Helsinki University of Technology
Department of Chemical Technology
Laboratory of Physical Chemistry and Electrochemistry
Espoo 2004

A NOVEL BIOMEMBRANE MODEL FOR ELECTROCHEMICAL STUDIES. CHARACTERISATION AND APPLICATIONS.

Annika Mälkiä

Dissertation for the degree of Doctor of Science in Technology to be presented with due permission of the Department of Chemical Technology, for public examination and debate in Auditorium Ke2 at Helsinki University of Technology (Espoo, Finland) on November 5, 2004 at 1 p.m.

ISBN 951-22-7282-2 (printed)
ISBN 951-22-7302-0 (URL: http://lib.hut.fi/Diss/)

Otamedia Oy
Espoo 2004

Abstract

This thesis describes the development and characterisation of a novel biomembrane model suitable for electrochemical investigation, and its applicability in studies of biologically related phenomena. The model system consists of a lipid monolayer, deposited at a polarisable liquid – liquid interface with the Langmuir – Blodgett technique. Combinations of ac and dc electrochemical techniques with theoretical models are employed to obtain information on the phase transfer and membrane interactions of charged therapeutics at the monolayer-covered interface. The thesis comprises six publications and a thorough introduction to biological and model membranes.

The first part of the thesis surveys background literature with relevance to the present work. Basic properties of biological membranes are discussed and existing biomembrane models reviewed. The presented literature serves as a reference point for the development and evaluation of the model system described in this thesis, and is frequently referred to in the discussion of the results.

In the second part of the thesis, the essential results of the publications are summarised. Instead of an article-by-article approach, results from various publications are jointly presented under appropriate headings. The development, characterisation and applicability of the model system are discussed. In the last-mentioned section, emphasis lies on methods applicable to drug development and delivery. To this end, the usefulness of the model system in the construction of pH – potential diagrams, determination of drug membrane activity, and studies of drug transfer through polyelectrolyte multilayers, is investigated. Finally, in view of the obtained results, the future prospects of the system are assessed.

Preface

The work described in this thesis has been carried out at the Laboratory of Physical Chemistry and Electrochemistry, Helsinki University of Technology during May 2000 – September 2002 and October 2003 – May 2004. I gratefully acknowledge the financial support from the Academy of Finland, the National Technology Agency of Finland (TEKES) and the ESPOM graduate school.

I would like to express my sincere gratitude to:

Professor Kyösti Kontturi, my supervisor, for sharing his interest and knowledge in the field of electrochemistry.

Dr. Peter Liljeroth, co-author, for an enjoyable collaboration, for his scientific enthusiasm and for always having time for stimulating discussions.

Emeritus Professor Göran Sundholm, whose interesting lectures on electrochemistry had a significant impact on my choice of field; and Professor Arto Urtti, for introducing me to the fascinating world of biopharmacy.

Co-authors, Drs. Anna-Kaisa Kontturi, Vincent Cunnane and Christopher Slevin, and Ms Martina Toiminen for fruitful collaboration.

Dr. Bernie Quinn for inspiring discussions, encouragement and sharing her scientific experience; and all the past and present members of the Laboratory of Physical Chemistry and Electrochemistry, for providing a pleasant and supportive working atmosphere.

Further thanks are due to:

My friends, for being there for me during my PhD studies, especially Salla, Piitu, Tuomas, Ilpo, Pete and Anki.

Félix, Elvira, Carolina and the rest of the UMH staff: for introducing me to the world of neuroscience, and making my stay in Alicante unforgettable.

Finally, my warmest thanks to my family:

Monica, Hannu, Jussi, Mommo and Mummi, for their encouragement, support and understanding; and to Krisse, for his literally never-ending patience.

Espoo, May 21, 2004.

Annika Mälkiä

Table of contents

List of publications i
Statement of the author's role ii

1. Introduction 1

2. Background to the present work 2
2.1 Biological membranes 2
2.1.1 Structure and composition 2
2.1.2 Electric properties 6
2.2 Model membranes 12
2.2.1 Unsupported bilayer lipid membranes 13
2.2.2 Liposomes 18
2.2.3 Solid-supported bilayer lipid membranes 18
2.2.4 Polymer-supported bilayer lipid membranes 27
2.2.5 Lipid monolayers 33

3. Aim of the work 48

4. The present work 49
4.1 Development 49
4.2 Characterisation 50
4.3 Applications 53
4.3.1 Drug partition 53
4.3.2 Membrane activity 56
4.3.3 Polyelectrolyte multilayers 58
4.4 Future prospects 60

5. Conclusions 61

List of abbreviations 62
List of symbols 64
References 65

List of publications

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals.

I. Liljeroth, P., Mälkiä, A., Cunnane, V.J., Kontturi, A.-K., Kontturi, K., 2000. Langmuir – Blodgett monolayers at a liquid – liquid interface. *Langmuir* 16, 6667-6673.

II. Mälkiä, A., Liljeroth, P., Kontturi, K., 2001a. Drug transfer through biomimetic Langmuir – Blodgett monolayers at a liquid – liquid interface. *Anal. Sci.* 17 Supplement, i345-i348.

III. Mälkiä, A., Liljeroth, P., Kontturi, A.-K., Kontturi, K., 2001b. Electrochemistry at lipid monolayer-modified liquid – liquid interfaces as an improvement to drug partitioning studies. *J. Phys. Chem. B* 105, 10884-10892.

IV. Malkia, A., Liljeroth, P., Kontturi, K., 2003a. Membrane activity of ionisable drugs – a task for liquid – liquid electrochemistry? *Electrochem. Commun.* 5, 473-479.

V. Malkia, A., Liljeroth, P., Kontturi, K., 2003b. Membrane activity of biotechnological peptide drugs. *Chem. Commun.*, 1430-1431.

VI. Slevin, C.J., Malkia, A., Liljeroth, P., Toiminen, M., Kontturi, K., 2003. Electrochemical characterization of polyelectrolyte multilayers deposited at liquid – liquid interfaces. *Langmuir* 19, 1287-1294.

Statement of the author's role

Annika Mälkiä has planned and performed all the experimental work in papers I – V. The interpretation of results and the preparation of the manuscripts for papers I – V has been carried out jointly with Peter Liljeroth. In publication VI, the experimental work was carried out jointly by Mälkiä and Toiminen. Mälkiä contributed to the planning of the experimental work as well as the preparation of the manuscript.

Espoo, May 21, 2004.

Kyösti Kontturi

Professor

1. Introduction

Since the realisation that lipid bilayers comprise the fundamental structure of all biological membranes, they have been the subject of numerous experimental and computational studies. As a result, membrane models of variable complexity and destination have emerged, some aiming at elucidating structural details of the bilayer membrane and others striving to mimic its functions.

The motive behind the present work was to develop a well-defined model membrane for electrochemical studies of membrane activity and permeation of biologically and therapeutically relevant compounds. To get an insight into what might constitute a well-defined model membrane, structural details of biological membranes are briefly discussed in the first part of this thesis. Many of these will be of relevance when discussing the results of the work presented in the latter section. Furthermore, existing model systems of biological membranes are reviewed, the emphasis being on approaches applicable to electrochemical studies. In the following, the term "biological" membrane refers to a membrane of a living organism, such as that of a cell or an organelle.

In the second part of the thesis, a novel methodology for constructing lipid model membranes at liquid – liquid interfaces with the Langmuir – Blodgett technique is presented. The effects of structure and composition on the membrane properties are investigated employing electrochemical methods. The applicability of the system to probe drug lipophilicity and membrane activity is studied using both conventional and biopharmaceutical therapeutics.

2. Background to the present work

2.1 Biological membranes

2.1.1 Structure and composition

Despite their different functions, all biological membranes have a common general structure. Their core is formed by a thin film of lipid and protein molecules that are held together mainly by non-covalent forces. The lipid molecules are arranged in a continuous bimolecular layer with a thickness of approximately 50 Å (Alberts et al., 1994). The lipid barrier exhibits remarkable physical properties such as low viscosity, deformability and the ability to self-seal (Devaux, Seigneuret, 1985).

The bilayer structure of the membrane lipids was first shown by Gorter and Grendel (1925). Ten years later, Danielli and Davson (1935) proposed that the lipid bilayer is sandwiched between proteins. The Danielli-Davson model was the basis of biomembrane research, until Singer and Nicolson introduced their fluid mosaic model in 1972. The fluid mosaic model describes the cellular membranes as two-dimensional, viscous solutions of lipids and proteins at thermodynamic equilibrium. The model introduced the concept of peripheral and integral proteins. The lipids and proteins were thought to organise randomly through lateral diffusion in the plane of the bilayer, but to have restricted rotational mobility.

The fluid mosaic model still acts as the framework when considering the dynamic and fluid structure of biological membranes, but certain revisions have been made since its introduction. It is today well established that the lipid/protein bilayer consists of lateral domains with different lipid compositions (Mouritsen, Jørgensen, 1994; Welti, Glaser, 1994) and that the cytoskeleton imposes restrictions on the movement of several membrane proteins (Luna, Hitt, 1992; Jacobson et al., 1995).

Lipid composition

Most cell membranes are composed of approximately 50 w-% lipids and 50 w-% proteins. The lipid composition is always heterogeneous, including lipids with a predominantly structural role such as cholesterol and the phospholipids, as well as functional lipids like the glycolipids. In addition, membranes of animal cells contain

substantial amounts of neutral lipids and free fatty acids, which are important metabolic intermediates (Sackmann, 1995a; Devaux, Seigneuret, 1985).

The phospholipids constitute more than half of the total lipid mass in most cell membranes. They can be divided into five classes: phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), phosphatidylinositols (PI) and sphingomyelins (SPHM). Phospholipids and glycolipids are commonly characterised by the structural features of their head groups (chemical composition, charge), their polar backbone (glycerol, sphingosine) and their hydrocarbon chains (number of chains, number of carbons/chain, chain saturation). The various combinations of these constituents result in the existence of thousands of different biological lipids, several hundred of which are present in the same membrane (Devaux, Seigneuret, 1985). Figure 2.1 shows the structure of one of the most abundant phosphatidylcholines in biological membranes, dipalmitoyl phosphatidylcholine (DPPC).

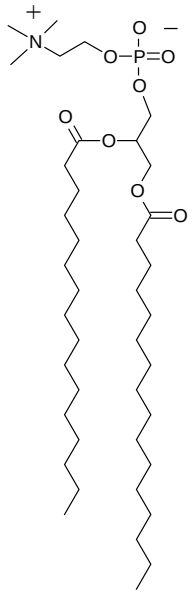

Figure 2.1. Structure of DPPC, dipalmitoyl phosphatidylcholine.

Different mammalian cells exhibit a remarkably similar plasma membrane lipid composition, characterised by a high content of structural lipids. Small but significant differences in composition exist between the various membranes of a cell (Sackmann, 1995a). The glycolipids, for example, can be found almost exclusively in the plasma membrane, where they contribute to the negative surface charge of the cells. Phosphatidylcholines abound in the membranes of the nucleus, the mitochondria and the endoplasmatic reticulum. In comparison with other cellular membranes, the mitochondrial membranes carry a significantly higher content of charged lipids (19 – 25 w-%).

Differences can also be found in the distribution of fatty acid between the various types of phospholipids. Phosphatidylcholines are mainly short chain lipids with a chain length of 16 – 18 carbon atoms. The majority of the sphingomyelins, on the other hand, are long chain lipids (C24) with none or one double bond. The remaining phospholipids (PE, PS, PI) are characterised by their high content of polyunsaturated chains (Sackmann, 1995a).

Phase behaviour

Lipids, such as many other rod-shaped organic molecules, exhibit thermotropic polymorphism, i.e. instead of undergoing a rapid transition from solid to liquid, they pass through intermediate phases as they are heated. These mesophases are known as the liquid crystalline phases. In contrast to liquids, molecules in the liquid crystalline phases exhibit orientational order (Petty, 1996). The most common mesophases of lipid bilayers are the fluid L_α-phase, the gel-phases $P_{\beta'}$ (rippled, tilted), $L_{\beta'}$ (tilted) and L_β (untilted), and the crystalline phase L_c (Seddon, Templer, 1995). The crystalline L_c -phase is formed by most phospholipids at low temperatures or hydrations and exhibits both short- and long-range order in three dimensions. In the presence of water, many lipids form gel phases at low temperatures. Their chains are still ordered in the all-*trans* conformation but they undergo hindered rotations around their long axis. The gel → fluid phase transition, also called the main or the chain melting transition, brings the hydrocarbon chains to a disordered state. Lipids in the fluid phase occupy an average molecular area of the order of 0.65 nm^2 (Sackmann, 1995b).

The biological lipid/protein bilayer is predominantly in a fluid state under physiological conditions. This is essential for cell growth to take place and for migrating cells (e.g. erythrocytes) to be able to travel through narrow body channels without losing their impermeability (Sackmann, 1995b). Bilayer softness is also important to membrane spanning proteins, as the functions of many of these involve conformational changes. In order to preserve the optimal fluidity of their cell membranes, many organisms are able to adjust their lipid composition in response to changes in environmental parameters, such as temperature. This type of behaviour has been found in bacteria like *Bacillus stearothermophilus* (Reizer et al., 1985) and *Escherichia coli* (Sinensky, 1974), but also in synaptic brain membranes of vertebrates (Kitajka et al., 1996).

Lateral and transverse order

The lipid-protein bilayer is not a structureless fluid, but exhibits certain lateral order. The formation of lateral domains is a result of interactions between the different molecular species in the lipid/protein bilayer. Domain formation can, for instance, be driven by the co-existence of lipids in different phases (Bloom, Mouritsen, 1995) or hydrophobic mismatch of lipids with considerably different chain lengths (Lehtonen et al., 1996). Another reason for domain formation is lipid-protein interactions, which can

cause lipid sorting by hydrophobic matching, as illustrated by Figure 2.2 (Dumas et al., 1997), or electrostatic interactions (Horváth et al., 1990). Domain formation can furthermore be induced and affected by external substances, such as polyelectrolytes (Macdonald et al., 1998), peptides (Gawrisch et al., 1995) and anaesthetics (Jørgensen et al., 1993).

The lateral heterogeneity of lipid bilayers and the connectivity of the membrane domains have important consequences for the functions of the cell membrane. The presence of domains has been reported to affect enzyme activity (Dibble et al., 1996; Hønger et al., 1996) and protein binding to the membrane surface (Kinnunen et al., 1994), as well as to play a part in cell division and membrane biogenesis (Welby et al., 1996). Simons and Ikonen (1997) recently proposed the existence of a particular type of microdomain, composed mainly of sphingolipid and cholesterol. These so-called rafts are believed to play an important part in the sorting and transport of lipids and proteins, as well as in signal transduction (Rietveld, Simons, 1998).

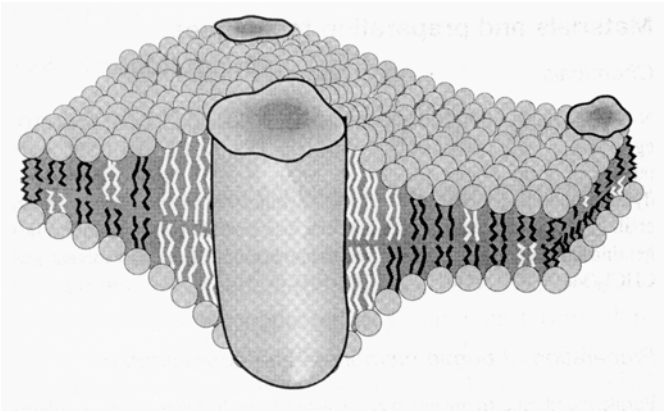

Figure 2.2. Drawing of lipid sorting by proteins. The hydrophobic matching principle implies that the lipid species that best matches the hydrophobic length of the protein will accumulate at its periphery. Reprinted with kind permission from Dumas et al. (1997) and the Biophysical Society.

Various experimental and theoretical studies have mapped the transverse structure of phospholipid bilayers (e.g. Hubbell, McConnell, 1971; Seelig, Seelig, 1974; Tu et al., 1996; Nagle et al., 1996, Hyvönen et al., 2003). The lipid head groups are thought to lie predominantly flat on the membrane surface. The hydrocarbon chains are highly ordered in the vicinity of the polar head groups, but the order diminishes the farther away from the head group one moves.

Marrink and Berendsen studied the transport of water (1994) and small molecules (1996) across a phospholipid bilayer using molecular dynamics (MD) simulations.

They described the phospholipid bilayer with the following four-region model: **region 1** starts at the point where the presence of the membrane surface begins to cause perturbations of the bulk water structure. The lipid headgroup density is low in this region. In **region 2,** the headgroup density is high and the water molecules are strongly bound. This region accommodates the major part of the glycerol backbones. **Region 3** contains the more ordered parts of the lipid tails. Hardly any water molecules are present in this region. Owing to the high tail density, the membrane in region 3 is viscous and resembles a soft polymer. Finally, **region 4** accommodates the major parts of the lipid chains. In this region, the tail density and viscosity is significantly lower than in region 3. Region 4 shows properties comparable to those of fluid decane.

The above-described transverse structure of a lipid bilayer has certain implications for its permeability towards solutes (Marrink, Berendsen, 1994; 1996). The local resistance to permeation is expected to be negligible in region 1 for most solutes. However, for very hydrophobic compounds, the slow diffusion in this region may cause it to become rate-limiting for the permeation process. Region 2 is characterised by a high head group density, which slows down diffusion. For hydrophilic solutes, this is overcome by the high dielectric permittivity of the region, which leads to an overall low permeation resistance. Owing to its low free volume and charge density, region 3 is expected to act as the rate-limiting barrier to permeation for a wide range of penetrants. Diffusion of small solutes is likely to occur via a hopping mechanism in this region. Region 4 is characterised by lower resistance to permeation than region 3 due to its more disordered nature.

2.1.2 Electric properties

The transverse structure of the bilayer causes the charged and dipolar lipid groups to be relatively fixed with respect to their orientation and location from the bilayer centre. Consequently, these charges and dipoles are only partially compensated by water dipoles and solution electrolytes, and a complex electric profile is generated over the membrane. This profile is composed of two components: the surface, or double-layer potential (ψ_0) and the dipole potential (ψ_d). In biological membranes, a further contribution to the electric profile is provided by the transmembrane potential difference ($\Delta\psi$). The three electric potentials are schematically illustrated in Figure 2.3.

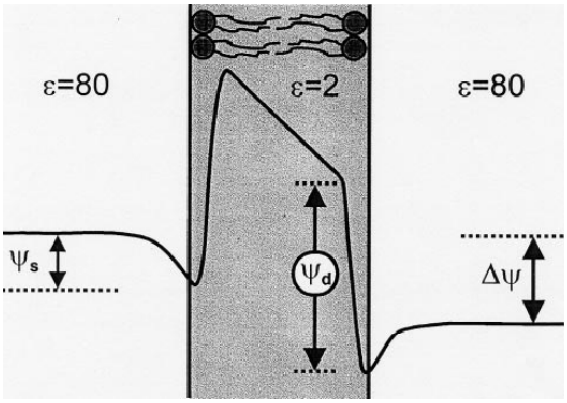

Figure 2.3. Schematic representation of the electric potential profile across a phospholipid bilayer showing the contributions of the surface potential (ψ_s), the dipole potential (ψ_d) and the transmembrane potential difference ($\Delta\psi$). Reprinted from Clarke (2001) with kind permission from Elsevier.

The surface potential

The surface potential profile, i.e. the potential difference between the membrane surface and the bulk aqueous phase, is commonly modelled with the classical Poisson-Boltzmann (PB) approach. The main difference between the various PB-based models is the treatment of the spatial distribution of the counterions. The simplest model is the Gouy–Chapman (GC) theory for the diffuse layer. In the GC theory, the membrane – water interface is treated as an infinitesimally thin surface onto which the lipid charges are uniformly smeared. The surface charge is counterbalanced by a diffuse layer of solution ions, which are modelled as point charges. According to the GC theory, the relationship between the surface charge density σ and the surface potential ψ_0 for a system with several ionic species present is as follows:

$$\sigma = \mathrm{sgn}(\psi_0)\sqrt{2\varepsilon_w kTN_A \sum_i c_{i,b}\left[\exp\left(-\frac{z_i e\psi_0}{kT}\right)-1\right]} \qquad (2.1)$$

where ε_w is the dielectric permittivity of water and $c_{i,b}$ is the bulk concentration of ionic species i.

It is well known that for large surface potentials, the GC treatment of ions as point charges results in unrealistically high ionic concentrations close to the membrane surface. The GC theory furthermore ignores the possibility of any chemical interactions between the ions and the surface. Stern modified the GC theory to account for the finite-size effect of the solution ions and the possibility of chemical binding. In the Gouy-Chapman-Stern (GCS) model, planes of closest approach are assigned for the

first layers of bound and non-specifically adsorbed ions. Beyond these layers, the ionic distribution follows the Gouy-Chapman picture of a diffuse layer based on point charges.

While the GCS model is clearly an improvement to the Gouy-Chapman theory, its picture of the surface structure differs significantly from that of a lipid membrane. A bilayer surface is neither homogeneous nor impenetrable, as portrayed in the GCS model. Molecular dynamics simulations indicate the thickness of the interfacial region of a DPPC bilayer to be over 1 nm (Marrink, Berendsen, 1994; Essmann et al., 1995) and the width of the head group distribution at half height to exceed 0.6 nm (Egberts et al., 1994). Small electrolyte ions are likely to associate with charged moieties of the lipid headgroups and affect the surface charge.

A common approach has been to model the bilayer surface with a GCS-based one-plane model, in which chemically bound ions are incorporated with the surface and the remaining ionic distribution obeys the GC result (McLaughlin et al., 1971; Lee, 1977; Eisenberg et al., 1979). In this approach, the surface density of adsorbed charge, σ_a, is obtained from the combination of the Langmuir adsorption isotherm and the Boltzmann relation. In the case of competitive binding of several ionic species to one type of binding site, one obtains:

$$\sigma_a = \frac{Ne\sum_i K_i z_i c_{i,b} \exp\left(-\frac{z_i e \psi_0}{kT}\right)}{1+\sum_i K_i c_{i,b} \exp\left(-\frac{z_i e \psi_0}{kT}\right)} \qquad (2.2)$$

where N is the surface density of binding sites and K_i is the binding constant of ion i.

The dissociation equilibrium of the lipid headgroups is ignored in many of the treatments on the surface electrostatics of lipid bilayers. Even in cases where the dissociation constant of the headgroups implies negligible or complete ionisation at bulk pH, it should be kept in mind that the surface concentration of protons differs from the bulk value according to the Boltzmann distribution. Consequently, the surface charge density of the lipid headgroups may be significantly different from what is expected on basis of the bulk pH. Successful estimates of surface potential values may be reached without accounting for the dissociation equilibrium of the lipid headgroup, when the models employed contain a large number of adjustable parameters. The deprotonation reaction may, for instance, be masked when assuming competitive binding of several ionic species. However, as demonstrated by Aguilella et al. (2000),

neglecting the dissociation equilibrium of the headgroups may lead to serious misinterpretation of experimental results.

The dipole potential

The dipole potential has been attributed to the transition region between the aqueous phase and the hydrocarbon interior of the membrane (Clarke, 2001). Its magnitude in a fully saturated phosphatidylcholine bilayer has been estimated to be 220-280 mV, positive in direction of the membrane interior (Clarke, 2001; Franklin, Cafiso, 1993). Bilayer dipole potentials have commonly been determined from the difference in conductance between the hydrophobic ions tetraphenylborate (TPB^-) and tetraphenylarsonium ($TPAs^+$), under the assumption that their hydration energies are identical (TATB assumption). Recently, it has been suggested that previous estimates should be corrected to 60-120 mV more positive due the inaccuracy of this supposition (Schamberger, Clarke, 2002).

