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Accounting for the size of molecules in determination of adsorption isotherms by XPS; exemplified by adsorption of chicken egg albumin on titanium

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The adsorption of chicken egg albumin on commercially pure titanium has been studied as a function of protein concentration, using X-ray photoelectron spectroscopy (XPS). The adsorption isotherm has been plotted using the increase in N 1s intensity and also by measurement in the decrease in the Ti 2p intensity as the adsorbed film reaches full coverage. It is shown that both sets of data are a good fit to the Temkin isotherm. The influence of the large size of the biomolecule is discussed and the isotherm is modified to take account of the molecular dimension according to the model proposed by Ratner and Paynter. The thicknesses of the adsorbed molecules are measured using atomic force microscopy (AFM) and it is shown that it is only when monolayer coverage has been reached that the molecules begin to take up the characteristic globular shape. Albumin reaches a coverage of 25% of a monolayer in solutions of only 10 ppb by volume, suggesting that it is easily bound to the TiO_2 surface. A complete monolayer is formed at a solution concentration of 100 ppm. The carbon 1s signal is used to estimate the surface free energy at different surface coverages using the model developed by Kinloch, Kodokian and Watts. The transformation from the initial coverage of hydrophobic contamination molecules to the hydrophilic surface presented by the adsorbed albumin film takes place over a range similar to that required to form the monolayer. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: XPS; adsorption isotherm; albumin; titanium

INTRODUCTION

The application of commercially pure (CP) titanium in biomedical devices is widespread. CP titanium is commonly used as the foundation for replacing teeth in dentistry, and is also used for orthopaedics because of its very good biocompatibility, as far as osseointegration capability is concerned, good mechanical properties, good workability, low toxicity and especially its superior corrosion resistance (being almost free of local corrosion).[1–4]

Much research has been carried out on adsorption of protein on the surface of implant materials. The adsorption of protein is accepted as the initial event occurring when implant materials come into contact with a protein-containing fluid. Since only the surface of an implant material is directly in contact with the host tissue, this interaction determines its biocompatibility. The process of adsorption is rapid; proteins are known to deposit onto implant surfaces within seconds of exposure to proteins in solution.[5–7] There are many proteins (albumin, fibrinogen and haemoglobin) present in the human body, performing specialised functions. However, protein adsorption is related to the nature of protein as a class and all are known to interact strongly with most solid surfaces.[8,9] The differing molecular weights of proteins give differing efficiencies in attaching to metal surfaces.[10,11] Albumin is a globular, extracellular protein that has a dimensions of $8 \times 8.7 \times 6$ nm and MW of 64 kDa. Haemoglobin has a similar molecular weight and is also a globular, intracellular protein but is built up from four myoglobin-like sub-units with overall dimensions of $5 \times 5.5 \times 6.5$ nm.[12] Fibrinogen is an extracellular protein with an elongated shape that has a dimension of $45 \times 9 \times 6$ nm and MW = 340 kDa. Albumin is the most common protein in the blood stream and is the first that covers and strongly binds to a titanium surface.[13,14] Although many protein adsorption studies have been carried out using different proteins, e.g. human serum albumin, bovine serum albumin and fibrinogen, which have focused on adsorption in relation to time, solution pH and physiological condition, there have been very few determinations of the actual adsorption isotherm for the adsorption of protein on titanium. The adsorption isotherm, a plot of the uptake at constant temperature of a compound as a function of its partial pressure (often expressed as a pressure relative to its saturation pressure) or, from a solution, as a function of its concentration, is one of the founding measurements

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of surface science. The isotherm is valued in surface science for the information it can give on monolayer coverage and on the mechanism of adsorption.[15] X-ray photoelectron spectroscopy (XPS) enables direct measurement of the surface uptake and thus is well placed to provide data for the isotherm to be plotted. The most relevant part of the isotherm is that for sub-monolayer coverage, and in the case of large molecules, such as the proteins described earlier, it could be assumed that the substrate is completely obscured so that the area covered is proportional to a marker atom in the protein, e.g. nitrogen, and the area remaining uncovered is proportional to a marker atom in the substrate, e.g. titanium.

Surface coverage, σ, is usually given as the surface concentration, related to the concentration at one monolayer. Thus, in the example of protein on titanium

$$I^{N}/I_0^{N} = \sigma \tag{1}$$

and

$$I^{Ti}/I_0^{Ti} = (1 - \sigma) \tag{2}$$

where I^{N}, I^{Ti} denote the XPS intensity of nitrogen or titanium respectively. The reference values, I_0^{N} and I_0^{Ti} denote, respectively, the N 1s intensity measured for a coverage of 1 monolayer or the Ti 2p intensity from the titanium substrate before adsorption of protein. Thus, the XPS determination of nitrogen and titanium concentrations enables two independent measurements of σ to be made as a function of solution concentration.

The problem with this attractive approach is the assumption that the molecules are so thick that their signal is 100% of the maximum attainable from the area that they cover and that the covered substrate is totally obscured. Paynter and Ratner[16] have considered this problem, proposing that a more complete relationship should be used. This splits the signal from the substrate into two parts: that from the covered area that is partially attenuated by the adsorbed molecules and that from the uncovered area that remains unattenuated. On this basis, Eqns (1) and (2) become Eqns (3) and (4) respectively.

Thus,

$$I^{N} = I_0^{N}(\sigma(1 - \exp(-d/\lambda \sin\theta))) \tag{3}$$

and

$$I^{Ti} = I_0^{Ti}(\sigma \exp(-d/\lambda \sin\theta) + (1 - \sigma)) \tag{4}$$

Again, the surface coverage given by σ can be obtained from nitrogen and titanium signals and gives a useful comparison of the validity of the data. When d, the thickness of the adsorbed molecule, is large compared to the effective attenuation length, λ, the exponential terms are negligible and Eqns (3) and (4) revert to the simple forms of (1) and (2).

