A Langmuir approach on monolayer interactions to investigate surface active peptides

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Abstract:–

The Langmuir Blodgett apparatus provides a versatile system for studying the interfacial properties of peptides and peptide-membrane interactions under controlled conditions. Using amphiphilic α-helical peptides to highlight studies undertaken, here we discuss the use of this system to provide information on the surface activity of peptides and describe the insights these studies give into biological function

Key words:

Amphiphilic peptide, Langmuir Blodgett, Maximum surface pressure, Monolayer, phospholipid, thermodynamic analysis.

Introduction

Amphiphilic α-helices play a fundamental role in modulating the interaction of proteins and peptides with asymmetric interfaces such as those found at membrane boundaries [1-2]. These structures are characterised by the spatial segregation of hydrophobic and hydrophilic amino acid residues about the α-helical long axis. This residue arrangement gives these α-helices an apolar face along one side of the helix, which is predominantly formed by residues such as phenylalanine, leucine and isoleucine, and a polar face, primarily formed by residues such as lysine, aspartic acid and glutamic acid, as is shown for the amphibian antimicrobial peptide, aurein 1.2, in Fig.(**1)** [3]. Possession of this amphiphilic architecture allows the apolar face of the α-helix to interact with the membrane hydrophobic core whilst concomitantly permitting its polar face to engage in electrostatic interactions with the membrane lipid head group region [4-6].

The spatial regularity of the residues within these structures has allowed a number of techniques to be developed for the prediction of structural and physiochemical parameters possessed by these amphiphilic α-helices from sequence data alone [2, 4-5, 7-11]. In particular, Eisenberg *et al.,* [12-13] quantified amphiphilicity by treating the hydrophobicity of successive amino acids in α-helical sequences as vectors. These vectors are summed in two dimensions, assuming an amino acid side chain periodicity of 100°, and the resultant provides the mean hydrophobic moment, $<\mu_H>$, which essentially provides a measure of α-helix amphiphilicity. In later work, Eisenberg *et al.,* [14] developed hydrophobic moment plot methodology, which plots $< \mu_H >$ as a measure of amphiphilicity against the mean hydrophobicity ($< H_0 >$) as a measure of the peptides affinity for the membrane interior. The location of these co-ordinate pairs is then used to predict whether an α-helical segment is

likely to be transmembrane, globular, obliquely orientated or surface active [15-16]. Other quantitative studies based on sequence analysis have shown that residue composition, sequence length, isoelectric point (IEP), net charge and hydrophobicity are all able to impact on the ability of α-helical segments to interact with membranes [2, 5, 7, 9-11]. With respect to peptide hydrophobicity, it has been noted that it is not only the magnitude that is important for function but also the arrangement of the peptide's residues in terms of arc size and the hydrophobicity gradient along the helical long axis.

Whilst a range of parameters, therefore, have been seen to influence membrane interactions, the mechanisms under-pinning peptide-membrane interactions remains poorly understood although it is well established that functionality depends upon the characteristics of both the participating amino acid sequence, peptide structure and the membrane composition. The initial step in membrane association is the binding of the peptide to the target cell membrane, which can involve protein receptors [17], lipid receptors [18], or relatively non-specific interactions with the head groups of membrane phospholipids as in the case of many amphiphilic α-helical peptides [3, 7, 19-23]. After binding, the ability of these amphiphilic peptides to partition into the membrane is strongly dependent on the lipids present in the target membrane. For example, Epand [24] observed that peptide partitioning into the membrane was affected by the ability of such surface-active peptides to alter the lipid polymorphism of a membrane, which in turn was dependent on the factors described above such as charge and amphiphilicity. Consideration of the peptide's molecular architecture and physiochemical characteristics are therefore particularly important in the design of therapeutic peptides since such parameters not only influence toxicity but also selectivity for

given membrane compositions. For example antimicrobial peptides must be able to differentiate between mammalian and microbial membrane compositions to ensure efficacy.

The lipid composition of membranes can be readily determined and it has been found that relatively simple lipid mixtures based on these compositions serve as good mimics of naturally occurring membranes as shown in recent studies on antimicrobial and anticancer peptides [3, 20, 25]. Lipid molecules are insoluble because of their amphiphilic nature and so form a monomolecular film or Langmuir monolayer at an air / water interface [26-28]. It has been found that the use of these lipid monolayers in Langmuir Blodgett troughs provides a system where membrane characteristics and experimental conditions can be varied in a controlled manner and used to investigate the interaction of proteins and peptides at a membrane interface.

Here, we briefly review the use of methodologies based on Langmuir Blodgett monolayers to characterise the membrane interactions of α-helical peptides at an amphiphilic phase boundary, including: membranes and the air / water interface.

Monolayers to study the surface activity of amphiphilic peptides

The ability of α-helical peptides to interact with membranes is usually associated with high surface activity (Table **1**) and is reflective of a general ability to respond to the environment at a phase boundary. Such surface activity can be investigated by observing the adsorption of peptides at an air / water interface. This ability has been extensively researched [29] and can be assessed using surface pressure measurements (π) as a function of time [30]. A major

example of a peptide able to form secondary structure at an air / water interface is melittin, which is considered to be one of the most potent, naturally occurring amphiphilic α-helical antimicrobial peptides [31-33]. The surface activity of melittin was first investigated at the air / buffer interface by Sessa *et al.,* [33] who reported a saturation pressure of 24.5 mN m^{-1} at concentrations as low as 0.3 μM, indicating that the peptide is highly surface active.

Based on its amphiphilicity, aurein 2.2, another antimicrobial peptide, would be predicted to be surface active and in Fig. (**2**), it is shown that at 4 μM, aurein 2.2 gives rise to surface pressure changes of 27.7 mN m^{-1}. These levels of surface pressure increase are comparable to other well characterised α-helical antimicrobial peptides that are active at the interface [3]. For example, a number of carboxypeptidase C-terminal protein membrane anchors were identified in the 1990s by Harris *et al.,* [34]. Using hydrophobic moment plot methodology, these latter authors predicted that peptides, P5 and P6, corresponding to the C-terminal membrane α-helical anchors of *Escherichia coli* penicillin binding protein 5 and 6 were strongly amphiphilic ($< \mu_H > \geq 0.5$, [35]) and candidates to form oblique orientated α-helices (Table **1**). Experimental determination showed that at concentrations of the order 5 μM, both P5 and P6 were highly surface active, inducing surface pressure changes of 33.7 and 22.3 mN m^{-1} respectively [35].

The surface activity of peptides is influenced by a number of intrinsic factors such as its sequence, structure and net charge [36] but also the external environment. For example, it is well established that pH is able to impact on secondary structure formation by peptides [37] and it is therefore important to take pH into account when investigating the surface activity of peptides. As an example, the effect of varying the pH of buffer on the surface activity of P5 and P6 was investigated by Harris *et al.,* [35] who showed that lowering the pH from pH 7 to

pH5 strongly increased the surface activity of the peptides, which correlated with a pH effect on their membrane binding activity. As another example, Pal *et al.,* [38] studied the adsorption behaviour of pepsin, a proteolytic enzyme, and showed that at concentrations of *circa* 1 μM, the peptide induced maximum surface pressure increase of 13 mN m^{-1}. However, these latter studies found that decreasing pH increased the rate of adsorption of the peptide at an air / water interface, resulting in elevated interfacial saturation rates. Pal *et al.,* [38] further showed that at its IEP, pepsin was insoluble but when the pH was shifted away from the IEP, the solubility of the peptide increased. The maximum surface activity of pepsin was observed at pH 2 and the minimum activity at pH 8. In response to such studies, it is common practice to use buffered subphases in experiments to measure surface activity. Maget-Dana [36] showed that the adsorption of proteins or peptides at an air / water interface is also influenced by other variables such as temperature, the subphase bulk composition and ionic strength and these factors must also be taken into consideration when determining surface activity.

