12

Biomimetic Model Membrane Systems Serve as Increasingly Valuable *in Vitro* Tools

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1. Introduction

Biological membranes contain a multitude of lipids, proteins, and carbohydrates unique for any given cell or organism, and are a critical component of many biological processes. Animal and cell cultures have been used to understand these biological processes at the membrane level and more traditionally, to assess toxicity. However, the complex composition does not allow understanding of the detailed role of each membrane component, such as individual lipid species. This insight can be obtained from using simplified model systems, which include various kinds of vesicles (unilamellar or multilamellar), micelles, monolayers at an air-water interface, planar lipid bilayers/black lipid membranes, bicelles (bilayered micelles) and supported bilayers. All systems allow detailed control of composition and experimental conditions, and have been used to mimic various different membrane types, such as mammalian and bacterial.

Using various physicochemical techniques including nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC), isothermal calorimetry (ITC), electron spin resonance, fluorescence spectroscopy, and X-ray diffraction, it is possible to investigate the mechanisms of membrane toxicity through differential changes in acyl chain melting temperature, membrane fluidity, and permeability of these different membrane models upon ligand binding. Moreover, the effects of ions (Na^+, K^+, Li^+, Ca^{2+}, Mg^{2+}, Ba^{2+}), toxic heavy metals (Hg^{2+}, Cd^{2+}) and a variety of drugs (e.g. Ellipticine for tumors and H1N1 virus or cyclosporine A to prevent graft rejection) have been evaluated on mammalian systems. For bacterial model membranes, the effects of antimicrobial peptides, antibiotics, the interaction of proteins with model membranes, and the insertion or reconstitution of membrane proteins into such systems have also been investigated.

When interpreting the results, it is important to note that some models may be better representatives of the natural membrane than others, and consequently, some results more relevant than others. Factors to consider include - but are not limited to - lipid composition, membrane curvature, or ionic strength of the solution, which all impart certain characteristics on the membrane model, influencing the results. Thus, while a single-component lipid model can be informative, it is important to consider its applications and limitations.

Overall, this chapter will provide insight as to the different lipid models used to mimic mammalian and bacterial membranes and how they have been found to be effective and useful research tools. Future development of these membrane models to more closely mimic

the composition and complexity of the natural membrane will provide further insight into the mechanisms of membrane processes in biological systems.

1.1 Membranes

As lipids are small amphiphilic molecules, there are three aspects that define the physical characteristics of a lipid: the polar headgroup, the hydrophobic acyl chains and the interface between them. There are several different lipid headgroup classes, each with unique chemical properties. Some biological headgroups are negatively charged and exhibit charge-charge repulsions, which result in larger effective cross-sectional areas (Cullis *et al.*, 1986). However, the charge, and thus the area, is subject to the experimental conditions. Changes in the pH of the solution can impart or eliminate charges from the lipid based on the specific pKa values of the headgroup. The presence of mono- or divalent cations can serve to shield or neutralize the charge-charge repulsions, thus decreasing their effective cross-sectional area and consequently altering the properties of the lipid (Tate *et al.*, 1991).

Unlike the polar headgroups, which can be altered by the environment, the behavior of the hydrophobic acyl chains is mainly based on their chemical structure. Acyl chains are typically 14 to 22 carbons long and can be fully saturated, mono-unsaturated, or poly-unsaturated. Length and degree of saturation play a major role in lipid packing and the behaviour of the membrane. Fully saturated lipids pack more tightly than lipids with unsaturated acyl chains, changing the fluidity, transition temperature, and the lateral membrane pressure profile. Longer chains also have greater van der Waals interactions that stabilize membranes (Birdi, 1988). In contrast, the increased cross-sectional area of unsaturated lipids enhances membrane fluidity (de Kruijff, 1997).

Membranes are known to play an important role in many crucial biological functions, be it as the cellular membrane or as barrier of intracellular compartments. The fluid mosaic model of biological membranes (Singer and Nicolson, 1972) was groundbreaking in the understanding of membrane dynamics and organization, and the main concept of free diffusion of lipid and protein molecules within a dynamic fluid bilayer is still relevant. Current research supports the fact that several proteins are sensitive to the presence of specific lipids, with some experiencing an increase in activity while others require the presence of certain lipids for proper membrane insertion or multimeric stability (van der Does *et al.*, 2000; van Dalen *et al.*, 2002; van den Brink-van der Laan *et al.*, 2004).

However, one of the main emphases of the fluid mosaic model was that proteins and lipids were free to diffuse within the membrane, distributed randomly throughout with no regions of distinct composition. Research now supports the existence of lipid domains, distinct regions of specific lipid composition within the fluid bilayer (Rietveld and Simons, 1998; Zerrouk *et al.*, 2008). These domains possess unique physical properties and could be vital for many cell processes such as signal transduction, cell adhesion, and the function of several membrane proteins (Simons and Ikonen, 1997; Harder *et al.*, 1998).

1.2 The mammalian membrane

Mammalian membranes are primarily composed of phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylserine (PS), phosphatidylethanolamine (PE), and cholesterol (Chol) lipid species in various ratios depending on cell type. The human erythrocyte membrane, one of the best characterized systems, is composed of 19.5% (w/w) of water, 39.5% of proteins, 35.1% of lipids, and 5.8% of carbohydrates (Yawata, 2003).

Lipids are asymmetrically distributed in the bilayer, in which 65-75% of PC and more than 85% of SM are found in the outer leaflet whereas 80-85% of PE and more than 96% of PS are found on the inner one (Zachowski, 1993). At physiological pH, SM, PC and PE are neutrally charged, PS is negatively charged and Chol is uncharged altogether. SM consists of a phosphocholine moiety ester-linked to the 1-hydroxy group of ceramide.

The zwitterionic PC makes up a large component of mammalian lipid model systems and therefore the membrane surface will primarily have a neutral charge. PE, another zwitterionic lipid species, can form the non-lamellar inverted hexagonal phase, affecting lipid-packing properties for membrane fusion or liposome budding. Negatively charged phospholipids like PS affect membrane functioning as the charge is influenced by pH and divalent ions like Ca^{2+} and Mg^{2+} (Vandijck et al., 1978). Moreover, PS has been shown to be an important lipid species in apoptotic processes in the presence of Hg^{2+}, for example (Eisele *et al.*, 2006).

It is important to mimic the fluidity properties of the biological membrane in mammalian model systems by varying the hydrophobic acyl chain in terms of length and saturation (e.g. palmitic acid versus oleic acid). Thus, egg PC, extracted from egg yolk, has also been used as it provides the required variety. Chol content in mammalian biomimetics may play an important role in modulating membrane fluidity and lipid raft formation (Simons and Toomre, 2000). Hence, by varying the composition of the lipid mixtures, these models will better mimic the heterogeneous nature of mammalian membranes.

1.3 The bacterial membrane

Based on the structure of their cell wall, bacteria are generally divided into two broad classes: Gram positive and Gram negative. The former includes those bacteria containing a single cell membrane surrounded by a thick layer of peptidoglycan, while the latter includes those with a thin layer of peptidoglycan surrounded by a second membrane (Dowhan, 1997). *E. coli* is a Gram negative bacterium, consisting of both an outer and an inner membrane. While the outer membrane is dominated by lipopolysaccharides, the inner membrane is composed of phospholipids PE, phosphatidylglycerol (PG), and cardiolipin (CL). PE is the most abundant species, making up 70-80% of the lipid portion of the inner membrane, while PG occupies 15-20% and CL roughly 5%, with these proportions varying depending on the mitotic state of and environmental stress imposed on the bacterium (Dowhan, 1997; Cronan, 2003). The different phospholipids impart unique physical properties on the membrane, which also facilitate bacterial adaptation to changing conditions. As mentioned, PE is a zwitterionic head group with both a positive and negative charge in neutral balance. The cross-sectional area of the headgroup is small compared to that of the acyl chains, and thus, while the conical-shaped PE lipids are part of a bilayer in the *E. coli* inner membrane, they also serve to create curvature stress. It has been shown that PE is an essential component in membrane protein assembly and enzyme function (Dowhan, 1997), and the non-lamellar propensity of some PEs may be an important factor in lipid-protein interactions in the membrane.

The second most abundant phospholipid, PG, has an anionic headgroup at physiological pH and corresponding charge-charge repulsions affect the physical properties of the bilayer. Like PE, PG has been shown to be required for important cellular functions, such as protein translocation across the *E. coli* membrane (Kusters *et al.*, 1991).

