REVIEW

Interfacial assembly of proteins and peptides: recent examples studied by neutron reflection

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Through reviewing a number of recent neutron reflection studies of interfacial adsorption of peptides and proteins, this paper aims to demonstrate the significance of this technique in studying interfacial biomolecular processes by illustrating the typical structural details that can be derived. The review will start with the introduction of relevant theoretical background, followed by an outline of representative biomolecular systems that have recently been studied to indicate the technical strengths of neutron reflection.

Keywords: neutron reflection; protein adsorption; peptide self-assembly; protein unfolding; biointerface; solid–water interface

1. INTRODUCTION

Since its first development in 1981, neutron reflection has been extensively used for investigating surface and interfacial phenomena (Hayter *et al.* 1981). Its early use was mainly in surface chemistry, solid films and surface magnetism (Penfold & Thomas 1990). Over the past 15 years or so, the technique has been extensively used for the interfacial characterization of surfactants, lipids, polymers and their mixtures (Lu & Thomas 1998; Thomas 2004; Lu *et al.* 2007). Since the late 1990s, its use in the study of different biointerfacial phenomena has also been reported. The biomolecular systems that have so far been studied include proteins (Lu *et al.* 1998*c,d*, 1999*a,b,c*, 2003*b*, 2005; Su *et al.* 1998*a,b,c*, 1999, 2000; Fragneto *et al.* 2000*b*; Holt *et al.* 2000; Nylander *et al.* 2001; Armstrong *et al.*, 2004; Cooper *et al.* 2005; Xu *et al.* 2006*b*, 2007, 2008; Pan *et al.* 2008), peptides (Holt *et al.* 2000; Lu *et al.* 2003*a*, 2004; Lad *et al.* 2007; Middelberg *et al.* 2008; Zhao *et al.* 2009), biosurfactants (Follows *et al.* 2007) and lipid bilayers (Fragneto *et al.* 2000*a*, 2001). A number of neutron reflection studies have also focused on interfacial interactions from binary mixtures of protein/surfactant (Lu *et al.* 1998*a,b*; Green *et al.* 2000*a,b*, 2001), protein or peptide/lipid (Schmidt *et al.* 1992; Lu *et al.* 2001; Miano *et al.* 2007; Valincius *et al.* 2008), protein/polymer (Murphy *et al.* 2000; Hollmann *et al.* 2007, 2008;

Ganzevles *et al.* 2008), DNA/polymer (Zhao *et al.* 2008*a*), DNA/surfactant (Zhang *et al.* 2008) and DNA/lipid (Wu *et al.* 2006). Several recent reviews have been dedicated to covering most of these systems (Lu 1999, 2002; Fragneto-Cusani 2001; Krueger 2001; Thomas 2004; Lu *et al.* 2007).

Interfacial adsorption of proteins and peptides underlies many technological applications. For example, preadsorption of functional peptides or extracellular proteins is widely used to enhance selective interactions between biomaterial surfaces and mammalian cells. On the other hand, decoration of biomaterial surfaces with polymeric segments such as poly(ethylene oxide) and polymethacrylate-based copolymers bearing phosphorylcholine (PC) groups inhibits non-specific protein adsorption, again improving surface selectivity and biocompatibility. A key aspect towards understanding and controlling dynamic processes of protein interfacial adsorption is the determination of their *in situ* interfacial structures (Fragneto *et al.* 1995). In this regard, extensive studies have been made to exploit the potential of neutron reflection, starting from the early pioneering studies of β -casein (Dickinson *et al.* 1993; Fragneto *et al.* 1995), lysozyme (Lu *et al.* 1998*c*, 1999*a*; Su *et al.* 1998*a,c*), BSA (Su *et al.* 1998*b*, 1999; Lu *et al.* 1999*c*) and HSA (Lu *et al.* 1999*b*). While these early studies have aimed at exploring the technical development, they have also demonstrated how surface hydrophobicity and chemical nature affected the amount and the thickness of adsorbed proteins by using substrate surfaces modified with self-assembled monolayers bearing alkyl chains (e.g. C₈, C₁₈ hydrocarbons; Fragneto *et al.* 1995; Lu *et al.* 1998*d*), phospholipids (Lu *et al.* 2001), C₁₅OH

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(Su *et al.* 2000) and PC-incorporated polymers (Murphy *et al.* 2000). The amount of protein adsorption was found to be at the minimum over the range of intermediate surface hydrophobicity, consistent with the feature observed by other researchers (Chapman *et al.* 2000, 2001; Ostuni *et al.* 2001, 2003). On surfaces that were very hydrophilic (e.g. bare SiO₂) and very hydrophobic (e.g. C₁₈ hydrocarbon), the amount of proteins adsorbed was similarly high, but neutron reflection revealed that the volume fraction distribution along the surface normal on the C₁₈-modified surface became very non-uniform and was similar to the ones formed by synthetic polymers, indicating surface-induced structural unfolding. In contrast, lysozyme and HSA layers adsorbed on the hydrophilic SiO₂ surface could be modelled by single uniform layer and the thicknesses were close to their respective short axial lengths, indicating that the adsorbed molecules retained their globular framework and were adsorbed sideways-on (Lu 1999, 2002). Thus, these early neutron reflection studies, though very limited, have revealed the main interfacial structural features of protein molecules adsorbed onto model interfaces. These interfacial features are coarse, but they help reveal the conformational orientation, deformation and possible structural unfolding of protein molecules at different interfaces.