It is also worth noting that conformation can itself affect adsorption. For example, Maget-Dana *et al.,* [39] showed that changing the conformation of a peptide from β-sheet to α-helix, increased both the rate of diffusion and adsorption of the peptide at an air / water interface, thereby enhancing its surface activity. In addition it has been recognised that peptides will tend towards their most amphiphilic structure at the interface [40] hence stabilization of such structures at a phase boundary can link to conformational change with many AMPs such as melittin forming random structures in solution but amphiphilic helices at the interface.

If peptide absorption occurs then it becomes possible to not only measure the pressure change noted above but to calculate the surface excess concentration of the interfacial peptide, Γ, which is calculated by applying the Gibbs' adsorption equation shown below [41]:

$$\Gamma = -\frac{1}{RT}\frac{\Delta\pi}{\Delta \ln c} \quad \text{.....Equation 1}$$

where R is 8.314 J mol^{-1} K^{-1}, T = 294 ºK, π is the interfacial pressure increase (mN m^{-1}) and c is the molar concentration of peptide in the sub-phase.

The value obtained from the surface excess concentration then enables the interfacial surface area per molecule, A, to be determined according to equation 2 where N is Avogadro's constant.

$$A = \frac{1}{N\Gamma} \quad \text{.....Equation 2}$$

The surface area per molecule can then be used provide data on the possible orientation of the peptide at the interface as demonstrated in a range of recent studies. Dennison *et al.*, [42], for example, investigated the surface activity of an amphiphilic α-helical peptide, VP1, which is homologous to a segment involved in the activation of the protease, m-calpain [43]. These studies found that the interfacial surface area per VP1 molecule was 0.453 nm^2, which supported further analysis showing that the peptide adopted a perpendicular orientation at the air / water interface. In another study, a β-sheet peptide, SIKVAV, was derived from the active region of PA22-2, a synthetic peptide from the laminin A chain sequence. Alminana *et al.*, [44] used Γ to show that the area per molecule of the peptide was 0.98 nm^2, which is consistent with other β-sheet peptides, which adopt a perpendicular orientation at the air / water interface [45].

Monolayers studies on the interaction of amphiphilic peptides with membranes

Since natural membranes are highly complex and contain a variety of lipids and proteins, a number of model systems have been developed to mimic the membrane environment and investigate how proteins and peptides interact with a lipid interface [46-47]. These studies have made it clear that subtle differences in the lipid composition of the target cell membrane can result in differences in the susceptibility of cells to the peptides activity and may influence the mode of membrane interaction [48]. With a strong capacity to model these subtle differences in membrane lipid composition, the monolayer technique has proved to be an invaluable approach to investigate the interactions of proteins and peptides with a lipid interface. As an example, a major focus of therapeutic research has been into the development of α-helical peptides as antimicrobial agents and monolayer studies have featured prominently in these investigations. In general, three main protocols have been used in characterising these peptide - lipid interactions and are known as: constant area assay, constant pressure assay and compression isotherm analysis.

The constant area assay

This protocol involves creating a lipid monolayer at the air / buffer interface. The lipid film area is then left constant and changes in surface pressure are monitored over time as peptides are added to the buffer subphase. An increase in surface pressure resulting from injection of a peptide into the subphase indicates an interaction between the peptide and the lipid film. Peptide molecules, which only interact with lipid head groups induce minimal surface pressure changes that are generally less than 3 mN m^{-1}. In contrast, surface pressure changes in the region of up to 10 mN m^{-1} are generally indicative of peptide insertion into the hydrophobic region of the lipid film [49].

Monolayers at constant area can be used to investigate specificity of a peptide for a particular lipid. The head group of a phospholipid is the first portion of the membrane encountered by the peptide and hence the size, charge and hydrophobicity of lipid head groups play an important role in the membrane selectivity exhibited by peptides. As an example, Lad *et al.,* [50] investigated the lipid interactions and selectivity of the α-helical defence peptides: melittin, magainin II and cecropin P1 for microbes (Table **2**). These cationic peptides were observed to interact much more strongly with monolayers formed from the anionic lipid 1,2 dihexadecanoyl-sn-glycerol-3-(phosphor-rac-(1-glycerol)) (DPPG) as compared to monolayers of the zwitterionic lipid, 1,2 dihexadecanoyl-sn-glycerol-3-phosphocholine (DPPC). These results suggested that in these cases the ability of the defence peptides to target microbes is electrostatically driven by columbic interactions between the net positive charge on the peptides and negatively charged lipid head groups on the surface of the microbial membrane [51]. It is well established that prokaryotic membranes carry a net negative charge due to the presence of anionic lipids such as phosphatidylglycerol (PG) and cardiolipin (CL) [52]. Furthermore, in Gram-negative bacteria, the outer leaflet of the outer membrane is composed of anionic lipopolysaccharide (LPS) whilst Gram-positive bacteria have an outer membrane consisting of peptidoglycan together with anionic teichoic and lipoteichoic acid. Such charge-charge interactions are therefore thought to be important in the initial targeting and binding of such peptides at the membrane surface.

The studies of Lad *et al.,* [50] also showed that in contrast to magainin II and cecropins, melittin induced much greater surface pressure increases in the presence of zwitterionic lipid and hence was less reliant on anionic head groups for binding (Table **2**). These results suggested that the ability of melittin to penetrate membranes was primarily driven by amphiphilicity with hydrophobic interactions playing a major role. This result helps explain

the observation that highly amphiphilic toxins such as melittin are active against both microbial cells and eukaryotic cells as it is the amphiphilic structure of the bilayer that drives peptide insertion rather than stabilisation of peptide structure at the interface by specific head group interactions. It was suggested by Lad *et al.,* [50] that the charge distribution along the α-helical structure of melittin was key to the interfacial behaviour of the peptide and it's mode of interaction.

Strongly supporting the findings of Lad *et al.,* [50], we present our own monolayer studies on aurein 2.2 in Fig. (**3**). It can be seen from Fig. (**3**) that the cationic peptide [25] has a high affinity for anionic 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine (DMPS) monolayers, inducing maximal surface pressure changes of 12 mN m^{-1}. These levels of interaction are consistent with disruption of the monolayer acyl chain region by the peptide and are comparable to those reported for other defence peptides (Table **2** and [3]). However, aurein 2.2 inserted into zwitterionic 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) at much lower levels, inducing pressure increases of *circa* 5.5 mN m^{-1}, suggesting that hydrophobic forces may play only a minor role in the membrane interactions of the peptide as has been suggested by monolayer studies on other defence peptides [53].

