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The application of monolayer studies in the understanding of liposomal formulations

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ABSTRACT

The study of surfactant monolayers is certainly not a new technique, but the application of monolayer studies to elucidate controlling factors in liposome design remains an underutilised resource. Using a Langmuir–Blodgett trough, pure and mixed lipid monolayers can be investigated, both for their interactions within the monolayer, and for interfacial interactions with drugs in the aqueous sub-phase. Despite these monolayers effectively being only half a bilayer, with a flat rather than curved structure, information from these studies can be effectively translated into liposomal systems. Here we outline the background, general protocols and application of Langmuir studies with a focus on their application in liposomal systems. A range of case studies are discussed which show how the system can be used to support its application in the development of liposome drug delivery. Examples include investigations into the effect of cholesterol within the liposome bilayer, understanding effective lipid packaging within the bilayer to promote water soluble and poorly soluble drug retention, the effect of alkyl chain length on lipid packaging, and drug-monolayer electrostatic interactions that promote bilayer repackaging.
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1. Introduction

1.1. Langmuir monolayer studies – the development of the system

A common idiom, 'pouring oil on troubled water', is a figurative way of suggesting that attempts are made to calm a contentious or problematic situation. This relates to the fact that a thin layer of oil can calm choppy water, a technique referred to as wave damping, where sailors poured oil onto the sea to prevent waves being formed. This method was first described by Aristotle and Plinius (Fulford, 1968). To achieve this effect, very little oil is required; it need only be a surface coating of 1 molecule thick, that is to say a monolayer. Marangoni effects are the basis of this wave damping effect produced by oil. The Marangoni effect is a phenomenon whereby movement of a liquid occurs due to local differences in the surface tension of the liquid (Kuroda et al., 2000). Sudden local increases in surface area lead to enhanced surface tension, resulting in a surface tension gradient which, in turn, promotes contraction of that area and thus, further surface area growth is prohibited. Basically, the Marangoni flow opposes the flow associated with the wave action.

Benjamin Franklin, having seen this phenomenon of wave calming behind ships on which the cooks used sea water to rinse the fat off dishes, undertook to scientifically investigate this further (Lyklema, 2000). On a lake near Clapham Common in London, Franklin noted that one teaspoon of oil was enough to calm several hundred square metres of the lake's surface, with the wind having a much reduced effect on treated areas of the water surface compared to the untreated parts (Franklin, 1774). Later, John Shields carried out large-scale wave-damping experiments in Scotland and lodged a patent based on this in 1879 (Lyklema, 2000). Lord Rayleigh also followed this research area, and he noted that water surface tension could be lowered by contamination and oil films. Although he had no method of exactly measuring the thickness of the films, he estimated them to be monomolecular, with a thickness of 1–2 nm, and noted that by using such films, information on the size of molecules was obtainable (long before the existence of molecules was generally accepted) (Lyklema, 2000; Rayleigh, 1890, 1899). However, the first surface pressure versus area measurements (as they are now referred to) were reported by Agnes Pockels (Pockels, 1891) a German Scientist, who made a basic surface balance in her kitchen. Using this system, she was able to determine surface contamination as a function of the surface area for different oils, and further observed that by com-

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pressing monolayers below a certain area, the surface tension falls rapidly.

Pockels methods were further developed by Irvine Langmuir with his film balance system, a method that still bears his name. One of the advantages the Langmuir trough offered was that a direct measurement of the film pressure could be derived from the deflection of a movable float, separating the film from clean water. Using his trough, Langmuir studied monolayer lipids more systematically and confirmed that the films are monomolecular. He also showed that the molecules in these monolayers are orientated on the aqueous surface, with the hydrophilic portion of the surfactants in contact with the liquid, whilst the hydrophobic region of the surfactants is pointing up towards the air (Langmuir, 1917, 1920).

1.2. Using film balances to determine interfacial pressures

The basic features of the original Langmuir trough remain in modern systems; as shown in Fig. 1, the setup consists of a Teflon trough containing the sub-phase (water or aqueous buffers) and two movable barriers. When a surfactant substance, dissolved in an organic solvent such as chloroform, is added drop-wise to the subphase surface, the solvent evaporates and a monolayer is formed on the surface of sub-phase (Box 1 ; Fig. 2). The formation of a monolayer on the surface between these barriers results, and by varying the position of the movable barriers, the surface area between these barriers can be controlled such that the monolayer may be compressed and the available surface area per molecule for the monolayer decreased (Fig. 1). This results in different interfacial tensions between the surface with the surfactant present (γ_m), and the 'clean' surface (γ_0); this difference is the interfacial pressure (Π). It can be measured by determining the force at the barrier, but is more commonly achieved by using the static Wilhelmy plate technique (Box 2). Therefore, by sliding the barriers across the interface, the monolayer is compacted, resulting in an increase in surface pressure. If the number of molecules in the monolayer is known, the surface pressure can be related to the interfacial area, or the average area per molecule, and surface pressure isotherms can be plotted until the compression on the monolayer is such that it collapses (Chechel and Nikolaev, 1990). Fig. 3 shows a schematic of a surface pressure isotherm. From such plots, a range of information can be gathered, including the orientation and/confirmation of molecules in the monolayer, their dimensional properties, and molecular interactions between mixed surfactant monolayers (Lyklema, 2000). In these plots, generally it is most useful to plot surface pressure against average area per molecule, and therefore the amount and concentration of surfactant initially added is required. However it should be noted that, whilst the Langmuir trough is commonly used to measure surface pressure/area curves, other methods are available (e.g. Dynarowicz-Latka et al., 2001).

1.3. Information gained from monolayer studies

From Fig. 3, various phases of the monolayer are shown: as the concentration of the molecules at the surface is increased (i.e. the distance between the barriers is contracted) the monolayer changes from a very dilute 'gaseous' monolayer (G), where the molecules are far apart and there is low interfacial pressure, to a 'liquid' state. Often there are two liquid states: a 'liquid expanded' (LE) and a 'liquid condensed' (LC) monolayer, which are determined by the proximity and orientation of the surfactants (Fig. 3B). Only a small reduction in the area is required to move the monolayer from the LC to the 'solid' (S) state. In this state, all the amphiphilic molecules are closely packed and the hydrophobic tails are aligned in parallel, with the area per molecule corresponding with the S-phase which is equal to the closed-packed molecular cross-sectional area (Lyklema, 2000). Further compression of the monolayer results in the monolayer collapsing (molecules breaking out of the monolayer, by forming micelles, or multilayers in the case of phospholipids, for example) which leads to a sharp break in

Box 1: How to make monolayers

Depending on the solubility of the surfactants used (i.e. Langmuir vs Gibbs monolayers), the procedure will vary. For soluble monolayers, the substance which will form the monolayer to be studied is dissolved in the subphase. Generally the surfactants used within liposomes form insoluble monolayers, and to prepare such monolayers a drop of the solution of the surfactant(s) in a volatile solvent is placed onto the aqueous interface such that it can spread spontaneously over the interface and subsequently evaporate (Fig. 2). Solvents that can be employed for this include e.g. chloroform, benzene, cyclohexane. It is generally recommended to employ the same solvent for all studies, as there have been reports of the choice of solvent influencing the Π–*A* curves. A typical protocol that can be adopted is as follows:

1. Prepare the lipid solution by dissolving an appropriate amount of lipid in chloroform as an organic solvent (e.g. 20 μg/ml).
2. Prior to commencing each measurement, the trough and barriers on the Langmuir trough should be washed: first rinse with warm soapy water then tap water and then pour ethanol onto the trough and barriers. Spread the ethanol all over the trough with a soft brush and try to collect any dirt. This may take around 2 min for a trough and 30 s for each barrier depending on the set-up. Then rinse them with distilled water and dry with lens/precision wipes.
3. Assemble the trough and barriers and, if using a water circulator, set the desired temperature for the subphase.
4. If using a Wilhelmy plate, this should be kept in methanol and the methanol must be evaporated by use of a naked flame prior to use. Typically the plates are made of platinum; however glass, quartz, mica and disposable filterpaper plates can also be used. In our experience, we found a platinum place to be the most effective and to clean these the plate was placed in the hottest part of the flame for a few seconds until the plate glows red-hot.
5. Connect the Wilhelmy plate to the balance. One third of the plate should be immersed in water.
6. Fill the trough with ultra pure deionised water so that the surface of the water subphase is a couple of millimetres above the edges of the trough.
7. It is generally recommended to expand the barriers to the maximum extension and then balance/zero the system. Then bring the barriers together; any change in surface pressure is a sign of presence of contaminant. Surface pressure of less than 0.2 mN/m can be considered sufficiently clean, but if it exceeds 0.2 mN/m the contamination should be removed by running the tip of an aspirator along the edge of barriers and the space between them to pick up the contaminants. Repeat the process until the subphase is clean of surface impurities.
8. Fill a precision syringe with the desired volume of prepared solution of monolayer material dissolved in a volatile organic solvent.
9. Gently push on the syringe to produce a droplet at the end of the needle and then touch the surface with the tip of the needle. Note: do not let the drop fall from the needle onto the subphase, as some of the sample might be lost by the formation of micelles in the subphase.
10. Wait for approximately 15 min for the solvent to evaporate, after which the measurement can commence.