Recent studies of protein and peptide adsorption have focused on molecular implications underlying current biotechnologies, such as biofilms, immunoassays, biosensors, drawing direct correlation between interfacial structure and bioactivity. This paper reviews representative examples of peptide and protein adsorption as studied by neutron reflection over the past few years, focusing on examples studied at the solid–solution interface. The aim is to illustrate the key structural features and technical strengths that can be offered by this technique. Where relevant, its complementary role in other interfacial techniques will also be emphasized.

2. NEUTRON REFLECTION

The principle of specular neutron reflection has been described in a number of reviews (Penfold & Thomas 1990; Lu *et al.* 1996, 2000; Penfold *et al.* 1997; Lu & Thomas 1998). The reflection of neutrons at a flat surface is comparable to light reflection. However, the availability of significantly shorter neutron wavelengths enables smaller layer structures to be probed, with resolution down to the atomic and molecular level perpendicular to the interface. Neutrons' refractive indices are related to their scattering lengths, which are the physical constants that vary from one isotope to other (Penfold & Thomas 1990; Penfold *et al.* 1997). The strong scattering by light atoms such as H, C, O and N emphasizes the advantageous feature of studying soft matter materials using this technique (Fragneto-Cusani 2001). In a neutron reflection experiment, neutron reflectivity, R , is measured as a function of wavevector transfer, Q , perpendicular to the reflecting surface or interface, where

$$Q = \frac{4\pi \sin \theta}{\lambda}, \quad (2.1)$$

where λ is the neutron wavelength and θ the glancing angle of the incidence beam. R is equal to the ratio of

the intensities between reflected and incoming beams, and in kinematic approximation it is given as

$$R(Q) = I_{\text{ref}(Q)} / I_{\text{inc}(Q)} \approx \frac{16\pi^2}{Q^2} |\rho(Q)|^2, \quad (2.2)$$

where $\rho(Q)$ is the one-dimensional Fourier transform of the scattering length density (SLD) profile, $\rho(z)$, normal to the interface:

$$\rho(Q) = \int_{-\infty}^{\infty} \exp(-iQz) \rho(z) dz. \quad (2.3)$$

Thus, these equations show that through Fourier transform neutron reflectivity is related to the SLD across the interface which is linked to interfacial chemical composition through the following equation:

$$\rho(z) = \sum_{i=1}^m b_i n_i(z), \quad (2.4)$$

where b_i is the scattering length and n_i the number density of the i th element in a given volume containing m different types of elements. Equation (2.2) is only approximate because when Q becomes very low, it gives inadequate account of multiple scattering and the relationship tends to deviate or break down.

Table 1 shows that the elements that comprise proteins or peptides have very similar scattering lengths with the exception of the value for hydrogen. Because proton (¹H) and deuteron (²H) have opposite signs of scattering lengths, deuterium substitution can be used in neutron reflection experiments to highlight the adsorbed interfacial layers differently. Because D₂O has an SLD of $6.35 \times 10^{-6} \text{ \AA}^{-2}$ and H₂O has an SLD of $-0.56 \times 10^{-6} \text{ \AA}^{-2}$, adjustment of the ratio of D₂O to H₂O is often used to obtain different SLDs so that the interfacial protein layers are highlighted differently. For example, mixing of 8.1 per cent D₂O into H₂O will result in a null reflecting water (NRW) with an SLD of 0. When neutron reflection is performed at the air–NRW interface, the water has no contribution to the specular signal. Thus, the entire signal arises from the interfacial layer or film. Thus, measurements under NRW or other similar contrasts may lead to more accurate determination of layer thickness and composition, but other parallel measurements under D₂O or a mixture of H₂O and D₂O can lead to further information such as the extent of mixing of peptide or protein with water. Deuterium labelling to parts of lipids and short peptides has been successfully used to reveal detailed information about local fragment distribution and its relation to other parts of the interface. Therefore, use of deuterated protein or peptide improves interfacial structural determination. This is particularly the case when dealing with the interfacial composition of binary or multiple component systems. Fully or partially deuterated peptides with moderate lengths can now be synthesized regularly. However, production of partially or fully deuterated proteins and DNAs is non-trivial. With the benefits of deuteration becoming increasingly realizable, more research activities will be dedicated into this direction in the next few years

Table 1. A list of common elements and their coherent scattering lengths (Sears 1992).

element (<i>i</i>)	scattering length (<i>b</i>) (10^{-5} Å)
C	6.65
(¹ H) H	−3.74
(² H) D	6.68
O	5.81
N	9.36
S	2.85
P	5.13
Si	4.15

(Meilleur *et al.* 2005; Budayova-Spano *et al.* 2006; Di-Costanzo *et al.* 2007; Liu *et al.* 2007; Laux *et al.* 2008).

