Invited Paper

Surface Analysis of Biomolecules: Unravelling biointerfacial interactions

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(Received: November 27, 2007; Accepted: March 24, 2008)

The ability to control interactions between biomolecules and surfaces influences a number of bioengineering applications. *In vivo* we desire materials that can prevent or initiate cellular adhesion, selectively adsorb proteins or mimic the structure of a cell surface. *In vitro*, high sensitivity proteomic and diagnostic applications require the immobilization of specific biomolecules without any loss of native function. In order to understand how the surface properties of a material influence the organization of biomolecules detailed characterization is required to establish the composition, structure and orientation. This paper will review how combining surface analytical techniques such as X-ray photoelectron spectroscopy (XPS), static secondary ion mass spectrometry (SSIMS) with biological assays has enabled the characterization of the type, amount, conformation, orientation and spatial distributions of proteins on biomaterial surfaces.

1. Introduction

As a discipline, bioengineering encompasses a diverse range of research interests but within this vast array of technologies there are a large number of fields that bring together materials technologies and biology. Obvious examples are biomaterials, tissue engineering and biosensors, but there are increasing numbers of technologies that are seeking new approaches based on materials and engineering technologies to optimise the processing of biological samples or assays (ie microfluidics applications in proteomics). The ability to characterise and/or manipulate biointerfacial events lies at the heart of this research.

The rapid, irreversible adsorption of proteins onto solid surfaces from biological media is a well-known phenomenon. Upon implantation of a biomedical device, for example, a layer of adsorbed protein immediately forms on the surface of the biomaterial [1]. This layer of adsorbed proteins directs subsequent biological responses to the material. The composition, concentration, conformation, orientation, and spatial distribution of adsorbed proteins all affect and mediate subsequent biological reactions to the surface. The past fifteen years have seen a rapid rise in techniques capable of probing the biointerface. Optical techniques like surface plasmon resonance (SPR) [2], optical waveguide lightmode spectroscopy (OWLS) [3] and ellipsometry [4] are all are all now used routinely to characterize biointerfaces. Atomic force microscopy (AFM) can be used to image single molecules, unfold proteins, monitor surface topography and measure the forces involved in protein-protein and protein-surface interactions [1]. One limitation of all these techniques is that they don't readily provide chemically specific information about the interface or interaction, making it difficult to unambiguously ascribe events in AFM force curves, SPR spectra or even features in an AFM image. This type of chemical surface analysis is typically the domain of XPS and Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS).

XPS has been used to detect and monitor the interactions of biomolecules with a range of both model and 'real' biomaterial surfaces [1,5]. Early work in this field and much of the underlying theory related to the detection of proteins has been reviewed in detail by Paytner *et al* [6]. Proteins contain mostly carbon, oxygen and nitrogen with lower levels of other elements such as metals,

Table 1. Elemental compositions of common biomolecules.

	Atomic % Composition			Atomic Ratios	
	C	O	N	N:C	O:C
Protein [a]	65.3	18.1	14.2	0.22	0.28
Lipid[b]	95.0	5.0	0.0	0.00	0.05
Mucin[c]	58.0	31.0	9.8	0.17	0.53

[a] Data derived from XPS spectra of thick human albumin film
[b] Theoretical composition of cholesterol ($C_{27}H_{48}O$)
[c] Data derived from XPS spectra of the glycosylated region of porcine submaxillary mucin (PSM, MUC1)

phosphorus and sulphur incorporated into specific structures. In theory, the presence of minor elements (sulphur, iron, etc.) in the protein may enable their detection on a surface, but in practice, the distribution of these elements within the protein and their low concentration often means that they are at or below the effective detection limits for most XPS instruments. The XPS detection of proteins at interfaces generally involves the detection of nitrogen directly from the adsorbed protein. Recent studies by Wagner *et al* correlated ^{125}I data with XPS data from adsorbed protein films [7]. The results showed that XPS detection limits for proteins could be as low as ~10 ng/cm^2 if the substrate did not contain nitrogen and that even in the worse case scenario, where the surface did contain nitrogen, it was possible to monitor changes in the amide contribution to the C1s spectrum and detect proteins to ~100ng/cm^2. Similarities in the elemental composition of most proteins do mean that XPS cannot be used to differentiate between proteins adsorbed from complex mixtures such as plasma or tears. Hence, biochemical assays such as enzyme–linked immunosorbent assays (ELISA) or radiolabelling with ^{125}I can be paired with XPS to characterize different species present in the adsorbed protein film [7-9].