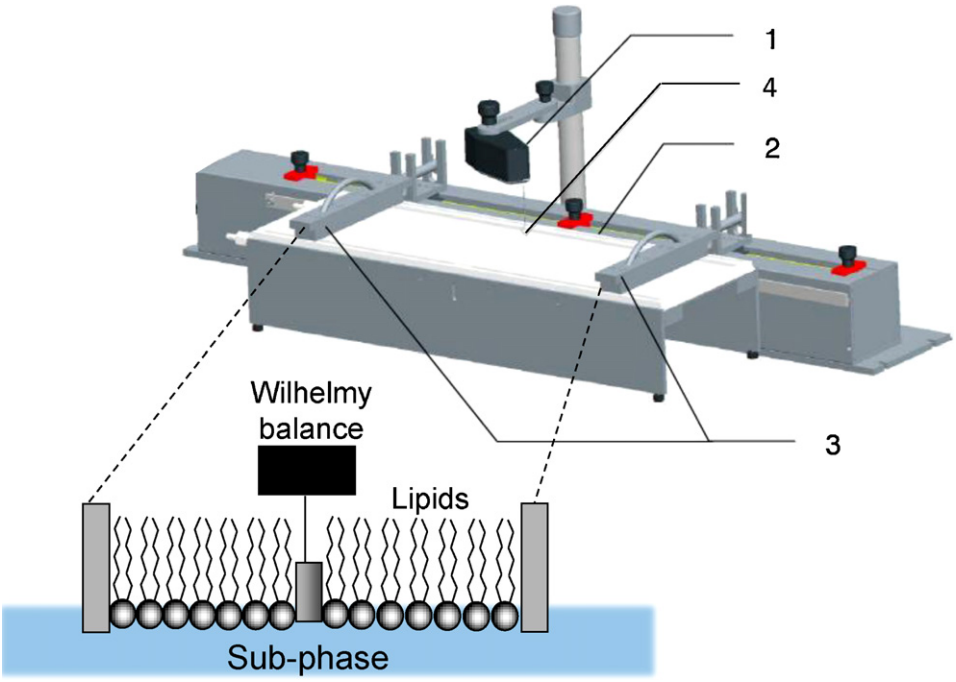

Fig. 1. Representation of Langmuir mini trough, kindly supplied by KSV Ltd and modified. Different parts of the instrument: (1) balance, (2) trough filled with clean subphase, e.g. water, (3) movable barriers, and (4) Wilhelmy plate.

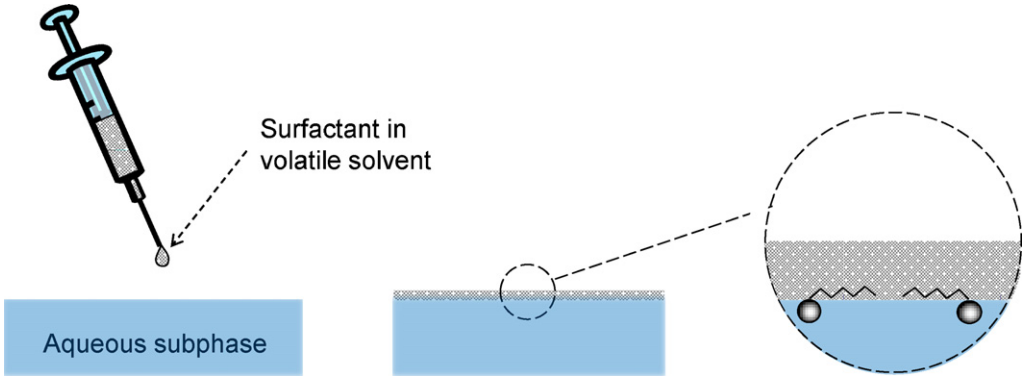

Fig. 2. Spontaneous spreading of a liquid of surfactant molecules (adapted from Lyklema, 2000).

Box 2: Measuring surface pressure

Surface pressure is a two dimensional analogue of pressure calculated by the following equation:

$$\pi = \gamma_0 - \gamma_m \quad (1)$$

where π represents surface pressure (mN/m), γ_0 is the surface tension of the subphase (e.g. water) in the absence of monolayer lipid, γ_m is surface tension in presence of monolayer lipid.

The surface pressure can be measured using a Wilhelmy plate in a Langmuir–Blodgett instrument. When the Wilhelmy plate is partially immersed in the subphase, various forces act on this plate: there are downward forces such as gravity and surface tension, and upward forces such as buoyancy due to the displacement of water. These forces are usually measured by a sensitive elector balance, which determines the change in the mass of the plate in the absence and presence of the monolayer.

the isotherm (Fig. 3B). As is shown in Fig. 3A, the transition between these phases is not always distinct and often more than one phase may be present. There is also a question as to the orientation of the surfactant tails in the gaseous phase: are they orientated flat on the interface (as in Fig. 3Bi), or are their tails out into the non-aqueous phase (Fig. 3Bii and iii). It is thought that situation (ii) may be entropically more favourable (Lyklema, 2000), but the overall molecular shape of the molecules in question would need to be considered and often option (iii) is used schematically to represent the general concept. Typical examples of isotherms from some common lipids and amphiphiles are shown in Fig. 4.

2. The use of monolayer studies in liposome research–example applications

As extensively reported, liposomes are bilayer vesicles, first described by Bangham et al. (1965) whilst studying cell membranes. They are vesicular structures consisting of hydrated bilayers, which form when phospholipids are dispersed in water (Bangham et al., 1965). Whilst there are very many reported variations on the theme (e.g. niosomes, virosomes, bilosomes, etc.), all

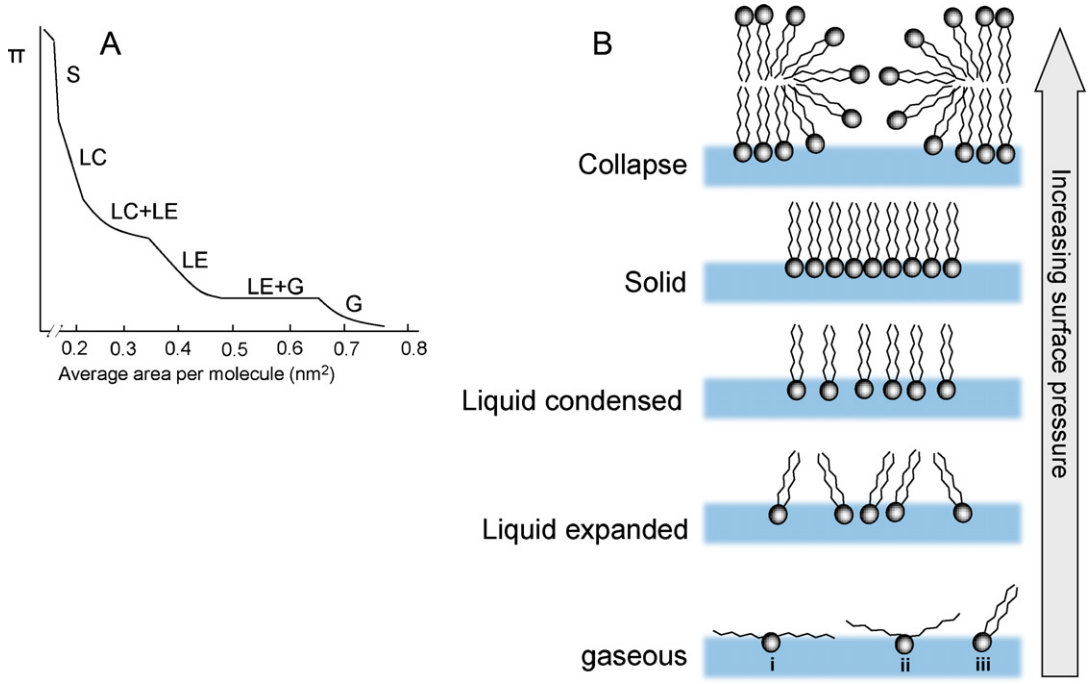

Fig. 3. (A) Schematic example of Π–*A* isotherm, exhibiting a variety of phases which can occur. G: gaseous, LE: liquid expanded, LC: liquid condensed, and S: solid. Curves like this are typical for lipid monolayers. (B) Schematic representation of lipid packaging at the monolayer interface.
Figure modified from Lyklema (2000).

