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Biomolecular characterization of glass surfaces

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Abstract

This paper introduces the concept of biomolecular characterization of inorganic surfaces. The choice of biomolecule is discussed followed by techniques that can be used to analyse the quantity of bound species, strength of binding, the nature of binding sites, conformational changes and the layer morphology. The prospects of modelling this data using a combination of molecular dynamics simulation and protein structural modelling and the correlation to measured data are outlined. The studies described in this paper are directed toward assessing the feasibility of biomolecular characterization, however, the data collected in the process are designed to also help elucidate our understanding of the interaction between biomolecular species and inorganic materials interfaces.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

The surface of glass in many instances is more important than the bulk: it is the surface that is often the determinant in the strength of a glass article. The surface is certainly a determining factor in the beauty of a glass article and it turns out to be probably the one place in which value can be added to the glass in a reasonable way, by engineering of the glass surface. There are multitudes of characterization techniques that can be used to look at the surface of glass provided the surface is reasonably flat, accessible or in great abundance compared to the volume. There has always been reflectance spectroscopy that could be used to examine the surface; both total or diffuse reflectance (DRIFTS) and specular reflectance (IRRS or IRRAS) depending on the sample and morphology. Surface analysis techniques such as secondary ion mass spectrometry (SIMS), x-ray photoelectron spectroscopy (XPS or ESCA) and, more lately, glancing angle x-ray diffraction (GIXR) and atomic force microscopy (AFM) can give a variety of chemical and physical data about the surface. In all cases, the data have to be very carefully assessed and evaluated. There is indeed a wealth of such information in the literature regarding many of these techniques. Unfortunately, the applicability of these techniques to

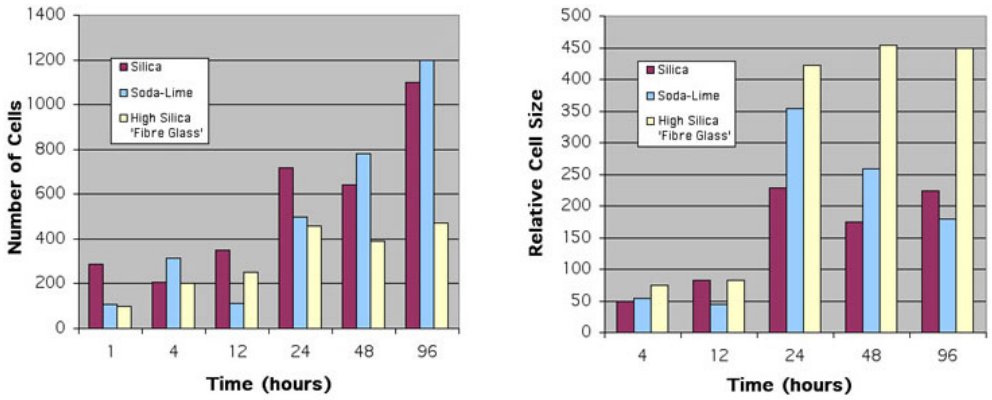

Figure 1. Relative cell size and relative number of cells as a function of time on polished glass samples.

all types of samples has been limited by their nature. In particular, the industries that make fibreglass generate small particles such as microspheres, or even the fibre optics industry, could use some techniques that might apply to their sample morphologies.

It has been known for many years that the measurement of gas adsorption gives information regarding the surface area of a sample and can be used with more reactive gases to chemically analyse a surface. Surface analysis groups such as those at the Pennsylvania State University have made headway along this path by using inverse gas chromatographic techniques which involve adsorbing gases to pristine surfaces and then measuring desorption profiles. These data are invaluable for examining the chemical nature of some of the surfaces but like the other techniques, this one is reliant on large surface areas. As an extension to this principle we have explored the possibility of obtaining more complex bonding information by using a well-characterized and yet more complex molecule.