The various proposals presented for the origin of the membrane dipole potential include the carbonyl residues of the lipid ester linkage (Pickar, Benz, 1978; Flewelling, Hubbell, 1986), the orientation of the lipid head groups and the polarised water associated with the membrane interface (Brockman, 1994; Gawrisch et al., 1992), as well as the functional group dipoles of the terminal methyl groups of the hydrocarbon chains (Vogel, Möbius, 1988). The lack of direct and non-perturbative measurement techniques to determine the partial dipole moments of these regions impedes the experimental resolution of the problem.

The formulation of a theoretical model for the dipole potential is hindered by the inadequate knowledge of the interactions from which it arises. The large change in the dielectric constant over a few Ångströms at the interface further complicates the picture (Brockman, 1994). However, the development of increasingly powerful computers and calculation algorithms has brought about new ways to study lipid bilayers through detailed computer simulations. Lipid bilayers have been modelled with a variety of approaches covering different time- and length scales. The three most common ones are based on mean and self-consistent field theories, Monte Carlo algorithms, and molecular dynamics (MD) calculations (e.g. Meijer et al., 1994; Pastor, 1994; Harries, Ben-Shaul, 1997; Tieleman et al., 1997). Particularly, the highly detailed MD simulations have shed light on the contribution of interfacial water to the dipole potential. The results obtained so far should still be regarded with some caution,

as despite the continuous improvement of computational resources, MD calculations are still too computer costly to be able to incorporate a bulk electrolyte.

Figure 2.4 (Tieleman et al., 1997) shows the simulated electrostatic potential profile across the interfacial region of a lipid bilayer and its unphysical separation into lipid and water contributions. The lipids give rise to a negative potential in the inside of the membrane; its magnitude is dependent on the type of lipid. For the case of DPPC, the negative sign of the lipid potential suggests an important contribution of the lipid head group dipoles, as the *sn*-2 chain carbonyl dipole moments are oriented with the positive charge towards the membrane interior (see Figure 2.1) making their contribution one of the opposite sign. The lipid potential, however, is counteracted by the dipole moments of the interfacially aligned water molecules. The orientational polarisation of interfacial water is in fact so strong that it overcompensates the sum of the lipid dipole moments, thereby inverting the dipole potential to positive inside the membrane. Several other groups have confirmed this result for the case of the uncharged lipid DPPC (Essmann et al., 1995; Shinoda et al., 1998; Stern and Feller, 2003).

The above observation was made for lipids with zero net charge. López Cascales et al. (1996) undertook a MD study of a bilayer of negatively charged dipalmitoyl phosphatidylserine (DPPS) in the presence of counterions. They found that the headgroup charge was mainly screened by the counterions. In addition, the PS headgroups exhibited much stronger interactions with neighbouring lipids than PC headgroups, resulting in a weaker tendency to be coordinated by water. As a result, the dipolar orientation of water did not fully compensate the negative (inside) potential generated by the lipids.

The transmembrane potential

The transmembrane potential, i.e. the potential difference between the intra- and extracellular side of all living cells, is a result of charge separation across the membrane. It arises from the concentration gradients of certain ionic species across the cell membrane and the selective permeability of the membranes towards some of these ions. The transmembrane concentration gradients are established by active transporter proteins, which move ions across the membrane against their concentration gradient. The selective permeability of membranes is mainly due to the presence of ion channels; proteins that allow certain ions to cross the membrane down their concentration gradients (Purves et al., 2001).

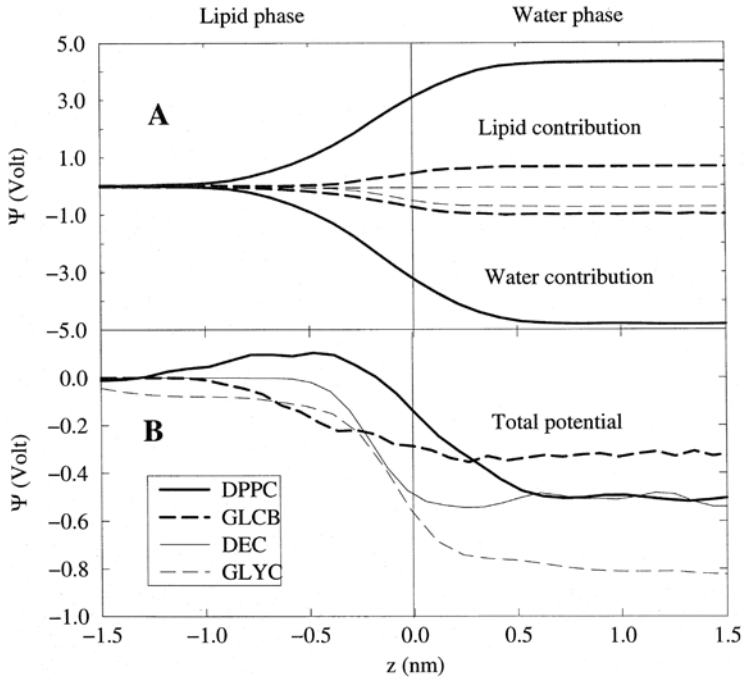

Figure 2.4. Electrostatic potential profile across the lipid – water interface from molecular dynamics simulations. Profiles are shown for three types of uncharged lipids: DPPC, *β*-decyl glucoside (GLCB) and dilauroyl glycerol (GLYC), and decane (DEC). (A) The upper half shows the contribution of the lipids and the lower half the contribution of the water molecules (the contribution from decane is zero). (B) The total electrostatic potential across the interface. Reprinted from Tieleman et al. (1997) with kind permission from Elsevier.

With regard to the transmembrane potential, it should be noted that cell membranes are classified as excitable or non-excitable. Whereas all cells respond to certain changes in their environment, excitable cells are characterised by their electrical signalling. Hille (2001) defines cells as excitable if they express voltage-gated calcium or sodium channels. Such cells exhibit rapid changes in their permeability as a function of potential, and launch so-called action potentials – the basis of electrical communication between cells. The magnitude of the transmembrane potential (at rest) depends on the cell type. Excitable cells, such as nerve and muscle cells have a resting transmembrane potential of approximately −90 mV (Hille, 2001).

As expected, the electric potentials that are present in bilayer membranes modify the model of Marrink and Berendsen (1996) in terms of permeability towards charged solutes. Surface charge and dipole density have been predicted to significantly affect

the permselectivity of the bilayer towards ionic permeants, both in the case of the hydrophobic interior or the polar head group zone constituting the rate-limiting step (Levadny et al., 1998; Aguilella et al., 1996). The large and positive dipole potential makes the bilayer interior more attractive to anions than cations (Meijer et al., 1999). In addition, the presence of a transmembrane potential difference has been found to increase ion partition into lipid vesicles (Mayer et al., 1985).

The electric properties of biological membranes play a fundamental role in the functions of the cell. The bilayer potential profile as a whole is essential for the functions of ion channels and other protein structures. The transmembrane potential furthermore constitutes the basis of signal transduction in excitable cells. Apart from regulating the surface concentration profile of charged species, the surface potential influences a variety of membrane-related phenomena, such as phase transitions (Lee, 1977), adhesion, aggregation and fusion (Tatulian, 1999). Several charged lipid species are known to play a part in protein recognition and signalling (Langner, Kubica, 1999). The membrane dipole potential affects the rate of transmembrane ion transport (Tatulian, 1993), domain behaviour (Klingler, McConnell, 1993) and fusion (Brockmann, 1994), as well as peptide insertion (Cladera, O'Shea, 1998) and enzyme activity (Maggio, 1999).

It should, however, be kept in mind that the picture presented in this section only concerns the electric properties of a lipid bilayer. In biological membranes, further contributions arise from the variety of proteins associated with the bilayer as well as the negatively charged glycocalix surrounding the outer leaflet of the cellular plasma membrane (Langner, Kubica, 1999).

2.2 Model membranes

The most thoroughly studied cell membrane is the human erythrocyte (red cell) plasma membrane. Erythrocyte plasma membranes constitute more feasible research objects than other biological membranes as they are only connected to the cytoskeleton (inner protein network), whereas most plasma membranes are also coupled to an extracellular protein film, the glycocalix (Sackmann, 1995a). Moreover, erythrocytes can be emptied of their intracellular contents through induced hemolysis to form so-called resealed erythrocyte ghosts. Such ghost cells, consisting only of the plasma membrane, the cytoskeleton and an intracellular solution, are regarded as valuable tools for the study of membrane properties (Jausel-Husken, Deuticke, 1981).

Nevertheless, the erythrocyte plasma membrane contains several hundred different lipid species and numerous types of membrane-associated proteins (van Golde et al., 1967; Devaux, Seigneuret, 1985) – a construction too complex for studying specific membrane functions on a molecular level. The surface activity and self-assembly of lipids in aqueous solutions has, however, enabled the preparation of model membranes where lipid and protein composition can be controlled and structure – function relationships established. In the following, existing model systems of biological membranes are reviewed, the emphasis being on approaches applicable to electrochemical studies.

2.2.1 Unsupported bilayer lipid membranes, BLM

Formation techniques

The first model membranes based on lipid bilayers were introduced in 1961 and described in Nature the following year (Mueller et al., 1962). These so-called black lipid membranes (the interference of light makes them appear dark) were originally formed by spreading a lipid solution in a small (Ø 1 mm) hole of a wall separating two aqueous compartments by brushing (Mueller et al., 1963). Evaporation or diffusion of the lipid solvent leads to thinning of the film to its final bilayer state (Figure 2.5). Later studies, however, have shown that this thinning of black lipid membranes continues until their breakdown, suggesting that the membranes are under constant change throughout their lifetime (Benz, Janko, 1976).

Accordingly, alternative, solvent-free methods to form unsupported planar bilayers have been developed to avoid retention of solvent in the bilayers and consequent time-dependent thinning. This has been achieved by the formation of a lipid monolayer at an air – water interface and the subsequent folding of the monolayer into a bilayer through an aperture in a substrate. Solvent-free monolayers can be formed by pipetting of lipid solution onto the water surface followed by solvent evaporation, or by adsorption from lipid vesicles in the bulk aqueous phase to the air – water interface. The apposition of the monolayers has been achieved either by lowering an aperture-containing substrate through the monolayer (Takagi et al., 1965; Tancréde et al., 1983) or by raising/lowering of the water level and thereby causing the monolayer to deposit on the substrate and cover the aperture (Montal, Mueller, 1972; Benz, et al., 1975; Nikolelis, Krull, 1992). In addition to being practically solvent-free, the monolayer-folding

technique enables the preparation of asymmetric membranes by the apposition of two monolayers with different lipid compositions.

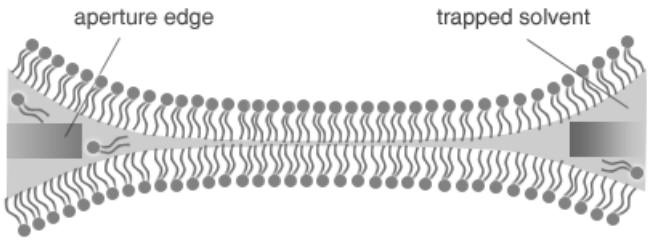

Figure 2.5. Schematic illustration of an unsupported BLM formed by spreading of lipid solution in a small aperture. The size of the lipids is considerably exaggerated in the figure.

Unsupported bilayer lipid membranes suffer from one serious drawback that hampers their use as model membranes in biological studies: they are extremely fragile, with lifetimes in the range of minutes to a few hours. One factor that contributes to this instability is the hydrostatic pressure difference that may form across the aperture during the deposition process. To this end, techniques for obtaining hydrostatically stabilised bilayers have been described (Vodyanoy et al., 1982), leading to increased mechanical stability of the membranes. Other attempts to increase the stability of BLMs include the polymerisation of the lipid headgroups (Benz et al., 1982) and the reduction of the cross-sectional BLM area, which has been effected by BLM formation in pores of microfiltration membrane filters (Thompson et al., 1982).

Whereas polymerisation of the lipid headgroups was found to impose strain on the bilayer, leading to the formation of holes in the membrane, the microfilter approach yielded BLMs with longer lifetimes and increased stability towards stirring. However, for reproducibility of the technique, a large number of single micro-BLMs with the same characteristics have to be formed simultaneously. In addition, a variable amount of the solvent used in spreading the lipid may become trapped in the BLMs, causing time-dependent thinning. Although Thompson et al. (1982) demonstrated the presence of single BLM structures by the insertion of a channel-forming compound that only yields functioning channels in bilayer structures, others have found that continuous thinning of solvent-spread BLMs takes place in almost every pore of the microfilter-

stabilised BLM (Ikematsu et al., 1996). In the latter study, the lifetime of the thinned membrane could be significantly increased by addition of cholesterol and Ca^{2+}.

Recent development in BLM research has seen the construction of lipid bilayers in microfabricated apertures of gold (Ogier et al., 2000) and silicon (Osborn, Yager, 1995a; Pantoja et al., 2001), with the possible scenario of micro-BLM arrays suited for mass production.

BLMs have been characterised with a variety of optical, electrochemical and spectroscopic approaches, the primary criterions being their specific capacitance and resistance. The capacitance, *C*, describes the ability of the lipid – water interface to store charge in response to a change in potential difference, and depends on the dielectric permittivity, thickness, and surface charge of the bilayer. The resistance, *R*, is the proportionality constant between current and potential given by Ohm's law, which in the context of lipid bilayers is a descriptor of membrane integrity. Depending on the method and the lipid, specific capacitances between 0.3 and 1 $\mu F/cm^2$ have been reported for unsupported BLMs, the capacitance of cell membranes being 1 $\mu F/cm^2$ (Nikolelis, Krull, 1992). For comparison, electrical characteristics of various BLMs are compiled in table 2.1.

Applications

The initial studies on BLMs were in large part devoted to shed light on the nature of bioelectric events in cellular membranes. Whereas the ionic basis of the action potential was known (Hodgkin, Huxley, 1952), the molecular mechanisms underlying the permeability changes were still a matter of debate. Mueller and Rudin carried out a series of studies on the voltage-dependent conductance of cyclic antibiotics embedded in BLMs, which provided a strong foundation for future studies of ion channels (Mueller, Rudin, 1967a, 1967b, 1968). A seminal contribution was made by Hladky and Haydon (1970), whose experiments on BLM-embedded gramicidin A provided a strong indication that the ion-selective pathways involved in cell signalling were not active transporter molecules but channels: membrane-spanning proteins forming a pore through which particular ions could move at high rates.

Table 2.1. Electrical properties of unsupported lipid bilayer membranes in substrate apertures. The grey fields indicate solvent-free bilayers. Membrane lifetimes are given where information was available.

Method	Lipid	Aperture diameter	C (μF/cm^2)	R (Ωcm^2)	BLM lifetime	Reference
Brushing from CH_3Cl / MetOH	Brain lipids	1 mm	1.0	10^8	24h	Mueller et al., 1963
Brushing from *n*-decane	PCs	1.6 mm	0.3 – 0.4	10^8		Benz, Janko, 1976
Monolayer folding		0.2 – 0.3 mm	0.9	10^6 – 10^8		Montal, Mueller, 1972
Monolayer folding	PCs	0.2 – 0.3 mm	0.5 – 0.7			Benz et al., 1975
Monolayer folding	DOPC*	0.2 – 0.3 mm	0.7			Benz, Janko, 1976
Monolayer hydrost. stab.	Egg-PC¤		0.8	10^8 – 10^9		Vodyanoy et al., 1982
Monolayer folding		0.32 mm	0.9	10^6 – 10^8	24 h, stable 4 h	Nikolelis, Krull, 1992
Monolayer folding	Soy-PC†	100 – 250 μm	0.6 – 0.7		< 8h	Baba et al., 1999
Microaperture in gold	Egg-PC	100 μm	0.6	10^6	20 min	Ogier et al., 2000
Microaperture in gold	Egg-PE	100 μm	0.3 – 0.7	10^7	5 h	Cheng et al., 2001
Microaperture in silicon	PE/PC/PS	50 – 200 μm	0.4 – 1.0	$5 \cdot 10^9$		Pantoja et al., 2001

* DOPC = dioleoyl phosphatidylcholine (C18:1); ¤ Egg-PC = mixture of phosphatidylcholines obtained from egg yolk; † Soy-PC = mixture of phosphatidylcholines obtained from soybean.

Channel reconstitution remains one of the most important applications of BLMs. Reconstitution enables the characterisation of various aspects of channel proteins, such as the mechanism of channel incorporation, the presence of various isoforms as well as

the determination of the ion selectivity, transport rate, voltage dependence and kinetics of the conductance caused by the membrane protein (Abrecht et al., 2000; Arora et al., 2000; Micelli et al., 2000; Tadjibaeva et al., 2000). In addition, channel reconstitution has been employed for the reproduction of a certain membrane function, such as the photoelectric effect of the retinal membranes (Nakanishi, 1995; Hong, 1997) and the odour reception of olfactory cells (Zviman, Tien, 1995).

Apart from ion channel studies, unsupported bilayer lipid membranes have been employed in diverse studies, ranging from photovoltage generation and transport in BLM/semiconductor particle systems (Rolandi et al., 1992) to electron transfer at, and across BLMs (Yamada et al., 1993; Cheng et al., 1996). During the past decade, BLM research has been directed towards the development of biosensors (Sugao et al., 1993; Nikolelis, Siontorou, 1995), the most recent efforts aiming at the construction of mass-producible BLM arrays for biosensor and high throughput drug screening assays (Osborn, Yager, 1995a; Cheng et al., 2001; Pantoja et al., 2001).

Patch-BLMs

In 1976, Sakmann and Neher introduced the "patch-clamp" technique, which was to revolutionise the field of electrophysiological experimentation (Neher, Sakmann, 1976). The procedure involves gently pushing a heat-polished glass micropipette tip (Ø ~1 μm) against the cell membrane and voltage-clamping the membrane patch, which closes off the mouth of the pipette. The technique was soon refined to achieve so-called “giga-seals”, pipette – membrane seals with resistances of 10 – 100 GΩ, which enabled the high-resolution measurement of picoampere currents through single ion channels (Hamill et al., 1981).

The discovery of the patch-clamp also had implications for BLM research. Several groups reported on the formation of planar lipid bilayers on patch pipettes (Suarez-Isla et al., 1983; Coronado, Latorre, 1983; Hanke et al., 1984). Patch-BLMs were formed by the subsequent movement of a filled, 1 μm patch pipette out of and into a solution covered by a lipid monolayer. The resulting BLMs exhibited pipette – membrane seals with a resistance ranging from 1 – 20 GΩ. Membrane capacitance was not measurable due to the large capacitance of the pipette. The applications of patch-BLMs have mainly focused on the reconstitution of ion channels. In comparison with patch-clamp experiments on real cells, reconstitution into artificial bilayers allows for the identification of less common ion channels, the conductances of which are masked by

voltage-gated, and abundant or high-conductance channels in biological preparations (Davenport, Tester, 2000).

Owing to their small membrane area and gigaohm seal, the current resolution of patch-BLMs is clearly superior to those formed in apertures of various substrates. Additional benefits include increased mechanical stability and small amounts of materials required for the experiments. Furthermore, patch-BLMs are completely solvent-free as no pre-treatment of the pipettes is necessary, whereas conventional BLMs formed through monolayer folding in substrate apertures often require the pre-treatment of the aperture surroundings with a hydrocarbon solvent. A drawback of patch-BLMs compared to conventional ones is the accessibility of only one aqueous compartment.

2.2.2 Liposomes

Simultaneously with the development of the black lipid membrane, other researchers were studying the behaviour of free phospholipids in aqueous solutions. Electron microscope experiments indicated that the lipids spontaneously formed so-called ‘smectic mesophases’ (closed bilayer membranes) in aqueous surroundings. In 1965, the first article utilising ‘smectic mesophases’ as cell membrane models was published (Bangham et al., 1965) and during the same year, they were renamed ‘liposomes’.

Liposomes have proven highly useful in a number of studies on membrane structure and function (Bangham, 1993), and have given rise to important pharmaceutical applications (Lasic, Needham, 1995; Rädler et al., 1997). However, the accessibility to only one side of the membrane limits the usefulness of liposomes for electrochemical approaches, with the exception of patch-clamp measurements. Consequently, liposomes will not be further discussed in this literature review. Nevertheless, they appear in various contexts in this work, often under the name of unilamellar lipid vesicles.

2.2.3 Solid-supported bilayer lipid membranes, ssBLM

Despite the various approaches employed to improve the stability of conventional BLMs, the unsupported bilayer remains an extremely fragile construction, rarely

retaining a stabile configuration for more than a few hours. Consequently, it is of limited use for protracted studies, and for practical applications such as biosensors and electronic devices. With the advent of the supported bilayer lipid membranes, not only has long-term stability improved, also the membrane configuration is more promising for incorporation into technological applications.

Formation techniques

Among the first to report on solid-supported BLMs were Tamm and McConnell (1985), who studied the formation of phospholipid bilayers on glass, quartz and silicon dioxide substrates. The ssBLMs were formed by subsequent deposition of two lipid monolayers employing the Langmuir – Blodgett (LB) and Langmuir – Schäfer (LS) techniques. In brief, the techniques involve the formation of a lipid monolayer at an air – water interface and the subsequent vertical (LB) or horizontal (LS) translocation of a substrate through the monolayer at a desired surface pressure. The LB and LS techniques have since been employed in a number of studies to form lipid bilayers on substrate materials such as SiO_2 (Osborn, Yager, 1995b), TiO_2 and $SrTiO_3$ (Starr, Thompson, 2000), glass (Sonnleitner et al., 1999) and mica (Benz et al., 2004). While these formation methods require the implementation of specific experimental instrumentation, their benefits include ease of automation and control of both the composition and the state of lipid organisation in each deposited monolayer.

Tien and Salamon (1989) proposed the formation of ssBLMs on the cross-section of Teflon-wrapped metal wires. This simple formation procedure involves cutting the wire in the presence of a BLM-forming solution, thereby revealing a fresh, hydrophilic substrate that attracts the polar groups of the lipids. The lipid-solution coated tip is subsequently transferred to an aqueous solution, where the self-assembly of the ssBLM to its final state occurs. This final state is not necessarily a bilayer structure, but mono- or trilayer structures have also been observed (Chiang et al., 1997; Passechnik et al., 1998).

Recently, an increasing amount of attention has been devoted to the vesicle unrolling technique, whereby ssBLMs are formed by the spontaneous adsorption and fusion of vesicles onto a hydrophilic surface (Keller et al., 2000; Starr, Thompson, 2000; Reimhult et al., 2003). An obvious benefit of the vesicle unrolling method is that it allows for the simultaneous incorporation of proteins into the bilayer without any

contact with denaturating solvents. However, in all of the studies cited above, some extent of adsorption of intact vesicles at the substrate was observed.

The improved mechanical and long-term stability combined with a more easily manoeuvrable configuration renders ssBLMs available to a more extensive repertoire of experimental techniques compared with their unsupported counterparts. The topography and stability of solid-supported BLMs formed on molecularly smooth mica sheets have been characterised by atomic force microscopy (AFM) and surface force measurements (Benz et al., 2004). The quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) techniques have been employed to shed light on the adsorption kinetics of vesicle unrolling onto substrates (Keller et al., 2000; Reimhult et al., 2003).

Fluorescence recovery after photobleaching (FRAP) and single molecule fluorescence microscopy have been widely used to provide information on the lateral diffusivity of the ssBLM lipids (Tamm, 1988; Groves et al., 1997; Starr, Thompson, 2000; Schmidt et al., 1996; Sonnleitner et al., 1999). Furthermore, the deposition of lipid bilayers onto metal or semiconductor substrates enables the application of a variety of electrochemical techniques such as cyclic voltammetry and impedance spectroscopy (Gu et al., 1996; Lindholm-Sethson, 1996; Haas et al., 2001; Wiegand et al., 2002). In addition to capacitance and resistance data, electrochemical techniques provide information on the kinetics of charge transfer, which can be related to membrane integrity and the presence of defects.