have the same basic bilayer construction. Since their first description as a possible drug delivery system (Gregoriadis and Ryman, 1971), these constructs have been extensively investigated and there are now several liposome-based products clinically used. Given their structure, it is not surprising that monolayer studies of phospholipids and other surfactants are undertaken and the findings extrapolated to liposomal bilayers. Indeed, such studies can be highly informative, giving insights into areas such as bilayer lipid packaging configuration (Ali et al., 2010; Dynarowicz-Latka and Hac-Wydro, 2004), drug–lipid interactions (Ali et al., 2010) and liposome stability (Christensen et al., 2008; Demel et al., 1998; Lambruschini et al., 2000). However, since they are effectively half a membrane, monolayer studies are less suited to study certain aspects, such as trans-membrane processes, and consideration to this overall difference in morphology should always be borne in mind.

2.1. Elucidating the role of cholesterol in liposomes – the supporting role of monolayer studies

Cholesterol (Fig. 4A) is a common ingredient in liposomal formulations and its beneficial role within liposomal bilayers is well recognised. Early studies, investigating the effect of liposome composition on drug retention (Gregoriadis and Davis, 1979), demonstrated that inclusion of 50% mol/mol cholesterol within a liposome formulation increased the stability and reduced the permeability of liposomal bilayers. At molar percentages of between 20% and 50%, depending on the nature of the phospholipids, cholesterol can dissolve within lipid bilayers, whereas at higher concentrations cholesterol can form crystal habits (Egelhaaf et al., 2003). This inclusion of cholesterol within liposomal bilayers has been shown to result in increased packing densities of phospholipids molecules (Semple et al., 1996), which is thought to result from the accommodation of cholesterol in the molecular cavities formed by surfactant monomers assembled into vesicles (Devaraj et al., 2002), as evidenced by monolayer studies and surface pressure measurements that show a decrease in effective area per molecule as the cholesterol content of the monolayer is increased (Rogerson et al., 1987). This space filling action, combined with the ability of cholesterol to complex with phospholipids, can reduce bilayer permeability to small hydrophilic solutes and ions (Demel et al., 1972; Papahadjopoulos et al., 1973) and their increased stability in biological environments (Gregoriadis and Davis, 1979). It was further shown (Gregoriadis and Senior, 1980; Kirby et al., 1980) that the presence of cholesterol in liposomal bilayers decreases, in proportion to its concentration, the loss of phospholipid molecules to plasma high-density lipoproteins [through lipid exchange (Sulkowski et al., 2005)] and hence reduces the subsequent loss of the entrapped solutes.

The effect of cholesterol on lipid packaging and on liposome bilayers is demonstrated in Fig. 5. By comparing the surface pressure-mean molecular area isotherms (Fig. 5A) of one-component films and mixtures of 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine (DSPC) and cholesterol at various ratios, the extent of geometrical interactions are seen. The Π–*A* isotherm for pure DSPC is in agreement with results collated by Cardenas et al. (Manosroi et al., 2003) with a molecular area calculated as 51.0 Å^2/molecule (Fig. 5A). For cholesterol, the practically linear increase of surface pressure up to point of collapse beyond 39.5 Å^2/molecule indicates a closely packed monolayer structure, which exists as a solid-phase during the compression. In the case of surfactant mixtures, all isotherms are found to lie between the ranges of those of the pure components (Fig. 5A) and the measured mean molecular areas for mixed surfactant monolayers (Table 1) are smaller than the theoretical predicted ones for the ideal mixtures, deviated negatively from the additive rule (Gregoria, 1973). This suggests a strong interaction between DSPC and cholesterol, highlighting the 'condensing effect' cholesterol has within the bilayer, which is attributed to the accommodation of cholesterol in molecular cavities generated via the assembling of lipids into vesicles (Hughes, 2005). The effect the inclusion of cholesterol has on the liposomal bilayers transition was also studied using dif-

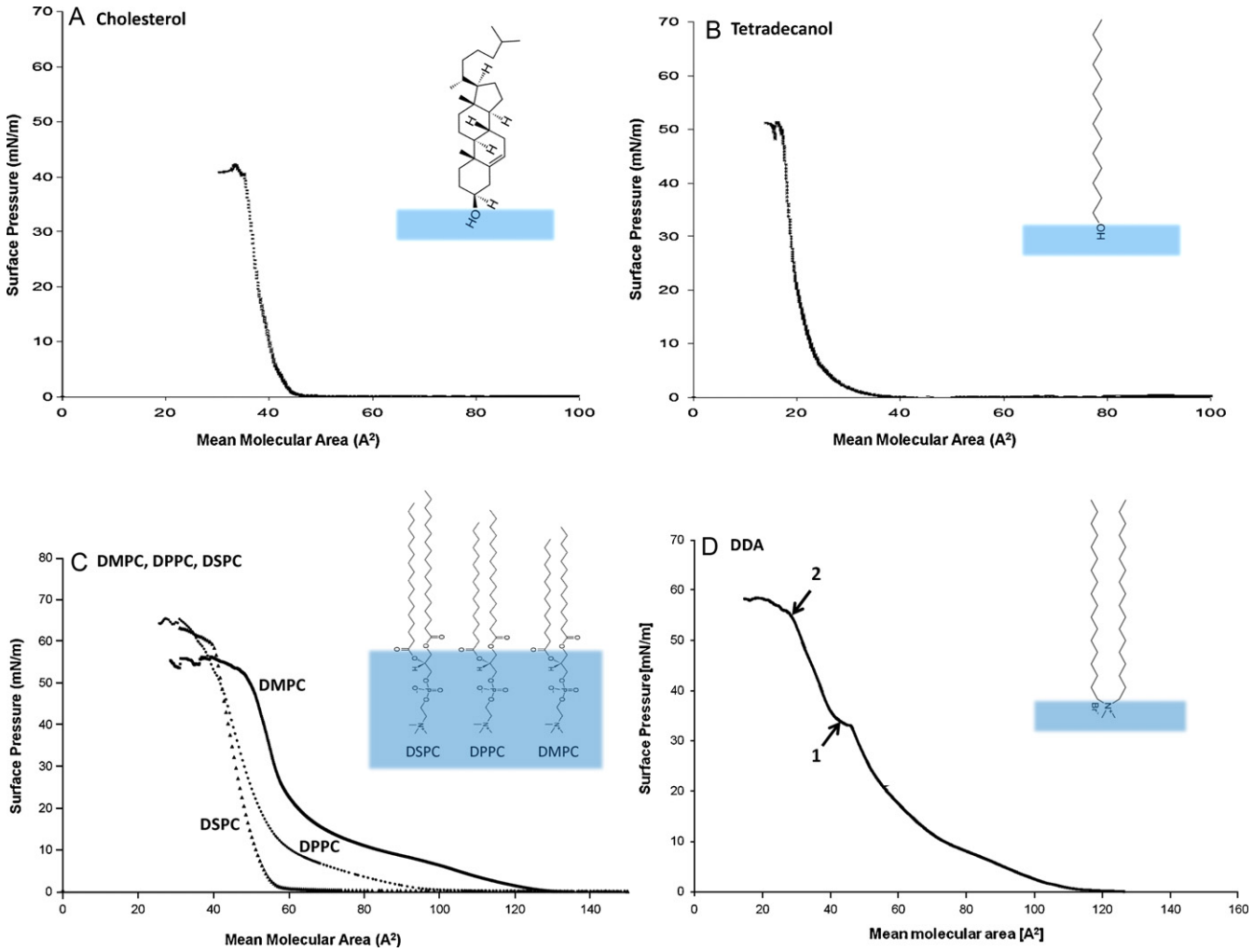

Fig. 4. The surface pressure–area isotherms of pure monolayers at the air/water interface (at 20 °C) for (A) cholesterol, (B) tetradecanol, (C) DMPC, DPPC and DSPC and (D) DDA where 1 denotes the transition from liquid-expanded to liquid condensed and 2 denotes monolayer collapse. Results are expressed as the means of three experiments ± S.D.