CL, also known as diphosphatidylglycerol, is the dimeric form of PG. CL has an anionic headgroup at physiological pH, but could potentially carry two negative charges under

certain conditions (pK1=2.8, pK2>7.5) (Kates *et al.*, 1993). It is unique with four instead of two acyl chains, which in bacteria are typically fully saturated and mono-unsaturated chains with 14, 16, or 18 carbons (Mileykovskaya *et al.*, 2005). The much larger cross-sectional area of the acyl chains compared to the headgroup promotes non-lamellar phase transitions (Lewis and McElhaney, 2009). This tendency to form transient, non-bilayer domains in the membrane is significant for many cellular processes (Rietvald *et al.*, 1994).
In *E. coli* phospholipids, 43% of the acyl chains are fully saturated palmitic acid (C16:0), while the remaining 57% are monounsaturated palmitoleic (C16:1) and oleic (C18:1) acids at 33 and 24%, respectively (Ingraham *et al.*, 1983). This lipid variety may allow for the formation of different polymorphic phases (Hui and Sen, 1989) and lipid domains within the membrane. It has been shown that the specific lipid headgroup and acyl chain composition is responsible for characteristic packing and phase transition behaviours in a lipid monolayer compression system (Kaganer *et al.*, 1999).

1.4 The importance of lipid model systems

Manipulating the lipid content, salt concentration, pH, and other factors of the model systems allows for a greater understanding of the interactions within the membrane (de Kruijff, 1997). The native biological membrane can be mimicked by using natural or synthetic lipids if the lipid composition of the cell type or organism is known. For example, lipids were extracted from erythrocyte membranes and purified by thin layer chromatography before being incorporated into model systems (Keller *et al.*, 1998). Different models have advantages to assess particular interactions. Lipid monolayers enable the study of interactions at the surface of a cell membrane whereas supported lipid bilayers and bicelles allow for the investigation of toxicant interactions with lipid headgroups and other moieties. Vesicles encapsulated with a fluorophore and planar lipid bilayers can also be used to look at metal and drug permeability. Generally, lipid model systems usually lack proteins, making them less fluid than biological membranes (Suwalsky *et al.*, 2000). However, numerous studies have employed single, binary and ternary lipid mixtures in protein-free models to study ion, heavy metal, drug and peptide interactions.

2. Applications of mammalian membrane models

2.1 Essential ions

Various ions such as Ca^{2+}, Zn^{2+} and Mg^{2+} are important in membrane-associated biological processes. Ca^{2+} is involved in resting and action potentials (Akerman and Nicholls, 1983); Zn^{2+} is a nutritionally required element that is central to enzyme function and membrane structure (Bettger and O'Dell, 1981); and Mg^{2+} plays an important role in regulating ion channels (Mubagwa *et al.*, 2007). Hence, the study of essential ions with different biomimetic systems can give insight to their role with the biological membrane.

2.1.1 Essential ions: vesicles

Vesicles can be unilamellar, small (SUVs) or large (LUVs), as well as multilamellar (MLVs) and are most often used to mimic biological membranes since they enclose an aqueous compartment. In conjunction with various physicochemical techniques, these model systems have been used to study ion interactions with lipid bilayers as a function of ion type and concentration, overall ionic strength and lipid structure (head group, acyl chains) as discussed below for simple and more complex matrices.

Dimyristoylphosphatidic acid (DMPA) MLVs have been used in DSC experiments to investigate Ca^{2+} binding which resulted in an increase of T_m from 50-65°C with a decrease in transition enthalpy (Blume, 1985). Furthermore, dipalmitoyl-PC (DPPC) and dioleoyl-PC (DOPC) systems were used for X-ray diffraction and force measurement studies on Ca^{2+} and Mg^{2+} binding (Lis *et al.*, 1981) that showed stronger Ca^{2+} binding at concentrations of 10 and 30 mM. The testing of additional divalent ions resulted in the following order of ion binding to DPPC bilayers: $Ba^{2+} < Mg^{2+} \simeq Co^{2+} < Ca^{2+} \simeq Cd^{2+} \simeq Mn^{2+}$, whereas for DOPC bilayers, $Mg^{2+} < Co^{2+} \simeq Ca^{2+}$. Subsequently, PC lipid species with varied acyl chain composition such as dilauroyl-PC (DLPC), dimyristoyl-PC (DMPC) and distearoyl-PC (DSPC) were compared. In 30 mM $CaCl_2$, the order of binding was determined to be DOPC < DLPC < DMPC $\simeq$ DSPC $\simeq$ DPPC in which Ca^{2+} bound better to longer and saturated acyl chains (Lis *et al.*, 1981). Furthermore, in the presence of 30 mM $CaCl_2$, egg PC bilayers were observed to undergo phase separation when subjected to osmotic stress (Lis *et al.*, 1981). This phenomenon was attributed to the differences in the acyl chains and was further confirmed with 1:1 mixtures of DOPC/DLPC as well as DMPC/DLPC and DOPC/DMPC to a smaller extent. The binary mixtures were shown to be in one phase in pure water and two distinct lamellar phases in 30 mM $CaCl_2$ using X-ray diffraction (Lis *et al.*, 1981).

Single-lipid containing MLVs, composed of DMPC or dimyristoyl-PE (DMPE), were used to study Zn^{2+}-membrane interactions (Suwalsky *et al.*, 1996). Zn^{2+} was shown to interact with DMPE and DMPC bilayers using X-ray diffraction at a concentration as low as 10^{-5} µM. 1,6-diphenyl-1,3,5-hexatriene (DPH) steady state fluorescence anisotropy and Laurdan general polarization values were also observed to increase in the presence of Zn^{2+} in a concentration-dependent manner, indicating a less fluid bilayer.

Effects of Ca^{2+} on binary lipid models of various negatively charged phospholipid MLVs with PC have been investigated using freeze-fracture electron microscopy, ITC and DSC (Vandijck *et al.*, 1978; Blume, 1985; Sinn *et al.*, 2006). Although PG, PS and PA all contain one negative charge, they were shown to exhibit distinct mixing behaviors in the presence of Ca^{2+} (Vandijck *et al.*, 1978). In DMPC/dimyristoyl-PG (DMPG) mixtures, excess Ca^{2+} neutralized the negative charge and shifted the phase transition peak to higher temperatures. For DMPC/dipalmitoyl-PG (DPPG), similar results were seen with a shift in the transition peak and, moreover, a lateral phase separation occurred upon the addition of two carbons to the PG acyl chains (Vandijck *et al.*, 1978). An increase in T_m was also observed for binary mixtures of DMPA/DMPC when Ca^{2+} was added (Blume, 1985).

In DMPC/dimyristoyl-PS (DMPS) systems, increasing concentrations of the PS lipids resulted in a mixture of two types of structures - vesicles and stacked lamellae/cylinders. Interestingly, in DMPC/DMPA matrices, gel phase immiscibility was observed in the presence of Ca^{2+} independent of the PC/PA molar ratio. Ca^{2+} interactions were strongest with PA followed by PS and then PG-containing model systems, showing that in addition to the negative charge, the size of the lipid headgroup also plays an important role (Vandijck *et al.*, 1978).

2.1.2 Essential ions: monolayers

Another frequently used model system is the lipid monolayer at the air-water interface, which allows for the study of surface processes e.g. lipid-ion interactions. Parameters such as lipid composition, subphase, pH and temperature can be controlled in order to better mimic biological conditions. Extracted animal cephalin, consisting primarily of PE and PS, has been used in monolayer model systems to study the effect of Ca^{2+} (Suzuki and

Matsushita, 1968). At a concentration of 10^{-3} M, Ca^{2+} expanded the monolayer on the water subphase. The same research group also extended this study by covering monovalent (Na^{+} and Li^{+}), divalent (Ca^{2+} and Mn^{2+}) and trivalent (Fe^{3+} and In^{3+}) ions (Suzuki and Matsushita, 1969). Monovalent and divalent ions expanded the monolayer whereas the trivalent ions had a condensing effect. It has been proposed that the condensing effect by the trivalent metal ions is due to the bridging of phospholipid molecules and the cavities that result from the movement of fatty acyl chains (Suzuki and Matsushita, 1969).