In some work undertaken at the request of the fibreglass industries our group looked at the fundamental behaviour of a standardized and well-characterized cell line when it came into contact with commercially available glasses in the silicate family. Chinese hamster ovary (CHO) cells were exposed to silica (S), sodium silicate (NS), soda lime silicate (SL), two Bioglass® (calcium phosphate modifier) compositions and two fibreglass compositions. The two fibreglass compositions, one with a high S content and the other with a high boron content, were labelled FS and FB respectively. The bioglass compositions were used as standards because of the wealth of data in the literature about the compatibility of these glasses with other cell types. The NS glass was selected mainly to see what effect the addition of one modifier ion had on the network in terms of biological behaviour. However the most interesting results were obtained for the S, SL and the high silica fibreglass composition (figure 1). A number of methods were used to examine the cell behaviour; one was total area coverage by cell matter as a function of time. In this regard the S, SL and FS fibreglass appeared identical, with the FB following a similar shaped curve, but with lower overall coverage. However, cell coverage has two components: one is the cell proliferation number while the other is the spread of individual cells or cell size. If the data are split in this manner one immediately sees that the fibreglass coverage is due entirely to different effects.

It is apparent that the coverage for the fibreglass is due to the relative cell size and not the proliferation, as opposed to that for the S and SL glasses. In addition, the cell shape was very different on the fibreglass. Cells have a resting stage when they are relatively rounded and then

an interphase where they undergo cell division. In interphase, the cells extrude pseudopodia, form attachment sites, and stretch to divide. The cells on the fibreglass, both low and high S, always looked like they were in interphase but did not readily divide (cf the population). This implied that the macromolecular recognition systems of the cells identified very different physicochemical surface features on the glasses. Cells are sensitive to both chemical and structural features, therefore just examining cell behaviour might not be sensitive enough to elucidate the surface morphology. However, it did occur to us that those proteins that form the interface between the cell matter and the surface might be used to characterize the difference in the surfaces that were revealed in the cell behaviour even on previously inaccessible surfaces.

2. Cherche le protein

If one works on the premise that a molecule could be used to characterize a surface, then it is pertinent to choose a well characterized molecule that can be examined in a variety of ways and that is conformationally sensitive regarding its binding sites. In addition, if one wants to subsequently try to model these processes using molecular dynamics simulation with energy minimization for molecular attachment it is also pertinent to choose a molecule which can either be used as a whole, or that can be fragmented both *in vivo* and *in silico* for binding and computer modelling purposes. Examples of these types of proteins that our group has looked at are streptavidin, human serum albumin (HSA), bacterial alkaline phosphatase (BAP), and fluorescently labelled goat anti-mouse immunoglobulin (IgG) in conjunction with mouse IgG.

2.1. Streptavidin

Streptavidin is a well-characterized molecule in the biological arena. It binds the small molecule biotin with one of the highest affinities known in biochemistry. This well-defined binding allows streptavidin to be examined orientationally. In short, once bound there are two clearly discernible orientations; one which leaves the biotin-binding site accessible, and one which does not. Intermediate orientations with lowered affinity are, of course, possible but beyond the first stage of our characterization system. Streptavidin is also naturally fluorescent allowing for another avenue for characterization.

2.2. Human serum albumin (HSA)

This is an abundant protein in blood and is therefore important for commercial applications of glass as a biomaterial or in biotechnology, therefore there are an abundance of other studies on the adsorption of HSA to many materials that are used in biomedicine and biotechnology. The commercial applications also mean that it can be obtained relatively easily and inexpensively with high purity.

2.3. Bovine pancreatic trypsin inhibitor (BPTI)

BPTI is a well-characterized protein consisting of only 58 amino acid residues ideally suited for molecular dynamics simulations due to its relatively small molecular weight. It can also be used for experimental investigations.