ferential scanning calorimetry (DSC) (Fig. 5B). The shape of the transition and the transition temperature (T_c; which refers to the gel–liquid crystalline phase transition) varies when mixtures of different hydrocarbon chain length phospholipids are compared to pure lipids (Taylor and Morris, 1995). From Fig. 5B, the transition temperature of the DSPC only liposomes can be seen at 54 °C, however with the addition of 33 or 50% cholesterol, this transition temperature is no longer detectable. In the absence of cholesterol, the hydrocarbon chains of the lipids in the bilayer crystallise into the rigid-crystalline phase, which results in the measured T_c for the DSPC liposomes (Taylor and Morris, 1995). On the addition of cholesterol, the T_c of the DSPC liposomes is removed, suggesting that no crystallisation of the hydrocarbon chains has taken place (Fig. 5B). In addition to the effect on bilayer transitions, the reduction in bilayer permeability that results from increasing cholesterol concentrations (up to 50%) is also shown in Fig. 5C.

The reduced membrane permeability of cholesterol rich liposomes compared to lower concentration formulations could be due to the presence of defects in the latter. As an amphiphatic substance, cholesterol is introduced to the phospholipid bilayers with its 3β-hydroxyl head group towards the aqueous layers and the rigid hydrophobic steroid ring locates itself next to the carbons of the phospholipid chains. This ability of cholesterol to condense the bilayer (as shown in Fig. 5A) and induce membrane-stability is thought to occur via an interaction between the rigid hydrophobic ring structure of the molecule and the alkyl side-chains of phospholipids, which ultimately decreases membrane permeability (Wiseman et al., 1993). Cholesterol, by increasing the orientation order of the relatively mobile hydrocarbon chains of

Table 1
The experimental and ideal extrapolated area and area compressibility of mixed and pure monolayers at the air/water interface (at 20 °C) by DSPC and cholesterol. Results are expressed as the means of three experiments.

	Extrapolated area at zero pressure (Å²/molecule)	Ideal extrapolated area at zero pressure (Å²/molecule)	Deviation from ideality (%)	Collapse pressure (mN/m)
DSPC	51.0	–	–	63.2
Cholesterol	39.5	–	–	44.2
DSPC:cholesterol (89:11%)	45.3	49.7	−8.9	59.3
DSPC:cholesterol (67:33%)	41.8	47.2	−11.4	42.5
DSPC:cholesterol (50:50%)	45.0	44.5	+1.1	23.2

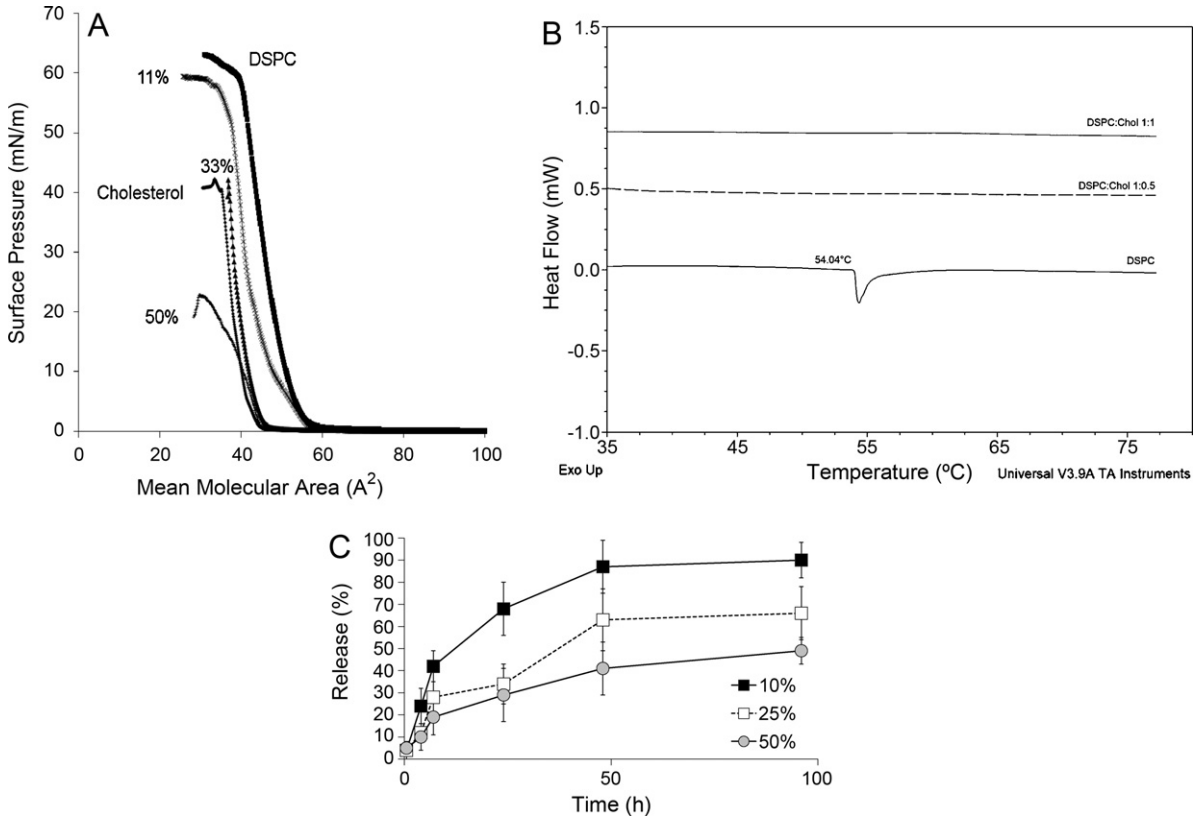

Fig. 5. Characteristics of DSPC and DSPC:cholesterol systems. (A) The surface pressure–area isotherms of single component monolayers and DSPC:cholesterol mixtures at the air/water interface (at 20 °C). (B) DSC scans of DSPC liposomes and DSPC liposomes with cholesterol at different ratios. Samples were scanned at 10 °C/min with 5 μg of sample in TA Q200 DSC. Water was used in the reference pan. (C) Release rate of carboxyfluorescein from MLV liposomes prepared with different ratios of DSPC:cholesterol at (at 20 °C). Results are expressed as the means of three experiments ± S.D.

liquid-crystalline phospholipid bilayers, decreases bilayer permeability, abolishes the gel-to-liquid phase transition endotherm of bilayers (Fig. 5B) and reduces the efflux of the entrapped drug, resulting in prolonged drug retention when present in sufficient quantity (Fig. 5C).

Liang et al. (2004) have also performed stability measurements of cholesterol containing liposomes of L-α-lysophosphatidylcholine (Egg PC) using atomic force microscopy (AFM), demonstrating that the addition of cholesterol to the phospholipid bilayer increased the membrane's mechanical stiffness, leading to increases in membrane cohesion. They concluded that cholesterol content determines the vesicle rigidity and thus its stability (Liang et al., 2004). In this instance, whilst the lipids have a low transition temperature, enhanced bilayer stability with the addition of cholesterol was again shown and, therefore, abolition of the liquid crystalline temperature is not a pre-requisite for enhanced stability.

2.2. Investigating potential alternatives to cholesterol

Although cholesterol incorporation into the liposome bilayer can enhance liposome stability, we have previously shown that the presence of cholesterol reduces drug loading within liposome bilayer potentially due to competition for space within the bilayer (Mohammed et al., 2004). Therefore, finding alternative liposome stabilisers, which do not reduce drug loading, would be beneficial.