Hexadecane/water emulsions containing DMPC or egg PC monolayers have also been used to investigate Ca^{2+}, Mn^{2+}, Cu^{2+} and Ni^{2+} binding to phospholipid molecules (Meshkov *et al.*, 1998). For DMPC monolayers, the ion binding constants (L mol^{-1}) at 25°C are 87, 21, 6, and 5.3 for Ca^{2+}, Mn^{2+}, Cu^{2+} and Ni^{2+} respectively. Interestingly, Cu^{2+} and Ni^{2+} had higher affinities for DMPC compared to egg lecithin monolayers (Meshkov *et al.*, 1998).

2.2 Heavy metals and neurotoxic cations

The toxic heavy metals mercury and cadmium are naturally mobilized from the earth's crust into the global environment, affecting the general population in many ways (Gailer, 2007). Cd^{2+} is an established carcinogen whereas chronic exposure to Hg^{2+} is linked to cardiovascular disease (Kostka, 1991; Huff *et al.*, 2007). It is important to study molecular interactions at the membrane to understand how these metals are involved in toxicity.

2.2.1 Heavy metals and neurotoxic cations: unilamellar vesicles

Binary lipid mixtures composed of DPPC/bovine brain PS (60:40) have been used to examine the effect of not only Ca^{2+} and Mg^{2+} but also Zn^{2+}, Cd^{2+} and Hg^{2+} using fluorescence spectroscopy (Bevan *et al.*, 1983). Phase transition temperatures (T_m) of the vesicles were determined using the fluorescence polarization of *trans*-parinaric acid methyl ester in which the free carboxyl group can interact with the divalent ions. Permeability studies were performed by encapsulating carboxyfluorescein and monitoring the increase of fluorescence due to dye release over time. 1.0 mM Ca^{2+} and Mg^{2+} as well as 0.1 mM Zn^{2+}, Cd^{2+} and Hg^{2+} were shown to increase the T_m of DPPC/PS vesicles. It was concluded that the ion-lipid interactions were a result of the PS molecules as there was little to no change in the T_m of pure DPPC vesicles (Bevan *et al.*, 1983). The permeability studies showed that the very same divalent cations that produced the greatest change in the T_m of the vesicles (Cd^{2+} and Zn^{2+}) were also the ones that altered the permeability of the vesicles and associated dye release (Bevan *et al.*, 1983).

SUVs made of PS or PS/DPPC were used to study the effects of neurotoxic cations such as Al^{3+} and Mn^{2+} in addition to Cd^{2+} (Deleers *et al.*, 1986). Membrane fusion was studied with PS vesicles by resonance energy transfer between N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-PE (NBD-PE) and N-(lissamine rhodamine B-sulfonyl)-PE. Carboxyfluorescein-encapsulated PS vesicles were used to follow dye leakage, and fluorescence polarization of DPH in DPPC/PS (8:2) vesicles allowed for the monitoring of membrane rigidification (Deleers *et al.*, 1986). Fusion, leakage and rigidity increases in lipid models were seen in the presence of Al^{3+}, Cd^{2+} and Ca^{2+}. Although both Al^{3+} and Cd^{2+} decreased fluidity, seven-fold lower concentrations of Al^{3+} were seen to increase DPH polarization compared to Cd^{2+}. At 25 μM of Al^{3+}, a concentration inhibiting choline transport in erythrocytes (King *et al.*, 1983), membrane effects were indeed observed in the model system. This correlation between biological and model systems supports the relevance of the latter.

The permeability of ternary lipid model systems was utilized to study the effects of toxicant-membrane interactions. Vesicles containing egg lecithin, diacetyl phosphate and Chol in the molar ratio of 1.0 : 0.1 : 1.0 were incubated with methylmercuric chloride and mercuric chloride to measure the leakage of a glucose marker over time (Nakada *et al.*, 1978). As the Chol content was decreased in the model system, the amount of leakage increased with mercurial concentrations of 0.1 μM. Several divalent cations such as Ba^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Pb^{2+}, and Zn^{2+} did not affect the permeability of egg lecithin/diacetyl phosphate/Chol vesicles (1.0 : 0.1 : 0.5), demonstrating that membrane leakage was specific for the two mercurial compounds (Nakada *et al.*, 1978).

Four-component lipid vesicles consisting of 35% 1-palmitoyl-2-oleoyl-PC (POPC) / 35% Chol / 15% 1-palmitoyl-2-oleoyl-PE (POPE) / 15% 1-palmitoyl-2-oleoyl-PS (POPS) have been used to investigate Hg^{2+} and Cd^{2+} binding affinity (Le *et al.*, 2009). As all the lipids had palmitic and oleic acyl chains, differences in metal affinity could be attributed to the nature of the headgroup. Using Phen Green ™ SK as a fluorescence probe, it was shown that Hg^{2+} preferentially binds to PS headgroups followed by PC and PE. In contrast, Cd^{2+} strongly prefers PE, followed by PC and PS.

2.2.1.1 Heavy metals and neurotoxic cations: multilamellar vesicles

A number of studies have used single component MLVs to investigate the effect of heavy metal interactions at the membrane surface to provide insight into the individual role of specific lipid classes. MLVs (DMPC and DMPE) were used with X-ray diffraction and LUVs (DMPC) with fluorescence spectroscopy to study the molecular mechanism of Hg^{2+} and Cd^{2+} interactions with the membrane (Suwalsky *et al.*, 2000; Suwalsky *et al.*, 2004). In addition, isolated resealed human erythrocyte membranes were analyzed by fluorescence spectroscopy, and perturbations of erythrocytes in the absence and presence of Hg^{2+} and Cd^{2+} were observed by scanning electron microscopy. Human erythrocytes incubated with 1 mM $HgCl_2$ exhibited both echinocyte and stomatocyte formation (Suwalsky *et al.*, 2000) whereas 1 mM $CdCl_2$ only induced echinocytes (Suwalsky *et al.*, 2004). According to the bilayer couple hypothesis, the shape induced in erythrocytes in the presence of heavy metals is due to the expansion of both monolayers in the membrane (Sheetz and Singer, 1974). Stomatocytes are formed when heavy metals interact with the inner monolayer and echinocytes upon interaction with the outer membrane surface. It was concluded that both Hg^{2+} and Cd^{2+} bind to the outer leaflet since echinocyte formation was most dominant, a result confirmed by X-ray diffraction. DMPC and DMPE MLVs, representing the outer and inner monolayer, generally showed a decrease in lipid reflection intensities in the presence of 10^{-5} - 10^{-1} M of Hg^{2+} and Cd^{2+}. A greater effect was observed with DMPC MLVs. The presence of Hg^{2+} and Cd^{2+} results in molecular disorder in the bilayer, affecting both the polar and acyl chain regions (Suwalsky *et al.*, 2000; Suwalsky *et al.*, 2004).

The effect of $HgCl_2$ at the membrane surface was investigated using DPH steady state fluorescence anisotropy to look at lipid acyl chain packing and Laurdan fluorescence spectral shifts via general polarization to observe interactions occurring in the phospholipid glycerol backbone (Suwalsky *et al.*, 2000; Suwalsky *et al.*, 2004). Hg^{2+} increased the DPH fluorescence anisotropy and Laurdan general polarization in erythrocyte membranes at 37°C and in DMPC LUVs at 18°C and 37°C (Suwalsky *et al.*, 2000). At 18°C, Cd^{2+} induced disorder in the DMPC bilayer whereas at 37°C the opposite effect was observed. At the higher temperature, the bilayer is in a more fluid state facilitating Cd^{2+}-phosphate interactions that result in an ordered state (Suwalsky *et al.*, 2004).

Cd-membrane interactions have been studied using single-lipid MLVs composed of DM- and DP-species of PC, -PS, -PA, -PG, and -PE as well as binary DMPE/egg PC (1:1) mixtures (Girault *et al.*, 1998). The physicochemical techniques used include ^{113}Cd-NMR to describe Cd^{2+} interactions at the membrane surface, DPH fluorescence polarization to look at changes in the acyl chain region and ^{31}P-NMR to monitor the mobility of the phosphate headgroup (Girault *et al.*, 1998).