This method, proposed by Ratner and Paynter, has been in existence since 1985, before scanning probe methods for measurement of thickness of adsorbed molecular films were generally available. Now, it is commonplace to examine a film in the scanning force microscope (AFM), but the possibility of combining this information with that from XPS so as to solve Eqns (3) and (4) for σ seems to have been overlooked by many workers in the field.

The final piece of information that is capable of contributing to an understanding of the adsorption isotherm is that of carbon peak shape. Frequently, the aliphatic signal of the adsorbed contamination is replaced by that for the more polar carbon of the adsorbed biomolecule. This transition, which represents a transition in surface energy, has been dealt with in the context of the failure of adhesive joints by Kinloch, Kodokian, and Watts.[17] The noteworthy feature of their approach is the structure that it gives for including the several components of the carbon 1s peak into a unified approach. This gives the possibility of a third, independent method of obtaining an adsorption isotherm and identifying the concentration at which a monolayer coverage is achieved.

In order to assess the contribution that this combination of methods might make to the understanding of protein adsorption, we have carried out a model experiment using adsorption of chicken egg albumin on CP titanium. This source of albumin, though not as well defined as those used in biocompatibility work, is used here as a model, as it serves to illustrate the value of the more complete approach and also reveals some remaining limitations. It is carried out in the recognition that those working in the field of biocompatibility use purified proteins and examine specific interactions. However, it is intended that this demonstration of principle will encourage the use of XPS to full advantage within their studies.

EXPERIMENTAL

Materials and procedure

Preparation of CP titanium

The materials that were used in this study were CP titanium disks Grade 2 (as per JIS H-4600), having a diameter of 9.9 mm and thickness of 1.5 mm, giving a total area of 200 mm^2. CP titanium has the following composition: H < 0.013%, O < 0.20%, N < 0.05%, Fe < 0.25%, Ti: the balance. Samples were wet-ground on 320, 500, 800, 1200, 2400 and 4000 silicon carbide abrasive papers followed by alpha polishing alumina 1 μm and finished with 0.3 μm. The polished samples were cleaned with acetone.

Preparation of albumin solution

Protein solutions were prepared by mixing fresh egg white with deionised water. Chicken's egg white is composed of water, 87.3 g; proteins, 11.1 g; lipid, 0.2 g; carbohydrate, 0.4 g and minerals, 0.7 g (contents per 100 g). Constituent proteins of albumen are ovomucoid, 11%; ovalbumin, 54%; ovotransferrin, 12–13%; lysozymem, 3.4–3.5%; G2 ovoglobulin, 1.0%; G3 ovoglobulin, 1.0%; ovoflavoprotein, 0.8%; ovostatin, 0.5%; cystatin, 0.05% and avidin, 0.05%.[18] The concentration of protein in egg white is thus approximately 11% (v)[19] and dilution factors were chosen to give a series of solutions having concentrations varying from 10^{-8} to 10^{-1} v/v.

Surface characterisation

XPS analysis

The surface of untreated and treated CP Ti disks were analysed by XPS (Thermo Electron Sigma-Probe spectrometer). Monochromatic Al Kα X-ray radiation at 1486.68 eV was used with 150-μm spot size and a power of 140 W. Survey spectra over a binding energy range of 0–1350 eV were obtained using a pass energy of 100 eV. Spectra of individual elements were obtained using a pass energy of 50 eV. The Shirley background was subtracted and the spectra fitted with mixed Gaussian/Lorentzian peaks using a non-linear least-squares method by means of GOOGLY software.[20] There was some nitrogen present on the 'clean' titanium surface and this amount (0.99At%) was subtracted as a zero-point error from each of the values measured for protein adsorption.

Protein adsorption isotherm

The adsorption isotherm of albumin on the titanium was determined by immersing a coupon of Ti in 100 ml of each one of the freshly prepared egg albumin solutions for 30 min. The polished titanium was immediately immersed in protein solutions in order to minimise high roughness of the native oxide layer, which may cause an error of estimation of the adsorbed protein thickness. Following immersion, each sample was rinsed with deionised water and dried over silica gel.

Atomic force microscopy (AFM)

Protein adsorption was studied using a Nanoscope IIIa AFM (Digital Instruments, Inc., Santa Barbara, CA). The instrument was operated in the tapping mode using silicon cantilevers oscillating with typical amplitudes from 20 to 100 nm at a resonance frequency between 310 and 320 kHz. The image sizes were $1 \times 1\ \mu m^2$ with 256×256 pixels. The samples were imaged at room temperature using AFM in air. Section analysis was performed to estimate the adsorbed protein thickness with the Nanoscope IIIa 5.12r3 software.

RESULTS

Surface composition of the polished surface

Figure 1 shows the XPS survey spectrum from a polished CP titanium sample. The spectrum is dominated by strong signals from Ti, O and C. Several papers have shown that during air exposure and polishing treatment, titanium oxide is spontaneously formed on the surface.[21–23] The carbon present is the contamination that may have come from solvents used in the cleaning process and polishing treatment.[24,25] In characterising the surface with adsorbed protein, it was assumed that the presence of nitrogen is indicative of the presence of protein;[26,27] thus the treated surfaces were characterised by the N 1s peak. N 1s is not observed on a polished titanium surface but, as shown in Fig. 2, it is present in the XPS survey spectrum of a sample treated in 10^{-8} v/v albumin solution. Thus, as shown by XPS, adsorption is already measurable at the lowest albumin concentration.