Applications

In terms of practical applications, the main focus in ssBLM research has been directed towards the field of biosensing. Biosensors employ biological operating principles, such as molecular recognition, for the specific detection of a molecular compound in an aqueous solution. The chemical-physical changes in the membrane properties that arise due to the recognition reaction, are transduced into an electrical signal for readout (Tien, Ottova, 1999). Solid-supported ssBLMs provide an attractive basis for biosensor development due to the following factors: (i) the presence of the lipid bilayer excludes hydrophilic compounds from reaching the detecting surface and producing undesired signals; (ii) simultaneously, the lipid bilayer acts as a matrix for embedding compounds that interact specifically with the detectable species, rendering the biosensor its selectivity. Such compounds are typically enzymes, ion channels and

antibodies, which require a fluid lipid bilayer environment for adopting their active conformation.

The numerous ssBLM-biosensor studies that have been reported in literature can be classified on the basis of their detection mechanism. In electrochemical ssBLM-biosensors, the detection can be based on the measurement of ionic currents in specific ion channels (Nikolelis et al., 1996; Cornell et al., 1997; Ivnitski et al., 2000) or the generation of electroactive species by enzymes embedded in the bilayer (Šnejdárková et al., 1997). Optical ssBLM-biosensors commonly rely on the surface plasmon resonance (SPR) technique, which detects surface binding through the change in the resonance angle of surface plasmons (Kuziemko et al., 1996; Heyse et al., 1998a,b; Puu, 2001). A third type of ssBLM-biosensor relies on the direct measurement of mass change, which is often quantified by the quartz crystal microbalance (QCM) technique (Janshoff et al., 1997).

Meanwhile, ssBLMs have been proposed for a number of purposes other than biosensors. Tien and co-workers have investigated self-assembled ssBLMs on indium-tin oxide (ITO) glass as a system for studying the light-induced properties of biomembranes and the development of biomimetic photoelectric devices (Feng et al., 1999; Gao et al., 2000). Furthermore, ssBLMs have been employed in structural studies of lipid bilayers, such as the reconstitution of membrane rafts (Dietrich et al., 2001; Saslowsky et al., 2002). Micropatterned ssBLMs have, on the other hand, been proposed for applications in high throughput screening, combinatorial chemistry and probing of living cell – synthetic membrane interactions (Groves et al., 1997; Hovis, Boxer, 2001; Groves, Dustin, 2003).

Despite the significant scientific interest, no commercial products based on ssBLMs have emerged so far. The lack of a facile formation technique and long-term structural integrity are some of the technical impediments standing in the way of practical applications. Further problem issues concerning ssBLM applicability are discussed in the following subsection.

Potential drawbacks

Despite their increased stability in comparison with unsupported BLMs, ssBLMs held together by van der Waals forces alone cannot be removed from the aqueous phase without loss of the outermost lipid monolayer (Benz et al., 2004). Halter et al. (2004) recently developed a series of DPPC derivatives, which undergo inter-leaflet

cross-linking after LB/LS bilayer deposition onto a glass coverslip. The formed lipid dimers that span the bilayer are believed to increase the ssBLM stability while preserving its fluidity. Whether or not these cross-linked ssBLMs are stable in air was not commented on, however. Conboy et al. (2003) proposed polymerisation of the ssBLM lipids to increase stability. Upon removal from water, the bilayer structure was only preserved in lipid bilayers where inter-leaflet polymerisation was possible. Significant loss of material was, nevertheless, observed, which was attributed to desorption of low molecular weight oligomers.

Furthermore, in comparison with conventional BLMs, solid-supported BLMs suffer from two potential drawbacks. Many studies employing LB/LS deposition or self-assembly of lipids at metal wire cross sections, report on the presence of membrane defects (Passechnik et al., 1998; Haas et al., 2001; Tamm, McConnell, 1985; Osborn, Yager, 1995b; Benz et al., 2004). Such defects, which arise partially due to substrate roughness (Hianik et al., 1995), will significantly reduce bilayer resistance and lead to the inability of the ssBLM to block redox reactions at the substrate (Yang, Klejin, 1999; Table 2.3).

Another issue of concern regarding ssBLM structure has been the loss of fluidity that the immobilisation onto a solid substrate may induce. To this end, lateral diffusion coefficients of ssBLM lipids have been measured. A compilation of results is shown in Table 2.2. The lateral diffusion coefficients of fluid phase phospholipids can be observed to lie within the same order of magnitude, regardless of preparation or measurement technique. Small differences may result from different measurement temperatures and lipids, as well as the length scale of the method employed (Vaz, Almeida, 1991). As investigated by Starr and Thompson (2000), the substrate material likewise exerts some influence on the lateral diffusion. For comparison, lateral diffusion coefficients measured in conventional BLMs (1, 10), hydrated multilamellar lipid crystals (7, 8), and large unilamellar vesicles (9), are also shown.

Lateral diffusion in ssBLMs does not appear to be dramatically reduced in comparison with multilamellar crystals and lipid vesicles, the latter of which are completely self-assembled, substrate-free bilayer constructs. On the other hand, the larger values observed in conventional, unsupported BLMs suggest the presence of retained solvent. Observations that lipids in glass- or quartz-supported BLMs diffuse at equal rates in both leaflets (Tamm, McConnell, 1985; Tamm, 1988) indicate the

Table 2.2. Lateral diffusion coefficients of solid-supported lipid bilayer membranes (2 – 6, grey fields); unsupported bilayer lipid membranes (1, 10); multilamellar bilayers (7, 8); as well as unilamellar bilayer vesicles (9) are shown.

Preparation	*T** (°C)	Lipid	Substrate	*D* (10^{-8}cm^2/s)	Ref. / *Method*
1. BLM, decane	23	POPE / POPC (7:3)	Ø 80 μm aperture in Teflon	20.6 ± 0.9	Sonnleitner et al., 1999
2. LB/LS (32mN/m)	23	POPE / POPC (7:3)	Glass	4.6 ± 0.1	*Single molecule fluorescence*
3. LB/LS (35mN/m)	RT	POPC	Fused silica TiO_2 $SrTiO_3$	1.4 ± 0.2 1.6 ± 0.5 2.1 ± 0.5	Starr, Thompson, 2000
4. Vesicle unrolling	RT	POPC	Fused silica TiO_2 $SrTiO_3$	0.6 ± 0.2 2.5 ± 0.8 3.1 ± 0.5	*FRAP*
5. LB/LS (36mN/m)	15 20 30	POPC	SiO_2	2.39 ± 0.08 2.9 ± 0.2 4.6 ± 0.1	Tamm, 1988
6. LB/LS (36mN/m)	15 20 30	DMPC	SiO_2	$< 10^{-2}$ 0.66 ± 0.02 3.8 ± 0.4	*FRAP*
7. Multilamellar crystals, MLC¤	15 20 30	DMPC	–	$< 10^{-2}$ $< 10^{-2}$ 6.4 ± 0.8	
8. MLC¤	18 29	DMPC	–	$< 10^{-2}$ 1 – 2	Fahey, Webb, 1978
9. Large bilayer vesicles	19 29	DMPC	–	~ 10^{-2} 1 – 2	*FRAP, fluorescence correlation spectroscopy*
10. BLM, hexane	17 24	DMPC	Electron microscopy grid	14 ± 3 14 ± 3	

*The gel to fluid phase transition temperatures for the lipids are –3ºC (POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine, C16:0-C18:1), 23.5ºC (DMPC, dimyristoyl phosphatidylcholine, C14:0) and 25ºC (POPE, 1-palmitoyl-2-oleoyl phosphatidylethanolamine), according to Cevc (1993). ¤The process involves the spreading of a lipid-solvent mixture on a glass plate, the subsequent evaporation of the solvent and the formation of hydrated, multilamellar bilayers in the presence of a humid atmosphere.

presence of a thin (10 – 20 Å), lubricating water layer between the support and the lipid headgroups, which enables the supported monolayer to retain some fluidity. However, other studies suggest that the effect of such a water layer is highly dependent on the properties of the substrate (smoothness, hydrophilicity). Hetzer et al. (1998) found that diffusion in fluid phase DPPC bilayers supported on spherical silica beads was slower in the monolayer closer to the support by a factor of two. Groves et al. (1998) report on the absence of lipid mobility in certain metal and metal oxide supported BLMs.

Although lateral mobility of ssBLM lipids can be preserved by the presence of a thin water layer, embedded transmembrane proteins often have hydrophilic sections that protrude significantly outside the lipid bilayer, and may thus become immobile or denatured upon contact with the solid support (Salafsky et al., 1996; Guidelli et al., 2001; Figure 2.6a). The lack of a well-defined ionic reservoir on the substrate side of the bilayer has been mentioned as an equally important drawback when studying membrane transport through ion carriers and channels (Raguse et al., 1998).

Hybrid ssBLMs

Hybrid ssBLMs were developed to improve some of the above-mentioned shortcomings of the original ssBLMs. In hybrid ssBLMs, the first monolayer is typically an alkanethiol monolayer, covalently attached to a metal substrate, onto which a phospholipid monolayer is deposited either by transfer from the air – water interface or through vesicle unrolling. Such thiol/lipid ssBLMs have been reported to be stable in air (Meuse et al., 1998) and constitute an improved barrier towards charge transfer (Plant, 1993; Lingler et al., 1997). Liquid mercury has been employed as an atomically flat and homogeneous support to yield reproducible and defect-free hybrid ssBLMs (Tadini Buoninsegni et al., 1998). However, the rigidity of alkanethiol/phospholipid ssBLMs is much higher than that of fluid biological membranes. In addition, the structure of thiol-based ssBLMs prevents the formation of a water layer between the bilayer and the metal support. Because of these conformational restraints, such mixed ssBLMs are unsuitable for the incorporation of integral proteins and studies of ion transport through ionophores (Guidelli et al., 2001).

In an attempt to overcome these drawbacks, while preserving the stability provided by the covalently bound alkanethiol monolayer, so-called thiolipids have been synthesised (Lang et al., 1994; Cheng et al., 1998; Steinem et al., 1998; Raguse et al.,

1998). The thiolipids are composed of lipid derivatives, extended at their polar headgroups by hydrophilic spacers, which terminate in a thiol or disulfide group for covalent binding to the substrate. When the first monolayer of the hybrid ssBLM is formed from such thiolipids, a more fluid bilayer results, allowing for the incorporation of functioning ion carriers (Steinem et al., 1998). Furthermore, to obtain a water-filled space between the bilayer and the solid support for accommodation of protruding integral protein sequences, hybrid ssBLMs have been constructed where the inner monolayer is a mixed layer of thiolipids and lipids (Cheng et al., 1998; Heyse et al., 1998a; Raguse et al., 1998; Figure 2.6b).

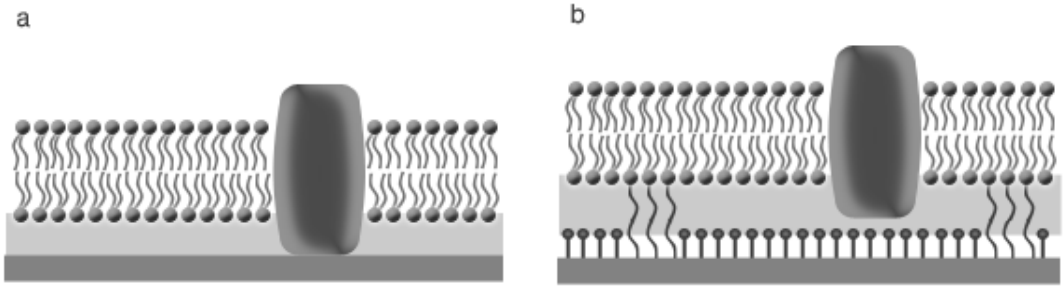

Figure 2.6 (a) A ssBLM on a hydrophilic surface. The thin, lubricating water layer between the substrate and the inner monolayer may preserve lateral mobility of the lipids. However, embedded transmembrane proteins may protrude significantly outside the bilayer and interact with the solid substrate. **(b)** A hybrid ssBLM where the inner monolayer is a mixed layer of thiolipids and lipids in order to create water-filled spaces between the bilayer and the solid support for accommodation of protruding integral protein sections. The surface-bound, short chain thiols with hydrophilic end groups are necessary for the lateral spacing of the thiolipids.

Hybrid ssBLMs have proven a successful environment for protein incorporation. Heyse et al (1998a) report on an elegant hybrid ssBLM-based assay to investigate receptor activation and interaction with G proteins by one-dimensional imaging surface plasmon resonance. Light-induced activation of the transmembrane photoreceptor rhodopsin (embedded as in Figure 2.6b) led to coupling with, and the subsequent dissociation of its surface bound G protein transducin, which was monitored by SPR. The same group also studied the incorporation of the pore-forming protein porin, into a hybrid ssBLM (Heyse et al., 1998b). Impedance spectroscopy was employed to observe the changes in porin conductance induced by the binding of ligands.

Knoll and co-workers (Schmidt et al., 1998; Naumann et al., 1999) employed hybrid ssBLMs, in which the thiolipid spacers consisted of oligopeptides, as an environment for protein embedding. SPR was used to follow the incorporation of the acetylcholine receptor, a ligand-gated ion channel protein, into such a bilayer, and the specific binding of the antagonist α-bungarotoxin to the protein surface. The same group also studied the incorporation of cytochrome *c* oxidase (COX) into the hybrid ssBLM. COX is the terminal component of the respiratory electron-transport chain, and is abundant in the inner mitochondrial membrane. It catalyses the enzymatic redox reaction between oxygen and cytochrome *c*, and actively transports protons against the gradient of electrochemical potential across the lipid membrane. Although this process can be studied by reconstitution of COX in liposomes (Steverding, Kadenbach, 1991), direct electrical measurements are not feasible due to the inaccessibility of the inner compartment. However, when embedded in hybrid, metal-supported ssBLMs, electrochemical techniques such as impedance spectroscopy can be employed to study COX activity in the form of transmembrane proton transport (Naumann et al., 1999).

Hybrid ssBLMs have furthermore been employed as substrates for the adsorption of biological membrane fragments containing ion pumps. Pintschovius and Fendler (1999) employed electrochemistry at an ssBLM to determine the kinetics of ATP- and Na^+ -binding to Na^+/K^+-ATPase, and the subsequent translocation of Na^+ through the latter.

The long-term stability and fluidity of the above described hybrid ssBLMs are promising for biosensor and drug development applications. However, these advantages are counteracted by the poor electrical properties of hybrid ssBLMs (Table 2.3). The low electric resistances (~ 10^4 Ωcm^2) of the membranes allow for appreciable non-specific fluxes of ions, leading to low signal to noise ratios.

In conclusion, ssBLMs are more stable in aqueous solution than their unsupported counterparts. Nevertheless, the proximity of the solid support may lead to decreased bilayer fluidity, and poses a problem particularly for the incorporation of transmembrane proteins. Thiolipid-based, hybrid ssBLMs maintain bilayer fluidity, allow for protein embedding, and exhibit desired long-term stability. However, most of the ssBLM approaches suffer to some extent from the presence of defects and pinholes, which presents a serious drawback for direct, electrochemical sensing.

Table 2.3. Electrical properties of solid-supported lipid bilayer membranes.

ssBLM	Support	Lipid	C (μF/cm^2)	R (Ωcm^2)	Reference
ssBLM	ITO	DMPC/DHADAB*/ chol (42:9:49)	0.53	$8.64 \cdot 10^4$	Gritsch et al., 1998
ssBLM	Ti/TiO_2	DPhPC/chol, egg-PC/chol, (7:3)	2.5 ± 0.1	–	Sinner, Offen-häusser, 1998
Hybrid	Hg drop	DOPC / thiol C14-C18	0.5 – 0.6	$10^7 – 10^8$	Tadini Buonin-segni et al., 1998
Hybrid, spacer	Gold	C18:1 – C24:1 PC DPEPC¤	0.48 – 0.68 0.58 – 0.62	$1 – 3 \cdot 10^5$ $1 – 5 \cdot 10^6$	Raguse et al., 1998
Hybrid, spacer	Gold	DMPC, DPPC, POPC/ thiolipid	0.6 – 0.8	$\sim 10^4$	Lang et al., 1994
Hybrid, spacer	Gold	POPC/ thiolipid	1.0 ± 0.2	$1.1 \cdot 10^4$	Steinem et al., 1998

* DHADAB = dihexadecyldimethylammonium bromide; † DPhPC = diphytanoyl phosphatidylcholine; ¤ DPEPC = 2,3-di-*o*-phytanyl-*sn*-phosphatidylcholine.

2.2.4 Polymer-supported bilayer lipid membranes, psBLM

Solid-supported BLMs have been successfully employed in a variety of structural and functional studies on lipid bilayers. However, with the exception of hybrid thiolipid-based ssBLMs, they have one serious shortcoming for modelling fluid biological membranes: the thin, lubricating water layer between the support and the bilayer may allow the lipids to preserve their lateral mobility, but integral proteins with large hydrophilic domains will interact with the substrate and become immobile, and possibly even denatured. Furthermore, due to the lack of a sufficiently large aqueous compartment on the substrate side of the bilayer, most ssBLM configurations preclude ion translocation.

Formation techniques

To overcome these drawbacks without compromising the stability provided by the solid support, polymer-supported BLMs were introduced. The first psBLMs emerged

simultaneously with the ssBLMs, and were constructed on hydrogel supports (Arya et al., 1985; Hongyo et al., 1987). In the approach adopted by Tien and co-workers (Lu et al., 1996; Yuan et al., 1996), an agar or agarose hydrogel is contained in a piece of Teflon tubing. The filled tube is cut and immersed in a lipid solution, whereby a psBLM is formed on the fresh, hydrophilic gel surface through self-assembly. In addition to a dynamic support, the hydrogel acts as a salt bridge between the bilayer and a reference electrode immersed in the hydrogel, thus enabling the use of electrochemical techniques in the characterisation of the psBLM. Such polymer-supported lipid membranes exhibited good mechanical stability and resistances indicative of low defect densities.

To maintain the conformation of ssBLMs, favourable for both biosensor development and a wide range of experimental techniques, psBLMs have been formed by separating the lipid bilayer from a solid support by a thin polymer cushion. Wong et al. (1999) studied the formation of DMPC bilayers on polyethylenimine (PEI) layers using various preparation methods. They obtained psBLMs where the polymer cushion had a PEI content of 10 – 20 vol-% and a thickness between 41 and 173 Å, depending on the formation method. Other investigated cushion materials include dextran (Györvary et al., 1999), chitosan (Baumgart, Offenhäusser, 2003), cellulose derivatives (Hillebrandt et al., 1999) and lipopolymers, i.e. polymers modified with lipid side chains (Baekmark et al., 1995; Sinner, Offenhäusser, 1998).

Recent work has focused on so-called tethered psBLMs, where the polymer cushion is covalently attached both to the solid support and the lipid bilayer (Figure 2.7). To this end, a variety of strategies have been adopted. In the approach by Seitz et al. (2000), the lipopolymer comprised of an acrylamide backbone modified with lipid side chains and disulfide moieties for spontaneous chemisorption to gold surfaces. The lipid bilayer was completed by vesicle adsorption onto the lipopolymer surface. Shen et al. (2001) employed the LB technique to pre-organise the alkyl side chain -containing polymer at the air – water interface, and to subsequently deposit the layer onto a solid substrate. Photochemistry was used to form a covalent bond between the benzophenone-modified substrate and the lipopolymer. Naumann et al. (2002) followed the photochemical attachment approach proposed by Shen et al. (2001), but used lipopolymers containing only one lipid moiety/polymer. The lipopolymer layer was formed with the LB technique, while the distal lipid monolayer was deposited with the LS method.

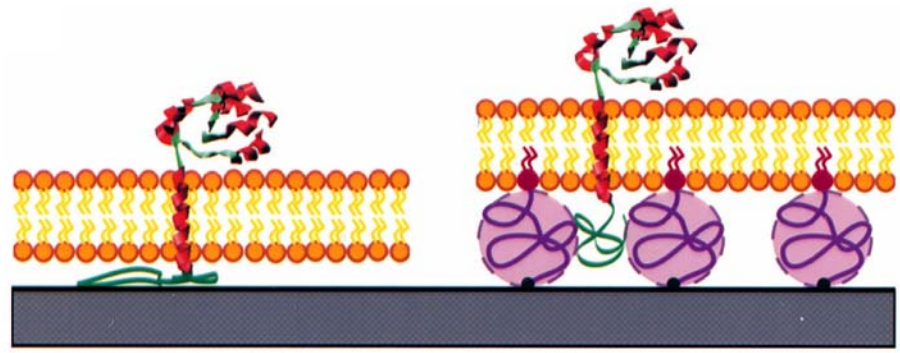

Figure 2.7. Schematic of protein incorporation into a ssBLM (left) and a psBLM (right). The polymer cushion allows for unhindered organisation of the protruding hydrophilic parts of the protein. Reprinted with kind permission from Wagner and Tamm (2000) and the Biophysical Society.

In the works by Wagner and Tamm (2000) and Munro and Frank (2004), a linear polyethyleneglycol (PEG) polymer was attached at its two ends to the substrate and a lipid molecule, respectively. This approach was designed to minimise the constraints on the single lipopolymer chains, and thus to enable the bilayer lipids to maintain their mobility. The choice of PEG as polymer was furthermore motivated by its negligible interactions with proteins and lipids. Another approach to have attracted recent attention is the use of polyelectrolyte multilayers (PEM) as polymer cushions for psBLM formation. In the studies by Cassier et al. (1999) and Wang et al. (2004) a PEM was formed by alternating adsorption of negatively charged poly(styrenesulfonate) (PSS) and positively charged poly(allylammonium) (PAH) from aqueous solution. After adsorption of 4 – 5 double layers (PSS/PAH), a lipid bilayer was deposited using LB/LS transfer or vesicle unrolling.

Finally, a related construction to the psBLM is the S-layer supported bilayer, where the BLM is separated from the solid substrate by crystalline bacterial surface layer (S-layer) proteins. S-layers are composed of a single protein or glycoprotein, and can be recrystallised directly on the solid substrate from aqueous solution (Györvary et al., 1999). While interesting, these model membranes will not be discussed in further detail in this work.

Applications

In accordance with the motivation behind their development, the major part of the psBLM studies has been directed towards the incorporation and characterisation of proteins. Ide and Yanagida (1999) employed a combination of electrochemical and optical techniques to demonstrate the successful reconstitution of single channels into agarose-supported bilayers. Hillebrandt et al. (1999) deposited polymer cushions of cellulose derivatives onto indium-tin-oxide (ITO) electrodes using the LB technique. Vesicle fusion was employed in the formation of the lipid bilayer and reconstitution of gramicidin channels. The bilayer properties as well as the cation selectivity of the ion channel were investigated with impedance spectroscopy.

Wagner and Tamm (2000) carried out an interesting study where they compared the lateral diffusion properties of two proteins reconstituted in tethered PEG-based psBLMs and quartz-ssBLMs. While the diffusion coefficients of the faster components ($\sim 10^{-8}$ cm^2/s) were not significantly larger in the psBLMs, the slower components, which were immobile in ssBLMs, exhibited restricted lateral diffusion of the order of 10^{-10} cm^2/s. This value is in accordance with the lateral diffusion coefficients for many integral proteins in cell membranes (Saxton, Jacobson, 1997). The restricted lateral mobilities of such proteins are thought to arise because of coupling to the membrane cytoskeleton (Jacobson et al., 1995), which in psBLMs may be closely mimicked by the polymer cushion. A similar result was reached by Goennenwein et al. (2003) who observed no lateral mobility of the large transmembrane protein integrin in glass-ssBLMs, but obtained a lateral diffusion coefficient of $0.6 \cdot 10^{-8}$ cm^2/s for integrin embedded in cellulose-psBLMs (mobile fraction 25%). The latter authors also employed the psBLMs for studies of vesicle adhesion on the membrane surface.