Using ^{113}Cd-NMR, Cd binding to lipids resulted in a decrease of the Cd (II)-free isotropic signal. Because of the slow exchange between the free and bound cadmium, lipid/water coefficients $\{K_{lw}$=(water vol./lipid vol.) x ($[Cd\ II]_{bound}/[Cd(II)]_{free}$)$\}$ at the lamellar gel (and fluid phase) were calculated to be: K_{lw} DMPC ~ K_{lw} egg PE ~ 2 $\pm$ 2 , K_{lw} DMPA = 392 $\pm$ 20 (505 $\pm$ 25), K_{lw} DMPG = 428 $\pm$ 21 (352 +17), and K_{lw} DMPS = 544 $\pm$ 27 (672 $\pm$ 34) (Girault *et al.*, 1998). Cd-lipid binding was observed to involve electrostatic interactions and more specifically, the phosphate group (Girault *et al.*, 1998). Fluorescence polarization experiments showed that the T_m increased for DPPG, DPPS, and bovine brain PS MLV systems in the presence of Cd (II) at R_i = [lipid]/[Cd] = 2. However, the gel-to-fluid phase transitions for DPPA, DPPS and DMPC/egg PC MLVs were suppressed with excess Cd (R_i=0.5). Salt concentrations of 0.8 and 1.8 M were used to reverse Cd-lipid interactions. Cd (II) affinities for negatively charged headgroups were determined as follows: PS>>PA>PG, due to the formation of $CdCl_n$ species (Girault *et al.*, 1998). Moreover, isotropic ^{31}P-NMR peaks, indicating non-lamellar phase formation, were observed for PG and the hexagonal phase was observed for egg PE lipid systems in the presence of Cd (II) at 24^{o}C, suggesting that the membrane has been reorganized. Hexagonal phase formation of egg PE has important toxicological implications as this lipid phase is involved in fusion and transport processes (Girault *et al.*, 1998).

Fluorescence quenching studies have used MLVs of single and two-component lipid mixtures to investigate Hg-lipid interactions. Egg PC and bovine brain PS extracts were used in the following membrane models: 100% PC, 100% PS, 25% PS / 75% PC, and 50% PS / 50% PC (Boudou *et al.*, 1982). Pyrene fluorescence labels were used to assess the accessibility of the bilayer core for mercury compounds, as the ratio of monomer and excimer emission peaks was used to determine the fluidity. At pH 9.5, CH_3HgCl quenched pyrene better than $HgCl_2$ with increasing PS concentration whereas $HgCl_2$ quenching occurred at a pH of 5.0 (Boudou *et al.*, 1982). In addition to the lipid composition and the charge of the polar headgroup, pH was determined to be an important factor affecting both the nature of the membrane and the species of mercury present (Boudou *et al.*, 1982).

MLVs of DPPC, egg PC, DMPA, bovine PS, DMPS, DPPG and binary mixtures of egg PC/DMPE (1:1) and DPPC/Stearoylamine (SA) (1:1) and fluorescence polarization were used to study the effects of $HgCl_2$ (Delnomdedieu *et al.*, 1989). Phase transitions of model systems containing bovine PS, DMPS or DMPE were abolished with 0.5-1 mM of Hg (II), which was attributed to interactions with the primary amine groups. The charge of the phospholipids was not involved since all three systems took this into account i.e. neutral (DMPE), negative (bovine PS, DMPS) or positive (DPPC/SA). In contrast, the T_m of DPPC/PS (60:40) vesicles in the presence of Hg (II) were interpreted as a charge interaction (Bevan *et al.*, 1983).

In a follow up study, ^{199}Hg-NMR was used to look at $HgCl_2$ binding to MLVs of PE, PS and egg PC (Delnomdedieu *et al.*, 1992). Although not truly representative of the biological membrane, the single-lipid model systems used confirmed previous results that the amine group is a common binding site in PE and PS lipids (Delnomdedieu *et al.*, 1992). xxx

The same authors used PS MLVs to study the effects of $HgCl_2$ and $Hg(NO_3)_2$. According to the chemical speciation diagram for mercuric chloride at pH 5.8-6.0 and pCl 3.0, the $HgCl_2$ species is present. From the ^{199}Hg-NMR study, the dissociation of the $HgCl_2$ provides Hg^{2+} for PE and PS binding and the two Cl^- ions compete for binding with the Hg^{2+}. $Hg(NO_3)_2$ dissociation in water avoids Cl^- competition in order to observe Hg-lipid binding on its own (Delnomdedieu and Allis, 1993). DPH fluorescence polarization results indicated that the phase transition was abolished and fluidity decreased in the presence of 0.05-4.75 mM $HgCl_2$ and 0.066-0.6 mM $Hg(NO_3)_2$. Increasing concentrations of NaCl were also shown to affect the ability of Hg (II) to interact with lipid binding sites. 10 mM NaCl prevented membrane perturbations of 0.5 mM $HgCl_2$ at pH 5.5 but only partially suppressed it at pH 7.1. Chloride ions do not compete with lipid binding sites when the amino group is deprotonated at neutral pH. The study by Delnomdedieu et al. is one of the very few that utilize both model systems and extracted erythrocyte membranes to look at Hg (II) interactions (Delnomdedieu and Allis, 1993). Data from the model systems were consistent with those from sonicated rat erythrocyte ghosts, showing that single-lipid MLVs can be useful in DPH fluorescence polarization experiments. The presence of Chol and proteins in the biological membrane did not offset the fluidity changes of the lipid bilayer induced by Hg (II) (Delnomdedieu and Allis, 1993). Fluidity will subsequently affect permeability and potentially the osmotic fragility of erythrocytes.

2.2.2 Heavy metals and neurotoxic cations: micelles

Both natural and synthetic lipids can be used to make micelles, with the latter being more widely used. Girault et al. used micelles and ^{31}P-NMR to study Hg (II) binding to lipid headgroups (Girault *et al.*, 1995; Girault *et al.*, 1996). Micelles (15 mM lipid) were prepared by Triton X-100 addition (10% w/v) to multilamellar vesicles. ^{31}P-NMR spectra were obtained for single lipid systems (PE, PS and PC) and binary lipid systems (PE/PC or PE+PC and PS/PC or PS+PC). Mixed micelles (phospholipid 1/phospholipid 2, 15 mM each) were prepared by mixing and stirring both lipid aliquots in chloroform, which was then evaporated, dispersed in acetate buffer and solubilized with Triton X-100. In the second method (phospholipid 1+phospholipid 2 micelles), each lipid was separately prepared in the same manner as the phospholipid 1/phospholipid 2, before being mixed to obtain 15 mM of each lipid. In the absence of Hg, the chemical shift values for PE, PS and PC were +0.30, +0.15 and -0.40 ppm respectively. However, in the presence of $HgCl_2$, peak areas decreased for all lipids with stronger effects for PE and PS compared to PC. Interestingly, a +0.30 ppm upfield shift, indicative of Hg-lipid phosphate interactions, was observed for PS in the presence of $HgCl_2$ but no chemical shifts occurred for PC and PE (James, 1975). PE and PC micelles show no change in the chemical shift because it is speculated that the distance between the phosphate and Hg binding moiety (amine group) is greater compared to PS due to different headgroup structure (Girault *et al.*, 1995; Girault *et al.*, 1996). Furthermore, binary micelles (PE/PC or PE+PC and PS/PC or PS+PC) showed a reduction in ^{31}P-NMR peak areas when $HgCl_2$ was added and MLVs (PS/PC and PE/PC) showed a decrease in chemical shift anisotropy values, again exemplifying Hg (II) specificity for PE and PS lipid headgroups, independent of the type of model system used (Girault *et al.*, 1995; Girault *et al.*, 1996).

A PE/PS lipid model system would allow the observed effect of Hg (II) binding to two different lipid headgroups. Unfortunately, this system could not be used because of the overlap of signal using ^{31}P-NMR. The use of more complex lipid systems is limited by the

capabilities of the physicochemical technique employed. Nonetheless, ^{31}P-NMR was able to determine that approximately 85% of $HgCl_2$ bound to phospholipids within 15 minutes, strongly suggesting that this metal adsorption to the lipid portion also occurs on the surface of biological membranes. Girault et al. also used egg yolk PC and DPPC micelles to show choline-specific binding by $HgCl_2$ (Girault *et al.*, 1996). This interaction was observed to be independent of acyl chain composition and more importantly, the Hg (II) affinity for PC is much less than for PE and PS. Delnomdedieu et al. were unable to detect any interactions between Hg (II) and the PC lipid headgroup using ^{199}Hg-NMR because of the higher concentrations of Hg (II) needed which may have masked the decrease in PC signal (Delnomdedieu *et al.*, 1992; Girault *et al.*, 1996).