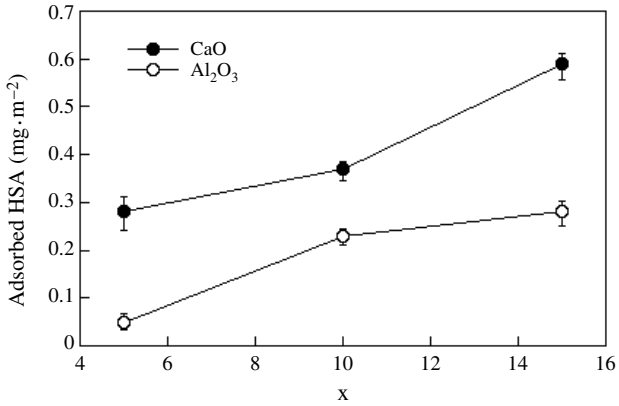

Figure 2. The binding capacity for HSA of sodium silicate glass substituted with alumina and calcia.

3. Techniques that can be used to examine protein behaviour on glass

3.1. Amount of protein bound

The total binding capacity of a material for a given protein can be measured in a number of ways. In principle, it is possible to measure the amount of protein missing from a solution, i.e. that which has bound to the sample. In this case, it is important to establish the correct concentration of protein in the initial solution. If there is too much protein in the initial incubating solution, then one will be measuring a small difference between two very large numbers. This is particularly true if you are trying to measure adsorption as a function of forming, and in the case of samples that may not have a very large surface area such as flat plate or cane. However, it is important to ensure that the available sites are all occupied. If the protein concentration is too low, errors could be introduced by a lack of coverage. Alternatively, the sample with the proteins can be extracted from the solution and then the proteins that are attached can be desorbed and measured. This latter method is probably more accurate, since one is comparing what might be a small signal to no signal, although care has to be taken not to lose protein during desorption. One way of removing the adsorbed proteins is to boil the sample in a buffer. The buffer can then be analysed for the protein content. However if one is using for example fluorescence as the probe for the protein concentration, care has to be taken that the removal method does not interfere with the lumophore used to assess the protein. There are numerous ways in which to measure protein concentration and thus binding capacity. Dependent on the concentration and accuracy of the test one can use a protein assay but these may have limited accuracy for some of the binding capacities in which we are interested. One method that is particularly good for desorption studies is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This is where the liquid containing the protein is placed in a sample well and on application of a voltage the charged protein is forced to migrate into the gel, its progress being determined by the molecular weight of the protein. Once in the gel, the protein is immobilized within the gel and can be developed using a stain sensitive to the protein in question. Silver marker stains are usually quite sensitive but unfortunately do not work well for streptavidin. The protein can be quantified by running in the same gel some 'lanes' that contain standards. The optical density of the stained gel can be measured quantitatively and by comparison to the standards the binding capacity of the material can be calculated. Figure 2

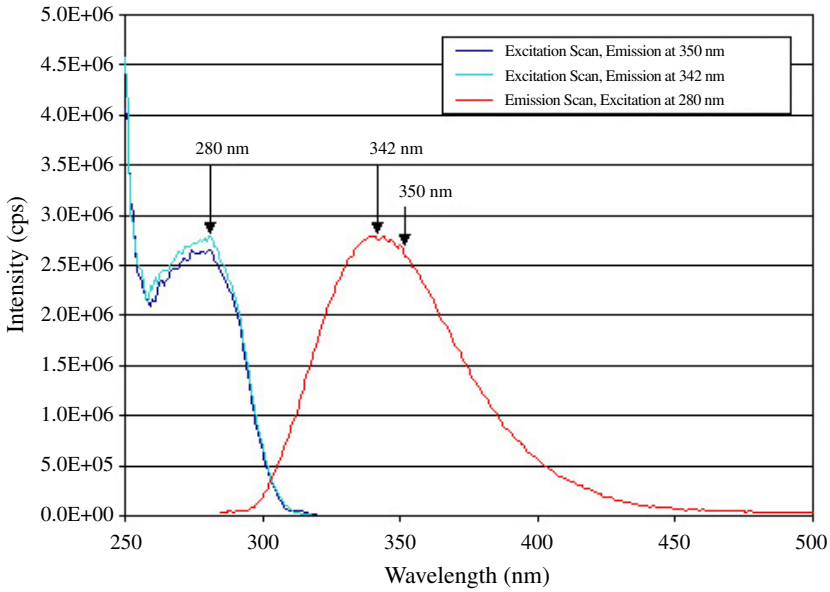

Figure 3. The excitation and emission spectra of streptavidin in solution (0.1 mg ml^{-1} concentration).