The kinematic approximation as outlined in equations (2.2)–(2.4) depicts a straightforward relation between neutron reflectivity in abstract space and interfacial structure in real space, and has been extensively used to analyse reflectivity profiles measured at the air–water interface by means of partial structure factors (Penfold & Thomas 1990; Lu *et al.* 1996, 2000; Penfold *et al.* 1997; Lu & Thomas 1998). However, the advantage of such an approach becomes less obvious when used for resolving the interfacial structure at the solid–water interface due to more complex structural correlations. In practice, neutron reflectivity profiles measured at the solid–water interface are mostly analysed using optical matrix formalism (Heavens 1965; Born & Wolf 1970). Data analysis based on optical matrix modelling usually starts with the calculation of a reflectivity profile from an assumed structural model, followed by comparison of the calculated reflectivity with the real data measured. The structural parameters are then varied in a least-squares iteration until the best fit is found. The parameters used include layer thickness (τ , often in Å) and its SLD (ρ , often in Å^{−2}). For any surface or interfacial layer that is non-uniform, a model consisting of two or more layers is usually tried to accommodate the inhomogeneous volume fraction distribution across the interface. For a peptide or protein layer that is fully immersed in water, its volume fraction in the layer can be obtained from the following equation:

$$\phi_p = \frac{(\rho - \rho_w)}{(\rho_p - \rho_w)}, \quad (2.5)$$

where ρ is the fitted SLD of the layer, ρ_w the SLD of water and ρ_p the SLD of the pure sample. The area per molecule (A , in Å²) can be calculated by

$$A = \frac{V_p}{\phi_p \tau}, \quad (2.6)$$

where V_p stands for the molecular volume (in Å³). The surface excess (Γ , in g m^{−2}) can subsequently be calculated by

$$\Gamma = \frac{10^{20} \text{MW}}{N_a A}, \quad (2.7)$$

where N_a is the Avogadro constant and MW the molar mass of the sample.

3. PROTEIN ADSORPTION

Extensive previous studies of protein adsorption have used model globular proteins such as albumins and lysozyme and this area of work has been well reviewed (Lu 1999). A number of recent experiments have focused on exploring the use of neutron reflection to study proteins bearing different structures and biological functions. For example, bilobal proteins such as glucose oxidase (GOx), lactoferrin (LF) and Y-shaped antibodies have been carefully investigated. Through work on adsorption of LF and antibodies, we aim to outline the main structural information about these large protein molecules as determined by neutron reflection.

3.1. Lactoferrin

Human lactoferrin (hLF) is a bilobal iron-binding protein with strong antimicrobial and antiviral activity. It is widely distributed in the tear, milk and blood fluids of vertebrates. The two globular lobes are linked by a single peptide chain. The dimensions of the protein in aqueous solution have been determined by small angle neutron scattering (SANS; Lu *et al.* 2005). The protein molecule was successfully modelled as a cylinder with a diameter of 47 Å. But the length of the protein was variable, depending on the salt concentration in the solution. In the presence of 0.3 M NaCl, the length was reduced to 105 Å, when compared with 190 Å in the absence of the salt (figure 1*b*(i)). However, the diameter of the protein did not change in the solutions. These structural features, when assessed against the crystalline structure of hLF, suggest that the molecule retains its globular framework in aqueous solution. The changes in the length of the cylinder as modelled by SANS reflected the distance variations in response to electrostatic interactions between the two lobes mediated by solution ionic strength.

Neutron reflection has been used for the *in situ* detection of interfacial protein folding and unfolding under different conditions (Lu *et al.* 2005). As already indicated, if the adsorbed protein retained its globular framework, it was likely to form a uniform layer with its thickness close to one of the globular dimensions. It was however found that at the air–NRW interface, the adsorbed hLF (at 10 mg l^{−1}) formed a top dense layer of some 14 Å containing 54 per cent of protein and an outer loose layer of 60 Å containing 20 per cent of protein, indicating the structural unfolding of its globular structure (figure 1*b*(ii)). With increasing hLF concentration, the thickness of both the layers increased, but the protein volume fraction of the top layer did not change although that in the outer loose layer showed some slight increase. When the concentration of hLF increased to 2 g l^{−1}, the top dense layer expanded to 26 Å and the bottom loose layer increased to 70 Å (figure 1*b*(iii)). While the volume fraction for the corresponding top outer layer did not increase significantly, the volume fraction in the bottom loose layer increased to 27 per cent. Parallel measurements, with the addition of 0.3 M NaCl to the solution, demonstrated that the addition of salt reduced both the thickness and the amount of the protein adsorbed (figure 1*b*(iv); Lu *et al.* 2005).