In addition, natural membranes have also been used to produce micelle systems. Brush-border membranes isolated from pig jejunum epithelial cells were solubilized with Triton X-100 to form micelle models and were used to study the effect of zinc and cadmium ions on membrane structure (Tacnet *et al.*, 1991). ^{31}P-NMR spectra of the micelles in the absence and presence of Zn^{2+} and Cd^{2+} showed both interacting with negatively charged PI and PS but they have different effects on enzymatic phospholipid degradation: Zn^{2+} was observed to prevent lipid hydrolysis whereas Cd^{2+} greatly altered the lipid structure.

2.2.3 Heavy metals and neurotoxic cations: monolayers

Monolayers using animal cephalin have been used to study the effects of Hg^{2+} and Cd^{2+} (Suzuki and Matsushita, 1969). With as little as 10^{-7} M for Hg^{2+} and 10^{-8} M for Cd^{2+}, these heavy metals were not only observed to expand the monolayer but $C_{1/2}$ values calculated (the metal ion concentration giving half of the maximum pressure change) showed a linear correlation between logarithms of $C_{1/2}$ values and logarithms of the acute lethal doses of the metal chlorides in rabbits or rats (Suzuki and Matsushita, 1969).

Single component lipid monolayers of DPPG, DPPC, lyso-PC, and SM have also been utilized to observe interactions of Hg ions with membrane phospholipids (Broniatowski *et al.*, 2010). In the presence of 500 μM $HgCl_2$ in the aqueous subphase, mercury ions were observed to interact more strongly to SM and lyso-PC monolayers. Although DPPC, lyso-PC and SM share the choline headgroup, different lipid backbone and side chain architecture also plays an important role in lipid-metal interactions.

Fatty acids such as stearic acid, octadecylamine, octadecanol, and octadecane-1-thiol monolayers have also been used to study Hg^{2+} binding at the membrane surface (Broniatowski and Dynarowicz-Latka, 2009). Hg^{2+} not only interacted with the –SH group but also with –COOH and $–NH_2$ groups which can be found in proteins and membrane lipids. Moreover, behenic acid (C22:0) monolayers have been used to study heavy metals (Dupres *et al.*, 2003). Cd^{2+} concentrations were varied from 10^{-7} to 10^{-2} M and experiments were carried out at three different subphase pHs: 5.5, 7.5 and 10.5. Pressure-area isotherms revealed that the packing density of the monolayer increased upon Cd^{2+} interaction.

2.2.4 Heavy metals and neurotoxic cations: black lipid membranes (BLMs)

The toxicant must cross cell membranes if it is to be distributed throughout the organism i.e. within erythrocytes circulating in the blood, storage cells in the target organs etc. (Boudou *et al.*, 1982). This process is dependent on membrane composition and surface charge, the ion size and speciation, and the external and internal environment in terms of pH and temperature (Boudou *et al.*, 1982). Hence, permeability studies provide insight on how toxicants exert membrane toxicity.

BLMs have been useful to study metal transport. Diphytanoyl-PC in decane (20 mg/mL) has been used to investigate Cd^{2+} and Tl^{+} permeability through the lipid membrane (Gutknecht, 1983). Permeability coefficients for the metal ions were Cd^{2+} ($<1.1 \times 10^{-11}$ cm/s) > Tl^{+} (1.8×10^{-11} cm/s) > Hg^{2+} ($<3.8 \times 10^{-11}$ cm/s) (Gutknecht, 1983). For neutral complexes, the permeability coefficient order was $HgCl_2$ (1.3×10^{-2} cm/s) > TlCl (1.1×10^{-6} cm/s) > $CdCl_2$ (4.1×10^{-8} cm/s) (Gutknecht, 1981; Gutknecht, 1983). The low permeabilities of $CdCd_2$ and TlCl are attributed to the more polar and ionic nature of these metals in comparison to $HgCl_2$ which is more nonpolar and covalent (Gutknecht, 1981).

Binary egg lecithin/Chol (1:1) BLMs have been used to study the diffusion of Hg^{2+}, the effects of chloride concentration and pH (Gutknecht, 1981). The ^{203}Hg tracer and conductance measurements were used to determine membrane permeabilities for the different Hg forms. For example, $HgCl_2$ is highly permeant with a permeability coefficient of approximately 10^{-2} cm·sec^{-1} which is 20 times higher than water and a million times more than Na^{+}, K^{+} and Cl^{-} (Gutknecht, 1981). The lecithin/Chol bilayer in a 1:1 ratio is a suitable model for erythrocyte membranes which shows how easily Hg^{2+} can cross the membrane, where it can subsequently lead to toxic events.

Membrane solutions containing egg PC, PS, PC/Chol or PC/PS mixtures (2% w/w of each component in n-decane) were used to study the $HgCl_2$ and CH_3HgCl transport through BLMs (Bienvenue *et al.*, 1984). It was shown that both mercury compounds cross the membrane in their neutral forms in which pH and salt play an important role. The same lipid model systems were used to monitor quenching interactions between mercury and pyrene that showed the importance of charge (Boudou *et al.*, 1982). The fluorophore pyrene embedded in PS-containing lipid systems was quenched by Hg^{2+} (Boudou *et al.*, 1982).

Initially, mercurial compounds are attracted by the charges in the membrane but then neutralization occurs, facilitating the transport of mercury compounds such as $HgCl_2$ and CH_3HgCl across the membrane (Bienvenue *et al.*, 1984). At pH 9.5 and low chloride concentrations, $HgCl_2$ is in the $Hg(OH)_2$ form whereas the chloride form is the main species found at pH 5.0 with small amounts of $HgOH^{+}$, $HgCl^{+}$, $HgCl_4^{2-}$, and $HgCl^{3-}$ (Hahne and Kroontje, 1973; Shin and Krenkel, 1976). CH_3HgCl is in the chloride form at pH 5.0 and CH_3HgOH at pH 9.5 with small quantities of CH_3Hg^{+} found at both pH values (Shin and Krenkel, 1976). In the PC/Chol BLM, diffusion decreased by 89% with $HgCl_2$ and 36% with CH_3HgCl when the pH was changed from 5.0 to 9.5 (Bienvenue *et al.*, 1984).

It was also hypothesized that translocation through the bilayer is limited by interactions at the membrane-solution interface after adsorption takes place (Bienvenue *et al.*, 1984). Diffusion of the mercurial compounds was observed to be PC < PC/Chol < PC/PS for $HgCl_2$ and PC/PS < PC < PC/Chol for CH_3HgCl. Chol, which usually decreases the permeability of the lipid bilayer, was shown to increase the permeability in this study (Bienvenue *et al.*, 1984). The presence of PS slightly increases the diffusion for $HgCl_2$ but not for CH_3HgCl. Increasing concentrations of NaCl decreased the transport of $HgCl_2$. Overall, neutrally charged $HgCl_2$ and CH_3HgCl were shown to cross the lipid bilayer and that the permeability of both mercury compounds was dependent on pH and chloride conditions (Bienvenue *et al.*, 1984).

The various lipid model systems have provided further insight as to how metals exert toxicity. Lipid-metal interactions are pH- and salt-dependent, affecting both the nature of the lipid and metal speciation. This is followed by the transport across the membrane where metals can subsequently interact with other intracellular components resulting in permeability changes, leakage etc.

2.3 Drugs

Research has focused on the determination of the physiological activity and how drugs influence the cell membrane e.g. transport, distribution, accumulation etc. In certain diseases, cell lipid composition may be altered. Examples shown below illustrate how biomimetic lipid systems can help in the design of better drugs.