shows the binding capacity for HSA as a function of composition substituting calcium oxide or aluminium oxide for sodium oxide in a NS glass. The binding capacity for the calcia-substituted glass is approximately three times higher than for the alumina-substituted glass.

For streptavidin to be analysed by SDS-PAGE an alternative stain must be used, or one can employ alternative techniques such as fluorescence spectroscopy. Some proteins such as streptavidin have naturally fluorescent residues, whereas other species such as IgG can be obtained commercially with fluorescent tags. Fluorescence is a very sensitive technique because unlike optical transmission, where, for a largely transparent sample we are analysing a small difference between two very large numbers. For fluorescence we are looking at the difference between no signal and a small signal. In fact, for strongly fluorescent species levels as low as picomolar concentrations may be observed. Figure 3 shows the natural excitation and emission peaks for streptavidin. The aforementioned advantage of streptavidin is that it binds the molecule biotin as a function of the orientation of the streptavidin molecule: the streptavidin can be further utilized via a fluorescently tagged biotin species. Hence fluorescence spectroscopy can aid in orientational quantification. Anti-mouse IgG can be purchased with an attached fluorphor fluorescin isothiocyanate (FITC) that can be used to measure the binding capacity of that protein, again either by measuring what has attached to the species or by measuring what has been left behind. It is worth noting that, for exquisite sensitivity, proteins may be labelled by I^{125} and quantified by autoradiography. We have not yet used this labelling method.

3.2. Binding strength

Binding strength can be measured in a couple of ways. One approach is to carry out temperature controlled native gel electrophoresis. Native gel electrophoresis allows proteins to stay in their natural state, thus denaturing chemicals are selectively eliminated from the buffer. This can be measured as a function of temperature. By this technique small particles such as microspheres

or fibres with proteins attached can be loaded into the wells of an electrophoresis gel and the gel run under an applied field at a single temperature. The temperature can be increased for subsequent gels to obtain a binding strength. An alternative method can be employed with complimentary proteins such as the streptavidin–biotin system where one can use competitive biotinylation. If one has a material which has a protein such as streptavidin attached, it is possible to purchase biotinylated beads which can compete for the streptavidin. Provided the strength of binding of the competing species is known, a benchmark, at least, for the binding strength can be found. Mass spectrometry techniques such as gas chromatography mass spectrometry (GCMS) or matrix assisted laser desorption ionization (MALDI) can also be used to measure binding strength (in GCMS one can either measure desorption directly or indirectly). Microcalorimetry is another technique that can be used to measure the binding strength of molecules to the surface; levels as low as nanowatts are measurable. However this will also be an average function of the binding sites. For specific binding strengths, site-to-site application of the chemical force microscopy (CFM) technique described below is particularly useful.

3.3. The nature of binding sites

Most of the techniques relating to the nature of binding sites are best carried out with flat samples, which is in fact why this molecular characterization is of interest. This is not a paper about general surface characterization but will include surface characterization pertinent to binding sites. Infrared spectroscopy has always been a popular choice for looking at the interaction between solid surfaces and organic molecules. Dependent on the sample one can use diffuse reflectance infrared or attenuated total reflectance. However care has to be taken with interpretation of the data. There has been quite a plethora of work on the characterization of molecular attachment, ranging from amino acid residues to polypeptide chains bound to surfaces, but on the whole the interactions at the interface are frequently masked by the presence of vibrations further along the molecule. Gas adsorption has been used before to probe acidic versus basic sites, as has titration. Both techniques can be open to misinterpretation however, for example leaching of the glass when carrying out pH titrations. Adaptation of AFM to measure the binding strength of particular sites on a surface can be achieved by using chemically functionalized tips, this is known as CFM. The useful thing about CFM is that one can buy or make tips that are modified with various molecules including proteins such as streptavidin. The only problem being that dependent on the technique used to modify the tip, it can look in reality like the tip has grown hair. This means that one may be measuring the attachment of more than one molecule to the surface. Figure 4 shows a trace from a CFM experiment with a tip that is coated with covalently bound streptavidin molecules. However the radius of curvature of the tip (85 nm) is such that there will be effectively active binding due to only one molecule.

