence of hydrophobic surface (c), other configurations will represent energy minima. These configurations place hydrophobic residues on the surface and expose hydrophilic residues to solution. The proximity of a hydrophilic surface (d), does not give rise to any new configurations that would lead to energy minimisation of the system.

are required. Although highly effective, this method of immobilisation is rarely implemented in non-research applications because it involves complex and time consuming wet chemical steps and limits the surfaces that can be used for immobilisation. Despite the fact that physical approaches yield unstable, non-robust and variable attachment that compromises the native conformation of immobilised proteins they dominate in commercially realised applications due to their simplicity and low cost.

Free radicals are often seen as the "bad guys" of biology, having been implicated in aging [11] and in many diseases arising from the malfunctioning of proteins[12]. However, evidence is emerging that shows their potential to do useful tasks such as immobilising proteins on surfaces while preserving protein function. An effective method of creating buried radicals is to treat an

organic polymer with energetic ions[13, 14]. Treating PTFE with energetic ions resulted in a very reactive surface which formed strong bonds with applied adhesives[15]. Organic polymer surfaces treated by energetic ions, either post-formation or during their deposition, strongly attached protein molecules from solution[10, 16-29]. Occasional speculation about a link between strong binding and free radicals can be found in the literature[16, 30, 31] but until now there has been little direct evidence and the crucial role of radical migration was not recognised.

In this paper, we review a new approach to the surface immobilisation of functional biomolecules[32] that relies on a reservoir of mobile free radicals, created under the surface during the ion irradiation of polymeric materials.

MATERIALS AND METHODS

Polymers for plasma processing were purchased from Goodfellow, UK. Enzymes for surface immobilization were purchased from Sigma Aldrich and Human recombinant tropoelastin was expressed in house as described in [33].

Plasma immersion ion implantation of polymer samples was carried out in an inductively coupled radio-frequency plasma. Samples were mounted on a stainless steel holder, with a stainless steel mesh of 150 mm diameter, electrically connected to the holder and placed 45 mm in front of the sample surface. Acceleration of ions from the plasma was achieved by the application of -20 kV high voltage bias pulses of 20 ms duration to the sample holder at a frequency of 50 Hz, unless otherwise specified. The sample holder was earthed between the pulses. The samples were treated for durations of 20 - 1600 s (800 seconds unless otherwise specified), corresponding to implantation ion fluence of $0.02 – 2.0 \times 10^{16}$ ions/cm^{-2}.

For deposition of ion treated plasma polymers on non-polymeric surfaces acetylene (purity 98% and flow rate 10 sccm) was injected as the polymer precursor and mixed with argon and nitrogen unless otherwise specified. A radio frequency (RF) electrode (bottom electrode) was used to generate plasma while the substrate holder (top electrode) was biased by a dc pulsed voltage source. Unless stated otherwise, the pulse voltage was -200 V at 10 kHz with a duty cycle of 10%. Substrates used included 316L stainless steel foil (25 µm thick) for ELISA and thin (12 µm thick) polyimide film for ESR measurements.

Electron spin resonance (Bruker Elexsys E500 EPR spectrometer) was used to detect unpaired electrons. The spectrometer operated in X band with a microwave frequency of 9.33GHz and a centre field of 3330G, at room temperature and was calibrated using a weak pitch sample in KCl and also with DPPH (a,a'–diphenyl-b-picrylhydrazyl). Samples for analysis were cut to a size of 5cmx5cm, rolled and placed into a quartz tube of 5 mm diameter.

Wettability was measured using the sessile drop method (Kruss DS10) with de-ionised water, glycerol, methylene diiodine and formamide. The surface energy and its components (polar and dispersic parts) were calculated using the Owens-Wendt-Rabel-Kaelble model. Atomic force microscopy (AFM) images of samples were collected on a PicoSPM instrument in tapping mode and analysed using the WSxM software (version 3, Nanotec Electronica S.L.).