2.3.1 Drugs: vesicles

Ellipticine [5,11-dimethyl-6H-pyrido(4,3-b)-carbazole] and its derivatives have been studied as potential cancer chemotherapy agents by using liposome model systems (Terce *et al.*, 1982; Terce *et al.*, 1983). MLVs of DMPG and DMPG/DPPC (8 μM and 5 μM) and SUVs of DPPG or DPPC were used as membrane models in addition to the natural *Micrococcus luteus* membrane (18% dimannosyldiglyceride, 65% cardiolipin, 12% PG and 5% phosphatidylinositol) to study the effects of Ellipticine (Terce *et al.*, 1982). UV spectra revealed that the drug stays in the hydrophobic environment and not in the aqueous medium. Ellipticine not only interacted with PG in a 1:1 ratio but at a pH of 10, the drug was not released from the lipid phase, emphasizing the importance of both electrostatic and hydrophobic interactions. In a subsequent study, PS/PC vesicles were used in electrophoretic mobility measurements at a 4:1 and permeability assays at 1:1 ratio to observe the effects of Ellipticine and its derivatives: amphiphilic 9-methoxyellipticine and dipolar 9-amino- and 9-hydroxyellipticine compounds. Electrophoretic measurements revealed that the drug-derivatives neutralized the surface charge almost completely, indicative of drug interactions with the membrane surface. Once the electrostatic barrier is lowered, the drugs can exert cytotoxic effects, leading to increased permeability. The fluorescence of 6-carboxyfluorescein was used to monitor dye leakage from liposomes and both 9-methoxyellipticine and 9-hydroxyellipticine increased dye leakage, although stronger effects were observed with the former. Maximum leakage was observed for 2×10^{-5} M 9-methoxyellipticine with liposome and resealed ghost models (Terce *et al.*, 1983).

2.3.2 Drugs: monolayers

Interactions of cyclosporine A (CsA) with the membrane have been investigated using binary mixtures of PC and Chol in monolayers (Soderlund *et al.*, 1999). CsA is a hydrophobic drug that is used to prevent graft rejections after organ transplants. Monolayers were prepared in circular wells drilled in Teflon and CsA penetration was indicated by surface pressure changes using the Wilhelmy plate method. The effect of CsA on the lateral distribution of the fluorescence probe 1-palmitoyl-2-(N-4-nitrobenz-2-oxa-1,3-diazol) aminocaproyl-PC (NBD-PC) in lipid monolayers was also studied using fluorescence microscopy. These experiments showed rapid insertion of CsA into egg PC and egg PC/Chol (1:1) monolayers as indicated by the increase in surface pressure. These changes, compared to the initial surface pressure, showed biphasic interactions of CsA with both types of monolayers. But Söderlund et al. showed that the morphology of lipid domains was dependent on the composition. NBD-PC is a useful fluorescent probe as it partitions into the boundaries between fluid and gel regions, imaged as light and dark areas in the fluorescence microscope (Soderlund *et al.*, 1999). Dark areas contain very small amounts of NBD-PC. CsA was observed to change NBD-PC distribution in DPPC/β-Chol (88:10) mixtures compared to DPPC monolayers (Soderlund *et al.*, 1999).

DPPC monolayers have also been used to mimic the surfactant layer lining the surface of the lung alveoli in order to study the interactions with gelatin based nanoparticles for the

purposes of drug delivery (Lai *et al.*, 2010). Using Brewster angle microscopy, the nanoparticles were shown to change the shape and reduce the size of the DPPC domains, thus these propensities need to be considered for potential pulmonary drug vehicles.

2.4 Antimicrobial peptides

Increased bacterial resistance to antibiotics has encouraged the investigation of other antibacterial approaches for biomedical research, including antimicrobial peptides (AMPs). They are generally short, amphiphilic structures containing 12-100 residues with several of those residues being positively charged (Jenssen *et al.*, 2006). AMPs decrease bacterial viability through several mechanisms, including altering gene expression, inhibiting nuclease activity, and by disrupting the bacterial membrane (Hui and Sen, 1989; Epand and Vogel, 1999; Jenssen *et al.*, 2006). AMPs do not interact with specific membrane receptors, but directly with the negatively charged bacterial membrane in many different ways e.g. by creating pores or regional disintegration, by acting as detergents or by disruption of the membrane potential (Epand and Vogel, 1999; Lohner and Prenner, 1999; Bechinger and Lohner, 2006). Among the most common methods to study the effects of membrane-disrupting antimicrobial peptides are dye leakage assays from bacterial membrane mimetic systems.

2.4.1 Antimicrobial peptides: vesicle controls

Vesicles are widely used models to investigate the structure of membrane-bound antimicrobial peptides (Mani *et al.*, 2006; Chongsiriwatana *et al.*, 2008; Sevcsik *et al.*, 2008; Tang and Hong, 2009). In particular, zwitterionic systems such as POPC LUVs have been used as a control mammalian system in the study of indolicidin and tritrpticin along with its derivatives (Andrushchenko *et al.*, 2008). ITC titrations of lipid suspension into peptide solutions revealed a stronger peptide binding affinity for POPE/POPG and *E. coli* membrane systems compared to the mammalian one.

Binary mixtures of egg PC/Chol (10:1) in SUVs have served as useful controls in the study of melittin diastereomers (Oren and Shai, 1997). LUVs of POPC/Chol (1:1) and POPE/POPG (3:1) mimic erythrocyte and bacterial membranes and have been used to study protegrin-1 (PG-1) (Mani *et al.*, 2006). This peptide is a disulfide-linked, β-hairpin antimicrobial peptide that is isolated from pig leukocytes (Kokryakov *et al.*, 1993). ^{1}H solid state NMR showed that PG-1 interacts with lipid acyl chains and headgroups in the bacteria-mimetic POPE/POPG (3:1) indicating peptide insertion in contrast to POPC/Chol (1:1) bilayers (Mani *et al.*, 2006). Furthermore, ^{19}F spin diffusion experiments indicated that PG-1 assembles into $(NCCN)_n$ multimers suggesting a β-barrel structure which is surrounding a water pore in the bacterial mimetic membrane. However, the N and C strands of PG-1 organize into tetramers, suggesting that β-sheets form at the mammalian erythrocyte membrane surface.

More complex three- and four-component mixtures have also been used i.e. POPC/POPE/Chol (65:25:10) in the absence and presence of 10% POPS prepared by freeze-thaw cycles and extrusion to study the effects of c[D-Pen^{2},D-Pen^{5}] enkephalin (DPDPE) and biphalin and to show how electrostatic interactions impact peptide permeability in the membrane (Romanowski *et al.*, 2002). DPDPE is a zwitterionic, cyclic enkephalin analogue whereas biphalin is a cationic, dimeric peptide. For the permeability assay, the sample of liposome encapsulated peptides were dialyzed and the amount of peptide released into the

buffer was determined by a fluorescence assay, which measured the reaction product from the peptide amino group with fluorescamine (ex380/em475) (Romanowski *et al.*, 2002). Partition coefficients were obtained using equilibrium dialysis experiments and the fluorescence measurements of the peptide Tyr and Trp residues (Romanowski *et al.*, 1997). Interestingly, increased surface charge by addition of POPS showed increased biphalin binding but reduced translocation of the peptide across the bilayer. Conversely, only a small decrease in permeability was observed for DPDPE with increased negative charge. These results show how the negative charges in the biological membrane can affect peptide membrane transport processes and must be taken into account when designing peptide drugs (Romanowski *et al.*, 2002).

2.4.2 Antimicrobial peptides: monolayer controls

Monolayers have become important tools to investigate antimicrobial peptide interactions with the lipid membrane (Maget-Dana, 1999). Single lipids such as DPPC and DPPG have been used in monolayers (20 μL of a 0.5 g/L) to see if peptide interactions occurred (Lad *et al.*, 2007). Melittin is a non-specific membrane peptide whereas magainin II and cecropin P1 are known to specifically interact with prokaryotic cell membranes. Surface pressure-area isotherms of the lipid monolayers were obtained in the absence of the various peptides. The barriers were then fixed in position to obtain the desired surface pressure and the peptide solution was then introduced into the subphase through a custom-made metal tube incorporated below the buffer surface. A greater increase in surface pressure indicated binding between the lipid headgroup and the cationic peptide. The melittin, and the antimicrobial peptides magainin II and cecropin P1 (0.7 μM) were shown to interact more with DPPG over DPPC monolayers (Lad *et al.*, 2007).

Mixtures of POPC/Chol have also been used to test synthetic peptides Phd1-3, which span the carboxy-terminal region of human β-defensins HBD 1-3 (Krishnakumari and Nagaraj, 2008). The peptide analogues have a single disulfide bridge and have exhibited antibacterial activity. No significant increase in pressure in POPC/Chol (7:3) was observed for all three peptides in contrast to POPC/POPG (7:3) indicating no interaction with the mammalian model (Krishnakumari *et al.*, 2006).