By knowing the spring constant of the tip, and by measuring the deflection from the trace, one can obtain the force with which the molecule that is attached to the tip is bound to a point on the surface. Therefore the binding map can be correlated with the topographic map of the surface. One can also measure with other tips to determine acidic and basic sites and obtain a map, which can again be related to the topographic map.

3.4. Measurement of conformational changes

One of the potentially most powerful features of using molecules to characterize surfaces has to do with conformational changes that may accompany molecular attachment to a surface. There

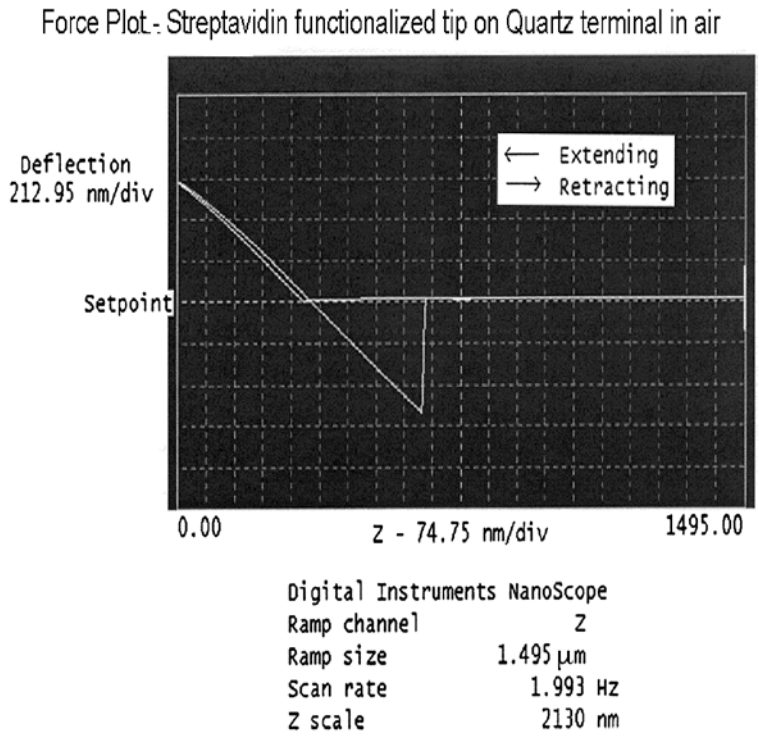

Figure 4. CFM trace with streptavidin functionalized tip on a quartz terminal.

are a number of ways to assess this. In particular, if one is dealing with a biologically functional molecule, activity is often affected by the immobilization process. For example, a protein that catalyses a chemiluminescent reaction may be quenched by adsorption-mediated changes in protein folding. Alternatively, if the molecule is naturally photoluminescent then the signature peaks may shift or may have different intensity profiles when the residues associated with the luminescence are compromised. The polarization properties of molecules may also change. Figure 5 shows the circular dichroism spectrum of a bioluminescent molecule that has been trapped in a sol gel matrix. In this case the immobilization caused the band at 210 nm to be disrupted which corresponds to a change in the beta sheet structure of the protein.