To further study the ability of aurein 2.2 with eukaryotic membranes, we present data in Fig. (**4**) obtained by the use of mixed monolayers, which involve the spreading of a multiple component lipid solution onto a subphase such experiments are frequently used to mimic the surfaces of cell membranes [54-55]. In Fig. (**4**), a DMPS / DMPC mix (1:3 v/v) in chloroform was spread onto a buffered subphase to mimic mammalian erythrocytes [56-57]. At an initial surface pressure of 30 mN m^{-1}, aurein 2.2 was found to rapidly interact with the

DMPS / DMPC monolayer, inducing maximal surface pressure changes of 4.5 mN m^{-1} (Fig. **4**). These levels of surface pressure change are comparable to those expected for lipid head group interactions rather than deep membrane penetration and are consistent with the observation that aurein 2.2 is non-haemolytic and inactive against eukaryotic cells [58]. It is well established that eukaryotic membranes are enriched in zwitterionic phospholipids such as phosphatidylcholine (PC), sphingomyelin (SM) and phosphatidylethanolamine (PE) [59] along with sterols, which can also attenuate peptide binding and insertion [60]. As can be seen from Fig (**4**), the addition of anionic component within a zwitterionic system rapidly increases binding by the antimicrobial peptide implying that in this case anionic lipid is likely to be a key factor in supporting aurein selectivity for microbial cells.

Mixed monolayers have also been used to study the membrane interactions of peptides in other therapeutic capacities. Stefin B is used as a model amyloidogenic protein in studies on the mechanism of amyloid fibril formation and its related cytotoxicity [61]. Studies on the interaction of the peptide with mixed lipid monolayers comprised of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-glycerol (DOPG) were undertaken and it was found that stefin B interacted strongly with these monolayers [62]. These results led to the suggestion that the interaction of prefibrillar oligomers / aggregates formed by stefin B with acidic lipid membranes resulted in pores in these membranes, a process, which contributed to cellular toxicity [63-64].

The constant pressure assay

In the case of the constant area assay, the increased pressure induced by peptide insertion can in itself become limiting, preventing the insertion of additional molecules**.** The second protocol to investigate peptide interactions with monolayers involves keeping the lipid film surface pressure constant and measuring the increase in the area of the film as the peptide is injected into the subphase. This experimental setup allows the change in area per molecule of lipid to be calculated upon interaction of the peptide with the lipid monolayer.

The use of constant pressure assays in conjunction with radiolabeled lipid or other tags on proteins/peptides have been used by a number of researchers to characterise protein adsorption and lipid interaction [65-67]. Peptides are rendered radioactive either by radioactively labelling an amino acid side chain or residue and if the specific activity is known the number of molecules bound can be ascertained. Harris *et al.*, [35] used a radiolabeled peptide, P4, to gain insight into its ability to form α-helical structure in the presence of a DOPG monolayer. At surface pressures of 20 mN m^{-1}, a molecular area of 330 $Å^2$ was calculated, indicating that P4 aligned parallel to the plane of the monolayer head group region at higher surface pressures. However, at surface pressures of 30 mN m^{-1}, a molecular area of 180 $Å^2$ was determined implying that P4 inserted perpendicular to this plane and penetrated the acyl chain region. The use of constant pressure assays therefore enables studies of binding and insertion to be undertaken while the pressure variable is controlled so giving insight to its effect on lipid association.

Using a variation on this technique, Ishitsuka *et al.,* [68] used constant pressure assay to investigate the effects of protegrin-1, a porcine β-sheet defence peptide, on different head group and tail group lipids by quantifying the relative area change once the peptide had been added to the system. Based on increased areas per lipid molecule, protegrin-1 was found to possess a high affinity for lipid with anionic head groups such as PG, implying that this affinity results from electrostatic interactions between the lipid and cationic properties of the peptide. The insertion results obtained from monolayers composed of lipids with different tail groups showed that an increase in tail group packing led to decreased protegrin insertion [68]. As another example, Dennison *et al.,* [3] investigated the role of anionic lipid in the interactions of aurein 1.2 with T98G glioma cell membranes. At constant pressure, in the presence of anionic lipid, there was an increase in area per lipid molecule when aurein 1.2 was introduced to the system. These results indicated that the peptide had a strong affinity for anionic lipid, which supported constant area experiments using the same lipid / peptide systems and suggested that this affinity may feature in the peptide's anticancer activity. Dennison *et al.,* [42] also used constant pressure assays to investigate the ability of VP1 to penetrate DMPC / DMPS monolayers, mimetic of eukaryotic membranes, at different molar ratios. These studies showed that as the levels of anionic lipid in the monolayer increased, the area per molecule increased. Regression analysis showed a strong correlation ($R^2 = 0.96$) between anionic lipid content and increased area per molecule. A comparison was made between these latter studies and constant area experiments using the same peptide / lipid monolayer system and a similar pattern was observed. At constant area, as the level of anionic lipid in the DMPC / DMPS monolayer was increased, the maximal level of VP1 surface pressure increased. Again, regression analysis showed a strong correlation ($R^2 = 0.97$) between these parameters.

Compression isotherm analysis

The most basic and widely used technique to characterise a monolayer is the surface pressure - area isotherm [36]. As a monolayer is compressed, there is a change in the molecular packing and the film undergoes several phase transitions [69]. These phase changes can be identified by monitoring surface pressure as a function of the area occupied by the film. These phase transitions are classified as: I. gaseous (G); II. liquid expanded (LE); III. intermediate or transition; IV. liquid condensed (LC) ; and V. solid (S). According to Harkins *et al.,* [70] and Dervichian [71] lipid compressibilities in each state are very different. Compressibility of a monolayer can therefore be assessed and lipid phase transitions observed by using moveable barriers to gradually decrease the surface area although the compression rate must be slow enough to ensure that changes occur under thermodynamic equilibrium conditions [36]. As the surface pressure increases, the surface area decreases until a point is reached where it is not possible to increase the pressure any further before the collapse point of the monolayer is reached [41]. By the use of microscopy techniques such as Brewster angle microscopy (BAM), atomic force microscopy (AFM) and fluorescence microscopy, the different phases of the lipid film under compression can be visualised [72-73]. Fluorescence microscopy for example has been applied to membrane studies by doping the monolayer with low concentrations of fluorescent lipid thereby providing a lipid probe [67] that can be used to image structural changes induced in the film by peptide interaction. For example, Gidalevitz *et al.,* [74] used fluorescence microscopy to show that protegrin 1 inserted into lipid monolayers resulting in the disordering of the lipid packing.

In BAM, the microscope is mounted onto a Langmuir trough allowing visualisation of the film organisation. Using this technique, Gehlert *et al.,* [72] investigated the morphology of

the collapse states of different glycerol amide lipids. The results indicated that small changes in the molecular chain and head group had a significant effect on the morphology and the packing characteristics of the molecule. In another example of BAM use, Volinsky *et al.*, [75] studied alamethicin, a fungal defence peptide that is believed to form pores in target membranes, and it was found that the peptide aggregated at different points along the compression isotherm [75]. The BAM images from these studies also confirmed the immiscibility of alamethicin with a DMPC monolayer and the authors were able to propose that toxicity may require phase segregation of alamethcin and the membrane lipid following peptide adsorption. Similar results have been seen using AFM to study polymyxin B, which is a bacterial defence peptide used to treat various Gram-negative bacterial infections [76]. AFM is widely used in conjunction with monolayer studies and involves transferring the film onto a mica substrate [76]. This microscopy technique enables high resolution topographical analysis of the lipid film and enabled Clausell *et al.*, [76] to investigate the interactions of *E. coli* lipid membranes with polymyxin B. The *E. coli* monolayers showed characteristic images of liquid condensed films and in the presence of polymyxin B, changes in monolayer morphology were observed. In the presence of peptide, the images revealed 'flower like' structures ~ 120 nm diameter protruding from the lipid monolayer ~ 0.7 nm. Further use of AFM in conjunction with thermodynamic data suggested that these morphological changes may involve phase separation between polymixin B and membrane lipid as part of the toxins mechanism of action.