3. Applications of bacterial membrane models

As previously discussed, different lipid composition can alter the physical and chemical characteristics of the membrane. The changes in behaviour exhibited in the different model systems provide information on the mechanism of action exhibited by these peptides upon interaction with natural bacterial membranes. Illustrated below are several examples of biomimetic systems used to study bacterial membranes, with a focus on antimicrobial peptides and the membrane protein EmrE. While these examples are by no means an exhaustive list of the systems used, they serve to illustrate how different lipid compositions can be used.

3.1.1 Antimicrobial peptides: vesicles

Using a simplified model system to represent the bacterial cell facilitates the study and thus the understanding of the complicated molecular interactions involved in antimicrobial activity. Vesicles have been widely used to study the effect of lipid composition on the

ability of AMPs to disrupt the membrane. Dye leakage assays take advantage of the encapsulation properties of vesicles and have been extensively used for testing the activity of AMPs on model membranes. Biomimetic vesicles are loaded with a fluorescent dye, such as calcein, and assayed for AMP-induced leakage (Kendall and MacDonald, 1982; Patel *et al.*, 2009). Differences in AMP activity in response to different mimetics can be assayed by the extent of dye release.

One of the interesting aspects is the understanding of the mechanism through which AMPs differentiate between target and host membranes, thereby avoiding or minimizing host toxicity. A major factor is the increased abundance of anionic lipids in bacterial membranes compared to eukaryotes, giving the cell an overall negative charge providing a natural electrostatic target for the cationic AMPs. A calcein leakage assay was used to study the effects of lipid charge on the activity of magainin, a widely characterized AMP (Matsuzaki *et al.*, 1989) whereby magainin induced significant leakage in negatively charged PS vesicles, compared to neutral PC vesicles or positive 9:1 (mol/mol) PC/SA. The negatively charged bacterial lipid PG behaved in a similar manner to the PS, emphasizing the importance of an electrostatic interaction with the positively charged AMP (Matsuzaki *et al.*, 1989; Lohner and Prenner, 1999).

A more accurate representation of the *E. coli* membrane was provided by using a headgroup ratio of 75:20:5 POPE/DPPG/CL in SUVs and LUVs (Glukhov *et al.*, 2005) to study the differential targeting of novel cationic AMPs to bacterial versus mammalian membranes. They found that the initial attraction between the positive AMP and negatively charged bacterial membranes was an important component of bacterial/mammalian selectivity. DSC has been used to elucidate the impact of the peptide interactions on the physical properties of the membrane, specifically the effect of AMP binding on the lipid phase transitions from gel to liquid crystalline (Prenner *et al.*, 1999). Using PC, PE, and PG vesicles, it was found that gramicidin S reduced the temperature and increased the cooperativity of the main phase transition of PG, but had moderate effects on the phase behaviour of PC, and practically no effect on PE (Prenner *et al.*, 1999). From these results, it follows that AMPs interact differently with the various lipid species, and that specific lipid interactions may be important for activity.

Along with the negative charge of the membranes, there are other components that could enable AMP differentiation between host and target membranes. In the presence of gramicidin S, a strong antimicrobial peptide, vesicles containing (4:1 mol/mol) PC/Chol showed less dye leakage than PC vesicles alone (Prenner *et al.*, 2001). Vesicles composed of 3:1 POPE/POPG were used to monitor the effect of zwitterionic phospholipids and sterols on membrane destabilization by the synthetic AMP pleurocidin-amide (Mason *et al.*, 2007). These authors also found that the presence of sterols can reduce AMP destabilization of a membrane, even if the membrane has anionic character. This decreased activity in Chol-containing vesicles could be a possible mechanism for the selectivity of antimicrobial peptides, explaining why AMPs are highly selective towards bacterial membranes, which do not contain sterols (Mason *et al.*, 2007).

There are several other factors that are involved in the lipid-protein interaction in the membrane, including the properties of the AMPs themselves. In 2001, Zhang et al. studied whether AMPs with varying structures and activities had similar mechanisms of action for inducing membrane leakage, as well as lipid redistribution and peptide translocation (Zhang *et al.*, 2001). They measured AMP-induced lipid flip-flop in unilamellar PG/PC (1:1) vesicles (in 10 mM Tris-HCl, 150 mM NaCl, and 1 mM NaEDTA, pH 7.5) asymmetrically

labeled with 0.5 mol% C_6-NBD-PC in the inner leaflet. Calcein release was tested as a measure of membrane leakiness, and also taken as a control to ensure that the lipid flip flop observed was not simply due to bilayer disruption. Peptide translocation was measured using chymotrypsin-entrapped LUVs of 50% egg PC, 45% egg PG, and 5% dansyl-PE (in 150 mM NaCl, 20 mM HEPES, pH 7.4). It was determined that lipid flip-flop, membrane leakiness, and peptide translocation could not individually be related to AMP structure or charge, concluding that many factors must be involved in AMP activity which have a varying impact on membranes (Zhang *et al.*, 2001). In a detailed investigation, it was shown that the ability of gramicidin S to differentiate between mammalian and bacterial membranes depended on many peptide parameters as well, such as solubility, beta-sheet content, self-aggregation propensity, hydrophobicity and amphiphilicity (Prenner *et al.*, 2005).

These examples illustrate how changing the properties of the model system can illuminate the interactions involved. Not all lipid systems used as bacterial models reflect the natural composition of a bacterial membrane, yet these models have been used to provide valuable information in regards to the behaviour and influence of AMPs on the membrane. Synthetic polydiacetylene (PDA) has been used in combination with lipids in bacterial membrane mimetics because of its colorimetric properties (Kolusheva *et al.*, 2000). PDA monomers are similar in structure to lipids, and vesicles exhibit colorimetric blue-red transitions when exposed to several environmental perturbations, including changes in surface pressure. When mixed with PDA, lipids tend to form bilayers in interspersed, segregated phases, creating a convenient model system of the lipid bilayer. Kolushiva et al. used 3:1:6 PE/PG/PDA and 3:1:6 PE/CL/PDA vesicles to create a simple screening assay to study the interaction of AMPs with the membrane (Kolusheva *et al.*, 2000). Using colorimetric analyses, the insertion of AMPs into the membrane was found to depend on bilayer composition, with higher insertion into the PG–containing system, consistent with observed biological data for levels of antimicrobial activity.

3.1.2 Antimicrobial peptides: monolayer

Although vesicles are a useful membrane mimetic for studying antimicrobial activity, other mimetic systems have provided valuable data, including monolayers. An increase in the surface pressure of a lipid monolayer at the air-water interface has been used as an indicator for AMP insertion and a method to study the probability of membrane disruption as the mechanism of action for different AMPs. In a fairly accurate representation of the headgroup ratio of *E. coli* phospholipids, Zhang et al. created a 78 : 4.7 : 14.4 PE/egg PG/CL mimic of the *E. coli* inner membrane, and studied the interaction of several natural and synthetic AMPs with the mixture as well as the individual monolayer constituents (Zhang *et al.*, 2001). Increasing peptide concentration resulted in a sigmoidal increase in the surface pressure of the mixture, indicating peptide insertion which is likely a cooperative event. As expected, the AMPs interacted more strongly with anionic monolayers, especially egg PG. Most of their AMPs had minimal interaction with zwitterionic lipids, however a few exceptions indicated that electrostatic interactions are not the only attraction between cationic AMPs and negatively charged bacterial membranes.

Planar lipid films have also been used combined with Brewster angle microscopy (BAM) to image the interaction of several AMPs with monolayers (Volinski *et al.*, 2006). While two AMPs were immiscible in 1:1 ratios of peptide:DMPC, valinomycin showed phase-separation in the monolayer. BAM was used to image valinomycin-induced changes to the

lateral film architecture in 1:1 DMPC/PDA. Further characterization using fluorescent NBD-PE and confocal microscopy also revealed significant changes to the DMPC-PDA membrane mimetic film (0.2 : 0.8 : 9 NBD-PE/DMPC/PDA) upon peptide addition since some AMPs preferentially formed distinct domains within the phospholipid portion of the mixed monolayer. Thus, phospholipid-PDA systems could be used, especially in conjunction with BAM, to elucidate surface phenomena of lipid-AMP interactions (Volinski *et al.*, 2006).