3.5. Analysing the protein layer

In principle, an absorbed layer of protein can be analysed using glancing angle x-ray reflectometry which, given a suitable standard for the density of the protein film, can be measured and modelled to give thickness, density and the number of layers. Figure 6 shows a glancing angle analysis of a streptavidin film on a S slide. In the absence of a more suitable model, a quick simulation was carried out using a simple chain of CH_2 over a single layer of SiOH. The result was that the fit was best for an approximate 50 Å protein layer over the hydroxyl layer. This is consistent with the fact that streptavidin is a molecule of approximate lateral dimension 40 Å. The question is even if one obtains all this information can it be modelled?

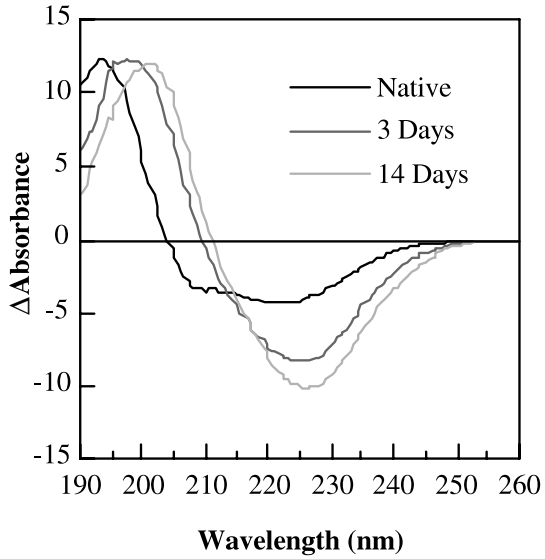

Figure 5. Circular dichroism spectrum of an enzyme encapsulated in a sol gel matrix.

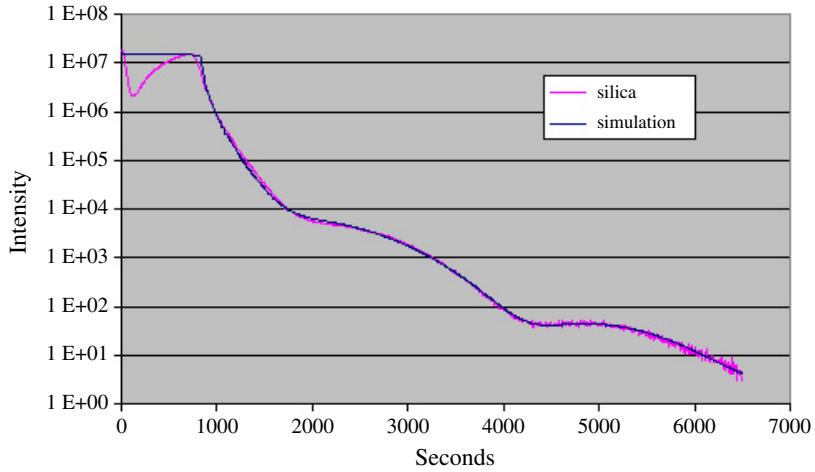

Figure 6. Glancing angle x-ray reflectance of streptavidin on a glass slide.

3.6. Molecular dynamic simulation

Given that one can reasonably accurately simulate the surfaces of glasses using traditional molecular dynamics, it should be possible to simulate the attachment of biological molecules to that surface. To discuss the progress of the former is a bit beyond the scope of this paper but suffice to say that in Cormack's group at Alfred University, MD surfaces of simple alkali silicate glasses have been created from what are believed to be representative MD simulations of the bulk by creating a 'notch' (removing some atoms from the model 'edge' and applying a force to split the structure in order to examine the surface that is created). Is this a real surface? Not exactly, but it might be equivalent to a surface created by breaking a glass in a