Recently within our laboratories, we have performed studies on solubilisation of drugs within liposomal bilayers and looked into using fatty alcohols as alternatives to cholesterol (Ali et al., 2010). The loading and release rates of diazepam, ibuprofen, midazolam and propofol (all poorly soluble drugs) when incorporated within the bilayer of multilamellar (MLV) liposomes formulated using a range of cholesterol (0–33 mol/mol%) or fatty alcohol (tetradecanol, hexadecanol and octadecanol) concentrations was investigated and correlated with the molecular packing of these surfactant mixtures in Langmuir monolayer studies. Unlike water soluble drugs, results of drug loading studies showed that increasing cholesterol content in liposome formulations reduces the drug loading in all four of the poorly soluble drugs tested. However, higher cholesterol content in the bilayer was shown to reduce drug release, similar as to what is expected for water soluble drugs. The presence of cholesterol in the formulation also changed the bilayer-loaded drug release kinetics from zero order to first order, with none of the formulations following a diffusion controlled matrix release in line with other bilayer-loaded systems (Hathout et al., 2007; Nounou et al., 2006).

Given the negative effect of cholesterol on drug bilayer loading, we investigated three different fatty alcohols as alternatives to cholesterol (Ali et al., 2010) based on their previous application in niosome systems (Devaraj et al., 2002). Bilayer loading of propofol showed no significant difference between liposome formulations containing cholesterol or those containing the fatty alcohols tested at lower concentrations (11 molar%). However, at higher concentrations (33%), liposomes prepared with tetradecanol (Fig. 4B) had drug loading of around 20% compared to ~12% for equivalent cholesterol formulations, indicating that cholesterol content had a more profound effect on bilayer drug loading than tetdradecanol (Ali et al., 2010). In terms of drug retention, tetradecanol formulations showed faster drug release profiles compared to their cholesterol counterparts.

To compare the geometrical interactions between lipid and fatty alcohol molecules versus those with cholesterol, monolayer studies

Table 2
Extrapolated area and collapse pressure of pure DSPC, DPPC, DMPC monolayers at the air–water interface at temperature of 20 °C. Results are expressed as the means of three experiments.

Lipid	Alkyl tail length (number of carbons)	Extrapolated area at zero pressure (Å^2/molecule)	Collapse pressure (mN/m)
DSPC	18	51.0	63.2
DPPC	16	55.8	64.3
DMPC	14	65.5	56.2

were undertaken. In the case of surfactant mixtures, as with cholesterol studies, all isotherms were found to lie between the ranges of those of the pure components (Ali et al., 2010). In both cases, the measured mean molecular areas for mixed surfactant monolayers (DSPC:chol and DSPC:tetradecanol) were smaller than theoretically predicted for the ideal mixtures, suggesting tetradecanol was also able to promote a 'condensing effect' similar to cholesterol. However, the condensing effect was more prominent with cholesterol compared to tetradecanol (Ali et al., 2010). This enhanced intercalation of the DSPC:cholesterol compared with DSPC:tetradecanol, detected using monolayer studies, could explain the higher release rates of propofol from the tetradecanol liposomes compared with the cholesterol (Ali et al., 2010).

2.3. Lipid packaging within bilayers: The effect of alkyl chain length

In addition to the effect of bilayer stabilisers such as cholesterol, the influence of lipid alkyl chain length on lipid packaging can be investigated by monolayer studies. For example, the effect of phospholipids alkyl chain length of lipids with identical polar head-group (DSPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (DMPC)) has been examined (Dynarowicz-Latka and Hac-Wydro, 2004). Similar data from our own studies are shown in Fig. 4C and Table 2.

Generally, the surface pressure for the transition from liquid-expanded to liquid-condensed phase increased with decreasing chain length (Barnes and Gentle, 2005). For DSPC, the transition from gaseous to liquid expanded phase occurred at an approximate molecular area of 58 Å^2/molecule and the film collapsed at ~63 mN/m (Fig. 4A). The DPPC isotherm revealed a more expanded shape with gaseous to liquid-expanded transition taking place at ~95 Å^2/molecule (Fig. 4C). The switch from liquid-expanded to liquid-condensed occurred at surface pressure of approximately 10 mN/m, with monolayer finally collapsing at about 64 mN/m. The DMPC isotherm was the least condensed and reveals that this lipid is in a liquid-expanded phase at the start of the compression process (~125 Å^2/molecule) and thereon converts to the liquid-condensed phase at around a surface pressure of 18–20 mN/m (Fig. 4A). The collapse pressure for DMPC is the lowest of the three lipids (around 56 mN/m), suggesting the lipid monolayer (and subsequently bilayers) to be least stable, as it has been reported that stability linearly correlates with the pressure at point of collapse (Dynarowicz-Latka and Hac-Wydro, 2004). The limiting mean molecular area for DSPC, DPPC and DMPC were 51.0, 55.8 and 65.5 Å^2/molecule, respectively (Table 2). The trend could be attributed to degree of cohesion between the chains. The longer the chain, the greater the van der Waal's attraction promoting stronger cohesion, thereby condensing the isotherm (Hac-Wydro et al., 2007). In this homologous series of long-chain compounds, the attraction between the chains will dictate how closely packed they arrange themselves. DSPC being the longest lipid, will have the strongest chain–chain interactions, resulting in closely packed chains (Gaines, 1966); this being reflected in its isotherm being steep and nearly straight. With decreasing chain length (i.e. DMPC < DPPC < DSPC), this cohesion depreciates, yielding films that become more 'gaseous' (Davies and Rideal, 1963). This work supports the rational of longer chain alkyl lipids producing more stable liposomes, since the longer alkyl chains, with stronger cohesion, results in less leakage from liposomes, as has been previously reported (e.g. Mohammed et al., 2004; Taylor et al., 1990). With reference to drugs incorporated within the liposomal membranes, this could support longer retention within the bilayers if good cohesion between the lipids is still supported.

2.4. Investigating the stability of liposomes using monolayer studies

In addition to their roles as drug delivery vehicles, liposomes are known to be highly effective vaccine adjuvants, such as those prepared from the cationic lipid dimethyldioctadecylammonium bromide (DDA) (Fig. 4D) and the immunostimulatory agent α,α′-trehalose 6,6′-dibenate (TDB) (Christensen et al., 2007; Kirby et al., 2008a,b). Combination of DDA/TDB and Ag85B-ESAT-6, which is a tuberculosis antigen, has consistently shown to produce good immune responses in animal studies (Henriksen-Lacey et al., 2010a,b; Holten-Andersen et al., 2004).

Focusing on the DDA/TDB system, both the stabilising interaction of TDB within the DDA bilayer, and the interaction of DDA/TDB at the aqueous interface with sugars (trehalose, glucose and sucrose) in an aqueous environment, was investigated using Langmuir monolayer studies (Christensen et al., 2008). Previous studies had shown that the incorporation of TDB into liposomes prepared from DDA effectively stabilised DDA liposomes, in terms of avoiding vesicle aggregation and antigen loss (Davidsen et al., 2005). To investigate this further; Langmuir–Blodgett studies were conducted. Monolayers of DDA or DDA/TDB mixtures were compared, and it was shown that the presence of TDB increased the surface pressure in the compressed state compared to that of pure DDA (67 mN/m vs 47 mN/m, respectively), suggesting that TDB was able to induce stronger interactions with the water phase than DDA (Christensen et al., 2008). This increased hydration of lipid/water interface with the presence of TDB supports the previous work demonstrating the enhanced stability shown by DDA/TDB liposomes compared to DDA only systems (Davidsen et al., 2005). Recently, monolayer studies were also employed to investigate the interactions of both DSPC and DDA with a new glycolipid α-galactosylceramide analogue, threitol ceramide, which successfully activates invariant natural killer T (*i*NKT) cells and overcomes the problematic *i*NKT cell activation-induced anergy (Kaur et al., in press). Langmuir–Blodgett studies suggest both DSPC and DDA stack within the monolayer co-operatively with threitol ceramide molecules, with no condensing effect. However, whilst there was no significant difference between the collapse pressure of DSPC and DSPC:threitol ceramide mixtures, the addition of threitol ceramide increased the collapse pressure of DDA 1:1 mixtures compared to DDA alone (Kaur et al., in press). This suggests that when cationic lipids are used, a mixed system may be preferential to improve liposome stability.