3.1.3 Antimicrobial peptides: supported bilayer

Understanding the mechanism through which AMPs disrupt the membrane requires insight into the orientation of the peptide within the lipid bilayer, which can be obtained by using supported lipid bilayers. These are two layers of lipids deposited as a bilayer onto a solid support, such as a glass slide, silica beads, or muscovite. An advantage of supported bilayers is their long-term durability and the ability for high definition imaging techniques due to their stable orientation (Henzler Wildman *et al.*, 2003; Davis *et al.*, 2009). Characterization methods include atomic force microscopy, solid-state NMR, and several fluorescence microscopy techniques (Henzler Wildman *et al.*, 2003).

Oriented lipid bilayers on glass plates were used to study the structure and mechanism of membrane disruption of LL-37, a human antimicrobial peptide that may be important in treating cystic fibrosis (Henzler Wildman *et al.*, 2003). Solid-state NMR was used to compare the orientation of LL-37 in zwitterionic DMPC and anionic DMPG to get insight into the potential mechanism of action. Although LL-37 showed no difference in surface orientation between the two systems, the cationic peptide exhibited greater disruption towards anionic bilayers. Moreover, when the two zwitterionic matrices POPC and POPE were compared LL-37 was more disruptive towards the smaller PE.

Comparisons between PC/PG and the more *E. coli*-like PE/PG systems were made using binary 4:1 (mol:mol) mixtures, whereby PE/PG behaved more like *E. coli* lipid extract. The role of acyl chain saturation was determined using monounsaturated and fully saturated PC and PG lipids, with an increase in membrane disruption in the less fluid, fully saturated bilayer. No effects of ionic strength or pH on LL-37 were observed. Combined with DSC results, these data suggest that LL-37 adopts the toroidal-pore model of membrane disruption (Henzler Wildman *et al.*, 2003).

3.2 Membrane proteins

The investigation of membrane protein insertion or orientation in model systems involves many experimental difficulties due to their hydrophobic nature, already severely limiting their purification. Thus, much less is known about membrane protein structure and function, or even the basic question such as their orientation within a membrane.

There are, however, a few proteins that have been purified and studied more extensively. For these membrane proteins, various lipid and detergent systems have been used to provide a hydrophobic environment to study their structure and function. For example, the structure and organization of the transmembrane retinal proteins rhodopsin and bacteriorhodopsin, as well as the conformation and activity of *E. coli* multidrug resistance protein EmrE, have been studied in several membrane mimetics (see below). However, as previously discussed, lipid composition may alter the physical characteristics of a membrane and affect how membrane proteins behave.

3.2.1 Membrane proteins: liposomes

SUVs have been used to study folding and ligand binding of membrane proteins. Native lipid vesicles have been used to investigate the refolding mechanisms of the well-studied *Halobacterium* retinal protein bacteriorhodopsin by circular dichroism and absorption spectroscopy (Popot *et al.*, 1987). Denatured by SDS before renaturing into lipid vesicles, a two-stage refolding mechanism of bacteriorhodopsin was determined since stable transmembrane helices are formed first before structural rearrangement results in the proper tertiary structure (Popot *et al.*, 1987). Later fluorescence spectroscopy studies using DPPC vesicles, and micelles composed of DMPC and the detergent CHAPS or DMPC/DHPC (di-7:0-PC) show that bacteriorhodopsin forms two intermediate folding stages followed by two-step retinal binding to form native bacteriorhodopsin (Booth, 2000).

The rate of protein folding and insertion is affected by membrane characteristics, particularly the lateral pressure profile of the bilayer. As some lipid species prefer to form non-bilayer structures, their presence increases the lateral chain pressure. Using PC liposomes and increasing amounts of non-bilayer PE, a decrease in the rate of bacteriorhodopsin folding/insertion was found corresponding to the increase in lateral chain pressure (Curran *et al.*, 1999). Similarly, longer acyl chains and thus increased chain pressure decreased the rate of bacteriorhodopsin folding (Booth *et al.*, 1997). Hence systematical alteration of liposome composition allows for the assessment of the effects of each lipid.

SUVs have also been used to assay binding affinity and conformation of the integral membrane protein EmrE, a multidrug resistance transporter found in *E. coli* (Federkeil *et al.*, 2003; Sikora and Turner, 2005). Although EmrE solubilized into SUVs composed of *E. coli* lipid extracts is the best mimic of its natural membrane, detergents such as sodium-dodecyl-sulfate (SDS) and N-dodecyl-β-D-maltoside (DM) have been used as well as membrane mimetics. Fluorescence spectroscopy and circular dichroism were utilized to monitor the conformation of EmrE in different detergents and organic mimetic systems such as SDS, ethanol, and urea (Federkeil *et al.*, 2003). Trp fluorescence spectra of EmrE in such systems were compared to EmrE in SUVs composed of *E. coli* polar extract, whereby the DM systems had similar spectral properties to the bacterial lipid vesicles. While the secondary structure was similar for all systems, the tertiary structure of EmrE depended more on the environment based on the mobility and exposure of Trps (Federkeil *et al.*, 2003).

Moreover, ITC experiments of ligand binding to EmrE solubilized in *E. coli* extract SUVs or in either SDS or DM micelles were performed to determine the binding constant and stoichiometry for several ligands (Sikora and Turner, 2005). Both SUVs and micelles showed weak and non-specific binding of EmrE for its ligands. In addition, while multimerization is important for its ligand/proton anti-transport, these results suggest that EmrE monomers bind the ligand in a 1:1 ratio (Bay *et al.*, 2010).

3.2.2 Membrane proteins: monolayers

One of the major concerns of studying membrane proteins in monolayers has been protein denaturation due to the lack of a second, outer leaflet. However, it has been shown that under certain conditions, some proteins indeed maintain their native secondary structure and activity in these systems. Parameters such as initial surface pressure, compression speed and temperature influence protein stability in monolayers, and some proteins are more susceptible to denaturation than others (Boucher *et al.*, 2007). Infrared spectroscopy showed that the retinal membrane protein bacteriorhodopsin maintained its native

secondary structure under varying experimental conditions (see above), while the related rhodopsin was more susceptible to denaturation (Lavoie *et al.*, 1999). However, upon spreading at higher initial surface pressure rhodopsin maintained its structural integrity.
These are only a few examples of model systems used to study membrane proteins but they demonstrate how properly selected membrane biomimetics and experimental conditions allow the elucidation of the biophysical characteristics of lipid-membrane protein interactions.

4. Conclusion

Lipid models have enabled e.g. the study of lipid-lipid and lipid-protein interactions by mimicking the composition, curvature, electrostatic potential or permeability properties of biological membranes. Their continuous improvement in terms of complexity will further increase their suitability to reveal the structure and function of biological membranes and the multitude of processes and interactions involved. Thus, such systems provide an important tool for a more detailed understanding of structure and function of biomembranes and their interactions with biomolecules and drugs.

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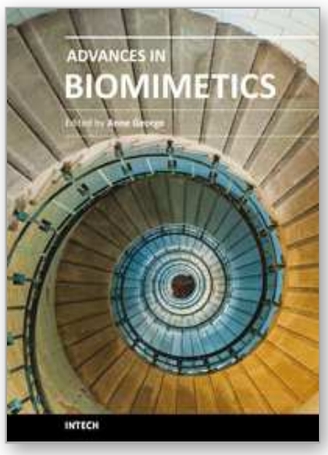

Advances in Biomimetics

Edited by Prof. Marko Cavrak

ISBN 978-953-307-191-6
Hard cover, 522 pages
Publisher InTech
Published online 26, April, 2011
Published in print edition April, 2011

The interaction between cells, tissues and biomaterial surfaces are the highlights of the book "Advances in Biomimetics". In this regard the effect of nanostructures and nanotopographies and their effect on the development of a new generation of biomaterials including advanced multifunctional scaffolds for tissue engineering are discussed. The 2 volumes contain articles that cover a wide spectrum of subject matter such as different aspects of the development of scaffolds and coatings with enhanced performance and bioactivity, including investigations of material surface-cell interactions.

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Mary T. Le, Jennifer K. Litzenberger and Elmar J. Prenner (2011). Biomimetic Model Membrane Systems Serve as Increasingly Valuable in Vitro Tools, Advances in Biomimetics, Prof. Marko Cavrak (Ed.), ISBN: 978-953-307-191-6, InTech, Available from: http://www.intechopen.com/books/advances-in-biomimetics/biomimetic-model-membrane-systems-serve-as-increasingly-valuable-in-vitro-tools

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