The concentrations of nitrogen on the titanium surfaces after adsorption of albumin from solutions of increasing strength are shown in Fig. 3(a) and (b). The expansion of data points using the logarithmic scale shows that over the range 10^{-8} to 10^{-4} v/v the adsorption isotherm is of the Temkin form,

$$X = aX_m \ln(bc) \tag{5}$$

where X is the uptake measured as at% N, X_m is the maximum uptake, c is the concentration in solution and a and b are constants. X_m can be determined as the mean of the final four points that appear to represent saturation coverage, giving it a value of 12.1 at%; it is shown in Fig. 3(b) as the best-fit horizontal line through these points. For comparison, the concentration determined by XPS of nitrogen in a dried film of albumin was 13 at% and the formula value for the albumin molecule is 16 at%. The values of a and b derived from the concentration range 10^{-8} to 10^{-4} v/v are 1.00 at%N and 1.98

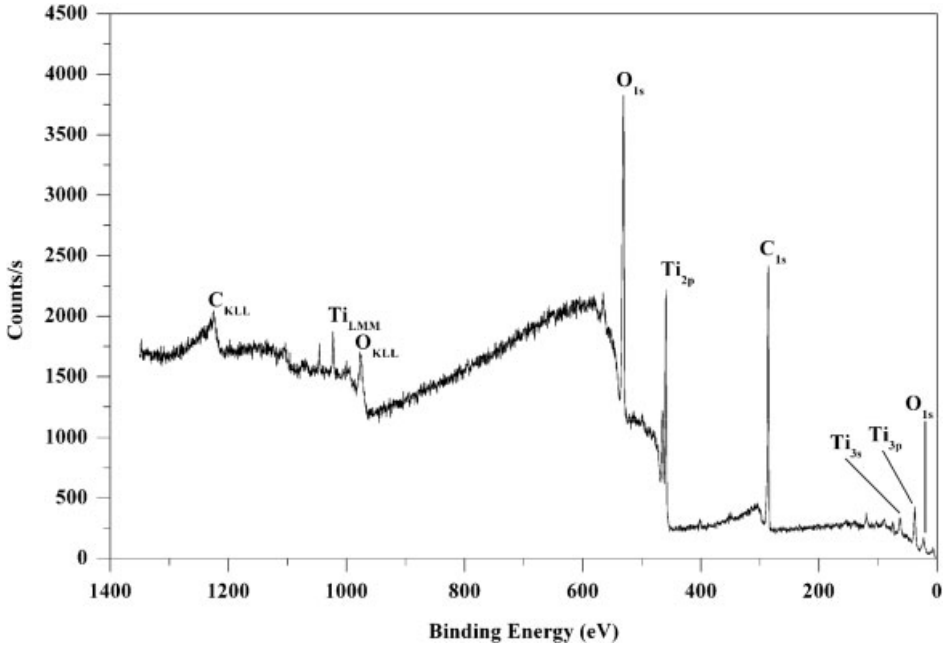

Figure 1. XPS survey spectrum for a polished CP titanium.

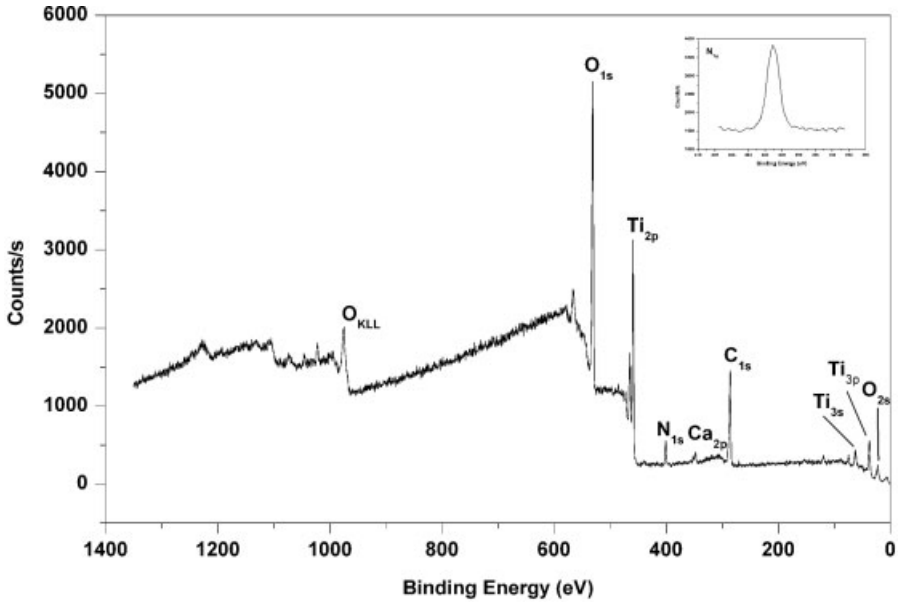

Figure 2. XPS spectrum for CP titanium in 1×10^{-8} v/v albumin solution.

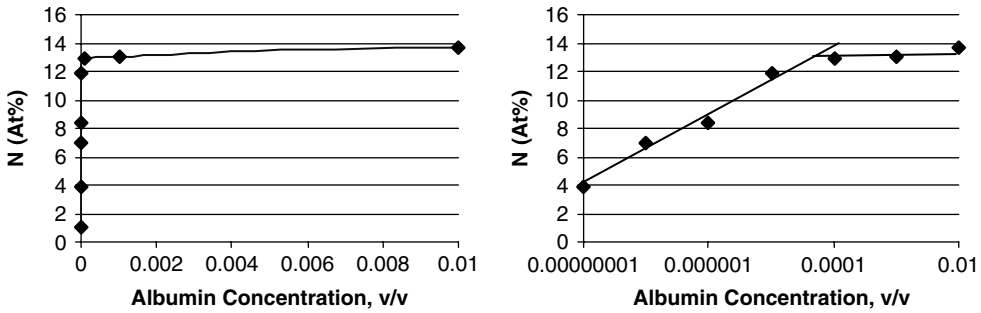

Figure 3. The surface concentration of nitrogen as a result of albumin adsorption, plotted as a function of solution concentration, (a) using a linear scale and (b) against a logarithmic scale.

v/v, respectively, shown as the best-fit line through these points on Fig. 3(a).