Properties

Polymer-supported BLMs were developed in order to combine the most important benefits of unsupported BLMs and ssBLMs: bilayer fluidity and stability, accessibility to a variety of characterisation techniques, and the possibility of incorporation and investigation of membrane proteins. While the last two have been discussed in the previous subsection, this subsection will deal with the physical properties of the psBLM.

As stated previously, the presence of a thin, lubricating water layer between the substrate and the inner monolayer in many ssBLMs grants the lipids some mobility. Therefore, it is not surprising that the lateral diffusion coefficients of psBLM lipids, as shown in Table 2.4, are comparable to those of ssBLM lipids. While the diffusion coefficients vary significantly between studies, inspection of works by the same group suggests that increased tethering of the inner monolayer restricts lipid mobility and that PEM-supported BLMs exhibit very slow lateral diffusion due to electrostatic interactions. Surprisingly, Wang et al. (2004) found that lateral diffusion of lipids in an anionic bilayer on a PEM was independent of the charge of the terminating PEM layer. This was attributed to the large flexibility of the outmost polyelectrolyte layers in water, enabling the interaction of the bilayer with the oppositely charged polyelectrolyte.

Although the stability issue has not been explicitly addressed for psBLMs, several studies describe them as being stable or exhibiting good mechanical stability (e.g. Novotny et al., 1996; Hillebrandt et al., 1999; Baumgart, Offenhäusser, 2003). The critical property for the application of psBLM for biosensor purposes is their electrical resistance. While protein reconstitution and ligand binding can be monitored with several surface techniques, electrochemical characterisation is the only direct technique for determination of ion channel activity. To this end, it is necessary that the defect conductance of the membrane be lower than that of the incorporated channel(s). Several groups have, employing optical techniques, observed the capability of psBLMs to self-heal, i.e. to spontaneously spread and cover defects (Hillebrandt et al., 1999; Wagner, Tamm, 2000; Baumgart, Offenhäusser, 2003).

However, few studies concern the electrochemical characterisation of psBLMs. Novotny et al. (1996) reported impressive electrical properties for their self-assembled, agar-supported bilayers of egg-PC ($C = 0.77 \pm 0.08$ μF/cm^2; $R \sim 10^8$ Ωcm^2), while the resistance of 2.2 kΩcm^2 obtained by Cassier et al. (1999) for PEM-supported DOPA bilayers suggests the presence of a considerable amount of defects. Hillebrandt et al. (1999) deposited a lipid mixture (same as Gritsch et al., 1998, table 2.3) on a cellulose cushion -covered ITO electrode. Employing impedance spectroscopy, they reached the reasonable values of 0.57 μF/cm^2 and $0.44 \cdot 10^6$ Ωcm^2, for the bilayer capacitance and resistance, respectively.

Table 2.4. Lateral diffusion coefficients of polymer-supported lipid bilayer membranes.

psBLM	Free lipid	Mol -% tetherer	*D* (10^{-8}cm^2/s)	Mobile fraction (%)	Reference
psBLM, agar	DPhPC	–	9 ± 5	–	Ide, Yanagida, 1999
ssBLM, glass	DMPC	–	1 – 1.5	–	Baumgart, Offenhäusser, 2003
psBLM, on dextran	DMPC	–	~ 1	–	
psBLM on dextran	DPPC	–	1.4 ± 0.2, inner 2.2 ± 0.2, outer	100 ± 4 98 ± 1	Györvary et al., 1999
Multilamellar crystals[¤]	DPPC	–	22 ± 1	–	Karakatsanis, Bayerl, 1996
ssBLM, on silica beads	DPPC	–	8 ± 1, inner 14 ± 1, outer	–	Hetzer et al., 1998
psBLM, no tetherer	DPPC	–	14 ± 2	–	Schmitt et al., 2001
ssBLM on glass	POPC	–	1.32 ± 0.07	77 ± 4	Wagner, Tamm, 2000
1-tethered, to lipid	POPC	3 10	1.04 ± 0.04 0.89 ± 0.05	68 ± 1 53 ± 5	*ibid*
2-tethered, at each end	POPC	3	0.89 ± 0.08	67 ± 1	*ibid*
2-tethered, side chains	egg-PC		~ 0.1	15	Shen et al., 2001
2-tethered, lipid at end	DMPC	5 10 30	17.7 9.7 1.1	100 80 20	Naumann et al., 2002
psBLM on PEM	DOPA[†] DMPC	– –	0.02 – 0.03 0.7 – 0.9	~ 50 > 95	Cassier et al., 1999
psBLM on PEM	DOPA[†] DOPC	– –	$(1.0 \pm 0.2)\cdot10^{-3}$ $(2.4 \pm 0.2)\cdot10^{-3}$	– –	Wang et al., 2004
psBLM on PEM	SOPS[*] SOPS/ POPC (3:1)		~ 0.12 ~ 0.21	> 85 > 85	Ma et al., 2003

In all studies, the diffusion coefficients were determined above the gel to fluid phase transition temperatures of the lipids. [¤] See table 2.2 for explanation; [†] DOPA = dioleoyl phosphatidic acid; [*] SOPS = 1-stearoyl-2-oleoyl phosphatidylserine (C18:0 / C18:1).

In conclusion, psBLMs are stable, fluid constructions that allow for reconstitution of proteins in their active conformation, and studies employing a multitude of experimental techniques. The thickness and fluidity of the polymer support can be adjusted with appropriate choice of materials. For the development of sensitive and selective biosensors, more attention needs to be directed to the electrical properties of psBLMs. In addition, the formation techniques of psBLMs are not significantly improved from ssBLMs. The simplest approach reported for psBLM formation is self-assembly of the polymer cushion followed by vesicle adsorption (Munro, Frank, 2004).

2.2.5 Lipid monolayers

Phase behaviour

The surface of a liquid always has excess free energy, which results from the different environments experienced by the molecules at the surface and those in the bulk. At thermodynamic equilibrium, the line force acting on the surface molecules is described by the surface tension γ, which at a planar surface is given by the partial differential:

$$\gamma = \left(\frac{\partial G}{\partial A} \right)_{T,P,n_i} \tag{2.3}$$

where G is the Gibbs free energy of the system, A is the surface area, and the temperature T, pressure P and composition n_i are held constant. Lipids, being amphiphilic molecules, have the tendency to orient themselves at gas – liquid, or liquid – liquid interfaces to minimise their free energy. As lipid molecules gather at an interface, they exert forces on each other and the solvent molecules, the magnitude of which depend on the packing density and properties of the molecules. The amount of interactions between the interfacial molecules is described by the surface pressure Π of the monolayer, which at equilibrium is defined as:

$$\Pi = \gamma - \gamma_0 \tag{2.4}$$

where γ_0 and γ are the surface tension in the absence and presence of the monolayer, respectively. Another important quantity that describes the state of the monolayer is its compressibility, χ:

$$\chi = -\frac{1}{A}\left(\frac{\partial A}{\partial \Pi}\right)_{T,P,n_i} \tag{2.5}$$

Whenever feasible, the simplest method of characterising a lipid monolayer is to measure its surface pressure as a function of the mean molecular area of the lipids (A_{lipid}). This is commonly carried out by compression of a monolayer at the air – water interface under constant temperature. The obtained relationship is called a surface pressure – area isotherm and reveals important information on the phase behaviour of the monolayer. This is exemplified by Figure 2.8, which shows isotherms of DPPC and DSPC (distearoyl phosphatidylcholine) recorded at the air – water interface.

At low surface concentrations, the lipid molecules exert negligible interactions on each other, and the monolayer is said to be in a gaseous state (G). Upon reducing the area of the monolayer, the lipids are forced into contact and the resulting intermolecular interactions induce a rise in the surface pressure. The nature of the lipids and the temperature of the experiment decide the phase behaviour of the monolayer. When the lipids consist of relatively short or unsaturated hydrocarbon chains, the monolayer enters the liquid expanded state (LE), where the polar headgroups are in contact with the aqueous phase but the orientation of the hydrocarbon chains is random. A direct transition from the gaseous state to a condensed phase (C) is observed for lipids with long, saturated chains or for shorter chain lipids at low temperatures (Petty, 1996). For some lipids, the condensed phase may also be reached upon further compression of the LE monolayer, often through a LE/C co-existence region as seen for DPPC in Figure 2.8.

In the C phase, the lipid order is significantly increased compared to the LE phase. The hydrocarbon chains are aligned parallel to each other, and the area per lipid approaches the sum of the cross-sectional areas of its hydrocarbon chains (Möhwald, 1995). Two separate condensed phases can be distinguished: the tilted condensed (TC) and the untilted condensed (UC). In the TC phase, the tails are uniformly tilted in relation to the monolayer normal, with tilt angles up to 30º. The tilted condensed phase exhibits relatively high compressibilities as the tilt angle can be reduced in response to a decrease in surface area. At higher pressures, the TC phase may transform into the UC phase, which is distinguished by normal tail alignment and a smaller compressibility compared to the TC phase (Kaganer et al., 1999).

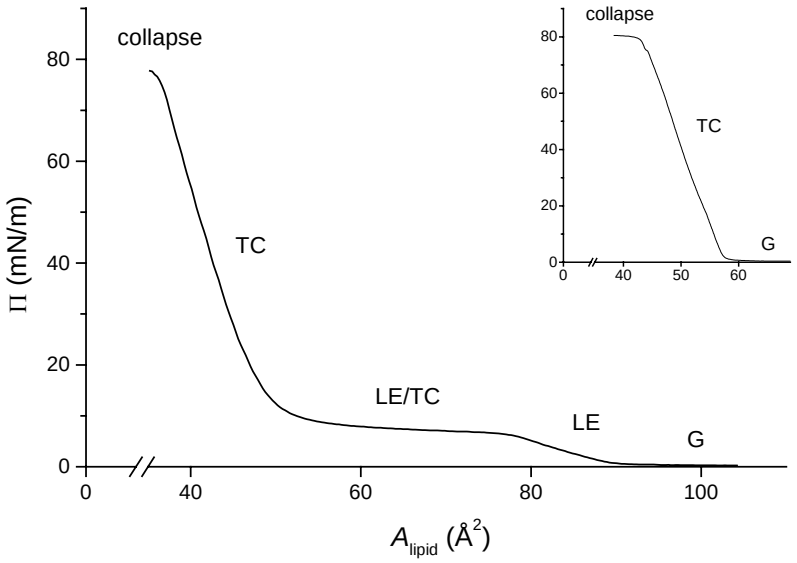

Figure 2.8. Large figure: Surface pressure – area isotherm of DPPC (C16:0). Upon compression at the air – water interface, the DPPC monolayer undergoes a transition from the gaseous phase (G) to the liquid expanded phase (LE). The tilted condensed phase (TC) is entered through a LE/TC co-existence region. Insert: Isotherm of DSPC (C18:0) at the air – water interface. The long-chain lipid DSPC undergoes a direct transition from the gaseous to the tilted condensed phase upon reduction of the mean molecular area of the lipid. The temperature in both experiments was 20ºC (Mälkiä, unpublished results).

For many lipids, intermolecular repulsion inhibits the formation of a condensed phase. In addition, a bulky headgroup may obstruct the reduction of the tilt angle upon compression, thereby preventing the monolayer from entering the untilted condensed phase. Both of the lipid monolayers presented in Figure 2.8 could be expected to be in the UC phase on basis of the high surface pressures reached. However, owing to the large PC headgroup, both DPPC and DSPC monolayers are known to remain tilted throughout the isotherm, exhibiting tilt angles close to 30º at surface pressures of 40 – 45 mN/m (Helm et al., 1987; DeWolf et al., 1999). Close examination of Figure 2.8 reveals a slight kink in the DSPC isotherm at a surface pressure around 20 mN/m, which does not cause a detectable decrease in compressibility. Plausible explanations for this repeatable feature include the presence of an impurity, or a transition to another tilted condensed phase, as observed for simple, long-chain fatty acids (Kenn et al., 1991; Shih et al., 1992).

If compression is continued, a point will be reached where the lipids can no longer pack more tightly and are forced out of the monolayer. This event is called the collapse of the monolayer and can be identified in the isotherm as a sudden change from low to infinite compressibility. The squeezed-out molecules may organise on top of the monolayer and form self-assembled multilayer structures. For arachidic acid, collapse has also been observed to resemble a nucleation and growth process (Gabrielli et al., 1976). In context with this, an important realisation to be made is that a compressed monolayer is in most cases not in thermodynamic equilibrium. The equilibrium spreading pressure (ESP), i.e. the surface pressure of a monolayer that is spontaneously formed when placing a three-dimensional lipid crystal in contact with the air – water interface, is of the order of a few mN/m (Möhwald, 1995). Consequently, above this pressure the monolayer should prefer to aggregate into crystals. The equilibrium process is, however, very slow, and as a result, monolayers can be compressed significantly above their ESP without nucleation taking place.

Monolayers vs. bilayers

The coupling between the opposing monolayers in a lipid bilayer is considered to be weak (Tocanne et al., 1994), which is corroborated by the observation that phase transitions in liposomes can occur in one leaflet independent of the other (Sillerud, Barnett, 1982). As a result, lipid monolayers have been widely employed as models for biological membranes. The monolayer constitutes a simple, controllable and well-defined system to study lipid – lipid and lipid – protein interactions, as well as other biological reactions in two dimensions.

An essential question when comparing bilayers and monolayers is the correspondence of their states. Whereas the concept of surface pressure is well-defined for planar lipid monolayers, a spontaneously formed lipid bilayer is in a tension-free state. Blume (1979) compared the phase transitions of phospholipid monolayers and bilayers, and concluded that the two membranes were in a corresponding state when the surface pressure of the monolayer was 30 mN/m. Similar values were reached by Marsh (1996, 30 – 35 mN/m) and by James et al. (1994, 25 – 30 mN/m), the latter of which approached the problem by comparing enzyme activity in phospholipid monolayers and bilayers. As many naturally occurring phospholipids contain unsaturated chains, the biological lipid bilayer is thought to be in a predominantly fluid phase. Accordingly, the viscosity and the diffusion coefficients in the LE phase of a

lipid monolayer are close to values derived for lipid bilayers in the L_α phase, but the two-dimensional compressibility of the LE phase is much larger (Möhwald, 1995).

The state of a lipid monolayer also determines its electric properties. The surface potentials of a lipid bilayer and monolayer of similar composition can be expected to be identical when the mean molecular area of the lipids is the same. Likewise, the magnitude of the dipole potential will depend on the lateral packing of the monolayer. Values between 270 mV and 411 mV have been measured for air – water monolayers of 30 – 40 mN/m (Cseh, Benz, 1998; Smaby, Brockman, 1990), which are comparable to dipole potentials observed for lipid bilayers, if these are corrected as proposed by Schamberger and Clarke (2002, section 2.1.2). Measurement of the boundary potential, e.g. the sum of the surface and dipole potentials, can be employed to monitor phase behaviour and adsorption of solution molecules to the monolayer (Cseh, Benz, 1999). While a change in monolayer packing can be observed in this way, the interpretation in terms of molecular orientation is not straightforward, as the structural contributions to the dipole potential are not well understood (see section 2.1.2).

The following sub-sections will describe the use of lipid monolayers as model membranes. As before, the emphasis will be on monolayers tractable with electrochemical techniques.

Air – water interfaces

Lipid monolayers have been extensively studied at the air – water interface. Frequently, studies have been carried out with the Langmuir technique, whereby the surface pressure of the monolayer can be controlled and monitored as a function of the mean molecular area of the lipids. In addition, a number of optical techniques have been implemented into the Langmuir setup, including fluorescence microscopy, Brewster angle microscopy (BAM) and polarised fluorescence microscopy (McConnell et al., 1984; Moy et al., 1986; Lösche et al., 1988; Flörsheimer, Möhwald, 1991; Hönig, Möbius, 1991; McConlogue, Vanderlick, 1997; DeWolf et al., 1999; Kane et al., 2000). The overwhelming majority of these studies have focused on characterising lipid domains in the LE/C co-existence region, either of a pure lipid or a lipid mixture. In addition, information on headgroup hydration and monolayer structure has been obtained with X-ray and neutron reflection studies (Helm et al., 1987; Bayerl et al., 1990). FRAP and single molecule fluorescence have been employed to study lateral diffusion in lipid monolayers, yielding values of $0.4 – 1.2 \cdot 10^{-8}$ cm^2/s for 30 mN/m

dimyristoyl and dilauroyl phosphatidylcholine (DLPC) monolayers (Tanaka et al., 1999; Forstner et al., 2001; Ke, Naumann, 2001). In addition, infrared spectroscopy, surface pressure and surface potential measurements have been used to elucidate the insertion and orientation of peptides in lipid monolayers (Shapovalov et al., 1999; Schwarz, Taylor, 1999; Ulrich, Vogel, 1999).

Electrochemical studies of monolayers at air – water interfaces are scarce. Recently, Unwin and co-workers carried out a series of work combining the Langmuir trough technique with scanning electrochemical microscopy (SECM). Using this approach, the permeation of oxygen across distearoyl phosphatidylethanolamine (DSPE) monolayers was investigated (Cannan et al., 2004). A LE phase DSPE monolayer was not observed to affect oxygen transfer, but upon entering the condensed phase it caused the transfer rate constant to decrease with increasing surface pressure. In another study, Zhang and Unwin (2002) employed a similar approach to investigate the lateral proton diffusion at DPPS and DPPC monolayers. The facilitating effect of a LE phase monolayer on interfacial proton diffusion was not observed, in contrast to previous reports (Gabriel, Teissié, 1991). Furthermore, as opposed to DPPS monolayers, lateral proton diffusion was not detected at DPPC monolayers. This may be attributed to the different hydration of the PS and PC headgroups, as mentioned in section 2.1.2.

While an indispensable asset for fundamental studies of lipid monolayers and an important tool in the construction of supported model membranes, the air – water interface is difficult to implement into a practically applicable biomembrane model. The nature of the interface renders the monolayer immovable and prevents permeation studies of non-gaseous compounds.

Solid – water interfaces

Lipid monolayers at the air – water interface are impractical for many experimental characterisation methods. Frequently, transfer onto solid supports is carried out to enable studies with techniques such as atomic force microscopy, scanning tunnelling microscopy and near-field scanning optical microscopy (Kolomytkin et al., 1991; Hollars, Dunn, 1998; DeWolf et al., 1999; Cordero et al., 2000; Cruz et al., 2004).

Reports of lipid monolayers on solid metal supports are rare. As a consequence, despite its liquid nature, the profoundly studied mercury drop electrode will be considered in this subsection. The first electrochemical studies of lipid monolayers at a mercury electrode were carried out by Miller and Bach (1969), who investigated the

properties of phosphatidylcholine monolayers transferred onto Hg drop electrodes from the air – water interface. Subsequently, Pospíšil et al. (1980, 1984) studied DPPC monolayers adsorbed on Hg from alcohol solution. Interfacial capacitance deduced from ac polarography measurements was found to be a sensitive means to characterise the phase behaviour of the adsorbed films. At negative electrode potentials, adsorption was particularly strong, resulting in the formation of a compact monolayer with a capacitance of $C = 0.8\ \mu F/cm^2$. This was attributed to interactions between the lipid and the supporting electrolyte cation Li^+.

Nelson and co-workers have carried out extensive studies of air – water monolayers transferred to the mercury drop electrode (Nelson, Benton, 1986; Nelson, Auffret, 1988a). The phospholipid monolayers were stable over a potential range of 1.5 V, and exhibited a 0.5 V wide capacitance minimum of $C \sim 2\ \mu F/cm^2$. This region, indicative of a compact and ordered monolayer, was found to shift in the negative direction when the drop area was increased and the membrane packing thus loosened. At potentials negative and positive of the capacitance minimum region, the monolayers underwent reorientation and became permeable to faradaic processes. The orientational changes could be explained with a self-consistent field theory (Leermakers, Nelson, 1990; Nelson, Leermakers, 1990).

In subsequent studies, it was shown that in the low-capacitance region, a DOPC monolayer inhibits the reduction of heavy metals at the electrode (Nelson, van Leeuwen, 1989), while significant reduction peaks were still observed in the presence of a compact monolayer of negatively charged phosphatidylserine (Nelson, 1993). The PS monolayer could be rendered more impermeable by neutralising the negative charge with a pH decrease or addition of Mg^{2+}. In contrast to the hydrated metal ions, reduction of the more hydrophobic oxygen and anthraquinone-2-sulphonate were found to occur readily in presence of the DOPC monolayer, indicating their ability to penetrate the lipid layer (Nelson, Auffret, 1988b).

Nelson (1991a, b) furthermore showed the monolayer-covered Hg electrode to be a feasible model membrane for studies of pore forming compounds. The selective permeabilities and transport mechanisms of gramicidin and ionophore A23187 could be demonstrated. Transport through the gramicidin channel was observed to resemble pore diffusion, and to occur both to and from the electrode surface. On the contrary, the A23187 ionophore was found to complex the transportable ion, which induced a conformational change in the monolayer and the subsequent release of the ion at the electrode surface. As a consequence, the transport took place in one direction only, and

the oxidation of the transported ion at the electrode was not observed. The first study of gramicidin-induced conductivity across a monolayer-covered Hg electrode employing SECM was recently reported by Mauzeroll et al. (2002).

Owing to its liquid nature, the mercury drop electrode provides a smooth support for a lipid monolayer. Furthermore, pore forming agents of monolayer thickness have been demonstrated to successfully incorporate into Hg-supported monolayers. Its suitability for electrochemical experimentation in combination with its stable nature renders the mercury/monolayer system a versatile model membrane. Downsides include the incompatibility with a vast number of non-electrochemical characterisation methods, the generation of toxic mercury waste and the preclusion of ion transfer studies.

Electrified liquid – liquid interfaces

In addition to the air – water and the mercury – water interfaces, a third phase boundary that has been employed in lipid monolayer studies is the interface between two immiscible liquids, formed upon contact of an aqueous and an organic solution. The main benefits of the liquid – liquid interface are its smooth, dynamic structure, its non-reactive nature and the possibility to observe ion translocation. By use of appropriate organic solvents, the liquid – liquid interface can be harnessed for electrochemical studies.

The first electrochemical studies at an interface between two immiscible electrolyte solutions (ITIES) were carried out a century ago by Nernst and Riesenfeld (1902). While this event opened up a new line of research (reviewed by Girault and Schiffrin, 1989), it was not until the groundbreaking work by Gavach et al. (1968) that the electrified ITIES began its rapid progress into the recognised field it is today. In brief, electrochemistry at the ITIES involves the contact of two liquid phases of poor mutual miscibility. In both phases an electrolyte is dissolved. If the same ion is present in the two phases, the electrolyte concentrations can be adjusted to fix the interfacial potential at a desired value and the interface is termed non-polarisable. On the other hand, as demonstrated by Gavach et al. (1968), if the electrolytes in the aqueous and organic phases lack a common ion, and are sufficiently hydrophilic and hydrophobic, respectively, the interface can be charged from an external source. Such an interface, termed polarisable, exhibits a potential region where little or no faradaic current flows, called the “potential window”.

The electrified ITIES can be exploited for a variety of electrochemical studies. In analogy with the solid electrode – electrolyte interface, the ITIES can be employed to investigate electron transfer (ET) reactions. While the solid electrode serves as the donor/acceptor of electrons, ET at the ITIES requires the presence of a redox couple in both of the liquid phases. However, unlike the solid electrode – electrolyte system, the ITIES is also applicable to studies of ion transfer (IT). The energy required for transferring an ion from one phase to the other is described by the standard transfer potential of the ion, $\Delta_{\mathrm{o}}^{\mathrm{w}}\phi_i^{0}$, defined as the difference of the solvation energies of the ion in the respective phases:

$$\Delta_{\mathrm{o}}^{\mathrm{w}}\phi_i^{0} = \frac{\Delta G_{i,\mathrm{tr}}^{\mathrm{w}\to\mathrm{o}}}{z_i F} = \frac{\mu_i^{0,\mathrm{o}} - \mu_i^{0,\mathrm{w}}}{z_i F} \tag{2.6}$$

where z_i is the charge, and $\Delta G_{i,\mathrm{tr}}^{\mathrm{w}\to\mathrm{o}}$ is the Gibbs free energy of transfer of species *i*; *F* is the Faraday constant; and $\mu_i^{0,\mathrm{o}}$ and $\mu_i^{0,\mathrm{w}}$ are the standard chemical potentials of ion *i* in the organic and aqueous phases, respectively. Another type of charge transfer only tractable through the ITIES methodology is facilitated ion transfer (FIT). In facilitated ion transfer, the transferring ion forms a complex with a ligand from the opposing phase at the interface, and the entire complex transfers into the receiving phase. The solvation energy of the complex is lower than that of the ion – hence the designation facilitated ion transfer.