Figure 1. (a) Reflectivity measured from surface layer adsorbed from 100 mg l^{-1} lactoferrin with 0.3 M NaCl . The continuous line represents the fit taking $18 \text{ \AA}$ out of $22 \text{ \AA}$ of the top layer out of water with the rest of the layer immersed. In contrast, the dashed line assumes that the entire layer is fully immersed in water. Error bars were omitted for clarity (Lu *et al.* 2005). (b) (i) Schematic representations of hLF structural changes with and without 0.3 M NaCl in solution; (ii) adsorption of hLF at 10 mg l^{-1} and (iii) 2 g l^{-1} at the air–NRW interface; (iv) adsorption of hLF at 10 mg l^{-1} at the air–NRW interface with 0.3 M NaCl ; (v) adsorption of hLF at 100 mg l^{-1} at the air– D_2O interface; (vi) assuming the entire layer is fully immersed in water. (c) Schematic representations of hLF adsorption at the air–water interface with the presence of lipid monolayer at the surface pressures (i) $\pi \geq 35 \text{ mN m}^{-1}$, (ii) $\pi \approx 15 \text{ mN m}^{-1}$ and (iii) $\pi \leq 8 \text{ mN m}^{-1}$.

A further advantage of neutron reflection was to study the structure under different isotopic contrasts of the solvent. Parallel measurements of neutron reflectivity profiles measured in D_2O and the mixed solvents revealed that the top layer was predominantly afloat, while the loose layer was fully immersed. The continuous line shown in figure 1a represents the best fit to the reflectivity profile of hLF adsorption from 100 mg l^{-1} measured at the same condition as described in NRW but now in D_2O . The best fit to the corresponding D_2O profile assumed that some $18 \text{ \AA}$ of the adsorbed layer was projected in air and the rest of the polypeptide distribution was fully immersed in water

(figure 1b(v)). The dashed line in figure 1a was calculated assuming that the entire protein layer was fully immersed in water (figure 1b(vi)). The poor fit from the fully immersed model confirmed the extent of partial layer immersion. The analysis of the D_2O measurement in the absence of salt also confirmed the predominant exposure of the top dense layer to air.

As an important bactericidal protein in tears, it is of practical importance to investigate its interaction with lipid structure. For simplicity, this process was modelled by studying hLF behaviour in the presence of a lipid monolayer on the surface of water. Neutron reflection measurements were subsequently undertaken to study the co-adsorption of hLF into a spread monolayer of dipalmitoylglycerol phosphatidylcholine (DPPC), mimicking the precocular tear film outer interface (Miano *et al.* 2007). The measurements were carried out at three different surface pressures (8 , 15 and 35 mN m^{-1}) of the lipid monolayer (figure 1c). Both the layer thickness and the amount of hLF were found to decrease with increasing surface pressure in the lipid monolayer. It was found that the DPPC monolayer effectively prevented hLF insertion at the surface pressure above 35 mN m^{-1} (figure 1c(i)), while a thick layer ($70 \text{ \AA}$) of hLF was detected at 8 mN m^{-1} (figure 1c(iii)). The co-adsorption of hLF into DPPC monolayer at 8 mN m^{-1} was also found to increase with time over the first 5 h .

The adsorption of hLF onto substrates such as teeth can effectively reduce the adherence and growth of hazardous microbes and sustain a healthy environment. But changes in sugar side chains may affect the stability of LFs and their adsorption behaviour. As already indicated, LF has antimicrobial power and is also a popular iron additive to infant milk powders. The demand for LF has stimulated the development of various recombinant technologies for an efficient and economical production of LF. One successful product is the recombinant rice lactoferrin (rLF; Nandi *et al.* 2005; Rachmawati *et al.* 2005). The rLF has the same amino acid sequence and three-dimensional structure as hLF. The difference between the two LFs is the different types of sugar side chains. In addition, the sugar side chains of rLF are shorter. It was found that hLF exhibited better hydration and solubility in aqueous solution than rLF. The unfolding temperature of hLF was higher than that of rLF. Different glycan chain sizes and structures also affected the amount of LF adsorption at the SiO_2 –water interface. The different adsorption is well exemplified in figure 2, where the reflectivity profiles of hLF and rLF adsorbed at the SiO_2 –water interface are compared (Pan *et al.* 2008). At a low concentration of 10 mg l^{-1} , both hLF and rLF formed a monolayer with a layer thickness of $47 \text{ \AA}$ and volume fraction of 13 per cent , indicating that the molecules adopted a flat-on orientation at the interface (figure 2c). When the concentration increased to 100 mg l^{-1} , an interfacial model consisting of three layers was adequate to fit the interfacial structure of LF adsorption. The first layer close to the oxide surface was a very narrow region ($6 \text{ \AA}$), while the middle and outer layers were much thicker ($60 \text{ \AA}$ each; figure 2c). The narrow inner layer contained rather low protein

Figure 2. Neutron reflectivity profiles measured from adsorption of (a) hLF and (b) rLF at 10 mg l^{-1} (open circles, offset one magnitude along the vertical axis for clarity) and 100 mg l^{-1} (open squares, offset two magnitudes along the vertical axis for clarity) in pH 7 phosphate D₂O buffer with $I = 20$ mM at the silicon oxide–solution interface. The continuous lines represent the best fits. The reflectivity measured from the D₂O buffer is shown as the dashed lines for comparison. (c) Schematic representation of the structure of LF layers formed at 10 mg l^{-1} (left) and 100 mg l^{-1} (right) at the hydrophilic silicon oxide–water interface (Pan *et al.* 2008).