In addition to the measurement of adsorbed nitrogen, the intensity of the Ti 2p peak was monitored. Figure 4(a–e) shows the curve-fitted Ti $2p_{3/2}$, $2p_{1/2}$ doublets. In each case, a Gaussian/Lorentzian function was used with a full width at half-maximum (FWHM) near 1.4 eV. The peak position was attributed to Ti^{IV}, showing that TiO_2 is the main constituent of the protective film on freshly polished Ti.[28,29] The low-intensity peak, Fig. 4(f) corresponding to immersion in 10^{-3} v/v albumin solution, is shown without curve fitting; similar spectra were found after exposure in 10^{-2} v/v and 10^{-1} v/v albumin. Figure 5 shows the Ti 2p peaks after immersion in (a) 10^{-8} v/v, (b) 10^{-7} v/v and (c) 10^{-3} v/v albumin solution on a common intensity scale to indicate how the Ti 2p peaks reduced in intensity with increasing concentration of albumin solution.

AFM

The surfaces used here were polished to 0.3 μm and had visually unblemished mirror finish. Nevertheless, for sub-monolayer coverage, it was difficult to ascribe surface features to albumin, rather than to oxide or metallographic topography. Analysis of the images, Fig. 6(a–d), gives a surface roughness (the peak to valley) of 1.5 nm at 10^{-4} v/v, a value that increases to 2.3 nm at 10^{-3} v/v, showing that this is due to the increased adsorption and thus associated with the albumin molecule. At 10^{-2} v/v and higher concentrations, the thickness of the molecule or molecular clusters, reaches a final value of 3 nm. Thus it is only close to monolayer coverage or beyond a monolayer that the thickness of the molecular deposit could be recognised. The AFM results illustrate the problems that will have to be addressed if the XPS adsorption data is to be corrected for the thickness of the adsorbed molecule.

DISCUSSION

The nitrogen isotherm

The uptake of protein, as illustrated in Fig. 3, has the form of the Temkin adsorption isotherm, Eqn (5). The importance of making measurements at very low concentrations and using a logarithmic scale is well illustrated by Fig. 3(b); a non-logarithmic plot of the same data, as shown in Fig. 3(a) is typical of chemisorption, but would be difficult to distinguish from a Langmuir isotherm, particularly if, as often happens, concentrations are only varied over one or two decades. The Temkin isotherm is based on a mechanism in which

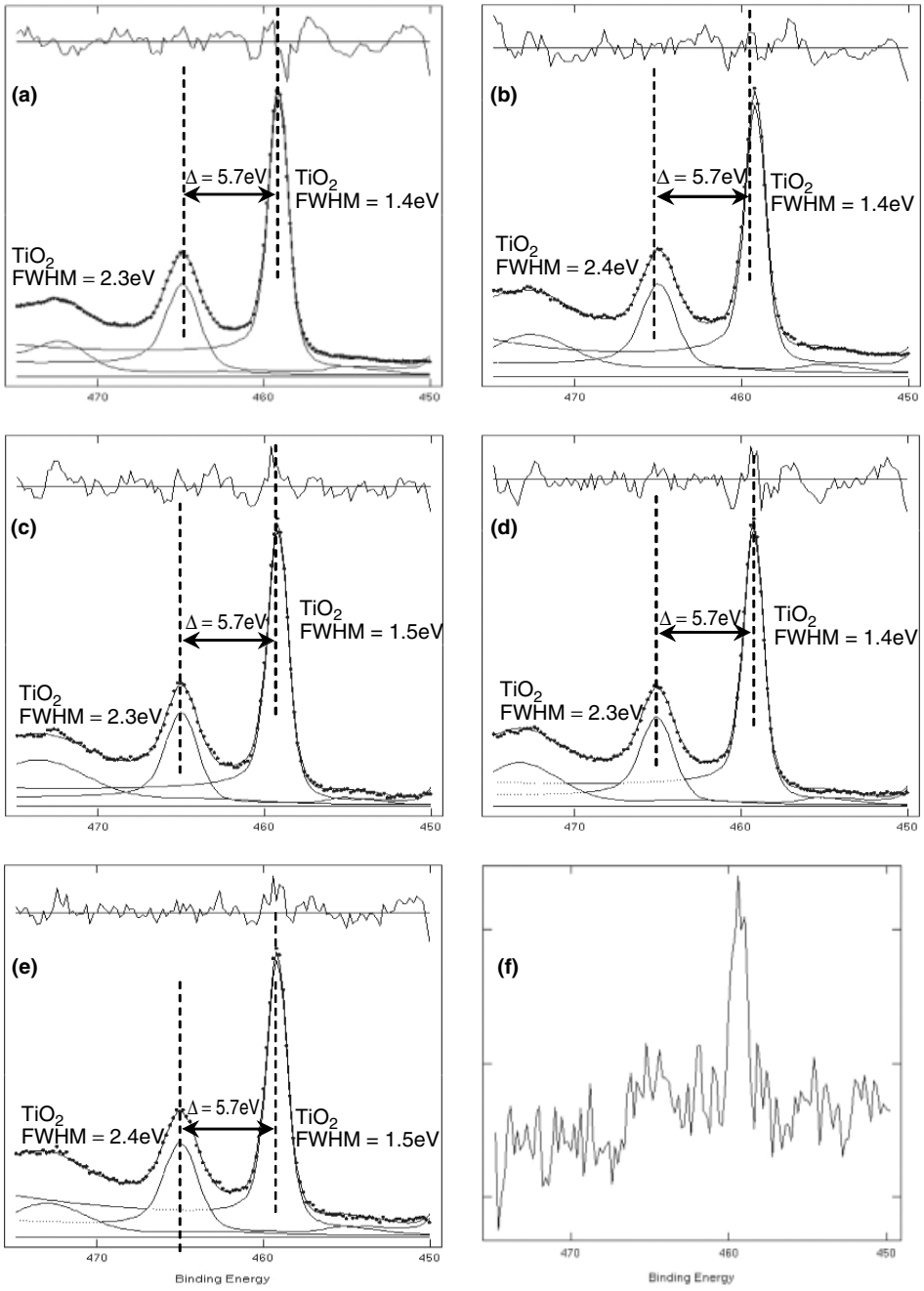

Figure 4. XPS spectra of the Ti 2p region for treated sample immersed in: (a) 1×10^{-8} v/v, (b) 1×10^{-7} v/v, (c) 1×10^{-6} v/v, (d) 1×10^{-5} v/v, (e) 1×10^{-4} v/v and (f) 1×10^{-3} v/v albumin concentration.