Attenuated total reflection Fourier transform infra-red (ATR-FTIR) spectroscopy was used to study changes in surface chemistry and detect the presence of immobilised protein. We used a Digilab FTS7000 FTIR spectrometer fitted with an ATR accessory (Harrick, USA) with trapezium Germanium crystal and incidence angle of 45º. The thickness of the measured layer was 400-800 nm. Samples undergoing SDS treatment prior to FTIR measurement were immersed for 1 hour in sodium dodecyl sulfate (SDS) 2% solution at 70C unless stated otherwise

and then washed in milliQ-water 3 times (20 mins each) at 23C. All samples were dried overnight prior to measurement. The intensity of the 1462 cm^{-1} methylene group vibration was used as an internal standard for normalisation. Changes in C=O group concentration was determined from the absorption intensity at 1720 cm^{-1}. The Amide A, I and II absorptions from the protein backbone were used to quantify protein. Deconvolution of the amide I line using the GRAMS software provided information on changes in protein conformation with Gaussian peaks at 1,694, 1,684, and 1,674 cm^{-1} representing β-turns; 1,661 and 1,649 cm^{-1} for α-helices;1,635 cm^{-1} for random structures; and 1,623 cm^{-1} for β-sheets.

A tetramethylbenzidene (TMB) assay was used to assess the activity of horse radish peroxidase immobilised on the polymer surfaces. After overnight incubation in the HRP buffer solution, samples were washed six times for 20 min in fresh buffer solution. And then clamped between two stainless steel plates separated by an O ring (inner diameter 8mm) which sealed to the sample surface. The top plate contained a 5-mm-diameter hole, enabling the addition of 75 microlitres of TMB (3,3',5,5' tetramethylbenzidine liquid substrate system for ELISA—Sigma T0440), an HRP substrate containing 0.012% hydrogen peroxide. After 30 s, 25 microlitre aliquots were taken and added to 50 microlitres of 2M HCl in a 100-microlitre cuvette, another 25 microlitres of TMB was added to bring the volume to 100 microlitres. The optical density (OD) at a wavelength of 450 nm was measured in transmission using a Beckman DU530 Life Science UV/vis spectrophotometer.

ELISA was used to quantify surface attached tropoelastin with primary anti-body, mouse anti-elastin antibody (BA-4), and goat anti-mouse IgG-HRP conjugated secondary antibody. The samples undergoing SDS treatment prior to ELISA were transferred to 5% SDS (w/v) in PBS and incubated at 90°C for 10 min. Non-specific binding to the polymer was blocked with 3% (w/v) bovine serum albumin (BSA) in PBS. The HRP label was detected by adding 0.75ml ABTS solution (40mM ABTS [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)] in 0.1M NaOAc, 0.05M NaH_2PO_4, pH5, containing 0.01% (v/v) H_2O_2) and measuring the absorbance at 405nm using a plate reader.

Results and Discussion

Free radicals have an unpaired electron and therefore an associated electron spin making them extremely reactive. Our strategy is to use plasma processes involving ion implantation to modify polymeric materials so that they contain a reservoir of stable and mobile free radicals. The stabilisation is achieved by the formation of π conjugated clusters on which unpaired electrons are delocalised and mobility through the structure occurs through hopping between adjacent clusters. This structure can be achieved in two ways. The first (shown schematically in Figure 2(a)) is by treating a carbon-based polymer using a technique known as plasma immersion ion implantation. Ions are accelerated into the surface by an electric field of the plasma sheath that forms around the sample holder. The second method (shown schematically in Figure 2(b)) is by the deposition of a plasma polymer film under moderate ion bombardment (up to 1 kV). This method can be used on non-polymeric materials. The electron spin density created by ion treatment is quantified using electron spin resonance (ESR). High concentrations of unpaired electrons are measured in both ion implanted (Figure 2(c)) and plasma deposited polymers (Figure 2(d)) for periods of many months after the ion treatment.