fragments (18% for hLF and 28% for rLF) due to the structural constraints of the bilobal molecules and possible electrostatic repulsion. The middle layer contained the main body of LF (42% for hLF and 52% for rLF). The layer at the outer water side contained loosely packed lobes and the protein volume fractions for the two LFs were the same (18%). These structural details indicated that the bilobal proteins retained their globular structure at the interface. The high protein fractions of rLF within the first two layers were consistent with the effect of shorter glycan side chains. It was also found that the amount of both LFs adsorbed at the interface reduced with increasing salt concentration. This observation was consistent with the trend obtained from other globular proteins such as lysozyme and HSA (Lu *et al.* 1998*d*; Su *et al.* 1998*b,c*, 1999).

3.2. Antibody

Interfacial immobilization of monoclonal antibodies is an important process in biotechnological applications such as fertility test pads, immunoassays and biosensors. Most of these techniques rely on the immobilized antibody to bind an antigen from the test sample. This step is usually followed by a second binding step from another antibody to offer the means of signal reading. Antibodies are Y-shaped molecules, each with two antigen-binding fragments (Fab) and one crystallizable fragment (Fc). Its dimensions are approximately $142 \times$

$85 \times 38 \text{ \AA}^3$ (Silverton *et al.* 1977). The antigen-binding sites are located at the N-terminal domains at the far ends of the Fab fragments. The sequence of this area varies in different antibodies and is called variable domains (Fv; figure 3*b*(i)). Immobilization of antibodies onto the solid substrate often results in different interfacial conformational orientations. Typical orientations are end-on (Fc attached to the support (figure 3*b*(iv))), head-on (Fabs attached to the support (figure 3*b*(iv))), sideways-on (one Fc and one Fab attached to the support (figure 3*b*(iii))) and flat-on (all three fragments attached to the support (figure 3*b*(ii))), or a combination of these (Lu *et al.* 2007). Different interfacial orientations can lead to different antigen-binding efficiencies because inappropriate orientations cause steric hindrance or unavailability of the active sites (Fv) for the antigen to access or bind from the solution side. The determination of *in situ* conformational orientations of the antibody adsorbed at the solid–solution interface represents a major challenge for many existing technologies. Neutron reflection is by far the most reliable technique and has been used for determining the interfacial orientations of monoclonal antibodies (Xu *et al.* 2006*a,b*, 2007; Lu *et al.* 2007).

The conformational orientations of a mouse monoclonal antibody and its binding capacity to the β unit of human chorionic gonadotrophin (anti- β -hCG) at the SiO₂–water interface have been investigated by neutron reflection and spectroscopic ellipsometry

Figure 3. (a) Uniform layer fits to the reflectivity profile in the presence of 2 mg l^{-1} of anti- β -hCG in D_2O with ionic strength at 20 mM and pH 7. The solid line was calculated with a layer thickness of $40 \pm 3 \text{ \AA}$ and a surface excess of 0.9 mg m^{-2} . The dashed and dotted lines were calculated with thicknesses of 85 and 142 $\text{\AA}$, respectively, mimicking side-on and end-on/head-on orientations, but with their respective SLDs varied to obtain the fits closest to the measured data (Xu *et al.* 2006b). (b) Schematic of the four representative conformational orientations of the antibody adsorbed on the silica–water interface.

(Xu *et al.* 2006b). Figure 3a shows the best fits to the measured reflectivity in the presence of 2 mg l^{-1} of anti- β -hCG in D_2O by assuming that the antibodies adopted ‘flat-on’ (solid line), ‘side-on’ (dashed line) and ‘end-on’ or ‘head on’ (dotted line) orientations (figure 3b). The best fit revealed a thickness of 40 $\text{\AA}$, in good agreement with the short axial dimension of the protein. This observation indicated that the molecules predominantly adsorbed flat-on with their Fc and Fab fragments lying flat on the surface. The layer was found to contain 15 per cent of the antibody with surface excess of 0.9 mg m^{-2} . Antibodies are robust and retain their three-dimensional structures upon adsorption onto the hydrophilic silica surface. Each antibody occupies about $12\,070 \text{ \AA}^2$. A mass of 0.9 mg m^{-2} will result in a surface coverage of 43 per

cent. Meanwhile, neither the best fits of ‘side-on’ nor ‘end-on’ orientations would fit the shape of the measured reflectivity profile.