sites of gradually increasing enthalpy of adsorption are occupied as the solution concentration increases.[15] At the lowest solution concentration the nitrogen signal, X, is about 25% of that corresponding to maximum coverage, X_m, i.e. $\sigma = X/X_m = 0.25$, where σ is the fractional coverage of the surface monolayer. The increase of intensity for the N 1s peak then corresponds to an increase in σ due to the gradual infilling of the available sites, with saturation being reached at a value, X_m, of 12–13% nitrogen. The isotherm is conventionally plotted as a function of coverage, using Eqn (1), as shown in Fig. 7.

In order to convert the isotherm to the more complete form of Eqn (3), thickness data from the AFM measurements is needed. Measurements of the surface topography over the ranges 10^{-8} to 10^{-4} v/v gave consistent readings of 1.5 nm, a value indistinguishable from that of the polished surface. Thus, it is presumed that at sub-monolayer coverage the molecules adopt a very flat conformation with a thickness that does not exceed 1.5 nm, and this value has been used for d in Eqn (3). Beyond monolayer coverage, the molecules are revealed, as they appear to adopt a more compressed and equiaxed conformation; a value of $d = 2.3$ nm has been used at 10^{-3} v/v and $d = 3$ nm for the higher concentrations. The effective attenuation length, λ, was taken to be 3.0 nm for the for the N 1s electron. The value of I_0^N was taken from the intensities corresponding to the best-fit line through the points for 10^{-4} to 10^{-1}, as shown in Fig. 3. The effect of the exponential term in Eqn (3) is to increase the apparent

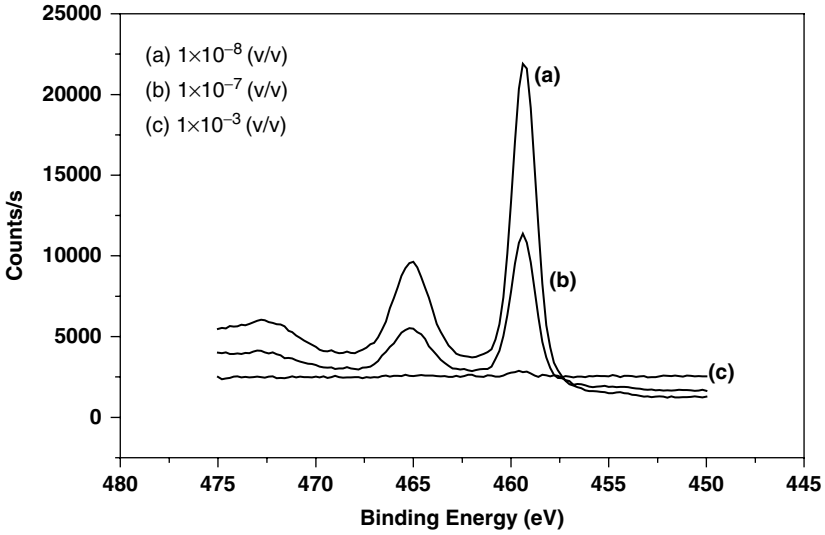

Figure 5. XPS spectra from Ti 2p region plotted on a common scale in order to illustrate the relative intensities obtained after immersion in a range of albumin concentrations.