Since the compressibility of a monolayer can be used to characterise the phase state of a monolayer [36, 77] thermodynamic analysis can be undertaken to characterise peptide or lipid isotherms. For a Langmuir monolayer, equilibrium elasticity is related to the

compressibility of the condensed monolayer phase [78]. The compressibility (C_s) of a monolayer at any area, *A*, is defined according to equation 3:

$$C_s = -\frac{1}{A}\left(\frac{\delta A}{\delta \pi}\right)_T \quad \textbf{Equation 3}$$

The compressibility properties can determined from the slope of a π-*A* isotherm. However, for a more precise measurement, the reciprocal of C_s is used to characterise the properties of the surface film and is called the compressibility modulus (C_s^{-1}), which is defined according to equation 4:

$$C_s^{-1} = -A\left(\frac{\delta \pi}{\delta A}\right)_T \quad \textbf{Equation 4}$$

As a reference, the phase state of a monolayer is characterised according to Table **3** [69].

An example of compression (π-*A*) isotherm analysis of a pure peptide film of aurein 2.2 is shown at Fig. (**5**). The collapse pressure of 37.8 mN m^{-1} is the highest pressure to which a stable monolayer can be compressed without detectable movement of molecules in the film [41]. The surface compression modulus was used to characterise a monolayer of aurein 2.2 using the methodology of Davies and Rideal [69]. According to these data, the peptide's elasticity ranged from 12.5 to 50 mN m^{-1}, indicating that the peptide was in the liquid expanded phase state, which is similar to the data observed in Fig (**5**). Sánchez-Martín *et al.*, [79] used the surface compressibility modulus to characterise the phase state of a monolayer formed from E1(145-162), a hepatitis G virus peptide. These studies showed that in this latter case the main arrangement of the isotherm was also a liquid expanded state with C_s^{-1} below 50 mN m^{-1}.

Even though Langmuir monolayer data does not directly provide structural conformation data, the surface cross-sectional area of peptides can be used to deduce their orientation at the interface, a technique, which has been accurately used to examine the interfacial orientation of lipids [41]. The surface behaviour of peptide monolayers is well characterised and it has been established that in general, α-helical peptides not under compression lie horizontally when spread onto a water subphase. However, assuming the collapse model proposed by Lavigne *et al.,* [80], it is possible to characterise the orientation of an α-helical peptide under compression. In this method at the lift-off point of the corresponding isotherm, the α-helices of these peptides are optimally aligned on the water surface so that their side chains come into maximal contact with each other [80]. Under these monolayer conditions, the residual area of the participating α-helical peptide can be calculated and used to deduce its orientation at the lift-off point. For example, in the case of an α-helical peptide lying parallel to the interface, its residual area at lift-off should correspond to the diameter of the α-helix formed by the peptide multiplied by the distance per residue along this α-helix. This orientation would be predicted for aurein 2.2 at lift-off, given that in an α-helical conformation, the expected length for the peptide would be 2.5 nm and its residual area would be 3.6 nm, which is in good agreement with the lift-off area indicated for aurein 2.2 in Fig. (**5)**. A peptide monolayer can be further compressed to its collapse point and it can be seen from Fig. (**5**) that at a collapse pressure of 37.8 mN m^{-1}, the maximum lateral packing molecular area of auren 2.2 was *circa* 1.75 nm^2. Based on this area, it would be predicted that the peptide was orientated perpendicular to the monolayer surface, given that the theoretical area of a α-helix so orientated to a surface is around 1.77 nm^2 [81-82]. Similar orientations at an air / water interface under collapse pressures have been reported for masculatin and citropin, which are α-helical antimicrobial peptides related to aureins [83]. This orientation change during compression is supported by conformational analysis undertaken by Brasseur *et al.,* [84] who

reported that gramicidin, which is a bacterial defence peptide, had an orientation parallel to the monolayer plane at low surface pressures but at collapse point adopted a perpendicular orientation. Langmuir monolayer data are not constrained to α-helices and can also help gain insight into other peptide structural conformations. For example, Almińana *et al.,* [44] showed that the SIKVAV peptide was able to form stable monolayers and that the overall shape of the corresponding peptide isotherm along with its extrapolated area of 0.72 nm^2 were in agreement with β-sheet conformation.

A number of factors can influence the shape of compression isotherms such as the composition of the subphase and pH. As an instance, Maget-Dana *et al.,* [85] showed that the pH of the subpase affected the shape of the isotherm of defensin A, which is a β-sheet defence peptide. These studies found that an increase in pH increased the stability of the defensin monolayer. More recently, research undertaken by Abriouel *et al.,* [86] on the effect of pH on bacteriocin AS-48, which is a bacterial defence peptide, showed that monolayer films were more stable at the air / water interface when the pH is close to the IEP of the peptide.

In addition to studies on peptide films, isotherm experiments can also be used to investigate peptide-lipid interactions [36, 38]. For example, Barzyk *et al.,* [87] investigated the association of N-23-T, an antimicrobial peptide derived from a bovine milk protein, with monolayers formed from DPPC and DPPG. It was observed that C_s^{-1} for isotherms of both lipids was 1.5 times higher in the presence of the peptide, indicating that N-23-T lowered the fluidity of these films. Most commonly, such isotherm experiments are performed by compressing a lipid monolayer, which has been spread onto a peptide-containing subphase.

Most recently, Glomm *et al.,* [88] compared this technique to three variants using bovine serum albumin (BSA) where: (i) BSA was spread on top of a phospholipid monolayer, (ii) BSA was spread onto a phospholipid film that had previously been compressed to a specific surface pressure, and (iii) a phospholipid film was compressed to a specific surface pressure and BSA was injected under this film. It was found that although each of the four methods produced different shaped isotherms, the introduction of the BSA did not significantly affect the collapse pressure of the isotherm [88]. However, Glomm *et al.,* [88] also used BAM images to provide information about the topography of the films for each of the four test methods, which showed that in contrast the film topography is highly dependent on how the protein is introduced into the phospholipid monolayer.

The effects of peptides on the thermal stability of mixed monolayers

The π-A isotherm provides useful information on the molecular packing in a lipid membrane by comparing the area occupied per lipid molecule at a given surface pressure. Information provided by studying the molecular packing of a single component monolayer is useful but the use of a mixed monolayers provides greater insight into the packing characteristics of the different components present. Thermodynamically analysing isotherm monolayer data for mixed systems can provide information on organisation, phase transition and molecular interactions as in the case of single component systems [36] but shows how these vary in the presence of peptide or with changing lipid composition. To achieve this result and gain a better understanding on the miscibility of a lipid system, the area of the system is compared to the area of each of its pure lipid component when maintained at the same surface pressure. Using these data, the mean molecular area for components within the mixed monolayer, $A_{12,}$ can be calculated by applying equation 5 [89-90]:

$$A_{12} = X_1A_1 + X_2A_2 \ldots\ldots\ldots\ldots \textbf{Equation 5}$$

where X_1 and X_2 are the molar fractions of the pure components present; A_1 and A_2 are the mean molecular areas for the individual components at the same surface pressure used to determine A_{12}.

Correlations between mean molecular area and molar fraction provide information on the molecular distribution, miscibility and interactions between the molecules in the monolayer [91]. Graphs of A_{12} versus molar fraction (x) are linear if the components in the monolayer are completely immiscible or they possess an ideal miscibility [89]. This methodology has been extended to undertake thermodynamic analysis of peptide-lipid interactions. For example, Sospendra *et al.,* [91] used this form of analysis to investigate the interaction of a synthetic viral peptide, AcVP3110, with dipalmitoyl phosphatidylcholine (DPPC) monolayers. The data showed that the lipid / peptide molar ratios deviated from ideal miscibility indicating that the interactions between the DPPC and AcVP3110 were repulsive. In contrast, more recently, Ambroggio *et al.,* [83] used a similar approach to investigate the peptide - lipid interactions of masculatin and citropin and found no significant deviations from ideal behaviour between these peptides and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) membranes.