The adsorption of anti- β -hCG at higher solution concentrations was also studied and the best fits to the measured reflectivity profiles are shown in figure 4a (Xu *et al.* 2006b). The D_2O profile and the data at 2 mg l^{-1} are plotted together for comparison. The monolayer model was found not to fit the data at higher concentrations. Instead, a model consisting of three layers was required to fit the data. At a concentration of 10 mg l^{-1} , the measured data could be well modelled using the thicknesses of 10 $\text{\AA}$ for the inner layer, 30 $\text{\AA}$ for the middle layer and 25 $\text{\AA}$ for the outer layer (figure 4b(i)), with their respective volume fractions of 0.22, 0.42 and 0.12. The total surface excess was found to be around 2.5 mg m^{-2} . When the solution concentration was increased to 50 mg l^{-1} , the inner layer thickness was increased to 14 $\text{\AA}$, and the outer layer thickness increased to 35 $\text{\AA}$, but the middle layer retained its thickness (figure 4b(ii)). The volume fraction of the inner layer increased to 0.3 and that for the outer layer dropped to 0.09. The volume fraction for the middle layer was found to increase slightly to 0.44. The total surface excess increased to 2.9 mg m^{-2} . The structural transition reflected by the three-layer structural changes indicated the twisting and tilting of segments within the adsorbed antibodies, driven by electrostatic repulsion between the molecules and resulted in a high surface excess.

The increased volume fraction and surface-packing density reduced the antigen accessibility due to the steric hindrance and the straining caused by electrostatic repulsion within the protein layer and between the protein and the substrate surface. This offered an explanation to the fact that the steady increase in surface excess was in sharp contrast to the fast decline in antigen-binding activity. The pH-dependent antibody adsorption at the hydrophilic SiO_2 –solution interface also confirmed that the steric hindrance was the main constraint on binding, restricting the access of the antigen to active sites within the antibody layer (Xu *et al.* 2007).

An elaborate study of the interfacial assembly of antibody-binding membrane protein arrays using polarized neutron reflection has been undertaken by Le Brun *et al.* (2008). In this work, the authors deposited onto a silicon substrate a layer of an alloy of iron and nickel that was magnetic and provided contrast between the different neutron spin states. Above the magnetic layer was a layer of gold to which the biological layer was fabricated. The protein OmpAZ is an outer membrane protein A and has a single cysteine residue in periplasmic turn to allow the protein to bind in a specific orientation to the gold surface. The space between protein molecules was filled with a layer of molecules of polyethylene glycol with a thiolalkane functional group. The OmpAZ protein had two SpA Z domains that could bind IgG antibodies in their constant regions. The application of a permanent external magnetic field induced a magnet moment in the magnetic layer along the same direction. The polarized neutrons provided a means of achieving an extra

Figure 4. (a) Plots of neutron reflectivity profiles at the SiO_2 – D_2O interface with anti- β -hCG concentration in the solution of 2 (plus symbols), 10 (crosses) and 50 mg l^{-1} (open circles). The solution pH and ionic strength were fixed at 7 and 20 mM, respectively. The reflectivity profile from the bare SiO_2 – D_2O interface is also shown for comparison (dashed line). The solid lines are the best fits. For clarity, error bars are shown for the 50 mg l^{-1} data only (Xu *et al.* 2006b). (b) Schematic of conformational orientations of the antibody on the silica–water interface at higher surface coverage matching to the concentration of the antibody at (i) 10 mg l^{-1} and (ii) 50 mg l^{-1} . The increased surface packing resulted in the fragment twisting and crowding, which reduced antigen accessibility from the solution side.

contrast, without hydrogen/deuterium labelling in the biological layer. The polarized neutron reflection studies revealed that the total thickness of the entire biological layer immobilized on the gold surface was close to 200 Å. The simultaneous fits with good fitting quality added confidence about the benefit of this approach in helping resolve multi-component interfacial structures, allowing for the modelling of rather complex layers containing component distributions of lipid, protein and solvent across the interface. Given that membrane protein studies are becoming increasingly popular and that membrane proteins require elaborate immobilization and stabilization, the gain of an additional

contrast through polarized neutron reflection will deserve further exploration for a wide range of interfacial membrane protein studies.

4. PEPTIDE ASSEMBLY

Neutron reflection is unique for revealing the structural features of peptide assembly at different interfaces and is highly complementary to other established techniques such as atomic force microscopy (AFM), circular dichroism and Fourier transform infrared (FTIR; Lu *et al.* 2003a, 2004; Zhao *et al.* 2008b, 2009). The technique has been used for characterizing interfacial adsorption from both biomimetic and designed peptides. The 15-mer peptide YYY15 (YVNAKQYYR-ILKRRY) was directly copied from the native sequence of a DNA-binding domain within a heteromeric transcriptional activator, HAP2, identified from yeast *Saccharomyces cerevisiae*, with tyrosine (Y) present at the 1st, 8th and 15th amino acid positions (Xing *et al.* 1994). Its point mutations at these three Y positions to tryptophans (W) (denoted as WWW15) led to the same length sequence except the replacement of the three Ys with the three Ws. These substitutions changed the delicate balance of helical stability of the peptides, resulting in different extent of peptide adsorption at the SiO_2 –water interface. Both peptides took α helical structures in phosphate buffer, with the five positively charged amino acids stacking on one side of the α helical structure and the hydrophobic amino acids projected on the other side. However, they behaved differently when undertaking interfacial assembly at the SiO_2 –water interface. Neutron reflection studies revealed that YYY15 formed a weakly adsorbed interfacial monolayer at pH 7 with a thickness of 26 Å, and a volume fraction of 19 per cent, indicating a loose interfacial packing. However, WWW15 adsorbed much more strongly at the interface. Data analysis revealed the formation of a well-structured peptide interface consisting of three layers, each having a thickness of 7–8 Å. The middle layer had a lower SLD ($2.5 \times 10^{-6} \text{ Å}^{-2}$) compared with the two outer layers (5.0 – $5.3 \times 10^{-6} \text{ Å}^{-2}$), suggesting the formation of a ‘sideways-on’ helical conformation (Lu *et al.* 2004). Hence, the substitution of the three Ys by the three Ws has promoted the stability of helical structure of the peptide at the interface. It was further demonstrated that WWW15 could well form dimers through interdigitation of the two WWW sides, with the charged groups facing the negatively charged substrate and the aqueous solution.