Owing to its dynamic, defect-free structure and its oil – water boundary, the ITIES has been recognised as a promising model for biological interfaces by several researchers over the years (e.g. Cremer, 1906; Koryta, 1979; Girault, Schiffrin, 1989). In addition, electrochemistry offers a direct and sensitive technique for probing interfacial phenomena, such as adsorption and charge transfer. Accordingly, electrochemistry at the ITIES has been employed in a variety of studies of biological nature, recent work including phase transfer behaviour of polysaccharides, polypeptide protamines and oligopeptides (Samec et al., 2003; Amemiya et al., 2003; Sawada, Osakai, 1999), electron transfer reactions involving cytochrome *c* and glucose oxidase (Lillie et al., 2002; Georganopoulou et al., 2000), detection of dopamine in the presence of an interfering ion (Arrigan et al., 2004), cation binding to DNA (Horrocks, Mirkin, 1998) and artificial photosynthesis (Lahtinen et al., 2000). In addition, Girault, Testa and co-workers have published a vast number of articles on the construction of pH – potential diagrams and the lipophilicity of ionisable compounds (Reymond et al.,

1996; 1999a). This topic, of great relevance to the fields of pharmacokinetics and drug design, will be further discussed in chapter 4.3.

In order to improve the biomimetic nature of the ITIES, lipid monolayers have been introduced at the phase boundary. While pioneering work in this area was carried out by Watanabe et al. (1968, 1971), Girault and Schiffrin (1984) are responsible for the first qualitative attempt to characterise phospholipid monolayers at electrified liquid-liquid interfaces. They studied the formation of egg-PC and egg-PE monolayers from 1,2-dichloroethane (DCE) onto an aqueous pendant drop by measuring the changes in surface tension. Adsorption was strongly potential-dependent for both lipids. For PC, two potential regions were distinguished: at more negative potentials (of the aqueous phase), the interfacial tension was constant with capacitances below 1 μF/cm^2, indicating a stable monolayer. At more positive potentials, the capacitance increased significantly. The authors proposed that this was due reorientation of the monolayer in combination with neutralisation of the phosphate group, which would render the lipid positively charged. Despite a pK_a value below 3.5 (Tatulian, 1993), potential-dependent protonation of the phosphate group was observed to take place at pH 5. The hypothesis of phosphate protonation was supported by the observation that at pH values over 8, the region of monolayer stability was significantly wider.

The most thorough work on lipid monolayers at the ITIES has probably been carried out by Kakiuchi et al. (e.g. 1987, 1989, 1990, 1992a), who employed ac polarography and impedance to study the behaviour of a number of phospholipids adsorbed at the planar water – nitrobenzene interface. On basis of the obtained capacitance – potential curves, the lipid monolayers were divided into two groups. Phosphatidylcholines with saturated chains of 18 carbons or more yielded minimum capacitances of 4 μF/cm^2 while those with less than 16 carbons/chain exhibited minimum capacitances of 10.5 μF/cm^2. DPPC (C16:0) was found to be an intermediate, with a minimum value of 7.5 μF/cm^2. In addition, the shapes of capacitance curves of the long-chain phosphatidylcholines were flatter, exhibiting minimum capacitances over potential regions of 100 mV, while the capacitance curves of the short-chain lipids were parabolic.

From these results, Kakiuchi et al. concluded that the long-chain monolayers were in a condensed state in the temperature range studied (5 – 30°C), while the short-chain monolayers were in a liquid expanded state, characterised by higher penetration of the organic solvent and ions into the hydrocarbon chains. DPPC was observed to be in the LE state at room temperature, but to undergo a temperature-induced phase transition to

a condensed state at 13 °C. The negatively charged DPPS monolayer was likewise in a LE state at room temperature, but was found to form 1:2 complexes with divalent cations, which caused a phase transition to a condensed or possibly even a solid crystalline phase. Kakiuchi et al. also investigated the shapes of the electrocapillary curves of DLPC and found that with increasing concentrations of DLPC, the potential of zero charge (E_{pzc}) was shifted towards more negative potentials. This behaviour was explained by specific adsorption of the aqueous electrolyte cations (Li^+) to the lipid headgroups. Furthermore, Kakiuchi et al. estimated that under their experimental conditions, potential-dependent protonation of the phosphate groups was not taking place, and postulated that the increase in surface tension and capacitance at potentials positive to the E_{pzc} was due to desorption of lipid molecules in combination with facilitated transfer of Li^+.

So far, this section has concerned monolayers formed by adsorption from the organic phase. While facile to implement, the disadvantages of this method are that the state of the monolayer cannot be controlled and the adsorbed amount of lipid is limited. Furthermore, adsorption equilibrium is reached slowly, in 1.5 – 2 hours (Kakiuchi et al., 1990, 1992a) and lipids will unavoidably be present in the organic phase. Grandell and co-workers (Grandell, Murtomäki, 1998; Grandell et al., 1999a) proposed an improvement to studies of monolayers at the ITIES in the form of a Langmuir trough, which enabled simultaneous surface area control and electrochemical measurements of a monolayer at the oil-water interface. In agreement with previous results, the stability of a DSPC monolayer was observed to be potential dependent and to decrease at more positive potentials. Interestingly, the authors noticed that the organic base electrolyte tetraphenylarsonium tetrakis(4-chlorophenyl)borate (TPAsTPBCl) interacts with the phospholipid acyl chains and increases their mean molecular area. Unfortunately, despite the intriguing prospects of this surface pressure -controlled ITIES, the approach suffered from a non-uniform potential distribution and instability of the monolayer, caused by desorption of the lipid to the organic phase and monolayer – organic solvent interactions.

Potential-driven ion transfer has been extensively studied at the monolayer-covered liquid – liquid interface. Egg-PC monolayers adsorbed from the organic phase have in many cases been reported to slow down (Koryta et al., 1982; Cunnane et al., 1988) or render completely irreversible (Girault, Schiffrin, 1984) the transfer of small cations, although such a retarding effect was not observed by Allen and Williams (1996). On basis of their studies on pure phospholipid monolayers, Kakiuchi et al. (1990, 1992a, 1992b) concluded that the change in monolayer phase affects the mechanism of ion

transfer. They proposed that in condensed phase monolayers, the solvent molecules are squeezed out of the hydrocarbon chain region, and consequently, the ion transfer in this region becomes rate-limiting due to hydrodynamic friction. On the contrary, the monolayers in the LE phase contain a significant amount of organic solvent in their hydrocarbon region. Therefore, for these monolayers the rate of ion transfer depends on the dehydration step at the interface. Kakiuchi et al. furthermore observed that at charged monolayers, the transfer of oppositely charged ions was enhanced.

More surprisingly, several studies have reported an increase in the apparent rate constant of ion transfer in the presence of a neutral monolayer at the ITIES (Table 2.5). Kontturi et al. (1997) concluded that in their studies ion transfer took place through less ordered domains in the monolayer, where hydrodynamic friction was negligible. The enhancement of ion transfer was attributed to the lowered surface tension of the water – DCE interface, induced by the presence of the lipid. Kakiuchi et al. (1992a) proposed a double layer effect resulting from specific adsorption of ions to the monolayer headgroups, and/or a restructuring of the interfacial solvent molecules, as plausible explanations for the increased rate constants. A Frumkin-type correction was, however, not able to account for the observed behaviour.

Manzanares et al. (2000) developed a theoretical model for a zwitterionic phospholipid monolayer at the ITIES. The model combined the electrical double layer correction to the Butler-Volmer equation with the solution to the Poisson-Boltzmann equation, and portrayed the phospholipid monolayer as two parallel charged planes at the interface. The model was able to successfully explain the enhancement of ion transfer observed in various experimental studies. The authors noted that the model was probably only applicable to expanded monolayers as it allowed electrolyte ions to unrestrictedly access the monolayer interior within their respective phases. However, no reference was made to the fact that despite the opposite contribution of the charged headgroup dipole, the total dipole potential of monolayers is known to be positive towards the hydrocarbon region of the monolayer (page 37).

At present, few studies have focused on the ion transfer across a monolayer at the liquid – liquid interface under *in situ* surface pressure control. Grandell et al. (1999b) employed cyclic voltammetry to study both anion and cation transfer through DSPC monolayers at a water – DCE interface, but observed no notable effect of the monolayer on the ion transfer kinetics. Zhang et al. (2003) conducted SECM studies of DSPC monolayers at a decane – water interface, and found that oxygen transfer across the monolayers was impeded at higher surface pressures ($\Pi > 25$ mN/m). The results

are not in discrepancy, since in the system studied by Grandell et al., the available surface pressure range was 0 – 15 mN/m, in which region Zhang et al. observed no retarding effect of the monolayer. In a study by Cannan et al. (2004), the presence of a DSPE monolayer at the decane – water interface only started to affect oxygen transfer at surface pressures above 40 mN/m, while at the air – water interface the transfer rate decreased in the presence of a 5 mN/m monolayer. The isotherms of the two monolayers suggested that the decane – water monolayer incorporates a significant amount of decane molecules at surface pressures below 40 mN/m, enabling uninhibited phase transfer. The implications of this result on conducting transport studies across solvent – containing BLMs were pointed out.

In addition to ion transfer, studies of electron transfer at monolayer-modified liquid – liquid interfaces have been reported. Employing cyclic voltammetry measurements, Cheng and Schiffrin (1994) found that an adsorbed egg-PC/cholesterol monolayer at the water – DCE interface inhibited the heterogeneous electron transfer reaction between the aqueous hexacyanoferrate(II/III) redox couple and organic redox couples to variable extent. The difference in the retarding effect of the monolayer was concluded to depend on the ability of the organic redox couple to approach the aqueous phase: large redox couples were excluded from the lipid monolayer, which led to complete inhibition of ET. Interestingly, when such a blocked redox couple was present in the organic phase together with a redox couple that was able to access the monolayer, the latter one was found to mediate electron transfer of the former one, which was evidenced as two subsequent current peaks in the voltammograms.

Bard, Mirkin and co-workers have used the more sensitive SECM technique to elucidate the effect of interfacially adsorbed phosphatidylcholine monolayers on the rate of electron transfer (Tsionsky et al., 1997; Delville et al., 1998; Liu, Mirkin, 2002). As found by Cheng and Schiffrin, the presence of the monolayer caused a decrease in the rate constant for electron transfer. The extent of the retarding effect due to the monolayer was concluded to depend on the length of the hydrocarbon chains of the monolayer lipids, and the driving force of the ET reaction, given by the sum of the interfacial potential drop and the difference between the standard potentials of the two redox couples. The incorporation of a lipid derivative, in which one chain was replaced with the linear 1,6-diphenyl-1,3,5-hexatriene (DPH) group, into the monolayer increased the rate of electron transfer. A plausible explanation for this observation involved electron delocalisation through the conjugated DPH chain, causing it to serve as an electron relay through the blocking monolayer; and the looser packing of the DPH-lipids, allowing for closer approach of the organic redox couple.

For monolayers with negatively charged headgroups (PS), the electron transfer rate was decreased. This was attributed to repulsion of the anionic aqueous redox couple, resulting in its lower interfacial concentration and a larger separation of the redox reactants. As found by Kakiuchi et al. (1990), addition of divalent cations induced a phase transition of the anionic monolayer, but on the contrary to the ion transfer rate (Table 2.5), the electron transfer reaction was enhanced. Scanning the monolayer surface in the x-y plane suggested that when the monolayer was formed in the presence of divalent cations, distinct domains of highly ordered lipids and domains rich in pinhole defects were formed. However, if the divalent cation was added subsequent to monolayer formation, a monolayer containing uniformly distributed pinholes resulted.

Table 2.5. Apparent standard rate constants of ion transfer, k, in the presence of a lipid monolayer at the ITIES.

Organic phase	Ion*	Lipid	c_{lipid} (μM)	T (°C)	k (10^{-2} cm/s)	Reference
DCE	TEA^+	Egg-PC	25 40 80	RT	0.75 0.38 0.15	Cunnane et al., 1988
Nitro-benzene	TEA^+	DLPC	10	25 15 5	≥ 50 6.2 4.3	Kakiuchi et al., 1992a
		DPPC	10	25 15 5	10.4 0.4 0.3	
		DBPC¤	10	25 15 5	2.6 0.9 0.7	
		No lipid	–	25 15 5	7.4 2.0 1.8	
Nitro-benzene	TMA^+	DPPS	0 20 (Li^+) 20 (Ca^{2+})	25	1.9 1.7 0.3	Kakiuchi et al., 1990

* TEA^+ = tetraethylammonium; TMA^+ = tetramethylammonium; ¤ Dibehenoyl phosphatidyl-choline, C22:0.

From the present chapter, it is apparent that a general, straightforward explanation for ion transfer kinetics at a monolayer -modified ITIES is not feasible. Factors such as composition, phase and charge of the monolayer, ion – monolayer interactions, nature of the organic solvent and the effect of applied potential are all able to affect the structure of the electric double layer. This, however, underlines the benefits of employing a simple model, such as a monolayer, in studies of electrochemical events at lipid membranes.

3. Aim of the work

The general purpose of the work presented in the following, was the development, characterisation and application of a biomembrane model that would be feasible for electrochemical studies. On the basis of the background literature presented in chapter 2, the following specific objectives were set:

(i) *Development.* The aim was to develop a model membrane that was stable, reproducible yet free from substrate-induced defects. Control of membrane composition and state was desirable. In addition, the model system was to allow for studies of transmembrane ion transfer.

(ii) *Characterisation*. The model membrane was to be characterised by a variety of electrochemical methods to assess its similarity to a biological membrane. Theoretical modelling was to be carried out in parallel to the experimental characterisation, to support and explain experimental observations. In addition, if implementable, optical and spectroscopic techniques would be employed in the characterisation.

(iii) *Applications*. The applicability of the developed model system was to be investigated. The probing of ion transfer, ion – membrane interactions and membrane activity of biotechnological drugs, such as peptides and oligonucleotides, were some applications of interest.

(iv) *Future prospects*. The future prospects of the developed model system are assessed. Does the biomembrane model hold promise for future biosensor applications? On basis of the obtained results, could the model system be improved in some way, with particular focus on problem issues in previous model membranes: stability, ease of formation, and biomimetic properties?

4. The present work

This chapter summarises the results of the publications listed at the beginning of the thesis, which will be referred to in the following sections by their Roman numerals.

4.1 Development (I)

A lipid monolayer was chosen as the model system in order to achieve better interpretability of results from electrochemical studies. For maximal controllability, the monolayer was prepared at the air – water interface with the Langmuir trough technique. Improved applicability and suitability for characterisation was achieved by depositing the monolayer on a substrate with the Langmuir – Blodgett method. The solid substrate contained a gelled organic solvent, which simultaneously served as a dynamic polymer support for the monolayer and an electrochemical half-cell. A number of substrate materials and geometries were investigated and three different half-cells were finally constructed with optimal properties for characterisation by cyclic voltammetry, ac impedance and fluorescence spectroscopy. The first-mentioned is schematically represented in Figure 4.1. Teflon was chosen for substrate material, as it is hydrophobic, shapeable, and inert in the experimental conditions of the study.

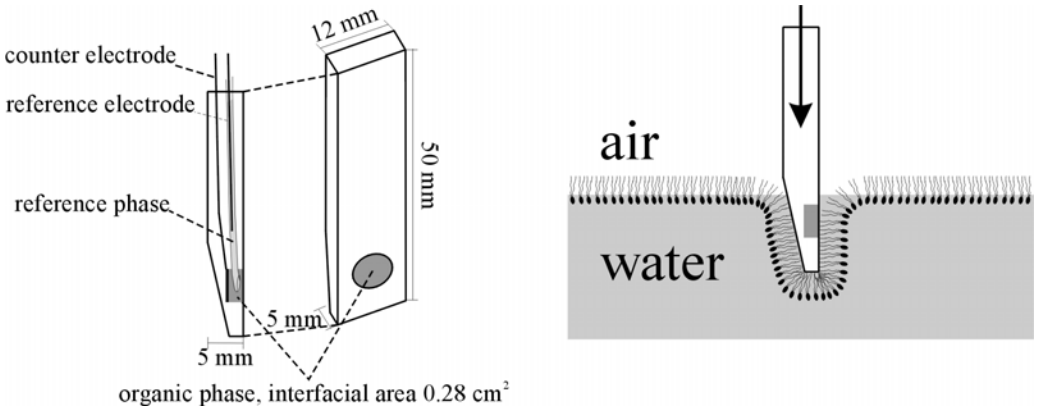

Figure 4.1. Schematic illustration of the organic half-cell employed in the dc cyclic voltammetry and ac voltammetry measurements. The right-hand figure demonstrates the function of the half-cell as a substrate during LB deposition. Reproduced from Publication III. Copyright 2001 American Chemical Society.

The organic phase was immobilised into a circular cavity in the substrate with 5 w-% PVC. The monolayer was formed by spreading a chloroform solution of lipid on the electrolyte – air interface. After evaporation of the chloroform, the monolayer was compressed to the desired surface pressure. Subsequently, the monolayer was deposited onto the gel-containing substrate by downward LB deposition, while maintaining the target surface pressure.

In all studies, the organic solvent was 2-nitrophenyl octyl ether (NPOE), the organic base electrolyte 10 mM tetraphenylarsonium or bis(triphenylphosphoranylidene)-ammonium -tetrakis(4-chlorophenyl)borate (TPAsTPBCl and BTPPATPBCl, respectively) and the aqueous phase a 10 mM chloride solution. The lipid monolayer was composed of DSPC, DPPC, DOPC, cholesterol or their mixtures. The experimental temperature in the presence of a monolayer was 20.0 ± 0.1 °C, while other experiments were conducted at an air-conditioned room temperature of 21 – 22 °C.

4.2 Characterisation (I – III)

The monolayer formation process was monitored by recording compression isotherms at the air – water interface. The success of the subsequent transfer of the monolayer onto the substrate was characterised by the transfer ratio, defined as the ratio of the required compression, in order to maintain target pressure during deposition, to the submerged substrate area. High surface pressures (50 – 60 mN/m) yielded transfer ratios of approximately 100%. At lower surface pressures, transfer was incomplete, which prevented the use of a 30 mN/m monolayer as our principal model membrane.

Interfacial capacitance was employed to characterise the obtained monolayers. To this end, cyclic voltammetry, ac voltammetry and ac impedance measurements were carried out. In the presence of base electrolytes only, the latter two methods yielded sensitive information on the potential-dependent properties of the monolayer, when interpreted with the simple Randles equivalent circuit. In all experiments, the Galvani potential difference across the liquid – liquid interface, $\Delta_{\mathrm{o}}^{\mathrm{w}}\phi$, was defined with respect to the potential in the organic phase, i.e.:

$$\Delta_{\mathrm{o}}^{\mathrm{w}}\phi = \phi_{\mathrm{w}} - \phi_{\mathrm{o}} \qquad (4.1)$$

Inspection of the obtained capacitance curves in publications I, II and III reveals a two-fold effect of the monolayer on the interfacial capacitance. In accordance with observations reported in section 2.2.5, in the presence of the monolayer, the overall capacitance is decreased, and the capacitance minimum is shifted towards more negative potentials. The drop in the capacitance is consistent with the formation of a compact layer of low dielectric constant at the interface, while the shift in the E_{pzc} reflects a change in the interfacial electrostatics. Qualitatively speaking, this would mean the introduction of a permanent positive charge and/or a dipole potential at the interface.

The potential-induced protonation of the lipid phosphate groups (section 2.2.4) was considered an unlikely event as in publication I, the aqueous phase was buffered to a pH of 8.3, which is at least 5 pH units above the pK_a value of the phosphate group in PC (Tatulian, 1993). In addition, no difference was observed between the capacitance minimum shifts in the presence of an unbuffered or buffered aqueous phase. Instead, adsorption of electrolyte cations to the lipid headgroups seems a more plausible explanation for the accumulation of permanent positive charge at the interface. The presence of a dipole potential in the lipid monolayer is a likely occurrence. In order to shift the capacitance minimum to a negative direction, the dipole potential should be positive towards the interface between the organic phase and the aqueous phase. This is in accordance with previously discussed findings on the sign of the dipole potential in PC mono- and bilayers (sections 2.1.2 and 2.2.5).

To test the above hypothesis, a simple electrostatic model was constructed for qualitative reproduction of the experimental capacitance curves. In the model, the interface was divided into three layers: the organic phase, the hydrocarbon region and the aqueous phase (Figure 4.2). The zwitterionic PC headgroups were situated on the boundary between the hydrocarbon region and the aqueous phase. The potential profile in the aqueous phase was modelled with the one-plane GCS approach, where adsorbing cations were incorporated into the plane of the zwitterions. The electrostatic profiles of the organic phase and the hydrocarbon region were modelled with the GC approach; however, the accessibility of organic cations to the latter phase was regulated with a partition coefficient. No form of dipole potential was incorporated into the model. While an obvious oversimplification, this was a conscious choice in view of the uncertainties concerning the structural contributions of lipid and water molecules, and the effect of applied surface pressure and an external potential difference on these contributions.

The above-described electrostatic model was able to account for the features in the experimental capacitance curves. The magnitude of the capacitance could be reproduced by appropriate adjustment of the partition coefficient of organic base electrolyte, the dielectric constant and the thickness of the hydrocarbon region, while the shift in capacitance minimum was largely dependent on the binding constant of aqueous cations to the lipid headgroups, reflecting the change in surface charge density.

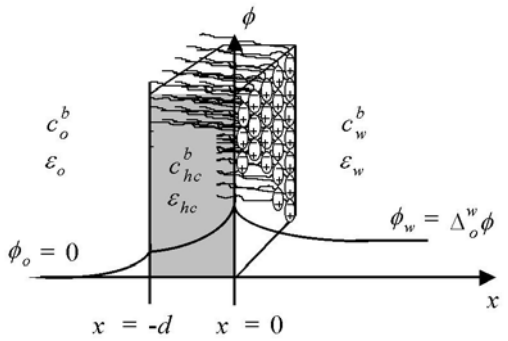

Figure 4.2. Schematic representation of the electric potential profile across the lipid monolayer at the liquid – liquid interface according to the three-layer model. Reproduced from Publication I. Copyright 2000 American Chemical Society.

The effect of lipid composition on the interfacial capacitance was investigated in publication II. Low capacitance values (2 – 3 μF/cm^2), indicating a condensed phase monolayer, were obtained for DSPC and DSPC/cholesterol (1:2 mol:mol) monolayers deposited at 50 – 60 mN/m. Incorporation of phospholipids with shorter or unsaturated chains (DPPC, DOPC) rendered the monolayer more fluid, which could be seen as an increase in the capacitance. The well-known condensing effect of cholesterol on long chain, saturated phospholipids (Sugahara et al., 2001; Radhakrishnan, McConnell, 2000) was evident in 40 mN/m monolayers, particularly in the binary mixtures between cholesterol and DSPC.

In relation to its structural integrity, the ability of the monolayer to block charge transfer reactions was investigated. Cyclic voltammetry measurements revealed neither inhibition nor enhancement of ion transfer kinetics in the presence of a condensed phase DSPC monolayer (publication I). While such a dense monolayer may have been expected to produce a pronounced blocking effect on charge transfer, the possibility of post-deposition defects has to be recognised. These could arise as a result of the strain that the boundary between the organic gel and the Teflon substrate imposes on the monolayer.

