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. 1998a,d, 1999a,b,c, 2003b, 2005; Su et al. 1998a,b,c, 1999, 2000; Fragneto et al. 2000b; Holt et al. 2000; Nylander et al. 2001; Armstrong et al., 2004; Cooper et al. 2005; Xu et al. 2006a, 2007, 2008; Pan et al. 2008), peptides (Holt et al. 2000; Lu et al. 2003a, 2004; Lad et al. 2007; Middelberg et al. 2008; Zhao et al. 2009), biosurfactants (Follows et al. 2007) and lipid bilayers (Fragneto et al. 2000a, 2001). A number of neutron reflection studies have also focused on interfacial interactions from binary mixtures of protein/surfactant (Lu et al. 1998a,b; Green et al. 2000a,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. 2008a), 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 B-casein (Dickinson et al. 1993; Fragneto et al. 1995), lysozyme (Lu et al. 1998c, 1999a; Su et al. 1998a,c), BSA (Su et al. 1998b, 1999; Lu et al. 1999c) and HSA (Lu et al. 1999b). 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. 1998d), phospholipids (Lu et al. 2001), C15 OH

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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 SiO$_2$) and very hydrophobic (e.g. C$_{18}$ hydrocarbon), the amount of proteins adsorbed was similarly high, but neutron reflection revealed that the volume fraction distribution along the surface normal on the C$_{18}$-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 SiO$_2$ 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 surface. Neutrons’ refractive indices are related to their scattering lengths, deuterium substitution can be used in neutron reflection experiments to highlight the adsorbed interfacial layers differently. Because D$_2$O has an SLD of $6.35 \times 10^{-6}$ $\text{A}^{-2}$ and H$_2$O has an SLD of $-0.56 \times 10^{-6}$ $\text{A}^{-2}$, adjustment of the ratio of D$_2$O to H$_2$O is often used to obtain different SLDs so that the interfacial protein layers are highlighted differently. For example, mixing of 8.1 per cent D$_2$O into H$_2$O 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 D$_2$O or a mixture of H$_2$O and D$_2$O 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.

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

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

\[ \rho(x) = \sum_{i=1}^{m} b_i n_i(x), \quad (2.4) \]

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 ($^1\text{H}$) and deuteron ($^2\text{H}$) have opposite signs of scattering lengths, deuterium substitution can be used in neutron reflection experiments to highlight the adsorbed interfacial layers differently. Because D$_2$O has an SLD of $6.35 \times 10^{-6}$ $\text{A}^{-2}$ and H$_2$O has an SLD of $-0.56 \times 10^{-6}$ $\text{A}^{-2}$, adjustment of the ratio of D$_2$O to H$_2$O is often used to obtain different SLDs so that the interfacial protein layers are highlighted differently. For example, mixing of 8.1 per cent D$_2$O into H$_2$O 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 D$_2$O or a mixture of H$_2$O and D$_2$O 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.

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

where $\lambda$ is the neutron wavelength and $\theta$ 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
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 1b(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 1b(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 1b(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 1b(iv); Lu et al. 2005).

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

<table>
<thead>
<tr>
<th>Element (i)</th>
<th>Scattering length (b) (10$^{-5}$ Å)</th>
</tr>
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<tbody>
<tr>
<td>C</td>
<td>6.65</td>
</tr>
<tr>
<td>$^1$H H</td>
<td>$-3.74$</td>
</tr>
<tr>
<td>$^2$H D</td>
<td>6.68</td>
</tr>
<tr>
<td>O</td>
<td>5.81</td>
</tr>
<tr>
<td>N</td>
<td>9.36</td>
</tr>
<tr>
<td>S</td>
<td>2.85</td>
</tr>
<tr>
<td>P</td>
<td>5.13</td>
</tr>
<tr>
<td>Si</td>
<td>4.15</td>
</tr>
</tbody>
</table>
The presence of lipid monolayer at the surface pressures (i) represents hLF adsorption at the air–water interface with the entire layer is fully immersed in water. (ii) Schematic representations of hLF adsorption at the air–NRW interface with 0.3 M NaCl; (iii) adsorption of hLF at 10 mg l$^{-1}$ at the air–NRW interface with 0.3 M NaCl; (iv) adsorption of hLF at 10 mg l$^{-1}$ at the air–D$_2$O interface; (v) 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) $\pi \geq 35$ mN m$^{-1}$, (ii) $\pi \approx 15$ mN m$^{-1}$ and (iii) $\pi \leq 8$ mN m$^{-1}$. (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$_2$O 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 Å) 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 glycans 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).