To further improve the longer-term stability of DDA:TDB liposomes and circumvent the need for 'cold-chain' supply, freeze-drying techniques have been considered (Christensen et al., 2007; Mohammed et al., 2010). The freeze-drying process can be harmful for liposomes, as liposome fusion and/or drug leakage can occur during the freezing, dehydration and subsequent rehydration process (Crowe et al., 1987, 1986). The use of cryo/lyoprotectants such are trehalose, sucrose and some amino acids can be added to the liposome suspension prior to the freeze-drying process to protect the liposomes (Crowe et al., 1994; Harrigan et al., 1990;

Mohammed et al., 2006; Sun et al., 1996; Wolfe and Bryant, 1999). The mechanism of action of these disaccharides is still not clear, with three different hypotheses being proposed: the water replacement hypothesis (Crowe et al., 1987), the glassy matrix theory (Wolfe and Bryant, 1999) and the kosmotropic effect theory (Branca et al., 1999; Koynova et al., 1997).

The theory of the water replacement hypothesis (Crowe et al., 1987) explains that the cryo/lyoprotectant sugar molecules replace the water on the membrane surface through interactions with the head groups of the lipids. Therefore, during the freeze-drying process the phospholipid head group spacing will be retained, supporting the bilayer structure, preventing drug leakage. This theory has been supported using Langmuir–Blodgett monolayer studies (Crowe et al., 1984; Demel et al., 1998; Lambruschini et al., 2000), DSC (Crowe et al., 1994; Sun et al., 1996), molecular simulation (Doxastakis et al., 2005; Skibinsky et al., 2005) and Fourier transform infrared spectroscopy (FTIR) (Crowe et al., 1994; Luzardo et al., 2000). With regard to the Langmuir–Blodgett studies, Demel et al. (Demel et al., 1998) investigated the effect of fructans (fructose polymers which protect plants from drought effect) on surface pressure of phospholipids and compared it with sucrose and trehalose, to show the role of saccharides in the protection against harmful effects of drought. In this study they looked at possible interactions of fructan in the sub-phase with membranes by using monolayer lipids of DPPC, 1,2-ditetradecanoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). Their results revealed that fructan has a similar, but more pronounced effect on surface pressure than trehalose or sucrose. Further, it was shown these saccharides were able to minimise and, in some instances, prevent phase transition in monolayer lipids, thereby helping lipid membranes to survive from harmful effects of dehydration and cooling or drought effect (Demel et al., 1998). Lambruschini et al., in a similar study, examined the effect of trehalose on monolayers of DPPC and demonstrated that trehalose interacts with polar headgroups of the lipid, causes expansion of the isotherm and obstructs the formation of the liquid condensed phase. They concluded that these data are in line with water replacement hypothesis, as trehalose forms hydrogen bonds with the membrane polar headgroups and replaces the water of hydration at the membrane-fluid interface; therefore, this maintains the headgroups at their hydrated position (Lambruschini et al., 2000).

Langmuir–Blodgett studies were also employed to investigate the interactions of sugars with the DDA:TDB/aqueous phase interface to elucidate further information regarding the ability of trehalose to stabilise DDA/TDB during freeze-drying (Christensen et al., 2008). Results revealed that the presence of trehalose in the aqueous sub-phase resulted in a further increase in surface pressure for DDA/TDB monolayers compared to DDA/TDB in water; however, a similar effect was not seen for DDA only monolayers, indicating that trehalose interacts with TDB in monolayer and not with DDA alone. A similar enhancement of the surface pressure at the collapsing point was found with DDA/TDB and sucrose, but not with glucose (Christensen et al., 2008). These direct interactions, between trehalose in the aqueous phase and lipids, may be a factor in the lyoprotection action offered by trehalose during the freeze-drying process. In line with the water replacement hypothesis, by increasing the concentration of bulk trehalose at the interface, a reduction in water at the interface could result (due to the kosmotropic properties of trehalose), thereby protecting the bilayers from dehydration (Christensen et al., 2008).

2.5. Electrostatic interactions of polyelectrolytes with interfaces

In addition to their outlined role as vaccine adjuvants, cationic liposomes are an appropriate vector for nucleic acid delivery (McNeil and Perrie, 2006; McNeil et al., 2010). The main considerations in these formulations are the electrostatic binding of the nucleic acids to the liposomes, and the interactions between the ensuing lipoplex with cell membranes. To investigate such interactions, monolayer studies again prove advantageous. There are several studies investigating the electrostatic interactions between DNA and monolayer lipid at the air/water interface (Cardenas et al., 2005; Sun et al., 2004; Vranken et al., 2002). For example, the interaction between DNA and the cationic lipid DDA at water/air interface was investigated by measuring the surface pressure and molecular area of the lipid in different sub-phases at different charge ratios (Sun et al., 2004). Using a combination of Langmuir–Blodgett and Brewster angle microscopy, they were able to show that the presence of DNA in the aqueous sub-phase resulted in a re-arrangement in the packing configuration of the lipids, a subsequent change in the morphology of the monolayer, an increased surface pressure for any given mean molecular area, and an increased collapse pressure for the lipid monolayers (Sun et al., 2004).

Cardenas et al., continued these studies by investigating the interaction between DNA with DDA or DSPC monolayers, and mixed DDA:DSPC monolayers at the air/water interface (Cardenas et al., 2005). Addition of DNA to the sub-phase resulted in the DDA Π–*A* isotherm shifting to a larger mean molecular area, and the disappearance of the liquid expanded-liquid condensed phase transition of DDA due to its electrostatic interactions with DNA. As would be expected, investigating the zwitterionic DSPC monolayer isotherm in the presence of DNA in sub-phase revealed no noticeable change in molecular area, nor surface pressure of DSPC monolayer in presence of DNA (Cardenas et al., 2005). When mixtures of DDA:DSPC, in two ratios (1:1 and 3:1), and in presence and absence of DNA were studied, the observed mean molecular area in the presence of DNA was smaller than the calculated ideal, potentially due to the electrostatic repulsion between the charged amino groups decreasing and subsequently the molecules can pack more tightly (Cardenas et al., 2005).

Whilst adsorption of moieties to the surface of bilayers can be exploited to improve drug loading and delivery, there are instances where surface adsorption is detrimental, for example in the case of interfacial reactions catalyzed by phospholipids (Brezesinski and Mohwald, 2003). Studying these enzymatic interactions using Langmuir systems has given new insight into the mechanisms of hydrolysis. Phospholipase A_2 (PLA_2) is a small calcium-dependent enzyme that is responsible for the selective hydrolysis of the *sn*-2 ester linkage of phospholipids to produce a fatty acid and a lysophospholipid (Menashe et al., 1981; Pieterso et al., 1974; Verger et al., 1973). PLA_2 is interfacially active and has a 10^4-fold higher activity for substrates in the form of micelles, vesicles or membranes compared to the substrate dispersed as monomers, and its enzymatic reaction starts by adsorption of the enzyme at the aggregate interface (Brezesinski and Mohwald, 2003). However, both the composition and phase structure of the bilayer have been shown to play an important role in dictating the hydrolysis efficiency of this enzyme. Using Langmuir studies in combinations with other methods, it has been shown that PLA_2 preferentially binds, and thus exhibits maximal enzymatic activity, at the phase boundaries between liquid expanded and liquid condensed (Brezesinski and Mohwald, 2003). In natural cell membranes and mixed liposome compositions these boundaries could be created by local differences in lipid composition whilst in single component DPPC monolayer a two-phase coexistence region between liquid expanded and condensed phases was produced at lateral pressures of approximately 6 mN/m (Brezesinski and Mohwald, 2003). However these conditions are not required for other phospholipase; in the case of phosphatidylcholine phosphohydrolase (PLD), a lipolytic enzyme that catalyzes the hydrolysis of phos-

pholipids to phosphatidic acids, the highest hydrolysis efficiency was found in the liquid expanded region of a DPPC monolayer (Brezesinski and Mohwald, 2003). The addition of charged phospholipids, such as phosphatidic acid, into a monolayer film had also previously been shown to enhance the penetration of the enzyme and its activity (Hirasawa et al., 1981) presumably due to the charged lipid expanding the monolayer. From the observation that the maximum activity of PLD (which, in contrast to PLA_2, acts in the hydrophilic region of the phospholipid) was found in the more disordered phase, the authors conclude that fluidity and defects in the monolayer structures are more important than a pre-orientation of the substrate induced by enzyme adsorption (Brezesinski and Mohwald, 2003). Therefore, it can be seen that with further investigations such as those outlined, more detail on the formulation options that could prohibit or accelerate (depending on the desired outcome) enzyme degradation of liposomes could be gained.