Once the miscibility of monolayer components has been established, a more detailed investigation into the thermodynamics of the monolayer can be undertaken to understand the specific interactions between the molecules forming the mixed system. A frequently used method for determining the relative stability of each component of the monolayer has been

developed by Goodrich [90, 92]. This method uses the Gibbs free energy of mixing (ΔG_{mix}), which represents the energy gain related to the mixing process for the pure components in a monolayer system and is calculated according to equation 6:

$$\Delta G_{mix} = \int [A_{12} - (X_1 A_1 + X_2 A_2) d\pi \quad \text{.....Equation 6}$$

where A_{12} is the molecular area occupied by the mixed monolayer A_1 and A_2 are the area of the individual pure components, X_1 and X_2 are the molar fractions of the individual components.

Positive values of ΔG_{mix} indicate that the process of mixing is not thermodynamically stable, which in a two-component monolayer would indicate that the interactions between the two components are weaker than the interactions of the pure components themselves. This analysis of mixed monolayers has been extensively used to study systems mimicking the lipid composition of bacterial membranes [22, 76, 93-94]. As an instance, Clausell *et al.,* [76] studied the interaction of polymyxin B with monolayers mimetic of *E. coli* membranes and thermodynamic analysis indicated that in the presence of peptide, $\Delta G_{mix} > 0$, which corresponds to non-favourable interactions which impacts on the effectiveness of lipid peptide mixing. More recently, research undertaken by Dennison *et al.,* [22] investigated the interaction of aurein 2.5 with *E. coli* and *Bacillus subtilis* membrane mimics showed that in the absence of peptide $\Delta G_{mix} < 0$ for *E. coli* lipid mimics and $\Delta G_{mix} > 0$ for *B. subtilis* lipid mimics showing the impact of lipid composition on membrane binding and insertion. Thermodynamic analysis of compression isotherms showed that the presence of aurein 2.5 destabilised both bacterial membranes ($\Delta G_{mix} > 0$) as expected for an antimicrobial peptide with lytic ability. Thermodynamic analysis has also been used to provide predictions into the mechanisms of peptide - membrane lipid interaction [94-95]. Hal18 is a subunit of the

antibacterial heterodimeric peptide, halocidin, and recently, Dennison *et al.,* [94] used isotherm analysis to investigated the interaction of Hal18 with bacterial membranes. Analysis of isotherms derived from *E. coli* membranes showed ΔG_{mix} was negative in the absence of the peptide and in contrast to aurein 2.5 became more negative in the presence of Hal18, indicating the addition of Hal18 had energetically favourable mixing. In combination with other monolayer data, Dennison *et al.,* [94] predicted that the action of Hal18 against *E. coli* is driven by a carpet-type mechanism of membrane interaction. However, isotherm analysis of *B. subtilis* monolayers showed that ΔG_{mix} was positive in the absence of Hal18 and became more positive in the presence of peptide [94], indicating that the peptide had a energetically unfavourable effect on mixing. These data supported a lytic mechanism of action against *B. subtilis*, driven by oblique structure in line with the data observed in the case of aurein 2.5 [94].

Further thermodynamic analysis of mixed monolayers can be undertaken using the interaction parameter (α), which provides a measure of the relationship between each molar fraction with the ΔG_{mix} at the different surface pressures. This parameter can be calculated [96] using equation 7:

$$\alpha = \frac{\Delta G_{mix}}{RT(X_1 X_2^2 + X_1^2 X_2)} \quad \text{.....Equation 7}$$

where X are the molar fractions of the monolayer lipid components, R = 8.314 J mol^{-1} K^{-1} and T = 294°K.

The stability and binding interactions of monolayers can be further investigated using the mixing enthalpy (ΔH) [96], which is given by equation 8:

$$\Delta H = \frac{RT\alpha}{Z} \text{.....} \textbf{Equation 8}$$

where R = 8.314 j mol-1 K-1 , T = 294 °K and Z is the packing fraction which is calculated using the Quickenden and Tan model [97].

Negative values of ΔH are a sign of attractive interactions between the individual monolayer components, in turn, implying that the monolayer is stable. However, positive values of ΔH indicate energetically unstable interactions between the individual lipid components of these membranes [36].

The thermodynamic analyses described above can be combined to investigate the overall thermal stability of mixed monolayers in the presence and absence of peptide and thereby provide information on the interactive forces between the lipid and peptide components of the monolayer. An example of such use is provided by considering our own compression isotherm data for aurein 2.2 / lipid monolayers, which are shown in Fig. (**6**). Using the isotherms shown in Fig. (**6**) ΔG_{mix} was calculated using equation 6 and examination of Table **4** shows that $\Delta G_{mix} < RT = 2444.316$ J mol^{-1}, indicating that deviations from ideal mixing behaviour in these model membranes are small [91]. Table **4** also shows that ΔG_{mix} was positive in the absence of aurein 2.2 but negative in the presence of the peptide, which indicates that the peptide will favourably interact with the lipid. The positive ΔH shows that

this has a thermodynamically stabilising effect on the membrane. Further thermodynamic analysis of the isotherms in Fig. (**6**) was undertaken and α and ΔH were calculated for these monolayers using equations 7 and 8. This analysis revealed that in the presence of aurein 2.2, α and ΔH were more negative, indicating that these membranes were thermodynamically more stable in the presence of peptide. This would imply the interaction of the peptide with the membrane is thermodynamically favourable.

Using a similar combination of thermodynamic analyses, Sospedra *et al.*, [91] investigated the effects of the AcVP3110 peptide on the thermal stability of mixed lipid monolayers, which were formed with the peptide included. Thermodynamic analysis of DPPC and dipalmitoyl phosphatidylglycerol / AcVP3110 mixtures revealed positive values for ΔG_{mix}, α and ΔH, which indicated positive deviations from ideal mixing and the presence of repulsive forces between the peptide and the lipid. However, in the case of monolayer mixes with AcVP3110 and the cationic lipid, stearylamine, low deviations from ideal mixing were observed depending on the amount of peptide present in the monolayer. At a peptide to lipid molar ratio of 0.8:0.2, negative values of ΔG_{mix}, α and ΔH were observed, indicating that this peptide to lipid ratio had a stabilizing effect on the monolayer presumably reflected in the packing of the components at theses ratios.

Other forms of thermodynamic analysis have been used to investigate the stability of monolayers. Zhao and Feng [98] investigated the stability of paclitaxel, an anticancer drug, and phospholipid monolayers. These studies found that ΔG_{mix} was not a convenient method to apply to their data because their isotherm data was more sensitive to surface pressure than molecular area. In response, Zhao and Feng [98] developed an alternative method to analyse

energy changes with varying surface pressure by calculating the excess Helmholtz energy as follows :-

$$\Delta A_m^{ex} = \int_{A0}^{A} [\pi_{12} - (X_1\pi_1 + X_2\pi_2)]dA \quad \text{.....Equation 9}$$

Where A_0 and A are molecular areas where π increases from zero at which the excess Helmholtz excess energy is calculated [41]. $\pi_{12,}$ π_1 and π_2 are the surface pressures of a mixed monolayer, pure lipid monolayer and pure peptide/drug monolayer. X_1 and X_2 imply the percentage lipid and peptide/drug in the mixed monolayer.