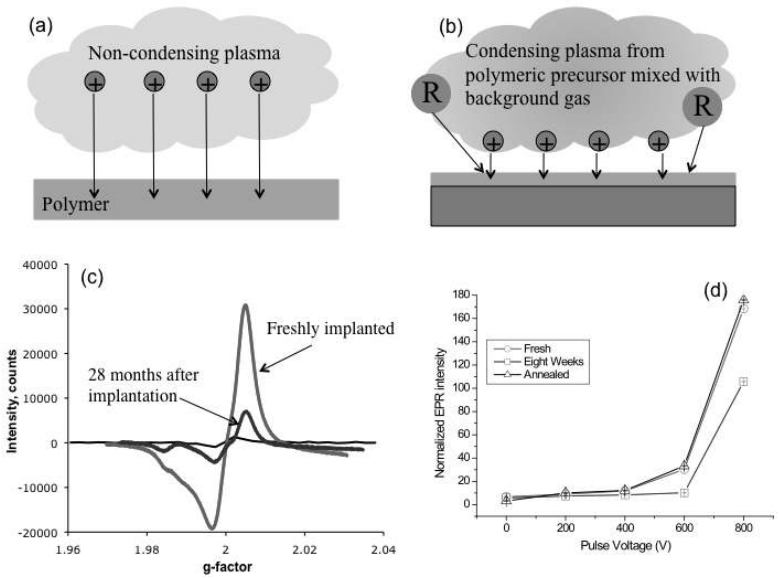

Figure 2: Schematic diagrams showing the methods we have employed to create free radical reservoirs. When the underlying material is a polymer (a) a high voltage (~20 kV) is applied in pulsed mode to accelerate ions from a surrounding non-condensing plasma. If the material is not a carbon rich polymer a plasma polymer film (b) is deposited under moderate ion bombardment applied in the form of a pulsed bias (up to 1 kV) during the deposition. (c) ESR signal from PIII treated PTFE showing the presence of free radicals. The red curve is for a freshly treated sample (20 mins after treatment), the blue curve for a 28-month old sample and the black signal is obtained from an untreated control sample. (d) ESR signal intensity as a function of pulse bias voltage applied during plasma polymer deposition. Measurements were taken 4 days after deposition (red circles), 8 weeks after deposition both before (blue squares) and after (black triangles) reactivation by annealing.

Figure 3(a) shows infra-red spectra revealing typical structural changes that occur in polymers upon ion implantation as a function of the ion implantation treatment time. The gradual reduction of the intensity of the characteristic polystyrene vibrations (1-5) shows that the polymer macromolecules are broken up and replaced by a carbonised structure that oxidises in the laboratory atmosphere (rise of peaks 8 and 9 respectively)[34, 35]. Figure 3(b) shows the evolution in treated polymers that typically occurs after removal from the plasma chamber. Reactions of free radicals, created under the surface by the ion impacts, with species in the atmosphere result in changes in surface energy and C=O IR adsorption bands that are correlated with changes in the electron spin density. The concentration of C=O groups on the surface increases during exposure to atmosphere because atmospheric oxygen reacts with radicals[36] in the polymer. The surface energy measured at the first time point is significantly higher than that of an untreated surface and then progressively decreases as high-energy radicals react with environmental oxygen. Such surfaces strongly attach protein molecules upon incubation in protein containing solution. There is minimal loss of the attached protein after washing with sodium dodecyl sulphate (SDS), a detergent capable of disrupting non-covalent interactions [16],

as observed by (c) protein amide peak absorbances in the infra-red and (d) enzyme-linked immunosorbent assays (ELISA) detecting the presence of the protein.