3. Conclusions

The purpose of this review was to illustrate the important role monolayer studies can play in understanding the bilayer mechanics of liposome formulation, in addition to standard characterisation studies generally undertaken (such as size and surface charge measurements, microscopy studies, thermodynamic properties, drug loading and release efficacy, stability and toxicity, as well as *in vitro* and *in vivo* studies, etc.) Whilst effectively considering only half a bilayer, in a flat rather than curved structure, Langmuir–Blodgett monolayer studies on lipid mixtures have shown to offer a variety of applications to support our continued appreciation of the (nano)mechanical properties of liposomes, and further underpin our understanding of liposomal drug delivery systems.

References

Ali, M.H., Kirby, D.J., Mohammed, A.R., Perrie, Y., 2010. Solubilisation of drugs within liposomal bilayers: alternatives to cholesterol as a membrane stabilising agent. J. Pharm. Pharmacol. 62, 1646–1655.

Bangham, A.D., Standish, M.M., Watkins, J.C., 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. 13, 238–252.

Barnes, Gentle, 2005. Interfacial Science: An Introduction. Oxford University Press Inc, New York.

Branca, C., Magazu, S., Maisano, G., Migliardo, P., 1999. Anomalous cryoprotective effectiveness of trehalose: Raman scattering evidences. J. Chem. Phys. 111, 281–287.

Brezesinski, G., Mohwald, H., 2003. Langmuir monolayers to study interactions at model membrane surfaces. Adv. Colloid Interface Sci. 100-102, 563–584.

Cardenas, M., Nylander, T., Jonsson, B., Lindman, B., 2005. The interaction between DNA and cationic lipid films at the air–water interface. J. Colloid Interface Sci. 286, 166–175.

Chechel, O.V., Nikolaev, E.N., 1990. Application of Langmuir–Blodgett-films as registrating layers of optical information carriers. Usp Khim+ 59, 1888–1903.

Christensen, D., Foged, C., Rosenkrands, I., Nielsen, H.M., Andersen, P., Agger, E.M., 2007. Trehalose preserves DDA/TDB liposomes and their adjuvant effect during freeze-drying. Biochim. Biophys. Acta 1768, 2120–2129.

Christensen, D., Kirby, D., Foged, C., Agger, E.M., Andersen, P., Perrie, Y., Nielsen, H.M., 2008. alpha,alpha′-Trehalose 6,6′-dibehenate in non-phospholipid-based liposomes enables direct interaction with trehalose, offering stability during freeze-drying. Biochim. Biophys. Acta 1778, 1365–1373.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., Aurell Wistrom, C., 1987. Stabilization of dry phospholipid bilayers and proteins by sugars. Biochem. J. 242, 1–10.

Crowe, J.H., Leslie, S.B., Crowe, L.M., 1994. Is vitrification sufficient to preserve liposomes during freeze-drying? Cryobiology 31, 355–366.

Crowe, J.H., Whittam, M.A., Chapman, D., Crowe, L.M., 1984. Interactions of phospholipid monolayers with carbohydrates. Biochim. Biophys. Acta 769, 151–159.

Crowe, L.M., Womersley, C., Crowe, J.H., Reid, D., Appel, L., Rudolph, A., 1986. Prevention of fusion and leakage in freeze-dried liposomes by carbohydrates. Biochim. Biophys. Acta 861, 131–140.

Davidsen, J., Rosenkrands, I., Christensen, D., Vangala, A., Kirby, D., Perrie, Y., Agger, E.M., Andersen, P., 2005. Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from M. tuberculosis (trehalose 6,6′-dibehenate)—a novel adjuvant inducing both strong CMI and antibody responses. Biochim. Biophys. Acta 1718, 22–31.

Davies, J., Rideal, E., 1963. Inlerfacial Phenomena. Academic, San Diego, Calif.

Demel, R., Geurts van Kessel, W., Van Deenen, L., 1972. The properties of polyunsaturated lecithins in monolayers and liposomes and the interactions of these lecithins with cholesterol. Biochim. Biophys. Acta (BBA) – Biomembr. 266, 26–40.

Demel, R.A., Dorrepaal, E., Ebskamp, M.J., Smeekens, J.C., de Kruijff, B., 1998. Fructans interact strongly with model membranes. Biochim. Biophys. Acta 1375, 36–42.

Devaraj, G.N., Parakh, S.R., Devraj, R., Apte, S.S., Rao, B.R., Rambhau, D., 2002. Release studies on niosomes containing fatty alcohols as bilayer stabilizers instead of cholesterol. J. Colloid Interface Sci. 251, 360–365.

Doxastakis, M., Sum, A.K., de Pablo, J.J., 2005. Modulating membrane properties: the effect of trehalose and cholesterol on a phospholipid bilayer. J. Phys. Chem. B 109, 24173–24181.

Dynarowicz-Latka, P., Dhanabalan, A., Oliveira Jr., O.N., 2001. Modern physicochemical research on Langmuir monolayers. Adv. Colloid Interface Sci. 91, 221–293.

Dynarowicz-Latka, P., Hac-Wydro, K., 2004. Interactions between phosphatidylcholines and cholesterol in monolayers at the air/water interface. Colloids Surf. B 37, 21–25.

Egelhaaf, R., Epand, R., Maekawa, S., 2003. The arrangement of cholesterol in membranes and binding of NAP-22. Chem. Phys. Lipids 122, 33–39.

Franklin, B., 1774. Of the stilling of waves by means of oil. Philos. Trans. Res. Soc. 64, 445–460.

Fulford, G., 1968. Pouring holy oil on troubled water. Isis 59, 198–199.

Gaines, G.L., 1966. Insoluble Monolayers at Liquid–Gas Interfaces. John Wiley & Sons Inc, New York.

Gregoria, G., 1973. Drug entrapment in liposomes. FEBS Lett. 36, 292–296.

Gregoriadis, Ryman, B.E., 1971. Liposomes as carriers of enzymes or drugs—new approach to treatment of storage diseases. Biochem. J. 124, 58P.

Gregoriadis, G., Davis, C., 1979. Stability of liposomes in vivo and in vitro is promoted by their cholesterol content and the presence of blood cells. Biochem. Biophys. Res. Commun. 89, 1287–1293.

Gregoriadis, G., Senior, J., 1980. The phospholipid component of small unilamellar liposomes controls the rate of clearance of entrapped solutes from the circulation. FEBS Lett. 119, 43–46.

Hac-Wydro, K., Wydro, P., Jagoda, A., Kapusta, J., 2007. The study on the interaction between phytosterols and phospholipids in model membranes. Chem. Phys. Lipids 150, 22–34.

Harrigan, P.R., Madden, T.D., Cullis, P.R., 1990. Protection of liposomes during dehydration or freezing. Chem. Phys. Lipids 52, 139–149.

Hathout, R.M., Mansour, S., Mortada, N.D., Guinedi, A.S., 2007. Liposomes as an ocular delivery system for acetazolamide: in vitro and in vivo studies. AAPS PharmSciTech 8, 1.

Henriksen-Lacey, M., Bramwell, V.W., Christensen, D., Agger, E.M., Andersen, P., Perrie, Y., 2010a. Liposomes based on dimethyldioctadecylammonium promote a depot effect and enhance immunogenicity of soluble antigen. J. Controlled Release 142, 180–186.

Henriksen-Lacey, M., Christensen, D., Bramwell, V.W., Lindenstrom, T., Agger, E.M., Andersen, P., Perrie, Y., 2010b. Liposomal cationic charge and antigen adsorption are important properties for the efficient deposition of antigen at the injection site and ability of the vaccine to induce a CMI response. J. Controlled Release 145, 102–108.

Hirasawa, K., Irvine, R.F., Dawson, R.M., 1981. The hydrolysis of phosphatidylinositol monolayers at an air/water interface by the calcium-ion-dependent phosphatidylinositol phosphodiesterase of pig brain. Biochem. J. 193, 607–614.

Holten-Andersen, L., Doherty, T.M., Korsholm, K.S., Andersen, P., 2004. Combination of the cationic surfactant dimethyl dioctadecyl ammonium bromide and synthetic mycobacterial cord factor as an efficient adjuvant for tuberculosis subunit vaccines. Infect. Immun. 72, 1608–1617.