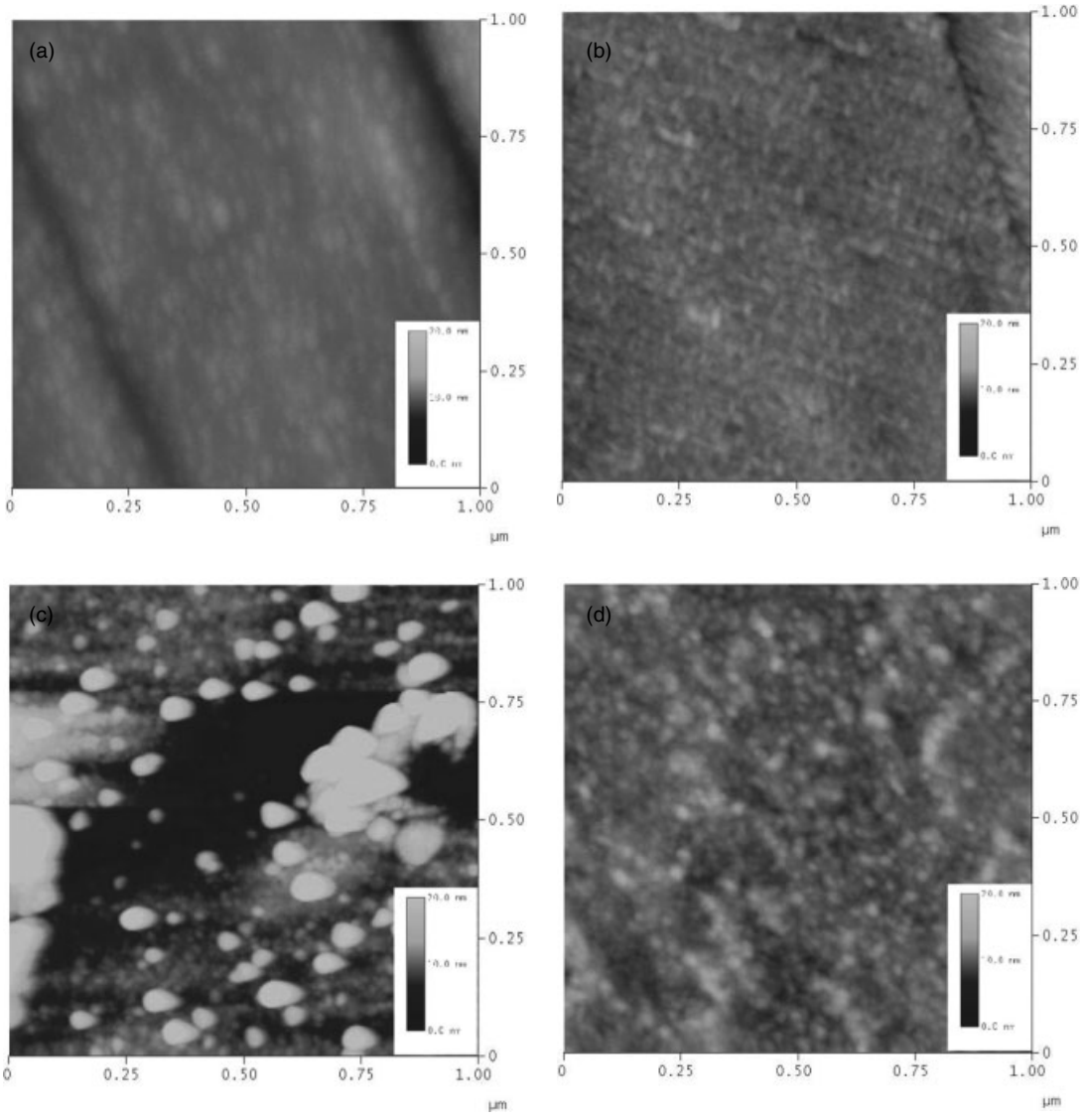

Figure 6. AFM images of the surface after differing stages of adsorption: (a) 1×10^{-5} v/v, (b) 1×10^{-4} v/v, (c) 1×10^{-3} v/v and 1×10^{-1} v/v albumin concentration.

coverage. Thus, renormalisation to give a coverage of unity is required; this was done using the value for 10^{-4} v/v, the point at which monolayer coverage was found to occur in the uncorrected isotherm.

A comparison of the nitrogen adsorption isotherms given by Eqns (1) and (3) is presented in Fig. 7. The curves are identical over the sub-monolayer portion, because over this region the molecules are presumed to have a uniform thickness of 1.5 nm independent of coverage. Both models lead to the conclusion that the Temkin isotherm is the appropriate model for the adsorption process of proteins on metallic surfaces, as was already suggested by Johnson and Arnold.[30] In this case, the Temkin isotherm is obeyed with a goodness of fit, R^2, of 0.98. Beyond monolayer coverage the molecules gradually assume a globular shape and the increasing value of d inserted in Eqn (3) leads to an apparent decrease in coverage. This is an artifact and results from the fact that once $\sigma = 1$, Eqn (3) reduces to the familiar form of the Beer–Lambert relationship and the intensities are more correctly expressed in terms of thickness instead of coverage.

The titanium isotherm

The estimation of coverage based on measurement of the titanium signal is obtained using Eqns (2) and (4). In this case the important reference point is the Ti 2p intensity, I_0^{Ti}, for the surface prior to adsorption of protein; all other parameters are as used in the discussion of the nitrogen adsorption. I_0^{Ti} is difficult to measure as other molecules inevitably adsorb on the surface, even when no added protein is present in the test solution. It can be obtained from Eqn (4) for any case in which the Ti 2p intensity is measured for a known level of coverage. Here, we can use the coverage measured by nitrogen uptake. In the present case, we have used the Ti 2p intensity measured after immersion in 10^{-8} v/v solution for which the nitrogen isotherm gives a coverage of $\sigma = 0.24$. Figure 8 shows the curves after normalization to give monolayer coverage at 10^{-4} v/v. The uptake of protein, measured by reduction in the Ti signal, increases more rapidly than required by the Temkin isotherm. However, it must be remembered that the reduction in Ti intensity is not specific for protein adsorption and it is likely that this reflects competing adsorption of the many other types of molecules present in chicken egg albumin. A comparison of the non-normalised data obtained using Eqns (3) and (4), Fig. 9, shows that there is good agreement between the measurements as monolayer coverage is reached; both curves peak at a concentration of 10^{-4} v/v, an indication that monolayer coverage is reached at this point.

Overall, it is clear that correction for the thickness of the adsorbed molecule has little effect before monolayer coverage is reached. At this coverage the molecule begins

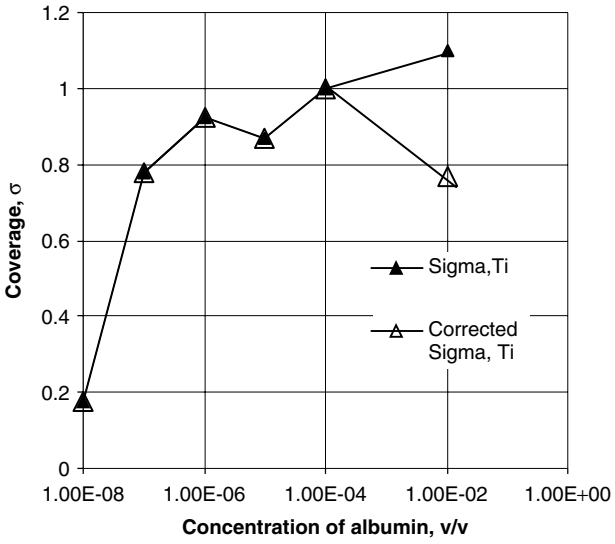

Figure 8. Adsorption isotherms obtained on the basis of Ti 2p intensities using Eqn (2), filled symbols and Eqn (4), open symbols. The curves were normalised to give monolayer coverage at a solution concentration of 10^{-4} v/v.