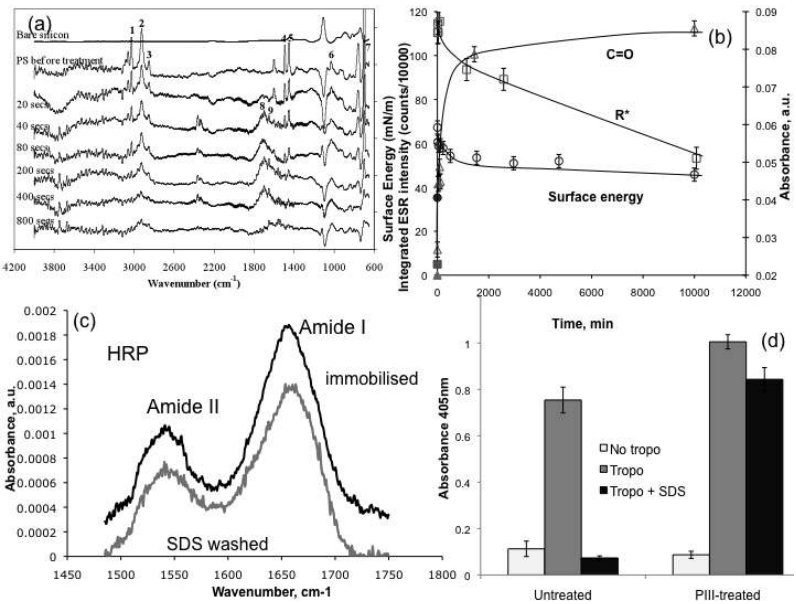

Figure 3: ATR-FTIR spectra of ion implanted polystyrene (a) show that the polystyrene macromolecular structure is gradually degraded, dehydrogenated and replaced by a carbonized structure that is oxidized on exposure to atmosphere. The spectra (top to bottom) are taken from a bare silicon wafer; untreated polystyrene; polystyrene treated for 20, 40, 80, 200, 400 and 800 seconds. (b) The surface energy, normalized carbonyl group absorbance from FTIR ATR and ESR intensity of free radicals as a function of storage time for PIII treated LDPE (20 keV, 20μs pulses at 50Hz in nitrogen). (c) FTIR ATR spectra of HRP enzyme immobilised on UHMWPE treated as in (b) to a fluence of 1e16 ions/cm^2 (UHMWPE background subtracted). Protein attached during incubation (black spectrum, upward shifted) is virtually 100% retained after SDS washing (red spectrum) [2% SDS solution, 70C for 1 hour]. (d) ELISA confirms that tropoelastin physisorbed from 20 μg/ml solution (grey bars) is retained only on the PIII treated PTFE (right) after SDS washing. The white bars indicate the assay background signal without tropoelastin in solution.

Figure 4 shows a characteristic curve describing the resistance to elution for a SDS washing protocol used by Kiaei et al [31] to remove albumin from a range of untreated polymers and plasma polymer surfaces. A clear trend (shown by the curve) with surface energy is apparent, with the strongest adsorption on the most hydrophobic (lowest energy) surfaces. Note that the room temperature SDS protocol employed by Kiaei et al does not remove all of the physisorbed protein. Data from plasma immersion ion implantation (PIII) treated polymers (red squares) and untreated polymers (blue diamonds) where a range of washing protocols were employed is also shown. Aggressive SDS protocols at 70-90C completely elute protein from very hydrophobic surfaces, such as PTFE. The PIII treated surfaces typically show 50-100% protein retention

despite being quite hydrophilic. This indicates that physical forces alone cannot be responsible for the robust protein attachment observed on the ion implanted surfaces implying that a covalent linkage is formed. The ability to covalently immobilise onto a strongly hydrophilic surface is a key advance that allows the retention of protein bioactivity by providing an environment conducive to retaining native conformations.