Hughes, G.A., 2005. Nanostructure-mediated drug delivery. Dm-Dis. Mon. 51, 342–361.

Kaur, R., Chen, J., Dawoodji, A., Cerundolo, V., Garcia-Diaz, Y.R., Wojno, J., Cox, L.R., Besra, G.S., Moghaddam, B., Perrie, Y. Preparation, characterisation and entrapment of a non-glycosidic threitol ceramide into liposomes for presentation to invariant natural killer T cells. J. Pharm. Sci., in press, doi:10.1002/jps.22500.

Kirby, C., Clarke, J., Gregoriadis, G., 1980. Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. Biochem. J. 186, 591–598.

Kirby, D.J., Rosenkrands, I., Agger, E.M., Andersen, P., Coombes, A.G., Perrie, Y., 2008a. Liposomes act as stronger sub-unit vaccine adjuvants when compared to microspheres. J. Drug Targeting 16, 543–554.

Kirby, D.J., Rosenkrands, I., Agger, E.M., Andersen, P., Coombes, A.G., Perrie, Y., 2008b. PLGA microspheres for the delivery of a novel subunit TB vaccine. J. Drug Targeting 16, 282–293.

Koynova, R., Brankov, J., Tenchov, B., 1997. Modulation of lipid phase behavior by kosmotropic and chaotropic solutes—experiment and thermodynamic theory. Eur. Biophys. J. 25, 261–274.

Kuroda, T., Nakata, S., Nakamura, T., Ishii, M., Neya, K., Inomoto, O., Ohya, T., Kai, S., Fujii, K., Hayashi, D., 2000. The Marangoni effect and its artistic application. Forma 15, 203–204.

Lambruschini, C., Relini, A., Ridi, A., Cordone, L., Gliozzi, A., 2000. Trehalose interacts with phospholipid polar heads in Langmuir monolayers. Langmuir 16, 5467–5470.

Langmuir, I., 1917. The constitution and fundamental properties of solids and liquids. II. Liquids.1. J. Am. Chem. Soc. 39, 1848–1906.

Langmuir, I., 1920. The mechanism of the surface phenomena of flotation. Trans. Faraday Soc. 15, 62–74.

Liang, X., Mao, G., Ng, K.Y., 2004. Mechanical properties and stability measurement of cholesterol-containing liposome on mica by atomic force microscopy. J. Colloid Interface Sci. 278, 53–62.

Luzardo, M.C., Amalfa, F., Nunez, A.M., Diaz, S., Biondi De Lopez, A.C., Disalvo, E.A., 2000. Effect of trehalose and sucrose on the hydration and dipole potential of lipid bilayers. Biophys. J. 78, 2452–2458.
Lyklema, J., 2000. Langmuir monolayers. In: Fundamentals of Interface and Colloid Science, 1st ed. Academic Press, London, pp. 3.15–13.19.
Manosroi, A., Wongtrakul, P., Manosroi, J., Sakai, H., Sugawara, F., Yuasa, M., Abe, M., 2003. Characterization of vesicles prepared with various non-ionic surfactants mixed with cholesterol. Colloids Surf. B 30, 129–138.
McNeil, S.E., Perrie, Y., 2006. Gene delivery using cationic liposomes. Expert Opin. Ther. Patents 16, 1371–1382.
McNeil, S.E., Vangala, A., Bramwell, V.W., Hanson, P.J., Perrie, Y., 2010. Lipoplexes formulation and optimisation: in vitro transfection studies reveal no correlation with in vivo vaccination studies. Curr. Drug Deliv. 7, 175–187.
Menashe, M., Lichtenberg, D., Guiterrezmerino, C., Biltonen, R.L., 1981. Relationship between the activity of pancreatic phospholipase-A2 and the physical state of the phospholipid substrate. J. Biol. Chem. 256, 4541–4543.
Mohammed, A.R., Bramwell, V.W., Coombes, A.G., Perrie, Y., 2006. Lyophilisation and sterilisation of liposomal vaccines to produce stable and sterile products. Methods 40, 30–38.
Mohammed, A.R., Bramwell, V.W., Kirby, D.J., McNeil, S.E., Perrie, Y., 2010. Increased potential of a cationic liposome-based delivery system: enhancing stability and sustained immunological activity in pre-clinical development. Eur. J. Pharm. Biopharm. 76, 404–412.
Mohammed, A.R., Weston, N., Coombes, A.G., Fitzgerald, M., Perrie, Y., 2004. Liposome formulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability. Int. J. Pharm. 285, 23–34.
Nounou, M.M., El-Khordagui, L.K., Khalafallah, N.A., Khalil, S.A., 2006. In vitro release of hydrophilic and hydrophobic drugs from liposomal dispersions and gels. Acta Pharm. 56, 311–324.
Papahadjopoulos, D., Jacobson, K., Nir, S., Isac, I., 1973. Phase transitions in phospholipid vesicles fluorescence polarization and permeability measurements concerning the effect of temperature and cholesterol. Biochim. Biophys. Acta (BBA) – Biomembr. 311, 330–348.
PietersoF Wa, Vidal, J.C., Volwerk, J.J., Haas, G.H.D., 1974. Zymogen-catalyzed hydrolysis of monomeric substrates and presence of a recognition site for lipid-water interfaces in phospholipase-A2. Biochemistry 13, 1455–1460.
Pockels, A., 1891. Surface tension. Nature 43, 437–439.
Rayleigh, L., 1890. Measurements of the amount of oil necessary in order to check the motions of camphor upon water. Proc. R. Soc. London 47, 364–367.
Rayleigh, L., 1899. Investigations in capillarity: the size of drops. – The liberation of gas from supersaturated solutions. – Colliding jets. – The tension of contaminated water-surfaces. – A curious observation. Phil. Mag. 48, 321–337.
Rogerson, A., Cummings, J., Florence, A., 1987. Adriamycin-loaded niosomes: drug entrapment, stability and release. J. Microencapsul. 4, 321–328.
Semple, S.C., Chonn, A., Cullis, P.R., 1996. Influence of cholesterol on the association of plasma proteins with liposomes. Biochemistry 35, 2521–2525.
Skibinsky, A., Venable, R.M., Pastor, R.W., 2005. A molecular dynamics study of the response of lipid bilayers and monolayers to trehalose. Biophys. J. 89, 4111–4121.
Sulkowski, W.W., Pentak, D., Nowak, K., Sulkowska, A., 2005. The influence of temperature, cholesterol content and pH on liposome stability. J. Mol. Struct. 744, 737–747.
Sun, L., Xu, M., Hou, X.L., Wu, L.X., 2004. In-situ observation of the aggregated morphology and interaction of dialkyldimethylammonium bromide with DNA at air/water interface by Brewster angle microscopy. Mater. Lett. 58, 1466–1470.
Sun, W.Q., Leopold, A.C., Crowe, L.M., Crowe, J.H., 1996. Stability of dry liposomes in sugar glasses. Biophys. J. 70, 1769–1776.
Taylor, K.M.G., Morris, R.M., 1995. Thermal-analysis of phase-transition behavior in liposomes. Thermochim. Acta 248, 289–301.
Taylor, K.M.G., Taylor, G., Kellaway, I.W., Stevens, J., 1990. Drug entrapment and release from multilamellar and reverse-phase evaporation liposomes. Int. J. Pharm. 58, 49–55.
Verger, R., Mieras, M.C.E., Dehaas, G.H., 1973. Action of phospholipase a at interfaces. J. Biol. Chem. 248, 4023–4034.
Vranken, N., Van der Auweraer, M., De Schryver, F.C., Lavoie, H., Salesse, C., 2002. Formation of highly oriented domains of a thiacarbocyanine dye in a monolayer at the air–water interface. Langmuir 18, 1641–1648.
Wiseman, H., Quinn, P., Halliwell, B., 1993. Tamoxifen and related compounds decrease membrane fluidity in liposomes. mechanism for the antioxidant action of tamoxifen and relevance to its anticancer and cardioprotective actions? FEBS Lett. 330, 53–56.
Wolfe, J., Bryant, G., 1999. Freezing, drying, and/or vitrification of membrane–solute–water systems. Cryobiology 39, 103–129.