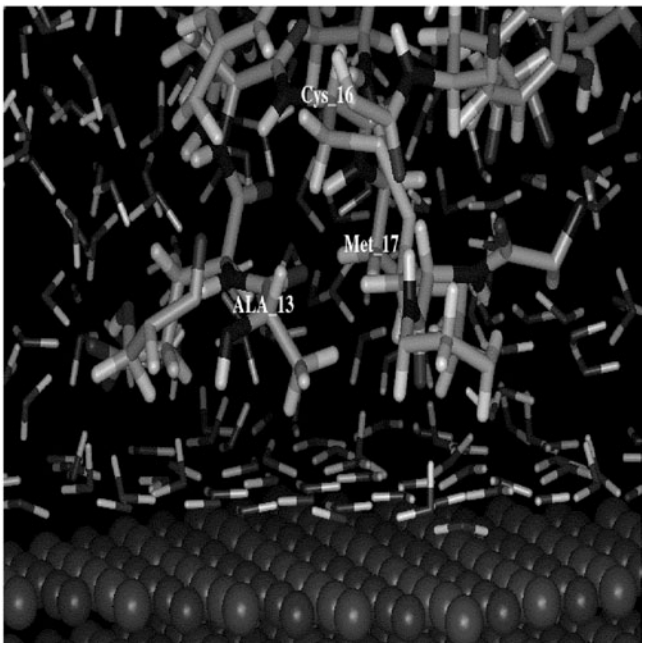

Figure 7. Partial view of BPTI orientation 2. Note the highly structured water at the MgO surface adjacent to the 'hydrophobic pocket' formed by the Ala–Cys–Met domain, whereas the hydration water at the edge of the protein–material interphase interacts with the bulk solvent.

high vacuum. Realistic glass surfaces have yet to be created with hydroxyl species, but this work is well underway. Because of the enormous implications for biomolecular medicine, physical biochemists have made great strides in modelling protein structure. The challenge in biomaterials modelling is to integrate protein modelling and materials modelling software algorithms. A further complication results from the fact that most material surfaces of interest in biomaterials applications are in an aqueous environment. These challenges have been taken up by a number of computational chemists including the group of Alastair Cormack which, in collaboration with one of us (AHG), has developed an integrated system that uses a mixture of commercially available and custom software to model the adsorption of small proteins such as BPTI to very well-known single crystal surfaces such as magnesia and quartz which can be represented with hydrated surfaces. While the system needs further refinement, a realistic picture of this interphase is beginning to emerge. The predicted binding energies are congruent with measured values for protein adsorption, which is certainly encouraging. When one recognizes that the 3D structure of a protein is often stabilized by as little as 15–50 kcal mol^{-1} (less than one covalent C–C bond in vacuum), it is clear that molecular dynamics will play an essential role in developing *in silico* systems that accurately predict protein adsorption. As shown in figure 7, most contacts between the adsorbed protein and the material surface will, as expected, be mediated by the hydration sheath. The energetics of protein binding in various orientations can be calculated and compared to experimentally determined values.

One can always show colourful pictures of large protein species landing on the nice ordered (perfect) surface but in order to extract information that can be correlated to measurements one has to analyse the protein conformation. One example of how this can be done is to try to quantify the conformation and this has been studied quite extensively by the protein chemists.

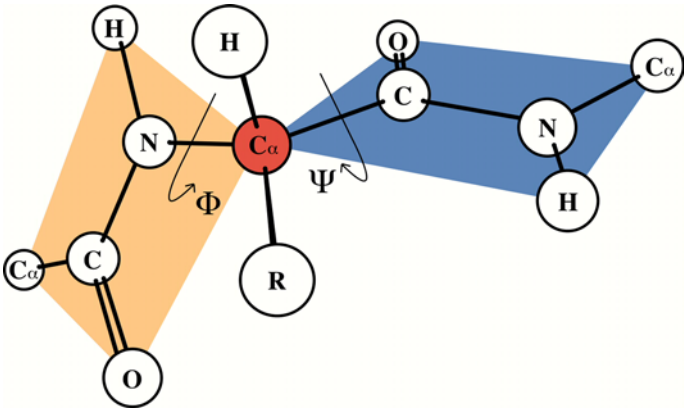

Figure 8. A generic dipeptide unit (i.e. two amino acid groups bonded) is shown, and defines two dihedral angles Φ and Ψ.