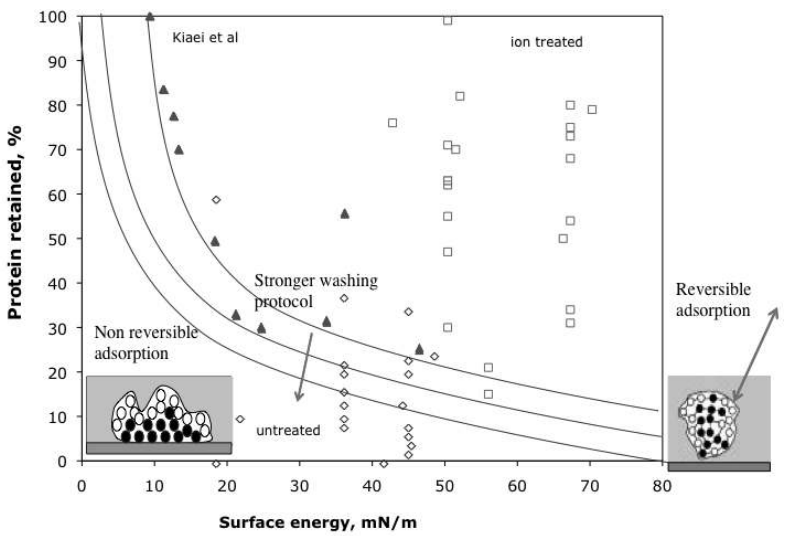

Figure 4: Percent of protein retained after SDS washing (various solution strengths and temperatures) as a function of surface energy for polymeric surfaces. Data is taken from Kaiei *et al* [31] (green triangles) and current work (red squares and blue diamonds). The top curve shows is a trend curve for the data of Kaiei *et al* and the lower curves show shifts in the trend expected for more rigorous SDS washing. Untreated controls washed with stronger SDS protocols are shown as blue diamonds. The points (red squares and one green triangle) lying above and to the right of the trend curves show exceptional protein retention given the hydrophilic nature of these surfaces.

We propose a model in which the covalent binding takes place via a reaction between an amino acid residue on the protein and a free radical on the ion treated polymer surface that is created by the diffusion to the surface of an unpaired electron from a reservoir below the surface. The first step is the physisorption of a protein on the surface and the second step is the formation of a covalent bond between a protein residue and a radical group (illustrated schematically in Figure 5(a)). There are two relevant time constants, one for the diffusion of proteins in solution to the surface and the second for the diffusion of the unpaired electrons from the reservoir to the surface. These processes are governed by the following two coupled differential equations:

$$\frac{dNp}{dt} = \frac{(N_{psites} - Np)}{\tau_1} \qquad (1)$$

$$\frac{dNc}{dt} = \frac{(FNp - Nc)}{\tau_2} \tag{2}$$

where N_p is the number of physisorbed protein molecules per unit area and N_c is the number of covalently immobilised protein molecules per unit area. t_1 is a constant that depends linearly on the number density and diffusion coefficient of the molecules in solution and on the sticking coefficient of physisorbed molecules on the surface. t_2 is a constant that depends linearly on the number density of unpaired electrons and on the diffusion coefficient of the unpaired electrons in the modified region of the polymer . N_{psites} is the number of sites available for physisorption per unit area and F is the fraction of physisorption sites that are accessible to radicals diffusing from the interior reservoir. These equations have the following solutions:

$$N_p = N_{psites}(1 - e^{-t/\tau_1}) \tag{3}$$

$$N_C = FN_{psites}\left(1 - \frac{\tau_1 e^{-t/\tau_1}}{\tau_1 - \tau_2} - \frac{\tau_2 e^{-t/\tau_2}}{\tau_2 - \tau_1}\right) \tag{4}$$

Experiments were conducted to test the predictions of the model. We treated PTFE films with a plasma immersion ion implantation (PIII) process. Voltage pulses of 20kV were applied to a mesh over the films for 20 microseconds at a frequency of 50Hz to provide the energetic ion bombardment from a nitrogen plasma. After treatment, the samples were incubated in a 20 mg/ml solution of the extra cellular matrix protein, tropoelastin, for a range of times. After removal from solution they were washed in fresh buffer and the surface immobilized tropoelastin was assayed using ELISA. The model predicts a time dependence of the form in equation 3 of the amount of protein physisorbed from solution. Equation 3 was used to fit the relationship of optical density obtained from ELISA (proportional to the amount of bound protein) and incubation time as shown in Figure 5(b).