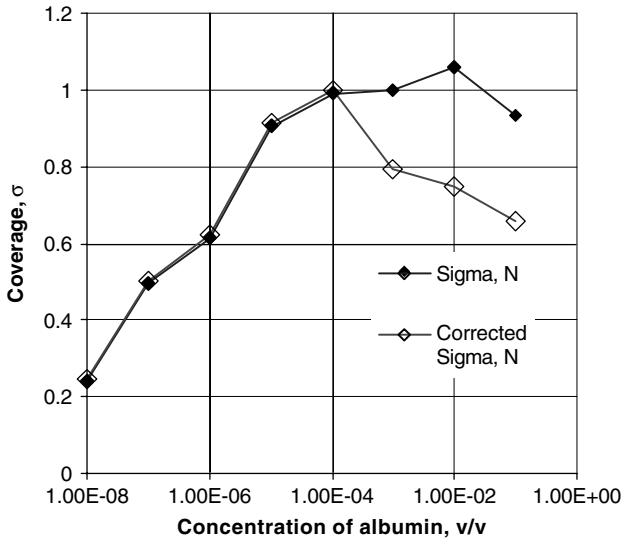

Figure 7. Adsorption isotherms obtained on the basis of nitrogen 1s intensities using Eqn (1), filled symbols, Eqn (3) and open symbols. The curves were normalised to give monolayer coverage at a solution concentration of 10^{-4} v/v.

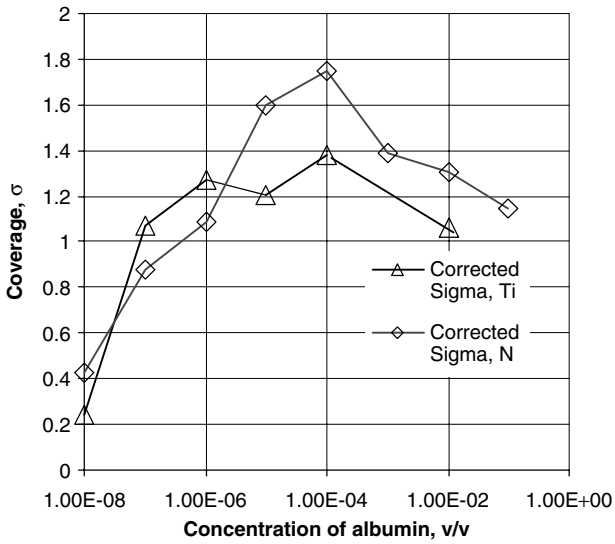

Figure 9. A comparison of the adsorption isotherms measured using the N 1s and Ti 2p signals, interpreted using Eqns (3) and (4).