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$_2$O 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$_2$O. The best fit to the corresponding D$_2$O profile assumed that some 18 Å of the adsorbed layer was projected in air and the rest of the polypeptide distribution was fully immersed in water.
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; Su et al. 1998, 2007).

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 $142 \times 85 \times 38 \, \text{Å}^3$ (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 3b(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 3b(iv))), head-on (Fabs attached to the support (figure 3b(iv))), sideways-on (one Fc and one Fab attached to the support (figure 3b(iii))) and flat-on (all three fragments attached to the support (figure 3b(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. 2006a,b, 2007; Lu et al. 2007).

The conformational orientations of a mouse monoclonal antibody and its binding capacity to the $\beta$ unit of human chorionic gonadotrophin (anti-$\beta$-hCG) at the SiO$_2$–water interface have been investigated by neutron reflection and spectroscopic ellipsometry.
measured reflectivity in the presence of 2 mg l\(^{-1}\) anti-\(\beta\)-hCG. Antibodies are robust and retain their three-dimensional structures upon adsorption onto the hydrophilic silica surface. Each antibody occupies about 12 070 Å\(^2\). A mass of 0.9 mg m\(^{-2}\) will result in a surface coverage of 43 percent. 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-\(\beta\)-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\(_2\)O 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 Å for the inner layer, 30 Å for the middle layer and 25 Å 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 Å, and the outer layer thickness increased to 35 Å, 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

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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 (YVNAKQYYRLKRKY) 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 $\alpha$ helical structures in phosphate buffer, with the five positively charged amino acids stacking on one side of the $\alpha$ 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}$ Å$^{-2}$) compared with the two outer layers (5.0–5.3$\times$10$^{-6}$ 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 $\beta$ hairpin peptides at the air–water interface using different ratios of H$_2$O and D$_2$O 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 $\beta$ sheet as predicted from computer modelling. From the reflectivity profiles measured in D$_2$O, 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.

Figure 4. (a) Plots of neutron reflectivity profiles at the SiO$_2$–D$_2$O interface with anti-$\beta$-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$_2$O 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.
A recent neutron reflection study has also been devoted to probing the interfacial assembly of designed surfactant-like peptides using V$_6$K$_1$ and V$_6$K$_2$ as examples (Zhao et al. 2009). In resemblance to conventional surfactants, peptide V$_6$K$_2$ 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 hV$_6$hK$_2$, isotopically labelled dV$_6$hK$_2$ (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–SiO$_2$ 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 (D$_2$O, H$_2$O) revealed that the densely packed peptide layer was comprised of a sandwiched peptide bilayer with their hydrophobic tails (V$_6$) attracted to each other and the cationic head groups (K$_2$) 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 SiO$_2$–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-(phospho-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 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 fibrilligenesis 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

![Figure 5](http://rsif.royalsocietypublishing.org/doi/abs/10.1098/rsif.2009.0322)

Figure 5. (a) Two-layer model fits to the reflectivity profiles measured at the silica–water interface for hV$_6$hK$_2$ in D$_2$O (triangles, green), dV$_6$hK$_2$ in D$_2$O/H$_2$O mixture (ρ = 2.07 × 10$^{-6}$ Å$^{-2}$) (diamonds, blue), dV$_6$hK$_2$ in H$_2$O (squares, red) and dV$_6$hK$_2$ in D$_2$O (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).
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 $\beta$-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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