In parallel, a group of equivalent samples was subjected to rigorous SDS washing prior to ELISA detection of the tropoelastin. The protein detected in this case is covalently bound and thus would be expected to show the time dependence predicted by equation 4. A fit of this data by equation 4 is shown in Figure 5(b). The parameters determined in the fitting of equation 3 (N_{psites} and t_1) were used in the fit of equation 4 with F=1, leaving only one free parameter, the time constant for covalent binding, t_2. The time constants, t_1 and t_2 were determined to be 4.3+1.2 minutes and 35+9 minutes respectively. The shape of the experimental curve for covalent attachment is distinctly different from that for physisorption, especially in its behaviour at short incubation times. The time dependences of both the physisorption and covalent attachment are well reproduced by the theory, confirming the presence of processes for covalent attachment with two different time constants.

Modelling the motion of unpaired electrons in the reservoir by kinetic theory gives the following equation for the time constant of covalent binding of adsorbed molecules, t_2:

$$\tau_2 = \frac{4FN_{psites}}{n_r \bar{v}_r S_r} \tag{5}$$

where n_r is the number density of unpaired electrons, $\bar{v}_r$ the mean velocity associated with their diffusion, and S_r the probability of interaction with an adsorbed protein that forms a covalent bond. The number density of free radicals in the reservoir beneath the surface was observed to decay with time as shown in Figure 2(c & d). This leads to an increase in t_2 for an aged sample. The increase in t_2 is expected to be greater than predicted on the basis of the reduction in n_r alone since both $\bar{v}_r$ and S_r may decrease with time. S_r would decrease as some of the surface becomes passivated by adsorption of atmospheric contaminants during storage. Radicals arriving at the surface at a site covered by contaminants will covalently bind to these rather than to a protein molecule. $\bar{v}_r$ may decrease over time because the unpaired electrons in environments allowing the highest mobility will have the highest rate of quenching.

To test the dependence predicted by the model on the sample's age, we compare in Figure 5(c) the dependence of the amount of protein covalently attached on incubation time for new and aged (448 days) PIII treated PTFE films. The time constant, t_2, for free radical binding found by fitting equations 3 and 4 to the data for the old sample was 3±1 days, two orders of magnitude greater than for the new sample. On the basis of the estimated reduction in free radical number density, n_r, obtained from the ESR spectra of Figure 2(c) there would be no more than a 10 fold increase in t_2. The remainder of the change is ascribed to decreases in $\bar{v}_r$ and S_r.

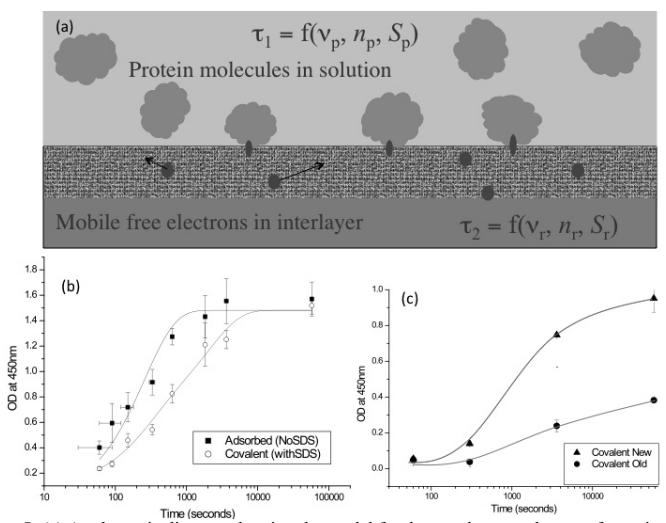

Figure 5: (a) A schematic diagram showing the model for the covalent attachment of protein molecules to the surface. There are two relevant time constants, one (τ_1) governing the adsorption of a protein layer on the surface and the second (τ_2) governing the flow of free electrons to the surface. Panel (b) shows the dependence of tropoelastin coverage on incubation time for PIII treated PTFE. The lines show the fits of equations 3 and 4 to the amounts adsorbed and covalently immobilised. Panel (c) shows how the time constant for covalent immobilisation increases with sample age.