When the more sensitive ac voltammetry technique was employed (publication III), the apparent capacitance curves, as obtained by analysis with the Randles equivalent

circuit, clearly indicated an effect of the monolayer on ion transfer. To elucidate the kinetic and mechanistic details of the transfer event, a theoretical model was constructed. While the model was slightly refined in later publications, its basic idea was to combine the Butler – Volmer description of ion transfer kinetics with the possibility of specific adsorption at the aqueous and organic sides of the interface.

Analysis of experimental ac voltammetry and impedance data with the theoretical model revealed a trend of decreasing ion transfer rate with increasing surface pressure for most compounds. This is in accordance with the assumption that the studied monolayers were in the condensed phase, as opposed to previously discussed observations of enhanced ion transfer at liquid expanded monolayers. An effect of monolayer composition on the transfer rate was not observed (Publication II). The outcome of interpreting experimental results with the model is further discussed in section 4.3.2.

Finally, in order to obtain independent information on the structure of the lipid monolayers deposited at the liquid – liquid interface, fluorescence anisotropy experiments were carried out. In brief, fluorescence anisotropy measures the difference in excitation and emission polarisation, thereby revealing the ability of a probe molecule to reorient during the lifetime of its excited state. The magnitude of the observed anisotropy describes the fluidity of the probe molecule environment. Owing to time limitations, only preliminary results were obtained, but this promising approach will be revived in the near future.

4.3 Applications

4.3.1 Drug partition (III – IV)

In publications III – IV, the bare liquid – liquid interface was employed for the determination of partition coefficients. The partition coefficient, P, is defined as the ratio of the activity of a single species of solute in two immiscible phases at equilibrium, and is frequently employed in drug development as a measure of lipophilicity. This property in turn, affects the ability of a drug to permeate through cellular membranes by passive diffusion. Conventionally, partition coefficients of neutral compounds have been obtained from bulk partition experiments between octanol and water (Leo et al., 1971), but lately the significantly more effective computational approaches have gained popularity (Mannhold, van de Waterbeemd,

2001). Despite some contradictory reports (Kürschner et al., 2000; Sugawara et al., 2002), passive biological permeation of ions has generally been assumed to be negligible and has thus received little attention in partition/permeation studies.

Kontturi and Murtomäki (1992) were the first to suggest liquid – liquid electrochemistry as a convenient means to obtain partition coefficients of ionisable compounds. The observed half-wave potentials of the transferring ions were connected to partition coefficients of the neutral species through the thermodynamic partition and dissociation equilibria (Murtomäki, Kontturi, 2002). Girault, Testa and co-workers (Reymond et al., 1999b; Caron et al., 1999) realised the importance of studying both neutral and ionic partition coefficients in different solvent systems to obtain information on hydrogen-bonding and charge delocalisation properties.

Furthermore, in analogy with the diagrams employed by Pourbaix in metal corrosion studies, Reymond et al. (1996, 1999a) developed the concept of pH – potential diagrams at the ITIES, which reveal the charge state and phase of a compound as a function of the solution pH and the Galvani potential difference. Such diagrams are particularly helpful for visualisation of the partition behaviour of multiprotic compounds with several, overlapping dissociation and partition equilibria. A concrete application for pH – potential diagrams can be found in the field of drug pharmacokinetics, where they could be employed to determine the predominant drug species on the basis of the pH and potential conditions. Recently, the approach was extended to allow for determination of pH – potential diagrams of highly hydrophilic and hydrophobic compounds by use of a supported liquid membrane (Ulmeanu et al., 2002).

In Publication III, the ionic partition coefficients of three conventional, cationic drugs were determined, and various theoretical and semi-empirical methods to estimate the partition coefficient of the neutral species from these results were investigated. In publication IV, the partition behaviour of four structurally similar aminoacridine derivatives was studied. pH – potential diagrams were constructed using cyclic voltammetry measurements to elucidate the predominance regions of the various species and to obtain values for the partition coefficients of the neutral species, P_D. To this end, the following equation was employed:

$$\Delta_o^w \phi_{1/2} = \Delta_o^w \phi_{DH}^{0'} + \frac{RT}{F} \ln \xi + \frac{RT}{F} \ln\left(1 + \frac{K_a^w}{c_H^w} + \frac{P_D K_a^w}{\xi c_H^w}\right) \qquad (4.2)$$

where $\Delta_o^w\phi_{1/2}$ and $\Delta_o^w\phi_{DH}^{0'}$ are the half-wave and the formal standard transfer potentials of the protonated drug (DH^+), respectively, and ξ stands for the square root of the ratio of the diffusion coefficients, $(D^w/D^o)^{1/2}$.

The pH – potential diagram for aminacrine is shown in Figure 4.3. The solid line represents the fit to equation (4.2). At pH values well below pH 10, which equals the aqueous pK_a^w value of aminacrine, the line illustrates the partition equilibrium of the protonated, cationic species DH^+. However, as the pK_a^w is approached, the observed half-wave potential becomes pH-dependent with a slope of 60 mV. This is indicative of an assisted proton transfer reaction, where the neutral drug from the organic phase reacts interfacially with a proton and thus facilitates its transfer into the organic phase (Reymond, 2001). Knowledge of the aqueous acid constant and the diffusion constants in both phases then allows for the calculation of the partition coefficient of the neutral species.

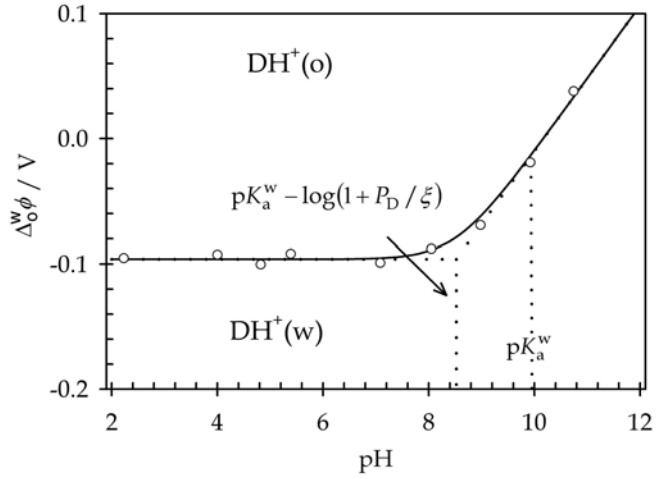

Figure 4.3. pH – potential diagram of aminacrine. The solid line represents the best fit to equation (4.2). Data from Publication IV.

Comparison of the NPOE – water partition coefficients for the neutral species with the corresponding octanol – water values revealed, that while both systems yielded qualitatively similar results, the two lipophilicity scales were not interchangeable. A plausible explanation was found in the different hydrogen-bonding ability of the solvents. Whereas the hydroxyl-group of octanol has both hydrogen-bond acceptor and donor potential, the nitro-group of NPOE is a typical hydrogen-bond acceptor. In this sense, NPOE can be considered a more biomimetic solvent than octanol, since the polar headgroups of lipids are predominantly hydrogen-bond acceptors.

Interestingly, the differences between the ionic and neutral partition coefficients in the NPOE – water system were rather small, especially in the case of proflavine. A likely explanation for this is the delocalisation of charge, which in the case of proflavine is particularly effective owing to its symmetrically substituted side rings (Figure 4.4). The strongly delocalised charge renders the ion more weakly hydrated, which explains the negligible decrease in lipophilicity upon ionisation.

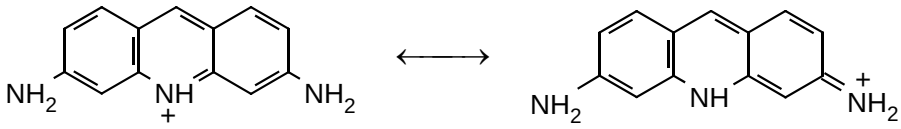

Figure 4.4. Schematic illustration of the resonance chemistry of proflavine.

In summary, the construction of biphasic pH – potential diagrams at the ITIES appears to be an informative and sensitive means of describing partition behaviour of ionisable compounds. Results obtained in the present work suggest that charge delocalisation effects may be sufficiently significant to render the ion suitable for biological permeation, a notion that warrants further studies to be carried out on the subject.

4.3.2 Membrane activity (II – V)

While the determination of partition coefficients is a routine procedure in drug development, the topic of membrane activity has received considerably less attention. The concept of membrane activity implies the tendency of a compound to interact with a biological membrane. In pharmaceutics, high membrane activity can present both a drawback and a benefit. Antimicrobial peptides rely on their membrane activity to bind to the cell surface of bacteria for subsequent insertion and permeabilisation of the bacterial membranes (Dathe, Wieprecht, 1999). The same tendency of binding and membrane insertion can be employed when designing peptides to act as vectors in drug delivery (Vié et al., 2000). Furthermore, Cantor (1997) proposed the alteration in the lateral pressure profile of biological membranes upon drug incorporation to be the mechanism of general anaesthesia. On the other hand, membrane active compounds can disrupt the barrier function of membranes and induce leakiness (Ollila et al., 2002; Grinius et al., 2002), with possible pathological consequences (Galisteo et al., 2000).

So far, the approaches to investigate the membrane activity of drugs include immobilised artificial membrane chromatography (Krause et al., 1999; Ollila et al., 2002), surface pressure and potential studies at air – water monolayers (Maget-Dana, 1999; Vitovič et al., 2004), as well as neutron diffraction (Jacobs, White, 1989) and circular dichroism (Dathe et al., 1996, 2001) measurements with lipid vesicles.

In publications II – IV, the use of the monolayer-covered liquid – liquid interface for probing membrane activity of conventional, ionisable drug compounds was investigated. Ac voltammetry and impedance measurements were analysed with the theoretical model described in chapter 4.2, which in combination with charge transfer accounted for the adsorption of the transferring ion on the aqueous and organic sides of the interface. In publications II and III, it was demonstrated that both tacrine and propranolol preferentially interacted with the headgroup region of an interfacially deposited DSPC monolayer, while metoprolol appeared to prefer the hydrocarbon tails. While a definite trend could not be established between adsorption and monolayer surface pressure, the presence of 67 mol-% cholesterol in the monolayer decreased tacrine interaction with the polar headgroup region. This is readily explained by the reduced surface concentration of interaction sites.

In a subsequent publication (IV), the theoretical model was refined to account for adsorption in two separate planes. The model employed the Langmuir adsorption isotherm with a linear dependence of the adsorption energy on the interfacial potential difference. Analysis of the ac impedance measurement data for four structurally similar cationic aminoacridine derivatives revealed that for the significantly more hydrophilic velnacrine, adsorption took place into one plane in the polar headgroup region. The more hydrophobic tacrine and aminacrine adsorbed in two planes, on each side of the lipid monolayer, exhibiting somewhat larger adsorption coefficients in the polar headgroup region. Proflavine, with an ionic partition coefficient similar to that of tacrine and aminacrine, was nevertheless strongly adsorbed in the headgroup region. This was postulated to result from the favourable location of its hydrogen-bonding groups enabling interactions with two adjacent monolayer lipids (Figure 4.4).

In publication V, the membrane activity of two structurally similar and electrically identical oligopeptides of pharmaceutical importance was investigated. As the peptides were too hydrophilic to undergo phase transfer to the organic phase, the capacitance curves obtained by ac voltammetry measurements were interpreted as reflecting only adsorption of the peptide to the interface. For quantitative interpretation, the electrostatic model presented in the following section (4.3.3) was employed.

While neither nafarelin nor LHRH (leutinising hormone -releasing hormone) was found to interact with a zwitterionic lipid monolayer, both peptides displayed membrane activity in the presence of a negatively charged membrane. Interestingly, while nafarelin differs from LHRH by just one amino acid, this was enough to result in a significant difference in membrane activity. Carrying a positive charge of 1.4, the surface concentration of adsorbed nafarelin was four times larger than that of LHRH. As the pH was increased to reduce the peptide charge to 0.9, LHRH became membrane inactive while nafarelin maintained its surface concentration. These findings support previous notions that the membrane activity of peptides depends on a fine balance between hydrophobic and electrostatic interactions (Seelig et al., 1993; Dathe et al., 1996, 2001).

Conclusively, the model system was demonstrated to provide a sensitive means to investigate membrane activity of both small ions undergoing phase transfer, and hydrophilic biopharmaceuticals constrained to the aqueous phase.

4.3.3 Polyelectrolyte multilayers (VI)

A current hot topic in physical chemistry, new publications on polyelectrolyte multilayers (PEM) emerge by the week. Of the numerous applications proposed for PEMs, biologically relevant ones include the formation of surfaces for cell adhesion studies (Berg et al., 2004) and substrates in polymer-supported lipid bilayers (section 2.2.4), as well as encapsulation materials in controlled-release drug formulations (Qiu et al., 2001; Michel et al., 2004).

Publication VI presents a novel approach to studying drug release through polyelectrolyte multilayers. PEMs were formed at the NPOE – water interface, where an anchoring layer of cationic lipid had previously been deposited. Layer-by-layer self-assembly of oppositely charged polyelectrolytes was employed to construct the multilayer. After each deposition cycle, ac and dc voltammetry was carried out to obtain information on the charge and thickness of the PEM as well as its resistance to ion transfer.

For interpretation of the ac voltammetry results, the electrostatic model described in section 4.2 was modified to account for the presence of the PEM at the interface. In the refined model, the lipid monolayer and the PEM form an ion-free layer where the potential drop is assumed to be linear. The excess charge of the layer, σ, is located in a plane d at the aqueous interface. Outside the PEM, the electrostatic properties of the

aqueous and organic phases are modelled with the GC approach. The interfacial potential profile, according to the theoretical model, is illustrated in Figure 4.5.

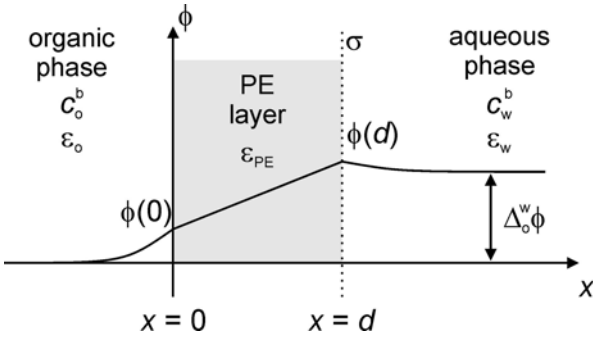

Figure 4.5. Schematic representation of the electric potential profile across the PEM-covered liquid – liquid interface. Reproduced from Publication VI. Copyright 2003 American Chemical Society.

The theoretical model was used to qualitatively reproduce the capacitance curves measured at the PEM-covered liquid – liquid interface. As in publication I, the dielectric permittivity and thickness of the layer governed the magnitude of the capacitance and the shape of the curve, while the surface charge determined the location of the capacitance minimum.

Dc cyclic voltammetry was employed to investigate the effect of the PEM on ion transfer. In the presence of a PEM with a negatively charged outermost layer, no decrease in the transfer rate of TEA^+ was observed until the deposition of the seventh polyelectrolyte layer. On the contrary, a positively charged last layer caused retardation of the ion transfer rate already upon the introduction of the fourth layer. The resistance to ion transfer could thus be concluded to contain both a steric and an electrostatic contribution. Moreover, comparison of TEA^+ and tacrine transfer across the PEM indicated an effect of the shape and charge delocalisation of the transferring ion on the apparent rate constant.

Electrochemistry across the PEM-modified liquid – liquid interface was shown to be a straightforward and informative approach to characterise the fundamental properties of the multilayer and to investigate its effect on drug transfer.

4.4 Future prospects

The methodology described in this thesis enables the formation of lipid monolayers of desired surface pressure and composition at the polarisable liquid – liquid interface. Electrochemical characterisation demonstrated the high reproducibility of such monolayers, and confirmed their stability over periods of 12 hours. While the approach allows for investigation of the monolayer properties as a function of its surface pressure, in practice, deposition of monolayers at less than 40 mN/m was not feasible owing to incomplete transfer. Plausible explanations for this include the substrate size and geometry, and in particular, the too low affinity of the lipid chains towards the substrate material Teflon. For improved transfer at lower surface pressures, the downscaling of the system in combination with a more lipophilic substrate material will be investigated.

The publications forming part of this thesis, demonstrate liquid – liquid electrochemistry to be a versatile tool in the characterisation of drug properties with relevance to drug delivery and pharmacokinetics. While the bare ITIES enables the determination of partition coefficients for both the neutral and charged species of ionisable compounds, ac electrochemical measurements can be combined with theoretical models to reveal the membrane activity of variable classes of therapeutics. The sensitivity of capacitance measurements to detect changes in membrane activity, induced by minor alterations in structure, holds promise for future applications in this field of biosensing.

Moreover, several approaches can be adopted to modify the monolayer-covered liquid – liquid interface. To improve the biomimetic nature of the model membrane, anionic polysaccharides can be assembled on the polar headgroups to resemble the glycosaminoglycan network of animal cells (Santos et al., 2004). Alternatively, as shown in the previous section, the monolayer can serve as an anchor for polyelectrolyte layers in studies of drug release kinetics.

Near future -goals include the incorporation and characterisation of pore-forming peptides in the model membrane system. In addition, the approach of employing complementary techniques, such as fluorescence anisotropy and fluorescence recovery after photobleaching, will be revived to obtain more detailed information on the structure and dynamics of the lipid monolayer at the liquid – liquid interface.

5. Conclusions

The aim of the thesis was to develop a novel methodology for constructing model biomembranes at polarisable liquid – liquid interfaces. To this end, a lipid monolayer was deposited at the NPOE gel – water interface with the Langmuir – Blodgett technique, rendering the monolayer stable, yet free from substrate-induced constraints, and allowing lipid packing and composition to be controlled. The phase transfer and membrane interactions of charged therapeutics at the monolayer-covered interface were monitored with ac and dc electrochemical techniques. Theoretical models were developed to explain experimental observations and extract quantitative data on ion transfer kinetics and interfacial adsorption.

The work presented in this thesis demonstrates liquid – liquid electrochemistry to be a useful tool in the characterisation of drug properties with relevance to drug delivery and pharmacokinetics. The bare ITIES enables the construction of pH – potential diagrams, yielding partition coefficients for both ionic and neutral species, thus elucidating the effect of charge on lipophilicity. In the presence of a monolayer, ac techniques reveal the membrane activity of both small ions undergoing interfacial phase transfer, and larger, highly hydrophilic biopharmaceuticals, which are constrained to the aqueous phase. Modification of the lipid monolayer with adsorbed polyions can be employed to mimic the extracellular matrix or the wall of controlled drug delivery devices.

The sensitivity and versatility of the developed model system, together with its unique combination of a controlled monolayer immobilised at an electrified liquid – liquid interface, provide a complementary approach to other methods aimed at investigating the behaviour of drugs at biological interfaces.

List of abbreviations

AFM	atomic force microscopy
BAM	Brewster angle microscopy
BLM	black lipid membrane / unsupported bilayer lipid membrane
BTPPATPBCl	bis(triphenylphosphoranylidene)ammonium tetrakis(4-chlorophenyl) borate
C	condensed phase
DBPC	dibehenoyl phosphatidylcholine, C22:0
DCE	1,2-dichloroethane
DHADAB	dihexadecyldimethylammonium bromide
DLPC	dilauroyl phosphatidylcholine, C12:0
DMPC	dimyristoyl phosphatidylcholine, C14:0
DOPA	dioleoyl phosphatidic acid, C18:1
DOPC	dioleoyl phosphatidylcholine, C18:1
DPEPC	2,3-di-*o*-phytanyl-*sn*-phosphatidylcholine
DPH	1,6-diphenyl-1,3,5-hexatriene
DPhPC	diphytanoyl phosphatidylcholine, C16:0, 4ME
DPPA	dipalmitoyl phosphatidic acid, C16:0
DPPC	dipalmitoyl phosphatidylcholine, C16:0
DPPS	dipalmitoyl phosphatidylserine, C16:0
DSPC	distearoyl phosphatidylcholine, C18:0
DSPE	distearoyl phosphatidylethanolamine, C18:0
ESP	equilibrium spreading pressure
ET	electron transfer
FIT	facilitated ion transfer
FRAP	fluorescence recovery after photobleaching
G	gas phase
GC	Gouy – Chapman
GCS	Gouy – Chapman – Stern
IT	ion transfer
ITIES	interface between two immiscible electrolyte solutions
ITO	indium – tin oxide
LB	Langmuir – Blodgett
LE	liquid – expanded phase
LHRH	leutinising hormone -releasing hormone

LS	Langmuir – Schäfer
MD	molecular dynamics
NPOE	2-nitrophenyl octyl ether
PB	Poisson – Boltzmann
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEG	polyethyleneglycol
PEI	polyethylenimine
PEM	polyelectrolyte multilayer
PI	phosphatidylinositol
POPC	1-palmitoyl-2-oleoyl phosphatidylcholine, C16:0 / C18:1
POPE	1-palmitoyl-2-oleoyl phosphatidylethanolamine, C16:0 / C18:1
PS	phosphatidylserine
psBLM	polymer-supported bilayer lipid membrane
QCM	quartz crystal microbalance
SAM	self-assembled monolayer
SECM	scanning electrochemical microscopy
SOPS	1-stearoyl-2-oleoyl phosphatidylserine, C18:0 / C18:1
SPHM	sphingomyelin
SPR	surface plasmon resonance
ssBLM	solid-supported bilayer lipid membrane
TC	tilted condensed
TEA^+	tetraethylammonium
TMA^+	tetramethylammonium
$TPAs^+$	tetraphenylarsonium
TPAsTPBCl	tetraphenylarsonium tetrakis(4-chlorophenyl)borate
TPB^-	tetraphenylborate
UC	untilted condensed

List of symbols

A	area
A_{lipid}	mean molecular area of lipid
C	capacitance
c_{b}	bulk concentration
D	diffusion coefficient
E_{pzc}	potential of zero charge
e	proton charge
F	Faraday constant
G	Gibbs free energy
i	chemical species, index
K	equilibrium constant
k	Boltzmann constant, standard rate constant of charge transfer
N	surface density of binding sites
N_{A}	Avogadro's number
P	partition coefficient, pressure
R	resistance, molar gas constant
T	absolute temperature
x	coordinate
z	charge number

χ	compressibility
$\Delta\psi$	transmembrane potential difference
$\Delta_{\mathrm{o}}^{\mathrm{w}}\phi$	Galvani potential difference
$\Delta_{\mathrm{o}}^{\mathrm{w}}\phi_{1/2}$	half-wave potential
$\Delta_{\mathrm{o}}^{\mathrm{w}}\phi_i^{0}$	standard transfer potential of ion i
ε	electrical permittivity
γ	surface tension
κ	inverse Debye length
μ^0	standard chemical potential
Π	surface pressure
σ	surface charge density
ψ_{d}	dipole potential
$\psi_{\mathrm{s}}, \psi_0$	surface potential

References

Abrecht, H., Wattiez, R., Ruysschaert, J.-M., Homblé, F., 2000. Purification and characterization of two voltage-dependent anion channel isoforms from plant seeds. *Plant Physiol.* 124, 1181–1190.

Aguilella, V.M., Mafé, S., Manzanares, J.A., 2000. Double layer potential and degree of dissociation in charged lipid monolayers. *Chem. Phys. Lipids* 105, 225-229.

Aguilella, V., Belaya, M., Levadny, V., 1996. Ion permeability of a membrane with soft polar interfaces. 1. The hydrophobic layer as the rate-determining step. *Langmuir* 12, 4817-4827.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., Watson, J.D., 1994. *Molecular biology of the cell.* 3rd edition. Garland Publishing, Inc., New York, pp. 477-506.

Allen, R.M., Williams, D.E., 1996. Dynamic effects at the interface between two immiscible electrolyte solutions. *Faraday. Discuss.* 104, 281-293.