Zhao and Feng [98] showed the excess Helmholtz energy of mixed phospholipid and paclitaxel monolayers was dependent on the composition and the molecular areas of the components within the mixed monolayers with for example, the stability of the monolayer depends on acyl chain length [98]. For DMPC monolayers, negative values of excess Helmholtz energy was observed indicating there are strong interactions between the two components. However, for 1, 2-distearoyl-sn-glycero-3-phosphocholine (DSPC), which has a longer chain length than DMPC, positive values of excess Helmholtz energy was observed. Based on these data, it was concluded that paclitaxel formed thermodynamically more favourable monolayers with lipids possessing shorter acyl chains due to their lower levels of van der Waals interaction as compared to longer acyl chains [98]. Zhao and Feng [99] also used this method to investigate the effect of lipid chain unsaturation and head group interactions with paclitaxel. Comparison between DSPC and 1, 2-dielaidoyl-sn-glycero-3-phosphocholine (DEPC) monolayers showed that unsaturation played a role in determining monolayer stability. Paclitaxel and DSPC form thermodynamically unstable monolayers and for DEPC monolayer systems negative values of excess Helmholtz energy were observed indicating a more stable system [99]. More recently, Yu *et al.*, [53] used this method for

calculating the excess Helmholtz energy of a synthetic antimicrobial peptide, V4, with monolayers formed from anionic 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (POPG) versus zwitterionic 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). For both POPG and POPC, in the presence of V4, negative values of the excess Helmholtz energy were observed, implying that the interactions between lipid and peptide were stable. Increasing the mol. percentage of V4 present from 0 to 50 % increased the levels of interaction between lipid and peptide, thereby enhancing the stability of the monolayer.

Conclusion

Lipid monolayers provide a model system to aid investigation into the adsorption and insertion mechanisms utilised by amphiphilic peptides at a phase boundary such as a cell membrane interface. The method can help our understanding of structure / function relationships especially if combined with other techniques such as fluorescence microscopy [100-101], BAM [100] and scanning electron microscopy [102-103]. Whilst there are limitations to the Langmuir Blodgett system such as its use of a single monolayer, lack of membrane curvature and simplified lipid systems, the monolayer technique provides a useful means to investigate a range of key biological processes involving peptide-lipid interactions. Physical and morphological analysis of monolayer films have helped identify the importance of key physiochemical properties on the efficacy of antimicrobial peptides and aided our understanding of the design parameters required for new therapeutic agents. Undoubtedly, the technique will continue to contribute to the elucidation of factors involved in a range of core biological processes linked to membrane function and lysis.

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Tables

Table 1. Examples of amphiphilic α-helical peptides active at an air / water interface. Also shown for these peptides are values of $< \mu_H >$, the mean hydrophobic moment, $< H_0 >$, the mean hydrophobicity and π, the surface pressure, which are all defined as in the text. A surface pressure change of ~ 22 mN m^{-1} is indicative of a strongly surface active sequence that also has lytic properties.

Segment	Primary sequence	$< \mu_H >$	$< H_0 >$	π (mN m^{-1})
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	0.57	0.25	24.5
Aurein 1.2	GLFDIIKKIAESF	0.71	0.21	30.0
Aurein 2.2	GLFDIVKKVVGALGSL	0.56	0.37	27.7
PBP5 C-terminus	GNFFGKIIDYIKLMFHHWFG	0.67	0.24	33.7
PBP6 C-terminus	GGFFGRVWDFVMMKFHHWFGSWFS	0.51	0.42	22.3

Table 2. Changes in surface pressure associated with the penetration of melittin, magainin and cecropin into phospholipid monolayers. The surface pressure, π, is defined as in the text and these data were taken from Lad *et al.*, [50].

Antimicrobial Peptide	Surface pressure, π (mN m^{-1})	
	DPPC	DPPG
Melittin	10	20
Magainin II	5	12
Cecropin P1	3	9

Table 3. The phase states of monolayers [69]. C_s^{-1}, the compressibility modulus, is defined as in the text.

Monolayer	**C_s^{-1} (mN m^{-1})**
Clean surface	0
Ideal	π
Protein	1 to 20
Liquid expanded	12.5 to 50
Liquid condensed	100 to 250
Solid	1000 to 2000

Table 4. Thermodynamic analysis of aurein 2.2 / lipid monolayer interactions. ΔG_{mix}, the Gibbs free energy of mixing, α, the interaction parameter, and ΔH, the enthalpy of mixing were all as defined in the text and were determined for lipid monolayers at varying surface pressure (π) in the absence (-A2.2) and presence (+A2.2) of aurein 2.2.

Π (mN m^{-1})	ΔG_{mix} (J mol^{-1})		α		ΔH (J mol^{-1})	
	- A2.2	+ A2.2	- A2.2	+ A2.2	- A2.2	+ A2.2
5	0.537071	-1.46	0.001181	-0.003	1.44	-3.92
10	0.910389	-3.29	0.002002	-0.007	2.44	-8.86
15	1.277555	-4.86	0.00281	-0.011	3.43	-13.07
20	2.42102	-6.43	0.005325	-0.014	6.50	-17.30

FIGURE LEGENDS

Figure 1. The secondary amphiphilicity of aurein 1.2, adapted from Dennison *et al.,* [3]. Fig.**1A** illustrates the three dimensional α-helical segregation of polar and apolar residues. Fig. **1B** illustrates a two-dimensional α-helical axial projection of aurein 1.2 with hydrophobic residues circled.

Figure 2. The surface pressure of aurein 2.2 as a function of peptide concentration.

Figure 3. Time course for the interactions of aurein 2.2 at a subphase concentration of 4 μM with monolayers formed from DMPS and DMPC.

Figure 4. The interaction of aurein 2.2 with DMPC/DMPS monolayers.

Figure 5. Compression isotherm of aurein 2.2

Figure 6. Compression isotherm analysis of lipid monolayers. Isotherms derived from a DMPC (a), DMPS (b) and DMPC/DMPS (c) in the absence (A) and presence (B) of aurein 2.2.