Proteins are not the only biomolecules of interest. The detection and characterisation of lipids, mucins and DNA at interfaces are critical to our understanding of biofouling [5], and the development of bioarrays [10,11], biosensors [12] and a range of other biotechnologies [13]. Using the same principles used for protein analysis, XPS has been used to quantitatively compare DNA immobilisation strategies and optimise surface coverage [10,11] and evaluate strategies for lipid immobilisation [14]. Table 1 lists the chemical compositions of a range of typical biomolecules [5]. Analysis of this data illustrates that deposition of any of these molecules at an interface would introduce distinct and differentiable changes in the elemental composition of a surface. As stated earlier, increases in the nitrogen to carbon ratio (N: C) are typically used as indicators of protein deposition, but significant changes in the oxygen to carbon (O:C) ratio can be expected with the deposition of lipids (ratio decrease) and mucins (ratio increase).

It is well established that contact lenses accumulate proteins, lipids and other tear components on their surface as well as in their matrix during even short period of wear [15] and XPS [5] has been used to monitor the early stages of protein adsorption on worn contact lenses. This accumulation is implicated in allergic and inflammatory reactions, mechanical irritation and microbial contamination, which in turn leads to infection [16]. Figure 1, shows the XPS atomic ratios produced from the analysis of worn contact lenses. It is evident from the increase in

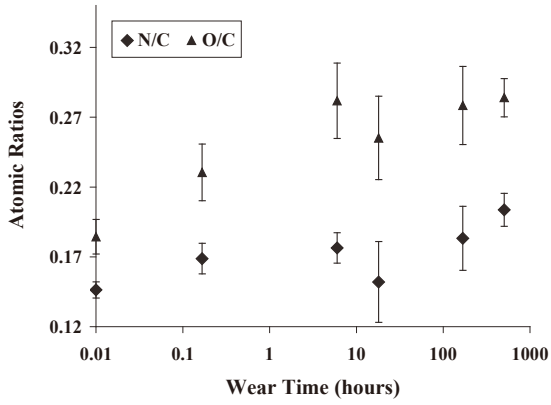

Figure 1. Variations in the XPS atomic ratios of Focus Night and Day® contact lenses with increasing lengths of extended wear.

the N/C ratio that protein rapidly adsorbs to the lens surface and reaches a peak after approximately 10 hours

of wear. Perhaps the most interesting point to note from this work is the change in composition at the 18-hour time point. In the study, contact lenses were worn continuously, even during sleep and this time-point represents lenses removed from the eye upon waking in the morning. The drop in both the O/C and N/C ratios suggests that in the closed eye environment, the contact lens adsorbed more carbon-rich lipid, but that when the eye is open, the composition of adsorbed species on the lens surface changes and becomes dominated by proteins and mucin. These results are interesting both in terms of comfort factors (lipids make the lens more hydrophobic) as well as fundamental issues associated with how the eye interacts with the lens in the closed eye environment.

The ability to eliminate protein adsorption at the surface of a material lies at the heart of many biotechnologies and medical devices. The sensitivity any bioassay relies on the ability to reduce the background signal. In many cases this background is caused by the non-specific adsorption of proteins or other ligands to the surface, creating a massive market for the development of materials and surface modifications that can prevent this type of protein adsorption [17]. Of course alongside the desire to develop these materials, is the need to be able to detect protein present at the surfaces with very high sensitivity [18].

The application of ToF-SIMS in the analysis of biomaterials and biological interfaces has historically revolved around the characterisation of polymeric interfaces. This has included the study of degradation pathways for biodegradable polymers, the monitoring of coating chemistries, detection of surface contamination and surface chemical characterization of co-polymer systems [1]. The surface sensitivity of ToF-SIMS has lead to its application in the detection and identification of biomolecules adsorbed at interfaces. Alongside the XPS studies, Wagner *et al* investigated the detection limits of ToF-SIMS and found that the technique was capable of detecting as little as 0.1ng/cm^2 of protein at a surface, making it 100 times more sensitive to protein adsorption than XPS [7]. This sensitivity has been utilized to compare a range of different non-fouling surfaces and evaluate different surface modification strategies [18]. Figure 2 gives an example of this strategy in the analysis of a region in a bioarray. The figure illustrates the presence of protein-related amino acid fragments on the plasma polymerized maleic anhydride regions of a patterned surface, while the PEO-like plasma polymerized tetraglyme regions show no evidence of protein.