Amemiya, S., Yang, X., Wazenegger, T.L., 2003. Voltammetry of the phase transfer of polypeptide protamines across polarized liquid/liquid interfaces. *J. Am. Chem. Soc.* 125, 11832-11833.

Arora, A., Rinehart, D., Szabo, S., Tamm, L.K., 2000. Refolded outer membrane protein A of *Escherichia coli* forms ion channels with two conductance states in planar lipid bilayers. *J. Biol. Chem.* 275, 1594-1600.

Arrigan, D.W.M., Ghita, M., Beni, V., 2004. Selective voltammetric detection of dopamine in the presence of ascorbate. *Chem. Commun.*, 732-733.

Arya, A., Krull, U.J., Thompson, M., Wong, H.E., 1985. Langmuir-Blodgett deposition of lipid films on hydrogel as a basis for biosensor development. *Anal. Chim. Acta* 173, 331-336.

Baba, T., Toshima, Y., Minamikawa, H., Hato, M., Suzuki, K., Kamo, N., 1999. Formation and characterization of planar lipid bilayer membranes from synthetic phytanyl-chained glycolipids. *Biochim. Biophys. Acta* 1421, 91-102.

Baekmark, T.R., Elender, G., Lasic, D.D., Sackmann, E., 1995. Conformational transitions of mixed monolayers of phospholipids and poly(ethylene oxide) lipopolymers and interaction forces with solid surfaces. *Langmuir* 11, 3975-3987.

Bangham, A.D., 1993. Liposomes: the Babraham connection. *Chem. Phys. Lipids* 64, 275-285.

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.

Baumgart, T., Offenhäusser, A., 2003. Polysaccharide-supported planar bilayer lipid model membranes. *Langmuir* 19, 1730-1737.

Bayerl, T.M., Thomas, R.K, Penfold, J., Rennie, A., Sackmann, E., 1990. Specular reflection of neutrons at phospholipid monolayers. *Biophys. J.* 57, 1095-1098.

Benz, M., Gutsmann, T., Chen, N., Tadmor, R., Israelachvili, J., 2004. Correlation of AFM and SFA measurements concerning the stability of supported lipid bilayers. *Biophys. J.* 86, 870-879.

Benz, R., Janko, K., 1976. Voltage-induced capacitance relaxation of lipid bilayer membranes. Effects of membrane composition. *Biochim. Biophys. Acta* 455, 721-738.

Benz, R., Prass, W., Ringsdorf, H., 1982. Black lipid membranes from polymerizable lipids. *Angew. Chem. Int. Ed.* 21, 368-369.

Benz, R., Fröhlich, O., Läuger, P., Montal, M., 1975. Electrical capacity of black lipid films and of lipid bilayers made from monolayers. *Biochim. Biophys. Acta* 394, 323-334.

Berg, M.C., Yang, S.Y., Hammond, P.T., Rubner, M.F., 2004. Controlling mammalian cell interactions on patterned polyelectrolyte multilayer surfaces. *Langmuir* 20, 1362-1368.

Bloom, M., Mouritsen, O.G., 1995. The evolution of membranes. In: Lipowsky, R. and Sackmann, E. (Eds.), *Structure and dynamics of membranes. From cells to vesicles.* Elsevier, Amsterdam, pp. 65-95.

Blume, A., 1979. A comparative study of the phase transitions of phospholipid bilayers and monolayers. *Biochim. Biophys. Acta* 557, 32-44.

Brockman, H., 1994. Dipole potential of lipid membranes. *Chem. Phys. Lipids* 73, 57-79.

Cannan, S., Zhang, J., Grunfeld, F., Unwin, P.R., 2004. Scanning electrochemical microscopy (SECM) studies of oxygen transfer across phospholipid monolayers under surface pressure control: comparison of monolayers at air/water and oil/water interfaces. *Langmuir* 20, 701-707.

Cantor, R.S., 1997. The lateral pressure profile in membranes: a physical mechanism of general anesthesia. *Biochemistry* 36, 2339-2344.

Caron, G., Steyaert, G., Pagliara, A., Reymond, F., Crivori, P., Gaillard, P., Carrupt, P.-A., Avdeef, A., Comer, J., Box, K.J., Girault, H.H., Testa, B., 1999. Structure-lipophilicity relationships of neutral and protonated β-blockers. Part I. Intra- and intermolecular effects in isotropic solvent systems. *Helv. Chim. Acta* 82, 1211-1222.

Cassier, T., Sinner, A., Offenhäuser, A., Möhwald, H., 1999. Homogeneity, electrical resistivity and lateral diffusion of lipid bilayers coupled to polyelectrolyte multilayers. *Coll. Surf. B* 15, 215-225.

Cevc, G., 1993. Appendix B: thermodynamic parameters of phospholipids. In: Cevc, G. (Ed), *Phospholipids handbook.* Marcel Dekker, Inc., New York, pp. 939-956.

Cheng, Y., Schiffrin, D.J., 1994. Redox electrocatalysis by tetracyanoquinodimethane in phospholipid monolayers adsorbed at a liquid/liquid interface. *J. Chem. Soc. Faraday Trans.* 90, 2517-2523.

Cheng, Y., Bushby, R.J., Evans, S.D., Knowles, P.F., Miles, R.E., Ogier, S.D., 2001. Single ion channel sensitivity in suspended bilayers on micromachined supports. *Langmuir* 17, 1240-1242.

Cheng, Y., Boden, N., Bushby, R.J., Clarkson, S., Evans, S.D., Knowles, P.F., Marsh, A., Miles, R.E., 1998. Attenuated total reflection Fourier transform infrared spectroscopic characterization of fluid lipid bilayers tethered to solid supports. *Langmuir* 14, 839-844.

Cheng, Y., Cunnane, V.J., Kontturi, A.-K., Kontturi, K., Schiffrin, D.J., 1996. Potential dependence of transmembrane electron transfer across phospholipid bilayers mediated by ubiquinone 10. *J. Phys. Chem.* 100, 15470-15477.

Chiang, K.-L., Krull, U.J., Nikolelis, D.P., 1997. Ellipsometric determination of the structure of surface-stabilized bilayer lipid membranes on silver metal. *Anal. Chim. Acta* 357, 73-77.

Cladera, J., O'Shea, P., 1998. Intramembrane molecular dipoles affect the membrane insertion and folding of a model amphiphilic peptide. *Biophys. J.* 74, 2434-2442.

Clarke, R.J., 2001. The dipole potential of phospholipid membranes and methods for its detection. *Adv. Colloid Interface Sci.* 89-90, 263-281.

Conboy, J.C., Liu, S., O'Brien, D.F., Saavedra, S.S., 2003. Planar supported bilayer polymers formed from bis-diene lipids by Langmuir – Blodgett deposition and UV irradiation. *Biomacromolecules* 4, 841-849.

Cordero, S.R., Weston, K.D., Buratto, S.K., 2000. Near-field microscopy of collapsed Langmuir – Blogett films. *Thin Solid Films* 360, 139-144.

Cornell, B.A., Braach-Maksvytis, V.L.B., King, L.G., Osman, P.D.J., Raguse, B., Wieczorek, L., Pace, R.J., 1997. A biosensor that uses ion-channel switches. *Nature* 387, 580-583.

Coronado, R., Latorre, R., 1983. Phospholipid bilayers made from monolayers on patch-clamp pipettes. *Biophys. J.* 43, 231-236.

Cremer, M., 1906. Über die Urasche der elektromotorischen Eigenschaften der Gewebe, zugleich ein Beitrag zur Lehre von den polyphasischen Elektrolytketten. *Z. Biol.* 47, 562-608.

Cruz, A., Vázquez, L., Vélez, M., Pérez-Gil, J., 2004. Effect of pulmonary surfactant protein SP-B on the micro- and nanostructure of phospholipid films. *Biophys. J.* 86, 308-320.

Cseh, R, Benz, R., 1999. Interaction of phloretin with lipid monolayers: relationship between structural changes and dipole potential change. *Biophys. J.* 77, 1477-1488.

Cseh, R., Benz, R., 1998. The adsorption of phloretin to lipid monolayers and bilayers cannot be explained by Langmuir adsorption isotherms alone. *Biophys. J.* 74, 1399-1408.

Cunnane, V.J., Schiffrin, D.J., Fleischmann, M., Geblewicz, G., Williams, D., 1988. The kinetics of ionic transfer across adsorbed phospholipid layers. *J. Electroanal. Chem.* 243, 455-464.

Danielli, J.F., Davson, H., 1935. A contribution to the theory of permeability of thin films. *J. Cell. Comp. Physiol.* 5, 495-508.

Dathe, M., Wieprecht, T., 1999. Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells. *Biochim. Biophys. Acta* 1462, 71-87.

Dathe, M., Nikolenko, H., Meyer, J., Beyermann, M., Bienert, M., 2001. Optimization of the antimicrobial activity of magainin peptides by modification of charge. *FEBS Lett.* 501, 146-150.

Dathe, M., Schümann, M., Wieprecht, T., Winkler, A., Beyermann, M., Krause, E., Matsuzaki, K., Murase, O., Bienert, M., 1996. Peptide helicity and membrane surface charge modulate the balance of electrostatic and hydrophobic interactions with lipid bilayers and biological membranes. *Biochemistry* 35, 12612-12622.

Davenport, R.J., Tester, M., 2000. A weakly voltage-dependent, nonselective cation channel mediates toxic sodium influx in wheat. *Plant Physiol.* 122, 823–834.

Delville, M.-H., Tsionsky, M., Bard, A.J., 1998. Scanning electrochemical microscopy studies of electron transfer through monolayers containing conjugated species at the liquid – liquid interface. *Langmuir* 14, 2774-2779.

Devaux, P.F., Seigneuret, M., 1985. Specificity of lipid-protein interactions as determined by spectroscopic techniques. *Biochim. Biophys. Acta* 822, 63-125.

DeWolf, C., Leporatti, S., Kirsch, C., Klinger, R., Brezesinski, G., 1999. Phase separation in phosphatidylinositol / phosphatidylcholine mixed monolayers. *Chem. Phys. Lipids* 97, 129-138.

Dibble, A.R.G., Hinderliter, A.K., Sando, J.J., Biltonen, R.L., 1996. Lipid lateral heterogeneity in phosphatidylcholine / phosphatidylserine / diacylglycerol vesicles and its influence on protein kinase C activation. *Biophys. J.* 71, 1877-1890.

Dietrich, C., Bagatolli, L.A., Volovyk, Z.N., Thompson, N.L., Levi, M., Jacobson, K., Gratton, E., 2001. Lipid rafts reconstituted in model membranes. *Biophys. J.* 80, 1417-1428.

Dumas, F., Sperotto, M.M., Lebrun, M.-C., Tocanne, J.-F., Mouritsen, O.G., 1997. Molecular sorting of lipids by bacteriorhodopsin in dilauroylphosphatidylcholine / distearoylphosphatidylcholine lipid bilayers. *Biophys. J.* 73, 1940-1953.

Egberts, E., Marrink, S.J., Berendsen, H.J.C., 1994. Molecular dynamics simulation of a phospholipid membrane. *Eur. Biophys. J.* 22, 423-436.

Eisenberg, M., Gresalfi, T., Riccio, T., McLaughlin, S., 1979. Adsorption of monovalent cations to bilayer membranes containing negative phospholipids. *Biochemistry* 18, 5213-5223.

Essmann, U., Perera, L., Berkowitz, M.L., 1995. The origin of the hydration interaction of lipid bilayers from MD simulation of dipalmitoylphosphatidylcholine membranes in gel and liquid crystalline phases. *Langmuir* 11, 4519-4531.

Fahey, P.F., Webb, W.W., 1978. Lateral diffusion in phospholipid bilayer membranes and multilamellar liquid crystals. *Biochemistry* 17, 3046-3053.

Feng, J., Ci, Y.X., Zhang, C.Y., Ottova, A.L., Tien, H.T., 1999. Photoelectric measurements of self-assembled and supported planar lipid bilayers: a new technique for studying apoptosis. *Electrochem. Commun.* 1, 145-147.

Flewelling, R.F., Hubbell, W.L., 1986. The membrane dipole potential in a total membrane potential model. Applications to hydrophobic ion interactions with membranes. *Biophys. J.* 49, 541-552.

Flörsheimer, M., Möhwald, H., 1991. Superimposed ordering transitions in phospholipid monolayers. *Coll. Surf.* 55, 173-189.

Forstner, M.B., Käs, J., Martin, D., 2001. Single lipid diffusion in Langmuir monolayers. *Langmuir* 17, 567-570.

Franklin, J.C., Cafiso, D.S., 1993. Internal electrostatic potentials in bilayers: measuring and controlling dipole potentials in lipid vesicles. *Biophys. J.* 65, 289-299.

Gabriel, B., Teissié, J., 1991. Proton lateral conduction along lipid monolayers is present only in the liquid-expanded state. *J. Am. Chem. Soc.* 113, 8818-8821.

Gabrielli, G., Guarini, G.G.T., Ferroni, E., 1976. On the mechanism of collapse of arachidic acid films at the water/air interface. *J. Colloid Interface Sci.* 54, 424-429.

Galisteo, M., Rissel, M., Sergent, O., Chevanne, M., Cillard, J., Guillouzo, A., Lagadic-Gossmann, D., 2000. Hepatotoxicity of tacrine: occurrence of membrane fluidity alterations without involvement of lipid peroxidation. *J. Pharmacol. Exp. Ther.* 294, 160-167.

Gao, H., Luo, G.-A., Feng, J., Ottova, A.L., Tien, H.T., 2000. Fabrication and photoelectric properties of self-assembled bilayer lipid membranes on conducting glass. *J. Photochem. Photobiol. B* 59, 87-91.

Gavach, C., Mlodnicka, T., Guastalla, J., 1968. Overvoltage phenomena at interfaces between organic and aqueous solutions. *C. R. Acad. Sci. C* 266, 1196-1199.

Gawrisch, K., Barry, J.A., Holte, L.L., Sinnwell, T., Bergelson, L.D., Ferretti, J.A., 1995. Role of interactions at the lipid-water interface for domain formation. *Mol. Membr. Biol.* 12, 83-88.

Gawrisch, K., Ruston, D., Zimmerberg, J., Parsegian, V.A., Rand, R.P., Fuller, N., 1992. Membrane dipole potentials, hydration forces, and the ordering of water at membrane surfaces. *Biophys. J.* 61, 1213-1223.

Georganopoulou, D.G., Caruana, D.J., Strutwolf, J., Williams, D.E., 2000. Electron transfer mediated by glucose oxidase at the liquid/liquid interface. *Faraday Discuss.* 116, 109-118.

Girault, H.H.J., Schiffrin, D.J., 1989. Electrochemistry of liquid-liquid interfaces. In: Bard, A.J. (Ed.), *Electroanalytical chemistry.* Marcel Dekker, Inc., New York, vol. 15, pp. 1-141.

Girault, H.H.J., Schiffrin, D.J., 1984. Charge transfer through phospholipid monolayers adsorbed at liquid-liquid interfaces. In: Allen, M.J. and Usherwood, P.N.R. (Eds.), *Charge and field effects in biosystems.* Abacus Press, Tunbridge Wells, pp. 171-178.

Goennenwein, S., Tanaka, M., Hu, B., Moroder, L., Sackmann, E., 2003. Functional incorporation of integrins into solid supported membranes on ultrathin films of cellulose: impact on adhesion. *Biophys. J.* 85, 646-655.

Gorter, E., Grendel, F., 1925. On biomolecular layers of lipids on the chromocytes of the blood. *J. Exp. Med.* 41, 439-443.

Grandell, D., Murtomäki, L., 1998. Surface pressure control of phospholipid monolayers at the water/1,2-dichloroethane interface. *Langmuir* 14, 556-559.

Grandell, D., Murtomäki, L., Kontturi, K., Sundholm, G., 1999a. Phospholipid monolayers studied by a combination of cyclic voltammetry and Langmuir techniques at the water | 1,2-dichloroethane interface. *J. Electroanal. Chem.* 463, 242-247.

Grandell, D., Murtomäki, L., Sundholm, G., 1999b. Ion transfer across a phospholipid monolayer adsorbed at the water | 1,2-dichloroethane interface under surface pressure control. *J. Electroanal. Chem.* 469, 72-78.

Grinius, L., Stanton, D.T., Morris, C.M., Howard, J.M., Curnow, A.W., 2002. Profiling of drugs for membrane activity using liposomes as an in vitro model system. *Drug Dev. Ind. Pharm.* 28, 193-202.

Gritsch, S., Nollert, P., Jähnig, F., Sackmann, E., 1998. Impedance spectroscopy of porin and gramicidin pores reconstituted into supported lipid bilayers on indium – tin-oxide electrodes. *Langmuir* 14, 3118-3125.

Groves, J.T., Dustin, M.L., 2003. Supported planar bilayers in studies on immune cell adhesion and communication. *J. Immunol. Meth.* 278, 19-32.

Groves, J.T., Ulman, N., Cremer, P.S., Boxer, S.G., 1998. Substrate – membrane interactions: mechanisms for imposing patterns on a fluid bilayer membrane. *Langmuir* 14, 3347-3350.

Groves, J.T., Ulman, N., Boxer, S.G., 1997. Micropatterning fluid lipid bilayers on solid supports. *Science* 275, 651-653.

Gu, L., Wang, L., Xun, J., Ottova-Leitmannova, A., Tien, H.T., 1996. A new method for the determination of electrical properties of supported bilayer lipid membranes by cyclic voltammetry. *Bioelectrochem. Bioenerg.* 39, 275-283.

Guidelli, R., Aloisi, G., Becucci, L., Dolfi, A., Moncelli, M.R., Tadini Buoninsegni, F., 2001. Bioelectrochemistry at metal | water interfaces. *J. Electroanal. Chem.* 504, 1-28.

Györvary, E., Wetzer, B., Sleytr, U.B., Sinner, A., Offenhäusser, A., Knoll, W., 1999. Lateral diffusion of lipids in silane-, dextran-, and S-layer-supported mono- and bilayers. *Langmuir* 15, 1337-1347.

Haas, H., Lamura, G., Gliozzi, A., 2001. Improvement of the quality of self assembled bilayer lipid membranes by using a negative potential. *Bioelectrochemistry* 54, 1-10.

Halter, M., Nogata, Y., Dannenberger, O., Sasaki, T., Vogel, V., 2004. Engineered lipids that cross-link the inner and outer leaflets of lipid bilayers. *Langmuir* 20, 2416-2423.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J., 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* 391, 85-100.

Hanke, W., Methfessel, C., Wilmsen, U., Boheim, G., 1984. Ion channel reconstitution into lipid bilayer membranes on glass patch pipettes. *Bioelectrochem. Bioenerg.* 12, 329-339.

Harries, D., Ben-Shaul, A., 1997. Conformational chain statistics in a model lipid bilayer: comparison between mean field and Monte Carlo calculations. *J. Chem. Phys.* 106, 1609-1619.

Helm, C.A., Möhwald, H., Kjær, K., Als-Nielsen, J., 1987. Phospholipid monolayer density distribution perpendicular to the water surface. A synchrotron X-ray reflectivity study. *Europhys. Lett.* 4, 697-703.

Hetzer, M., Heinz, S., Grage, S., Bayerl, T.M., 1998. Asymmetric molecular friction in supported phospholipid bilayers revealed by NMR measurements of lipid diffusion. *Langmuir* 14, 982-984.

Heyse, S., Ernst, O.P., Dienes, Z., Hofmann, K.P., Vogel, H., 1998a. Incorporation of rhodopsin in laterally structured supported membranes: observation of transducin activation with spatially and time-resolved surface plasmon resonance. *Biochemistry* 37, 507-522.

Heyse, S., Stora, T., Schmid, E., Lakey, J.H., Vogel, H., 1998b. Emerging techniques for investigating molecular interactions at lipid membranes. *Biochim. Biophys. Acta* 1376, 319-338.

Hianik, T., Passechnik, V.I., Sargent, D.F., Dlugopolsky, J., Sokolikova, L., 1995. Surface potentials and solvent redistribution may explain the dependence of electrical and

mechanical properties of supported lipid bilayers on applied potential and bilayer history. *Bioelectrochem. Bioenerg.* 37, 61-68.

Hille, B., 2001. *Ion channels of excitable membranes*, 3rd ed. Sinauer Ass., Sunderland (U.S.A.), pp. 441-470.

Hillebrandt, H., Wiegand, G., Tanaka, M., Sackmann, E., 1999. High electric resistance polyer/lipid composite films on indium-tin-oxide electrodes. *Langmuir* 15, 8451-8459.

Hladky, S.B., Haydon, D.A., 1970. Discreteness of conductance change in bimolecular lipid membranes in the presence of certain antibiotics. *Nature* 225, 451-453.

Hodgkin, A.L., Huxley, A.F., 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* 117, 500-544.

Hollars, C.W., Dunn, R.C., 1998. Submicron structure in L-α-dipalmitoylphosphatidylcholine monolayers and bilayers probed with confocal, atomic force, and near-field microscopy. *Biophys. J.* 75, 342-353.

Hong, F.T., 1997. Interfacial photochemistry of retinal proteins. *Prog. Surf. Sci.* 62, 1-237.

Hongyo, K., Joseph, J., Huber, R.J., Janata, J., 1987. Experimental observation of chemically modulated admittance of supported phospholipid membranes. *Langmuir* 3, 827-830.

Horrocks, B.R., Mirkin, M.V., 1998. Cation binding to DNA studied by ion-transfer voltammetry at micropipets. *Anal. Chem.* 70, 4653-4660.

Horváth, L.I., Brophy, P.J., March, D., 1990. Influence of polar residue deletions on lipid-protein interactions with the myelin proteolipid protein. Spin-label ESR studies with DM-20/lipid recombinants. *Biochemistry* 29, 2635-2638.

Hovis, J.S., Boxer, S.G., 2001. Patterning and composition arrays of supported lipid bilayers by microcontact printing. *Langmuir* 17, 3400-3405.

Hubbell, W.L., McConnell, H.M., 1971. Molecular motion in spin-labeled phospholipids and membranes. *J. Am. Chem. Soc.* 93, 314-326.

Hyvönen, M.T., Kovanen, P.T., 2003. Molecular dynamics simulation of sphingomyelin bilayer. *J. Phys. Chem. B* 107, 9102-9108.

Hønger, T., Jørgensen, K., Biltonen, R.L., Mouritsen, O.G., 1996. Systematic relationship between phospholipase A2 activity and dynamic lipid bilayer heterogeneity. *Biochemistry* 35, 9003-9006.

Hönig, D., Möbius, D., 1988. Direct visualization of monolayers at the air – water interface by Brewster angle microscopy. *J. Phys. Chem.* 95, 4590-4592.

Ide, T., Yanagida, T., 1999. An artificial lipid bilayer formed on an agarose-coated glass for simultaneous electrical and optical measurement of single ion channels. *Biochem. Biophys. Res. Commun.* 265, 595-599.

Ikematsu, M., Iseki, M., Sugiyama, Y., Mizukami, A., 1996. Lipid bilayer formation in a microporous membrane filter monitored by ac impedance analysis and purple membrane photoresponses. *J. Electroanal. Chem.* 403, 61-68.

Ivnitski, D., Wilkins, E., Tien, H.T., Ottova, A., 2000. Electrochemical biosensor based on supported planar lipid bilayers for fast detection of pathogenic bacteria. *Electrochem. Commun.* 2, 457-460.

Jacobs, R.E., White, S.H., 1989. The nature of the hydrophobic binding of small peptides at the bilayer interface: implications for the insertion of transbilayer helices. *Biochemistry* 28, 3421-3437.

Jacobson, K., Sheets, E.D., Simson, R., 1995. Revisiting the fluid mosaic model of membranes. *Science* 268, 1441-1442.

James, S.R., Demel, R.A., Downes, C.P., 1994. Interfacial hydrolysis of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-biphosphate by turkey erythrocyte phospholipase C. *Biochem. J.* 298, 499-506.