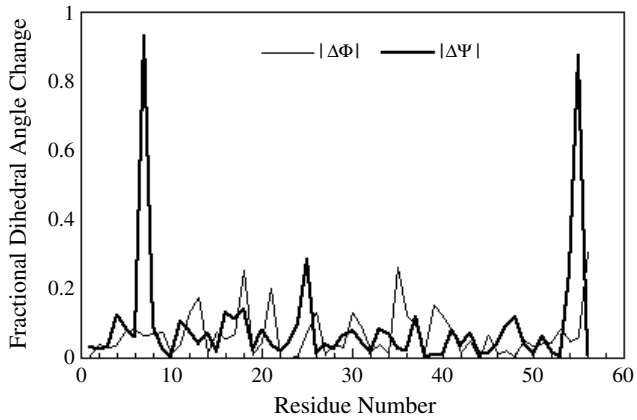

Figure 9. Change in dihedral angles Φ and Ψ on adsorption with residue number.

It is relatively easy to extract these two dihedral angles from the atomic coordinates obtained from the simulation, and the change in these angles before and after adsorption are plotted against the residue number as shown in figure 9. The quantities $\Delta\Phi$ and $\Delta\Psi$ have been normalized to 360° so the rotation in the residues in the main body are fairly conservative as a result of steric constraint. However the ends of the chain which have more steric freedom can rotate more freely.

Since the residues are reasonably well-characterized and their positions known from protein crystallography, conformational changes can be followed. In addition, one can look at the relative distance between residues and this would quantify the amount of folding that is occurring for the adsorbed protein. A difference tertiary contact map that demonstrates this is shown in figure 10.

4. Summary

What we have attempted to show here is the variety of information that might be accessed from examining the binding and conformation of a well-characterized biological molecule to

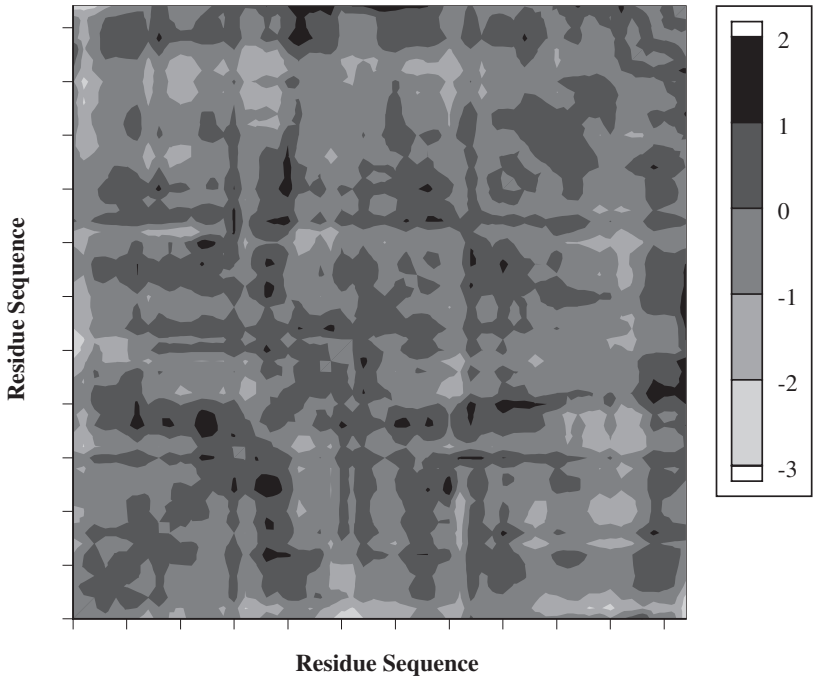

Figure 10. Difference tertiary contact map: white segments indicate minimum distortion whereas dark segments indicate significant distortion. The numbers on each side represent the residue number.

a glass surface. While interpretation of the data will not be trivial, the ability of the method to be applied to otherwise inaccessible surfaces could ultimately be very useful. Even if this does not end up as a universally useful characterization tool, the knowledge will certainly be applicable to the application of glasses in many fields of biotechnology.