The process is not without its problems though. The largest ions detected from any protein are the immonium ions (+NH2=CHR) from each amino acid (MW < 200). As a result of this fragmentation, the identification of proteins is often more like a jigsaw puzzle. Mantus et al. showed that a static SIMS spectrum of a protein film is comprised of at least two peaks from each of the twenty amino acids [19], as was the case in Figure 2, most authors select a few 'representative' peaks from the static ToF-SIMS spectrum for their protein data analysis. Lhoest [20] and Ferrari [21] were the first to apply multivariate analysis techniques (utilizing many of the peaks from the static ToF-SIMS spectra) to characterize the differences between static ToF-SIMS spectra of different adsorbed protein films and the amount of adsorbed protein, respectively. Since then, Wagner has expanded and shown that ToF-SIMS can be utilized for protein identification, detection of proteins from mixtures and quantification of protein mixtures [22,23].

While static ToF-SIMS analysis has been successfully applied to many studies of peptides and proteins, there remain a number of significant challenges to be resolved. A primary challenge is the requirement for UHV. While adsorption of a protein onto a surface may result in some denaturation, the removal of bound water and subsequent dehydration of the protein under UHV likely causes further denaturation. Cold stage static SIMS, in which the sample is maintained in a frozen-hydrated state during analysis, has been developed to circumvent this issue [24]. While a freeze fracture technique has been applied successfully to the analysis of cell membranes [25] it is yet to be applied specifically to the analysis of proteins or peptides. Issues related to the effects of vacuum on adsorbed proteins and peptides have been investigated and the surface sensitivity of static ToF-SIMS and its utility in probing protein conformation and orientation remains a focus of current research.

In a study examining ToF-SIMS spectra from proteins adsorbed at different temperatures, Tidwell et al have shown significant variations in the intensities of characteristic amino acid peaks [26]. These results suggested