Figure 1

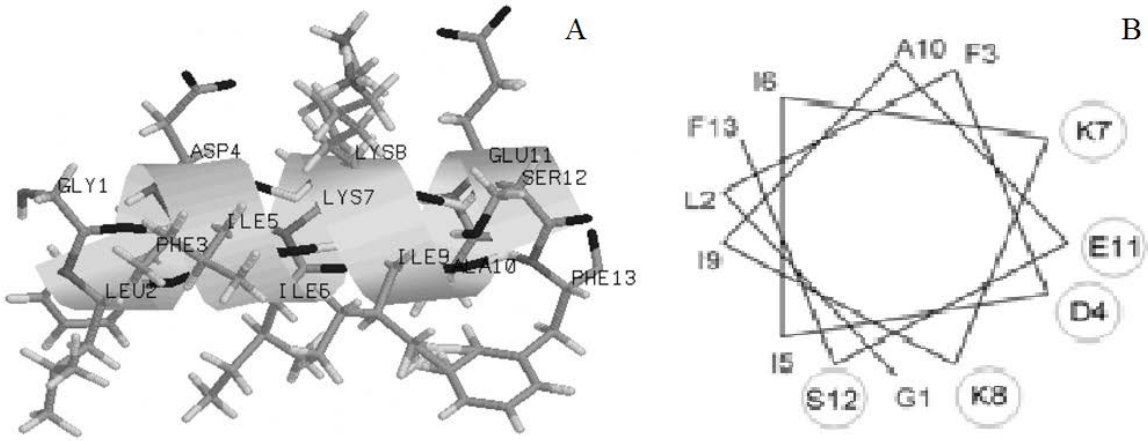

A
ASP4
LYS8
GLU11
GLY1
SER12
LYS7
ILE5
PHE3
ILE9
LEU2
ILE6
PHE13
B
A10
F3
I8
F13
K7
L2
E11
I9
D4
I5
S12
G1
K8

Figure 2

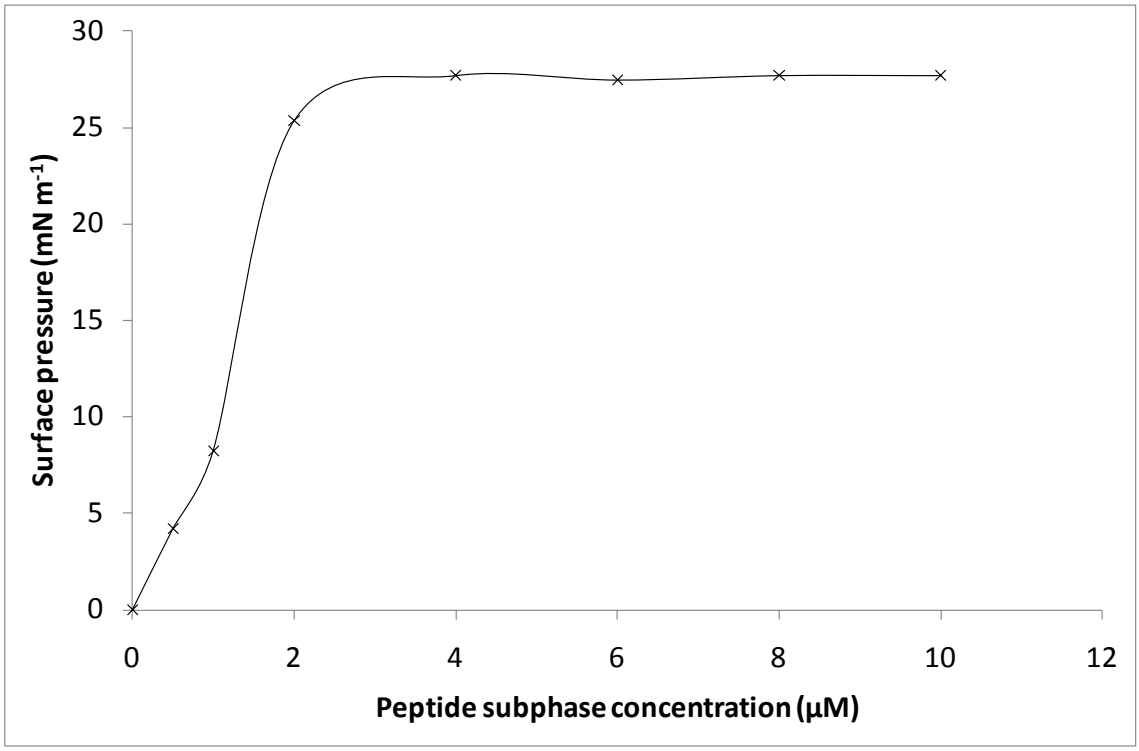

30
25
20
15
10
5
0
Surface pressure (mN m-1)
0
2
4
6
8
10
12
Peptide subphase concentration (µM)

Figure 3

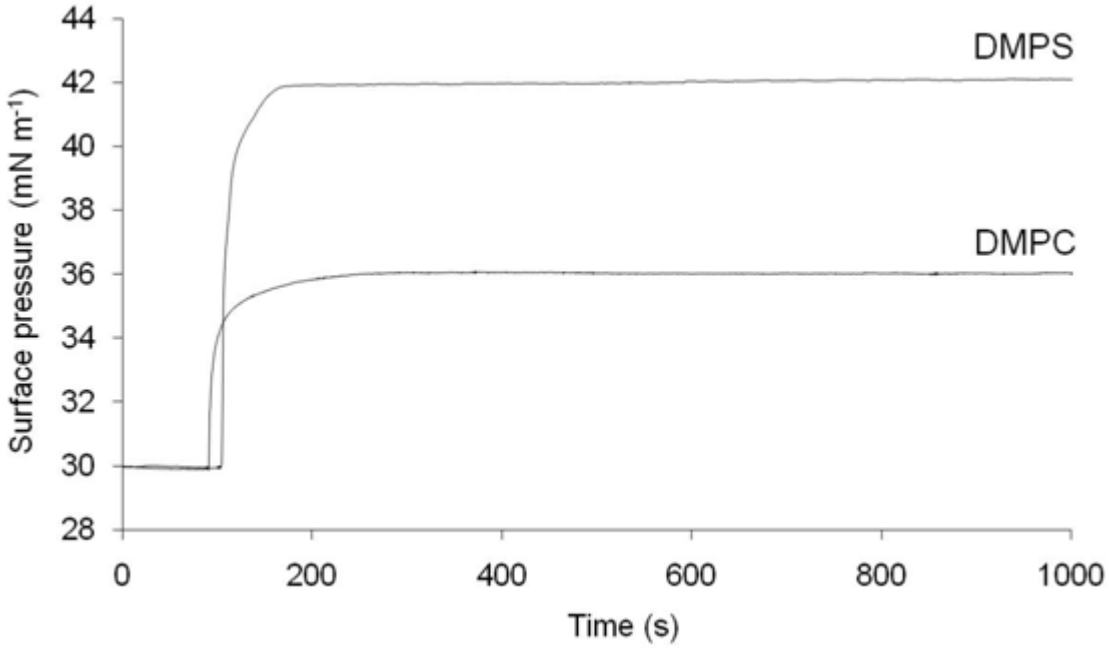

DMPS
DMPC
Surface pressure (mN m-1)
Time (s)
44
42
40
38
36
34
32
30
28
0
200
400
600
800
1000