Neutron reflection has also been used to determine the adsorption of β hairpin peptides at the air–water interface using different ratios of H_2O and D_2O to highlight the layer differently (Lu *et al.* 2003a). The peptides were found to form uniform layers of 8–10 Å, consistent with the formation of the single layer β sheet as predicted from computer modelling. From the reflectivity profiles measured in D_2O , it became clear that the peptide monolayers stayed almost entirely afloat in the air, with the hydrophobic side chains completely dry and the hydrophilic side chains in water only at their tips.

A recent neutron reflection study has also been devoted to probing the interfacial assembly of designed surfactant-like peptides using V₆K₁ and V₆K₂ as examples (Zhao *et al.* 2009). In resemblance to conventional surfactants, peptide V₆K₂ has six valines (Val or V) as hydrophobic tail, and two lysines (Lys or K) as hydrophilic head. In addition to the synthesis of the fully hydrogenated sample hV₆hK₂, isotopically labelled dV₆hK₂ (chain-deuterated sample) was also prepared to enhance the contrast within the interfacial region. Figure 5 shows the best two-layer fittings for the peptide layers adsorbed at the water–SiO₂ interface under three different contrasts. It was found from data analysis that the interfacial region was comprised of a dense inner layer of some 40 Å containing about 50 per cent peptide close to the oxide surface and a loose 40 Å outer layer containing some 8 per cent peptide on the solution side. The parallel AFM imaging revealed the coexistence of different sizes and shapes of holes within the main peptide layer and the insertion of a small number of large vesicular objects. AFM depth profiling revealed a thickness of 3–4 nm for the main layer and a similar thickness for the outer region across the large vesicles, thus depicting broadly consistent interfacial morphological features to neutron reflection. Further data analysis with the help of partial deuteration to the peptide and the variation of solvent isotopic contrasts (D₂O, H₂O) revealed that the densely packed peptide layer was comprised of a sandwiched peptide bilayer with their hydrophobic tails (V₆) attracted to each other and the cationic head groups (K₂) projected towards the oxide surface and the bulk water (table 2). This peptide bilayer structure was similar to that formed by conventional cationic surfactants when adsorbed at the same anionic SiO₂–water interface, indicating the dominant effect of hydrophobic interaction.

Neutron reflection has also been used to investigate the lipid-binding behaviour of antimicrobial peptides (Lad *et al.* 2007) and amyloid- β peptides (Chi *et al.* 2008; Valincius *et al.* 2008). By combining the measurements of surface pressure, FTIR spectroscopy and neutron reflection, Lad *et al.* showed that their cationic peptides (melittin, magainin II and cecropin P1) interacted more strongly with the anionic lipid 1,2-dihexadecanoyl-sn-glycerol-3-(phosphor-rac-(1-glycerol)) (DPPG) compared with the zwitterionic lipid 1,2-dihexadecanoyl-sn-glycerol-3-phosphocholine (DPPC), demonstrating the different effects of electrostatic interaction coupled with other effects implicated from molecular structures. Neutron reflection measurements revealed that all three cationic peptides penetrated DPPC lipid monolayers spread on the surface of water and the extent of penetration was dependent on the peptide, its concentration and surface pressure. Their studies also revealed that the mode of peptide binding to DPPG was dependent on the distribution of basic residues within the peptide helical structure, although in all cases adsorption below the lipid layer was dominant when compared with the insertion within the layer. The surface infrared and neutron reflection together revealed structural disruption to the DPPG lipid layer upon melittin adsorption, but such lipid disruption was

Figure 5. (a) Two-layer model fits to the reflectivity profiles measured at the silica–water interface for hV₆hK₂ in D₂O (triangles, green), dV₆hK₂ in D₂O/H₂O mixture ($\rho = 2.07 \times 10^{-6}$ Å⁻²) (diamonds, blue), dV₆hK₂ in H₂O (squares, red) and dV₆hK₂ in D₂O (circles, black). All the measurements were made at 500 mg l⁻¹, pH 7 and at 22–23°C. Symbols represent the measured data; solid lines represent the best fits for the two-layer model. For clarity, error bars are shown for one set of data only. (b) Schematic cartoon models to indicate the interfacial structure adsorbed from the peptide at the silica–water interface: (i) overall in-plane morphology of the adsorbed peptide showing a predominant peptide bilayer incorporating a few large vesicles; (ii) typical vertical section of the peptide interface; (iii) the detailed molecular packing structure in the dense bilayer (Zhao *et al.* 2009).

not observed for magainin or cecropin. In addition, melittin binding to both lipids was shown to be 50 per cent greater than for either magainin or cecropin. The infrared amide spectra also revealed that melittin adopted a helical structure only in the presence of lipid, whereas magainin and cecropin adopted a helical structure also at an air–water interface without the lipid layer present. This behaviour has been related to the different charge distributions on the peptide amino acid sequences.