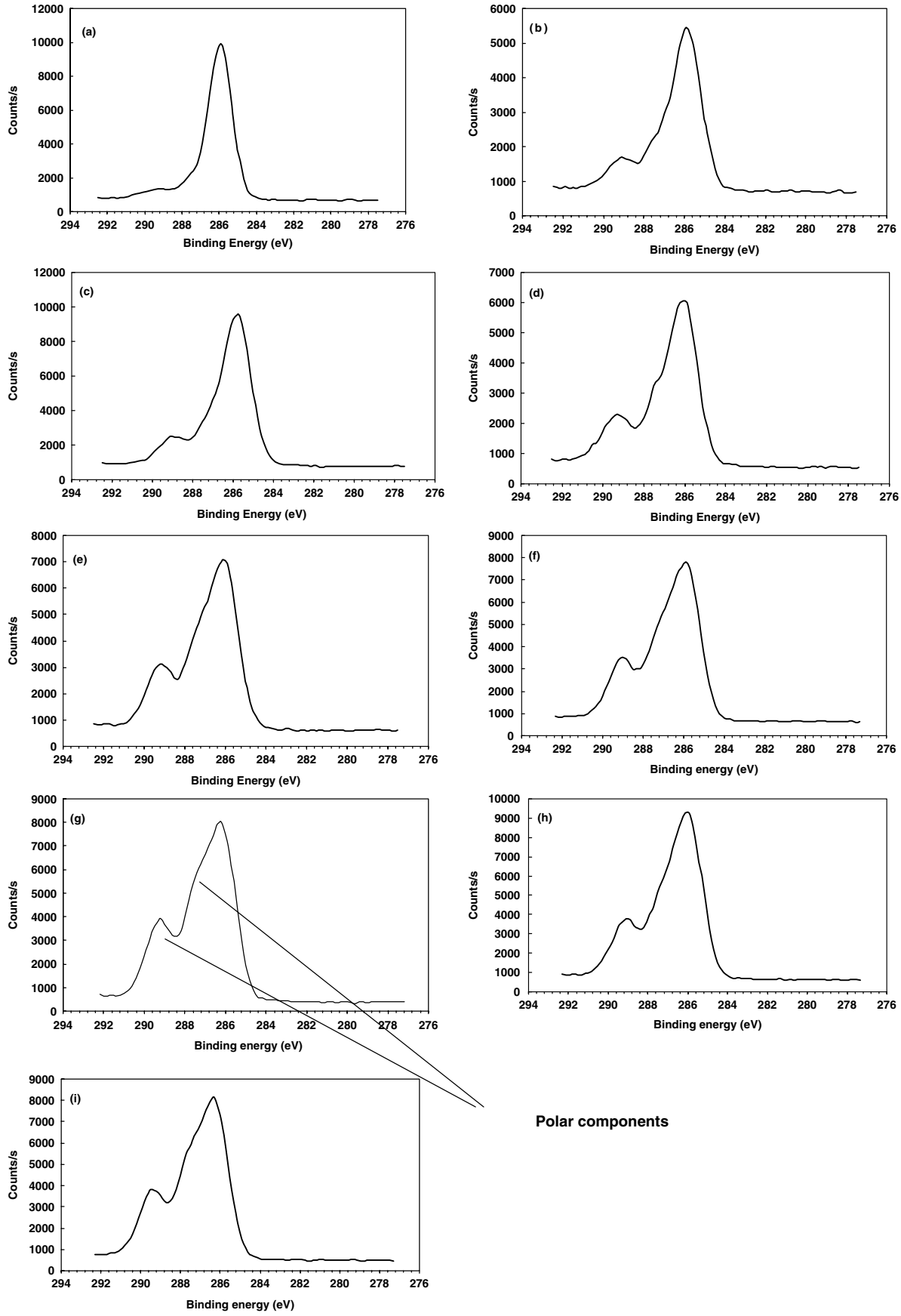

Figure 10. XPS spectra of the C 1s region illustrating the growth of polar organic components associated with the adsorbed albumin: (a) polished titanium and immersed in (b) 1×10^{-8} v/v, (c) 1×10^{-7} v/v, (d) 1×10^{-6} v/v, (e) 1×10^{-5} v/v, (f) 1×10^{-4} v/v, (g) 1×10^{-3} v/v, (h) 1×10^{-2} v/v and (i) 1×10^{-1} v/v albumin solution.

to assume a globular shape and, as shown in Figs 7 and 8, this does influence the interpretation of measured uptake. Evidence that the substrate signal transmitted through the adsorbed molecule has only a small influence on the overall adsorption data also comes from the shape of the Ti peaks in Fig. 4, which remained self-similar throughout the sub-monolayer range. It is a characteristic of peaks that have been depressed in intensity by scattering in an overlayer that they gain an energy-loss structure, causing them to be superposed on a rising background.[19] The background in these spectra is almost flat, showing that the thickness of adsorbed molecules is rather small and thus consistent with the AFM measurements over the sub-monolayer range.

Estimation of the surface free energy

Figure 10 shows a considerable change in the C 1s spectrum arising from the polar groups in the protein adsorbed on the titanium surface, as can be seen by comparison between Fig. 10(a), the virgin surface and Fig. 10(f), the point at which the monolayer is complete. Similar changes were seen in the O 1s spectra as the oxide signal is replaced by the carboxy groups. The strong polar constituent peaks of the carbon and oxygen spectra point to the formation of a hydrophilic surface, as might be expected for deposition from aqueous solution. Kinloch*et al*.[17] have shown how the intensity of the differing carbon groups can be interpreted in terms of the polar component of the surface free energy, γ^p. To make this correlation they take the weighted percentage dipole moment, Ω, and compare this value with the polar component of surface free energy, obtained by contact-angle measurements for oxidized polymer surfaces. The approximate correlation can be expressed as

$$\gamma^p(\%) = 1.72\ \Omega \qquad (6)$$

Ω is obtained as the sum of the product of the carbon type and the dipole moment. The peaks of Fig. 10 were fitted using peaks at 285, 286.3 and 288.1 eV. The peak at 285 eV was ascribed to aliphatic carbon with a dipole moment of zero, that at 288.1 eV to carboxylic groups with a dipole moment of 2.0 Debye units and that at 286.3 eV to the α-carbon bonds with zero dipole moment. An additional peak at 289.2 eV was required to fit the spectra at the two greatest concentrations. This peak was assumed to arise from adsorption of additional material after full coverage was reached and was not included in the calculation of free energy. As can be seen from Fig. 11, the adsorption of protein causes the polar component of free energy to increase from only 20% of the total surface energy to 70% of the total surface energy, the transition being complete at a concentration of 10^{-5} v/v.

CONCLUSION

This investigation of the adsorption of chicken egg albumin on titanium provides an example for the generic case of adsorption of molecules that are large with respect to the effective analysis depth for XPS. In this case, the widely used Beer–Lambert model for interpretation of intensities is inapplicable. Instead, the respective signals from substrate and adsorbate should be used to provide measurements of coverage. The adsorption of albumin has been shown to follow the Temkin isotherm. The logarithmic shape of the Temkin isotherm is associated with the strong chemisorption of the albumin molecule giving high uptake at low concentrations. Here, we have shown that the intensity of both nitrogen and titanium peaks corresponds to monolayer coverage at a concentration of a solution volume fraction of 10^{-4} (100 ppm) with the protein film reaching a surface concentration of nitrogen of 12%. The formation of this monolayer is accompanied by a transition from a surface free energy that is hydrophobic to one that is completely hydrophilic. The shape of the carbon 1s peak has been interpreted in terms of the contribution each component makes to the total Debye moment and hence to the surface free energy. The surface energy has been plotted in the form of an isotherm and independently places the maximum in surface energy at a concentration of 10^{-5} v/v. AFM shows that the shape of the molecule becomes apparent at concentrations greater than that required to form a monolayer.

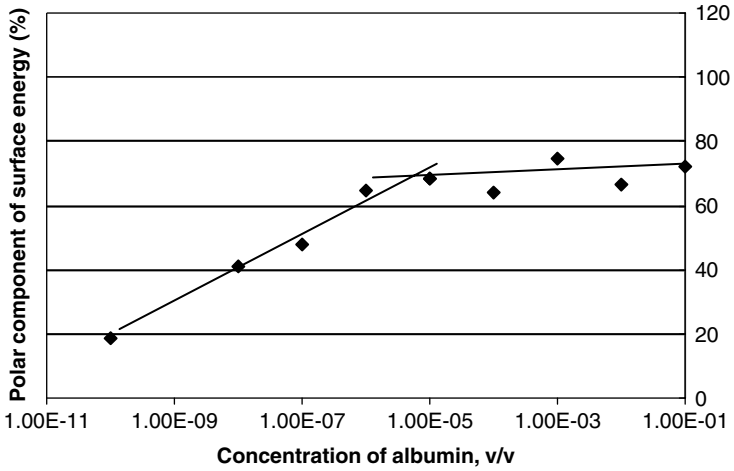

Figure 11. The increase in the polar component of surface energy as albumin is adsorbed on the surface. The value for the 'clean' surface has been plotted at 10^{-10} v/v to facilitate the use of a logarithmic scale.

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