Figure 6 shows the characteristics of covalently attached protein layers on polymer surfaces treated with energetic ions. A comparison with a protein layer physically adsorbed onto an

untreated polystyrene surface shows that a densely packed monolayer forms on the treated surface (Figure 6 (b)) while a layer with significant void fraction is observed on untreated polystyrene (Figure 6 (a)). The conformation of the protein on the treated surface is closer to a native conformation than that for the untreated polystyrene surface (Figure 6(c)). HRP enzyme bound to the treated surface shows significantly better resistance to removal by washing and better retention of enzyme activity than that adsorbed onto untreated UHMWPE (Figure 6(d)).

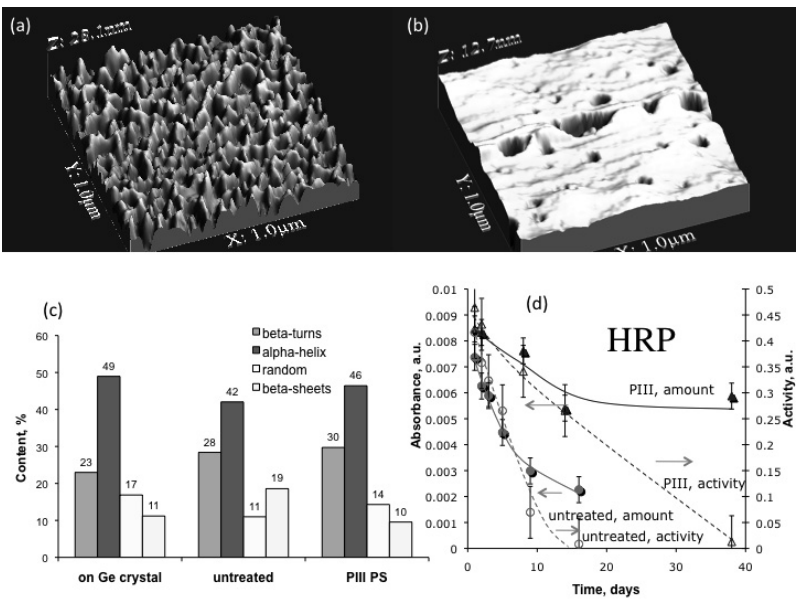

Figure 6: The AFM images show HRP protein layers on polystyrene films on silicon (scanned area is 1.0 x 1.0 mm). The coverage of protein is incomplete, with height indicative of aggregation, on the untreated surface (a – z axis is 28.1 nm) and forms a densely packed monolayer on the PIII treated (2.5e15 ions/cm^2, N_2 20 kV) surface (b – z axis is 12.7 nm). Ellipsometry and FTIR measurements indicate that there is a layer of protein with thickness ~ 9 nm on the surface as expected for a monolayer of HRP. The conformation (c), as determined by relative contents of beta-turns, alpha-helices, random coils and beta-sheets in IR amide I peak is closer to native on PIII treated polystyrene than when adsorbed on the untreated surface. (d) Amount (FTIR, solid symbols) and activity (TMB assay, open symbols) of HRP protein are both higher on PIII treated than on untreated UHMWPE at all times of storage in buffer.

Conclusions

We have developed a quantitative understanding of how unpaired electrons, mobile in a free radical reservoir, are effective in covalent immobilisation of protein molecules. The dependences of the rate of covalent immobilization on the age of the sample and the density of free radicals show that the irreversible protein immobilization observed is associated with free radicals that migrate from the a bulk reservoir to the surface.