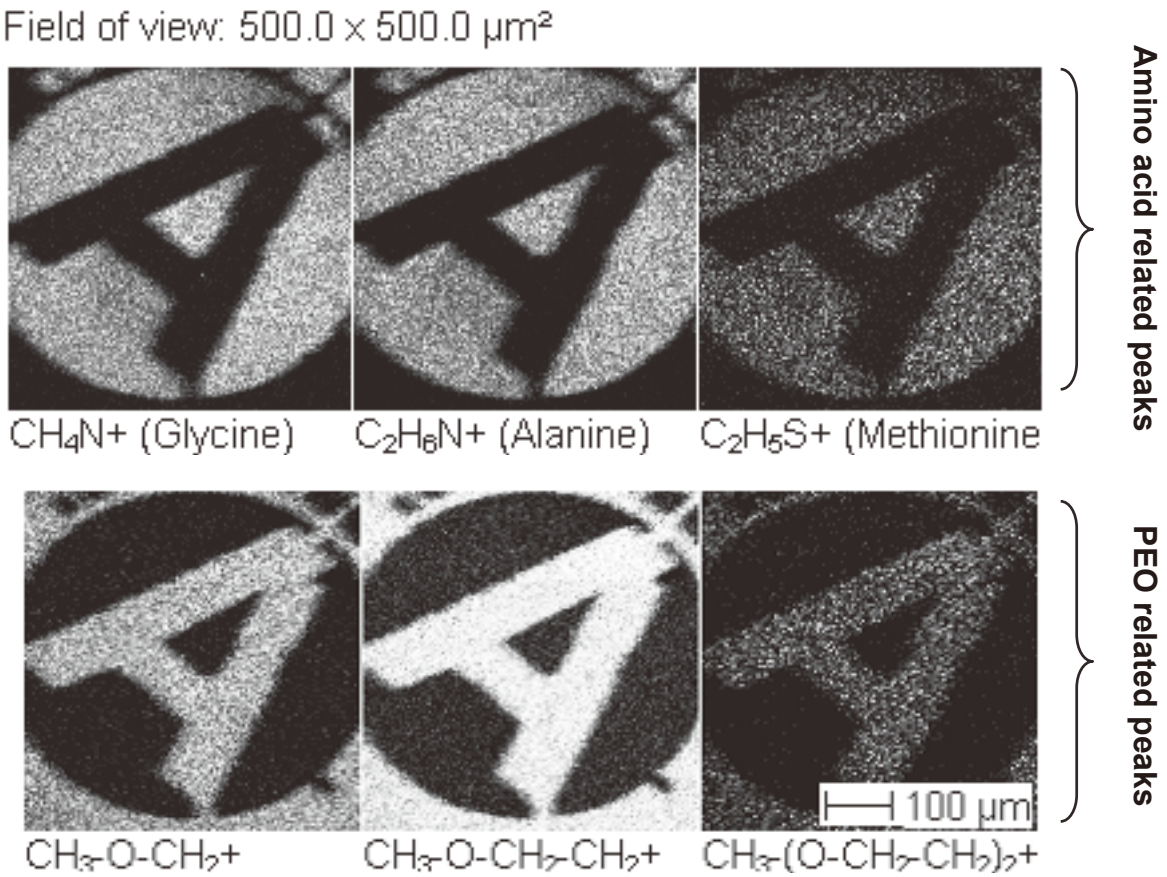

Figure 2. Positive ion ToF-SSIMS image (500 x 500µm image, Bi3++ source at 50keV) of plasma polymerised maleic anhydride films patterned on a plasma polymerised tetraglyme films. The sample was incubated in 1mg/ml human fibrinogen (PBS, pH 7.4) for 2 hours to enable the protein to couple with the anhydride groups.

that in addition to identifying the protein, analysis of static ToF-SIMS spectra could provide information about the orientation and conformation of the protein. Subsequent studies by Xia et al have indicated that it is possible to limit the dehydration effects of UHV on the conformation of proteins by incorporating sugar into the protein film [27]. Studies using bioassays, surface plasmon resonance (SPR) and static ToF-SIMS showed that antibody films dried in the presence of sugar adsorbed antigen at levels comparable to undried antibody film. In contrast, there was a significant reduction in activity of the adsorbed antibody when dehydrated without sugar. Comparison of the static ToF-SIMS spectra from the samples dried with and without sugar indicated that their surface amino acid composition were different. For films dried with sugar a relative enhancement of fragments from hydrophilic amino acids was detected, while for films dried without sugar a relative enhance of fragments from hydrophobic amino acids was detected [27].

In addition to spectroscopy, as illustrated in Figure 2, ToF-SIMS can be used in an imaging mode to chemically map the surface of a material. There is always a trade off between high special resolution and high mass resolution, but with the advent of liquid metal ion sources (eg Bi+), systems are typically capable spatial resolution of <1µm, while retaining nominal mass resolution. As a result, there is increasing interest in the application of ToF-SIMS for the chemical imaging of a range of patterned biomolecules on surfaces. Recent studies by Lee *et al* have demonstrated how the chemical state imaging capabilities of ToF-SIMS can be utilized to study DNA spotting procedures and the spatial distribution of DNA and its fluorescent tags on array substrates [28]. The results show how the presence of specific additives and buffer agents influence the doughnut effects and other discontinuities commonly seen in arrays. These

surface effects complicate the interpretation of the fluorescent readout, limiting the reproducibility and sensitivity of many bioarrays.

In addition to the high sensitivity of ToF-SIMS, significant developments in ion sources have shown that polyatomic (eg Bi_3) and cluster ion (C60) sources can significantly improve the molecular ion yield of both biological and polymeric materials. As a result, it has become possible to create images from larger molecular weight molecular fragments ie lipid molecular ions. Combined with the development of integrated freeze hydration stages for sample preparation, this has lead to increased activity in the application of ToF-SIMS in the analysis of cell membranes and other hydrated biological systems [29]. While these approaches are currently being focused on tissue analysis and disease detection, there is potential for their application to other areas of biotechnology including microfluidics, cell culture and tissue engineering.

In conclusions, XPS and ToF-SIMS are versatile and useful techniques for the characterization of adsorbed proteins and biomolecules. XPS instrumentation and protocols are well-developed and the technique is suitable for routine application. Interpretation and quantitation are well secured; coupled with experimental flexibility this makes it eminently suitable as a prime technique in biomaterials research. ToF-SIMS provides very low detection limits and information about the molecular structure of adsorbed proteins, but experimentation and data interpretation are more involved than for XPS. A key advantage of ToF-SIMS remains its ability to probe conformation and orientation of adsorbed proteins alongside its spatial imaging capability. By combining these UHV techniques with the more traditional biological assays and fluorescence imaging techniques, we can gain invaluable insight into the nature of biomolecules at surfaces and begin to unravel complex biointerface interactions.

2. References

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