Similar to antimicrobial peptides, Chi *et al.* have demonstrated that amyloid peptides (implicating the fibrillogenesis and toxicity of Alzheimer's disease) are also capable of the association and insertion into lipid monolayers mediated by electrostatic interactions (Chi *et al.* 2008). Their work based on neutron reflection combined with X-ray reflection and grazing-incidence X-ray diffraction revealed that amyloid peptides exhibited enhanced interactions with charged lipids compared with zwitterionic lipids, broadly

Table 2. Structural parameters obtained from four-layer model fitting to neutron reflectivity profiles measured at the silica–water interface. The oxide layer was determined from pure D₂O with $\tau = 12 \text{ \AA}$ and $\rho = 3.41 \times 10^{-6} \text{ \AA}^{-2}$. All the measurements were made at 500 mg l⁻¹, pH 7 and 22–23°C (Zhao *et al.* 2009).

sample/contrast	layer	τ (Å)	ρ ($\times 10^{-6} \text{ \AA}^{-2}$)	ρ_p ($\times 10^{-6} \text{ \AA}^{-2}$)	ϕ_p	ϕ_p	Γ (mg m ⁻²)
hV ₆ hK ₂ /D ₂ O	hV ₂ hK ₂	11	4.5	2.13	0.44	0.57	2.46
	hV ₄	9	2	1.54	0.90		
	hV ₂ hK ₂	11	4.5	2.13	0.44		
	hV ₆ hK ₂	70	6.1	1.84	0.06		
dV ₆ hK ₂ /D ₂ O	dVhK ₂	12	5.5	3.94	0.35	0.49	2.57
	dV ₅	14	6.55	6.63	0.71		
	dVhK ₂	12	5.5	3.94	0.35		
	dV ₆ hK ₂	40	6.29	5.59	0.08		
dV ₆ hK ₂ / $\rho = 2.07 \times 10^{-6} \text{ \AA}^{-2}$	dVhK ₂	13	2.4	3.07	0.33	0.43	2.36
	dV ₅	16	4.2	6.24	0.51		
	dVhK ₂	13	2.4	3.07	0.33		
	dV ₆ hK ₂	40	2.3	5.02	0.08		
dV ₆ hK ₂ /H ₂ O	dVhK ₂	13	0.4	2.52	0.31	0.44	2.44
	dV ₅	16	2.8	6.00	0.51		
	dVhK ₂	13	0.4	2.52	0.31		
	dV ₆ hK ₂	40	0	4.66	0.11		

consistent with the observation by Lad *et al.* indicating the role of electrostatic interaction. Anionic lipid DPPG induced crystalline ordering of the peptide at the membrane surface, mimicking the β -sheet structure in fibrils templated by the ordering effect of DPPG. These studies exploited the unique technical strength of neutron reflection in the determination of thickness and volume fraction of each interfacial component with the help of deuterium labelling. When used together with other techniques, the combined results provide a detailed characterization of the molecular structure and dynamic assembly that may underlie different biomolecular interfacial processes.

5. CONCLUSIONS

Neutron reflection has been demonstrated as a unique method for determining the *in situ* interfacial structural features of proteins and peptides at different interfaces from which protein orientation, deformation and unfolding can be inferred. Its current technical strength has mainly relied on its high depth resolution, highly complementary to other methods such as AFM imaging and profiling. In comparison with techniques such as ellipsometry, surface plasmon resonance, interferometry and light and X-ray reflection, neutron reflection benefits from the selective deuteration to solvent and biomolecules. This technical capability is particularly advantageous in studying biomolecular interactions, e.g. between protein and peptide and between protein and other molecules such as lipids or drug molecules. These combinations of interaction underpin many model systems for fundamental research and technological applications. Current biotechnologies such as biomedical engineering, healthcare, biosensors and immunoassays have already benefited from useful information unravelled by neutron reflection. Future work in this direction will help strengthen the development of the relationship between interfacial structure and the biological function of proteins, peptides and many

other biomolecular systems. Interfacial structure measurements will support the endeavour in understanding complex biomolecular interactions. Further development of polarized neutron reflection will aid the resolution of the complex structural information concerning the distribution of individual components and their relative locations across interfaces. Off-specular neutron reflection will enable in-plane morphological structures to be unravelled and will thereby open up new technical strengths in studying surface and interfacial assembly of proteins and peptides and their interactions.

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