Figure 4

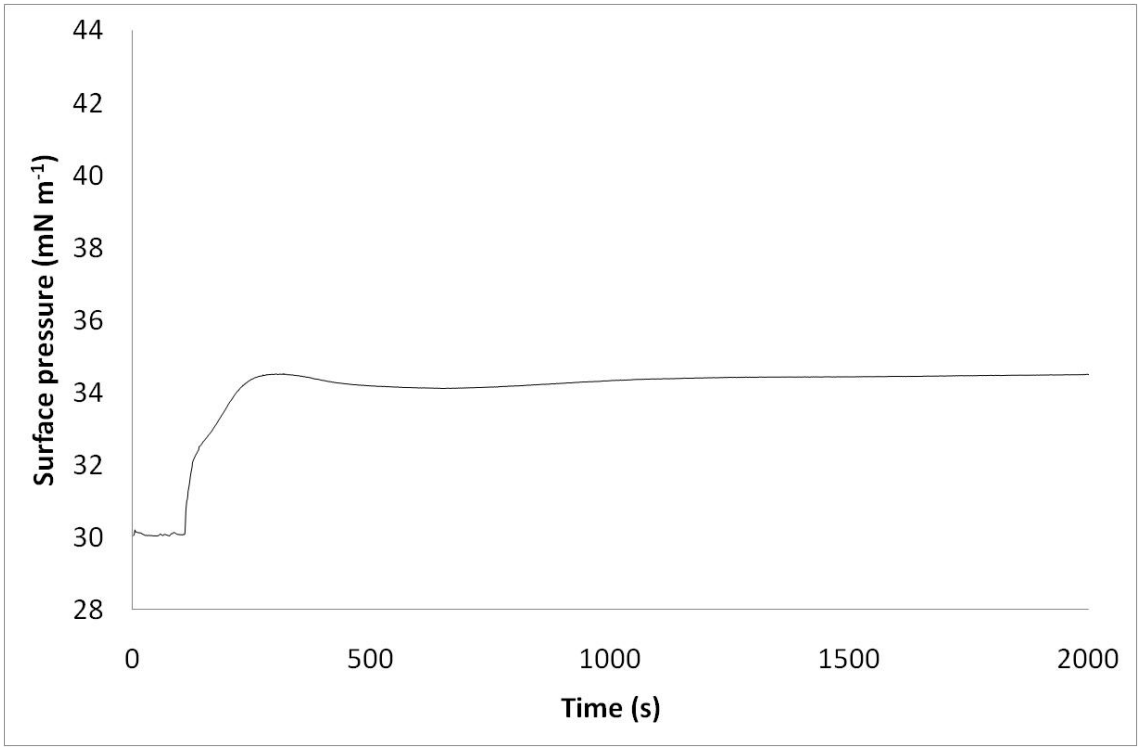

44
42
40
38
36
34
32
30
28
Surface pressure (mN m-1)
0
500
1000
1500
2000
Time (s)

Figure 5

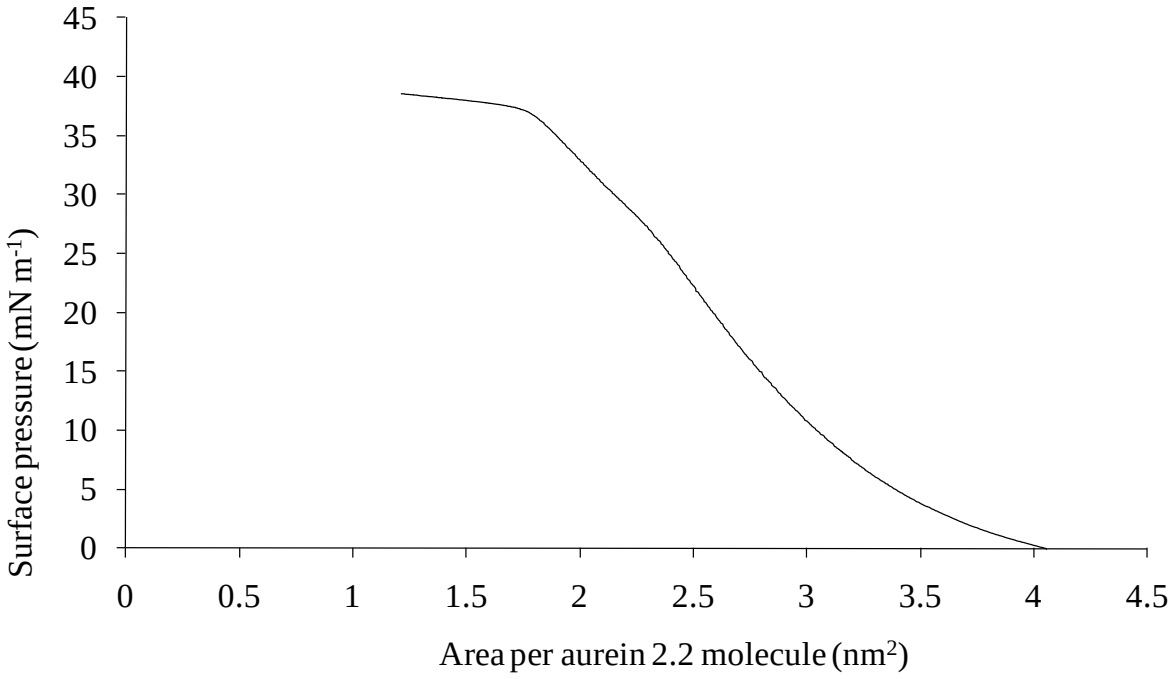
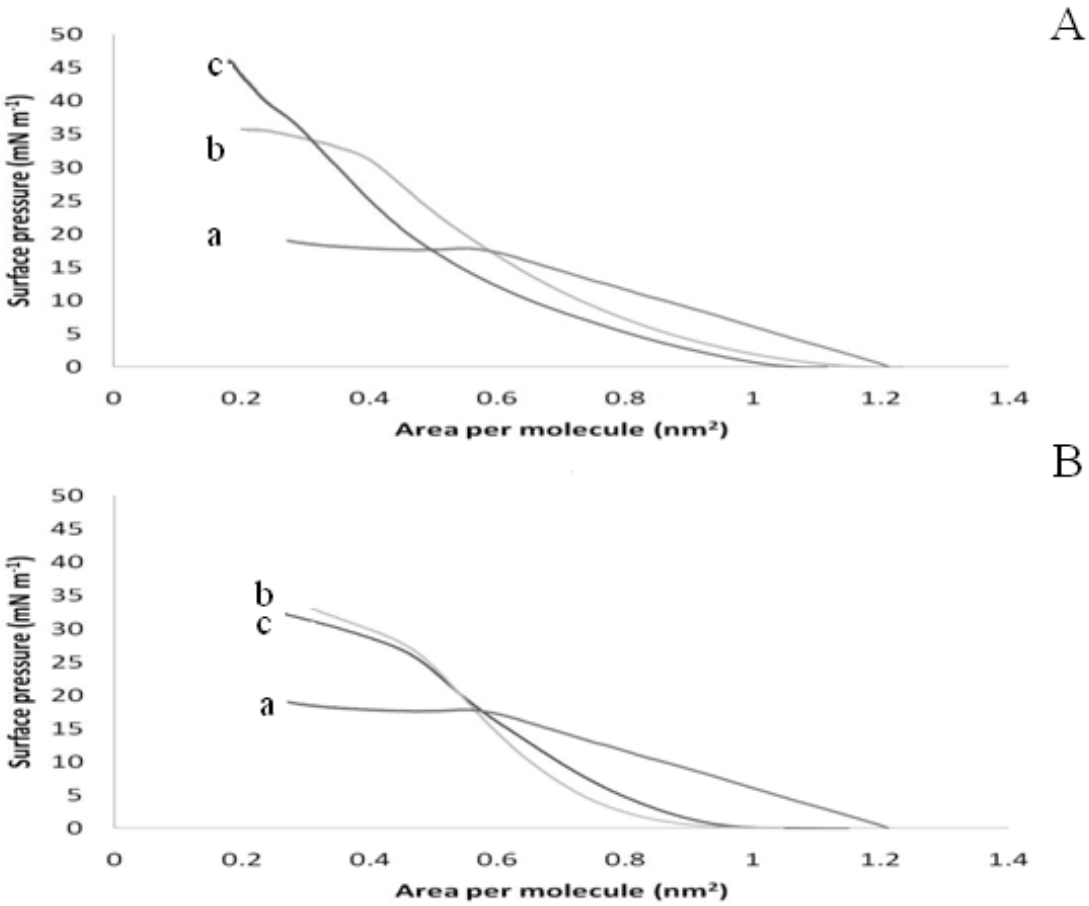

45
40
35
30
25
20
15
10
5
0
Surface pressure (mN m-1)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
Area per aurein 2.2 molecule (nm2)

Figure 6


A
Surface pressure (mN m-1)
50
45
40
35
30
25
20
15
10
5
0
c
b
a
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Area per molecule (nm²)
B
Surface pressure (mN m-1)
50
45
40
35
30
25
20
15
10
5
0
b
c
a
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Area per molecule (nm²)