Janshoff, A., Steinem, C., Sieber, M., el Bayâ, A., Schmidt, M.A., Galla, H.-J., 1997. Quartz crystal microbalance investigation of the interaction of bacterial toxins with ganglioside containing solid supported membranes. *Eur. Biophys. J.* 26, 261-270.

Jausel-Husken, S., Deuticke, B., 1981. General and transport properties of hypotonic and isotonic preparations of resealed erythrocyte ghosts. *J. Membr. Biol.* 63, 61-70.

Jørgensen, K., Ipsen, J.H., Mouritsen, O.G., Zuckermann, M.J., 1993. The effect of anaesthetics on the dynamic heterogeneity of lipid membranes. *Chem. Phys. Lipids* 65, 205-216.

Kaganer, V.M., Möhwald, H., Dutta, P., 1999. Structure and phase transitions in Langmuir monolayers. *Rev. Mod. Phys.* 71, 779-819.

Kakiuchi, T., Kotani, M., Noguchi, J., Nakanishi, M., Senda, M., 1992a. Phase transition and ion permeability of phosphatidylcholine monolayers at the polarised oil/water interface. *J. Colloid Interface Sci.* 149, 279-289.

Kakiuchi, T., Kondo, T., Kotani, M., Senda, M., 1992b. Ion permeability of dilauroylphosphatidylcholine monolayer at the polarized nitrobenzene/water interface. *Langmuir* 8, 169-175.

Kakiuchi, T., Kondo, T., Senda, M., 1990. Divalent cation-induced phase transition of phosphatidylserine monolayer at the polarized oil-water interface and its influence on the ion-transfer processes. *Bull. Chem. Soc. Jpn.* 63, 3270-3276.

Kakiuchi, T., Nakanishi, M., Senda, M., 1989. The electrocapillary curves of the phosphatidylcholine monolayer at the polarized oil-water interface. II. Double layer structure of dilauroylphosphatidylcholine monolayer at the nitrobenzene-water interface. *Bull. Chem. Soc. Jpn.* 62, 403-409.

Kakiuchi, T., Yamane, M., Osakai, T., Senda, M., 1987. Monolayer formation of dilauroylphosphatidylcholine at the polarized nitrobenzene-water interface. *Bull. Chem. Soc. Jpn.* 60, 4223-4228.

Kane, S.A., Compton, M., Wilder, N., 2000. Interactions determining the growth of chiral domains in phospholipid monolayers: experimental results and comparison with theory. *Langmuir* 16, 8447-8455.

Karakatsanis, P., Bayerl, T.M., 1996. Diffusion measurements in oriented phospholipid bilayers by ^{1}H-NMR in a static fringe field gradient. *Phys. Rev. E* 54, 1785-1790.

Ke, P.C., Naumann, C.A., 2001. Single molecule fluorescence imaging of phospholipid monolayers at the air – water interface. *Langmuir* 17, 3727-3733.

Keller, C.A., Glasmästar, K., Zhdanov, V.P., Kasemo, B., 2000. Formation of supported membranes from vesicles. *Phys. Rev. Lett.* 84, 5443-5446.

Kenn, R.M., Böhm, C., Bibo, A.M., Peterson, I.R., Möhwald, H., Als-Nielsen, J., Kjær, K., 1991. Mesophases and crystalline phases in fatty acid monolayers. *J. Phys. Chem.* 95, 2092-2097.

Kinnunen, P.K.J., Kõiv, A., Lehtonen, J.Y.A., Rytömaa, M., Mustonen, P., 1994. Lipid dynamics and peripheral interactions of proteins with membrane surfaces. *Chem. Phys. Lipids* 73, 181-207.

Kitajka, K., Buda, Cs., Fodor, E., Halver, J.E., Farkas, T., 1996. Involvement of phospholipid molecular species in controlling structural order of vertebrate brain synaptic membranes during thermal evolution. *Lipids* 31, 1045-1050.

Klingler, J.F., McConnell, H.M., 1993. Field-gradient electrophoresis of lipid domains. *J. Phys. Chem.* 97, 2962-2966.

Kolomytkin, O.V., Golubok, A.O., Davydov, D.N., Timofeev, V.A., Vinogradova, S.A., Tipisev, S.Y., 1991. Ionic channels in Langmuir – Blodgett films imaged by a scanning tunneling microscope. *Biophys. J.* 59, 889-893.

Kontturi, K., Murtomäki, L., 1992. Electrochemical determination of partition coefficients of drugs. *J. Pharm. Sci.* 81, 970-975.

Kontturi, A.-K., Kontturi, K., Murtomäki, L., Quinn, B., Cunnane, V.J., 1997. Study of ion transfer across phospholipid monolayers adsorbed at micropipette ITIES. *J. Electroanal. Chem.* 424, 69-74.

Koryta, J., 1979. Electrochemical polarization phenomena at the interface of two immiscible electrolyte solutions. *Electrochim. Acta* 24, 293-300.

Koryta, J., Hung, L.Q., Hofmanová, A., 1982. Biomembrane transport processes at the interface of two immiscible electrolyte solutions with an adsorbed phospholipid monolayer. *Stud. Biophys.* 90, 25-29.

Krause, E., Dathe, M., Wieprecht, T., Bienert, M., 1999. Noncovalent immobilized artificial membrane chromatography, and improved method for describing peptide-lipid bilayer interactions. *J. Chromatogr. A.* 849, 125-133.

Kuziemko, G.M., Stroh, M., Stevens, R.C., 1996. Cholera toxin binding affinity and specificity for gangliosides determined by surface plasmon resonance. *Biochemistry* 35, 6375-6384.

Kürschner, M., Nielsen, K., von Langen, J.R.G., Schenk, W.A., Zimmermann, U., Sukhorukov, V.L., 2000. Effect of fluorine substitution on the interaction of lipophilic ions with the plasma membrane of mammalian cells. *Biophys. J.* 79, 1490-1497.

Lahtinen, R., Fermín, D.J., Kontturi, K., Girault, H.H., 2000. Artificial photosynthesis at liquid|liquid interfaces: photoreduction of benzoquinone by water soluble porphyrin species. *J. Electroanal. Chem.* 483, 81-87.

Lang, H., Duschl, C., Vogel, H., 1994. A new class of thiolipids for the attachment of lipid bilayers on gold surfaces. *Langmuir* 10, 197-210.

Langner, M., Kubica, K., 1999. The electrostatics of lipid surfaces. *Chem. Phys. Lipids* 101, 3-35.

Lasic, D.D., Needham, D., 1995. The "Stealth" liposome: a prototypical biomaterial. *Chem. Rev.* 95, 2601-2628.

Lee, A.G., 1977. Lipid phase transitions and phase diagrams. I. Lipid phase transitions. *Biochim. Biophys. Acta* 472, 237-281.

Leermakers, F.A.M., Nelson, A., 1990. Substrate-induced structural changes in electrode-adsorbed lipid layers. A self-consistent field theory. *J. Electroanal. Chem.* 278, 53-72.

Lehtonen, J.Y.A., Holopainen, J.M., Kinnunen, P.K.J., 1996. Evidence for the formation of microdomains in liquid crystalline large unilamellar vesicles caused by hydrophobic mismatch of the constituent phospholipids. *Biophys. J.* 70, 1753-1760.

Leo, A., Hansch, C., Elkins, D., 1971. Partition coefficients and their uses. *Chem. Rev.* 71, 525-616.

Levadny, V., Aguilella, V., Belaya, M., Yamazaki, M., 1998. Ion permeability of a membrane with soft polar interfaces. 2. The polar zones as the rate-determining step. *Langmuir* 14, 4630-4637.

Lillie, G.C., Holmes, S.M., Dryfe, R.A.W., 2002. Electrochemistry of cytochrome *c* at the liquid – liquid interface. *J. Phys. Chem. B* 106, 12101-12103.

Lindholm-Sethson, B., 1996. Electrochemistry at ultrathin organic films at planar gold electrodes. *Langmuir* 12, 3305-3314.

Lingler, S., Rubinstein, I., Knoll, W., Offenhäusser, A., 1997. Fusion of small unilamellar lipid vesicles to alkanethiol and thiolipid self-assembled monolayers on gold. *Langmuir* 13, 7085-7091.

Liu, B., Mirkin, M.V., 2002. Electron transfer at liquid/liquid interfaces. The effects of ionic adsorption, electrolyte concentration, and spacer length on the reaction rate. *J. Phys. Chem. B* 106, 3933-3940.

López Cascales, J.J., Berendsen, H.J.C., García de la Torre, J., 1996. Molecular dynamics simulation of water between two charged layers of dipalmitoylphosphatidylserine. *J. Phys. Chem.* 100, 8621-8627.

Lu, X., Ottova, A.L., Tien, H.T., 1996. Biophysical aspects of agar-gel supported bilayer lipid membranes: a new method for forming and studying planar bilayer lipid membranes. *Bioelectrochem. Bioenerg.* 39, 285-289.

Luna, E.J., Hitt, A.L., 1992. Cytoskeleton – plasma membrane interactions. *Science* 258, 955-964.

Lösche, M., Duwe, H.-P., Möhwald, H., 1988. Quantitative analysis of surface textures in phospholipid monolayer phase transitions. *J. Colloid Interface Sci.* 126, 432-444.

Ma, C., Srinivasan, M.P., Waring, A.J., Lehrer, R.I., Longo, M.L., Stroeve, P., 2003. Supported lipid bilayers lifted from the substrate by layer-by-layer polyion cushions on self-assembled monolayers. *Coll. Surf. B* 28, 319-329.

Macdonald, P.M., Crowell, K.J., Franzin, C.M., Mitrakos, P., Semchyschyn, D.J., 1998. Polyelectrolyte-induced domains in lipid bilayer membranes: the deuterium NMR perspective. *Biochem. Cell Biol.* 76, 452-464.

Maget-Dana, 1999. The monolayer technique: a potent tool for studying the interfacial properties of antimicrobial and membrane-lytic peptides and their interactions with lipid membranes. *Biochim. Biophys. Acta* 1462, 109-140.

Maggio, B., 1999. Modulation of phospholipase A2 by electrostatic fields and dipole potential of glycosphingolipids in monolayers. *J. Lipid. Res.* 40, 930-939.

Mannhold, R., van de Waterbeemd, H., 2001. Substructure and whole molecule approaches for calculating log P. *J. Comput. Aided Mol. Des.* 15, 337-354.

Manzanares, J.A., Allen, R.M., Kontturi, K.K., 2000. Enhanced ion transfer rate due to the presence of zwiterionic phospholipid monolayers at the ITIES. *J. Electroanal. Chem.* 483, 188-196.

Marrink, S.-J., Berendsen, H.J.C., 1996. Permeation process of small molecules across lipid membranes studied by molecular dynamics simulations. *J. Phys. Chem.* 100, 16729-16738.

Marrink, S.-J., Berendsen, H.J.C., 1994. Simulation of water transport through a lipid membrane. *J. Phys. Chem.* 98, 4155-4168.

Marsh, D., 1996. Lateral pressure in membranes. *Biochim. Biophys. Acta* 1286, 183-223.

Mauzeroll, J., Buda, M., Bard, A.J., Prieto, F., Rueda, M., 2002. Detection of Tl(I) transport through a gramicidin-dioleoylphosphatidylcholine monolayer using the substrate generation – tip collection mode of scanning electrochemical microscopy. *Langmuir* 18, 9453-9461.

Mayer, L.D., Bally, M.B., Hope, M.J., Cullis, P.R., 1985. Uptake of dibucaine into large unilamellar vesicles in response to a membrane potential. *J. Biol. Chem.* 260, 802-808.

McConlogue, C.W., Vanderlick, T.K., 1997. A close look at domain formation in DPPC monolayers. *Langmuir* 13, 7158-7164.

McConnell, H.M., Tamm, L.K., Weis, R.M., 1984. Periodic structures in lipid monolayer phase transitions. *Proc. Natl. Acad. Sci. USA* 81, 3249-3253.

McLaughlin, S.G.A., Szabo, G., Eisenman, G., 1971. Divalent ions and the surface potential of charged phospholipid membranes. *J. Gen. Physiol.* 58, 667-687.

Meijer, L.A., Leermakers, F.A.M., Lyklema, J., 1999. Self-consistent-field modeling of complex molecules with united atom detail in inhomogeneous systems. Cyclic and branched foreign molecules in dimyristoylphosphatidylcholine membranes. *J. Chem. Phys.* 110, 6560-6579.

Meijer, L.A., Leermakers, F.A.M., Nelson, A., 1994. Modeling of the electrolyte ion – phospholipid layer interactions. *Langmuir* 10, 1199-1206.

Meuse, C.W., Krueger, S., Majkrzak, C.F., Dura, J.A., Fu, J., Connor, J.T., Plant, A.L., 1998. Hybrid bilayer membranes in air and water: infrared spectroscopy and neutron reflectivity studies. *Biophys. J.* 74, 1388-1398.

Micelli, S., Gallucci, E., Picciarelli, V., 2000. Studies of mitochondrial porin incorporation parameters and voltage-gated mechanism with different black lipid membranes. *Bioelectrochemistry* 52, 63-75.

Michel, M., Vautier, D., Voegel, J.-C., Schaaf, P., Ball, V., 2004. Layer by layer self-assembled polyelectrolyte multilayers with embedded phospholipid vesicles. *Langmuir* ASAP article, la049736q.

Miller, I.R., Bach, D., 1969. Structure and membrane properties of lecithin monolayers at the polarized mercury-water interface. *J. Colloid Interface Sci.* 29, 250-260.

Montal, M., Mueller, P., 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. USA* 69, 3561-3566.

Mouritsen, O.G., Jørgensen, K., 1994. Dynamical order and disorder in lipid bilayers. *Chem. Phys. Lipids* 73, 3-25.

Moy, V.T., Keller, D.J., Gaub, H.E., McConnell, H.M., 1986. Long-range molecular orientational order in monolayer solid domains of phospholipid. *J. Phys. Chem.* 90, 3198-3202.

Mueller, P., Rudin, D.O., 1968. Action potentials induced in biomolecular lipid membranes. *Nature* 217, 713-719.

Mueller, P., Rudin, D.O., 1967a. Action potential phenomena in experimental bimolecular lipid membranes. *Nature* 213, 603-604.

Mueller, P., Rudin, D.O., 1967b. Development of potassium - sodium discrimination in experimental bimolecular lipid membranes by macrocyclic antibiotics. *Biochem. Biophys. Res. Commun.* 26, 398-404.

Mueller, P., Rudin, D.O., Tien, H.T., Wescott, W.C., 1963. Methods for the formation of single bimolecular lipid membranes in aqueous solution. *J. Phys. Chem.* 67, 534-535.

Mueller, P., Rudin, D.O., Tien, H.T., Wescott, W.C., 1962. Reconstitution of cell membrane structure in vitro and its transformation into an excitable system. *Nature* 194, 979-980.

Munro, J.C., Frank, C.W., 2004. Adsorption of lipid-functionalized poly(ethylene glycol) to gold surfaces as a cushion for polymer-supported lipid bilayers. *Langmuir* 20, 3339-3349.

Murtomäki, L., Kontturi, K., 2002. Correction to 'Electrochemical determination of partition coefficients of drugs', J Pharm Sci 81:970-974, 1992. *J. Pharm. Sci.* 91, 900-901.

Möhwald, H., 1995. Phospholipid monolayers. In: Lipowsky, R. and Sackmann, E. (Eds.), *Structure and dynamics of membranes. From cells to vesicles.* Elsevier, Amsterdam, pp. 161-211.

Nagle, J.F., Zhang, R., Tristram-Nagle, S., Sun, W., Petrache, H.I., Suter, R.M., 1996. X-ray structure determination of fully hydrated L_α phase dipalmitoyl- phosphatidylcholine bilayers. *Biophys. J.* 70, 1419-1431.

Nakanishi, H., 1995. Artificial retina membranes. *Prog. Surf. Sci.* 49, 197-225.

Naumann, C.A., Prucker, O., Lehmann, T., Rühe, J., Knoll, W., Frank, C.W., 2002. The polymer-supported phospholipid bilayer: tethering as a new approach to substrate-membrane stabilization. *Biomacromolecules* 3, 27-35.

Naumann, R., Schmidt, E.K., Jonczyk, A., Fendler, K., Kadenbach, B., Liebermann, T., Offenhäusser, A., Knoll, W., 1999. The peptide-tethered lipid membrane as a biomimetic system to incorporate cytochrome c oxidase in a functionally active form. *Biosens. Bioelectr.* 14, 651-662.

Neher, E., Sakmann, B., 1976. Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature* 260, 799-802.

Nelson, A., 1993. Voltammetry of Tl^{I}, Cd^{II}, Cu^{II}, Pb^{II} and Eu^{III} at phosphatidylserine-coated mercury electrodes. *J. Chem. Soc. Faraday Trans.* 89, 3081-3090.

Nelson, A., 1991a. Electrochemical studies of thallium(I) transport across gramicidin modified electrode-adsorbed phospholipid monolayers. *J. Electroanal. Chem.* 303, 221-236.

Nelson, A., 1991b. Electrochemical studies of antibiotic 23187 (A23187) mediated permeability to divalent heavy-metal ions in phospholipid monolayers adsorbed on mercury electrodes. *J. Chem. Soc. Faraday Trans.* 87, 1851-1856.

Nelson, A., Auffret, N., 1988a. Phospholipid monolayers of di-oleoyl lecithin at the mercury / water interface. *J.Electroanal. Chem.* 244, 99-113.

Nelson, A., Auffret, N., 1988b. Phospholipid monolayers of di-oleoyl lecithin (di-O-PC) at the mercury / water interface. Effects on faradaic reactions. *J.Electroanal. Chem.* 248, 167-180.

Nelson, A., Benton, A., 1986. Phospholipid monolayers at the mercury / water interface. *J. Electroanal. Chem.* 202, 253-270.

Nelson, A., Leermakers, F.A.M., 1990. Substrate-induced structural changes in electrode-adsorbed lipid layers. Experimental evidence for the behaviour of phospholipid layers on the mercury – water interface. *J. Electroanal. Chem.* 278, 73-83.

Nelson, A., van Leeuwen, H.P., 1989. Metal voltammetry at dioleoyl lecithin (di-O-PC) coated mercury electrodes: Cd, Eu, Pb, V and Zn and the effect of electrolyte composition. *J. Electroanal. Chem.* 273, 183-199.

Nernst, W., Riesenfeld, E.H., 1902. Ueber elektrolytische Erscheinungen an der Grenzfläche zweier Lösungsmittel. *Ann. Phys. (Leipzig)* 8, 600-608.

Nikolelis, D.P., Krull, U.J., 1992. Reliable and facile method for preparation of solventless bilayer lipid membranes for electroanalytical investigations. *Talanta* 39, 1045-1049.

Nikolelis, D.P., Siontorou, C.G., 1995. Bilayer lipid membranes for flow injection monitoring of acetylcholine, urea, and penicillin. *Anal. Chem.* 67, 936-944.

Nikolelis, D.P., Siontorou, C.G., Krull, U.J., Katrivanos, P.L., 1996. Ammonium ion minisensors from self-assembled bilayer lipid membranes using gramicidin as an ionophore. Modulation of ammonium selectivity by platelet-activating factor. *Anal. Chem.* 68, 1735-1741.

Novotny, I., Rehacek, V., Tvarozek, V., Nikolelis, D.P., Andreou, V.G., Siontorou, C.G., Ziegler, W., 1996. Stabilized bilayer lipid membranes (BLMs) on agar – thin film electrode system support. *Mater. Sci. Eng. C* 5, 55-58.

Ogier, S.D., Bushby, R.J., Cheng, Y., Evans, S.D., Evans, S.W., Jenkins, T.A., Knowles, P.F., Miles, R.E., 2000. Suspended planar phospholipid bilayers on micromachined supports. *Langmuir* 16, 5696-5701.

Ollila, F., Halling, K., Vuorela, P., Vuorela, H., Slotte, J.P., 2002. Characterization of flavonoid-biomembrane interactions. *Arch. Biochem. Biophys.* 399, 103-108.

Osborn, T.D., Yager, P., 1995a. Formation of planar solvent-free phospholipid bilayers by Langmuir-Blodgett transfer of monolayers to micromachined apertures in silicon. *Langmuir* 11, 8-12.

Osborn, T.D., Yager, P., 1995b. Modeling success and failure of Langmuir-Blodgett transfer of phospholipid bilayers to silicon dioxide. *Biophys. J.* 68, 1364-1373.

Pantoja, R., Sigg, D., Blunck, R., Bezanilla, F., Heath, J.R., 2001. Bilayer reconstitution of voltage-dependent ion channels using a microfabricated silicon chip. *Biophys. J.* 81, 2389-2394.

Passechnik, V.I., Hianik, T., Ivanov, S.A., Sivak, B., 1998. Specific capacitance of metal-supported lipid membranes. *Electroanalysis* 10, 295-302.

Pastor, R.W., 1994. Molecular dynamics and Monte Carlo simulations of lipid bilayers. *Curr. Opin. Struct. Biol.* 4, 486-492.

Petty, M.C., 1996. *Langmuir-Blodgett films: an introduction.* University Press, Cambridge, 234 p.

Pickar, A.D., Benz, R., 1978. Transport of oppositely charged lipophilic probe ions in lipid bilayer membranes having various structures. *J. Membr. Biol.* 44, 353-376.

Pintschovius, J., Fendler, K., 1999. Charge translocation by the Na^+/K^+ -ATPase investigated on solid supported membranes: rapid solution exchange with a new technique. *Biophys. J.* 76, 814-826.

Plant, A.L., 1993. Self-assembled phospholipid/alkanethiol biomimetic bilayers on gold. *Langmuir* 9, 2764-2767.

Pospíšil, L., Müller, E., Emons, H., Dörfler, H.D., 1984. Determination of the lipid phase transition temperature from electrochemical double-layer capacity measurements at the mercury/solution interface. *J. Electroanal. Chem.* 170, 319-331.

Pospíšil, L., Kůta, J., Müller, E., Dörfler, H., 1980. The interfacial properties of lecithin homologues at the mercury/solution interface and the effect of thermally induced chain "melting" phase transition. *J. Electroanal. Chem.* 106, 359-369.

Purves, D., Augustine, G.J., Fitzpatrick, D., Katz, L.C., LaMantia, A.-S., McNamara, J.O., Williams, S.M. (Eds.), 2001. *Neuroscience*, 2nd ed. Sinauer Ass., Sunderland (U.S.A), pp. 43-56.

Puu, G., 2001. An approach for analysis of protein toxins based on thin films of lipid mixtures in an optical biosensor. *Anal. Chem.* 73, 72-79.

Qiu, X., Leporatti, S., Donath, E., Möhwald, H., 2001. Studies on the drug release properties of polysaccharide multilayers encapsulated ibuprofen microparticles. *Langmuir* 17, 5375-5380.

Radhakrishnan, A., McConnell, H.M., 2000. Chemical activity of cholesterol in membranes. *Biochemistry* 39, 8119-8124.

Raguse, B., Braach-Maksvytis, V., Cornell, B.A., King, L.G., Osman, P.D.J., Pace, R.J., Wieczorek, L., 1998. Tethered lipid bilayer membranes: formation and ionic reservoir characterization. *Langmuir* 14, 648-659.

Reimhult, E., Höök, F., Kasemo, B., 2003. Intact vesicle adsorption and supported biomembrane formation from vesicles in solution: influence of surface chemistry, vesicle size, temperature, and osmotic pressure. *Langmuir* 19, 1681-1691.

Reizer, J., Grossowicz, N., Barenholz, Y., 1985. The effect of growth temperature on the thermotropic behaviour of the membranes of a thermophilic Bacillus. Composition-structure-function relationships. *Biochim. Biophys. Acta* 815, 268-280.

Reymond, F., 2001. Transfer mechanisms and lipophilicity of ionizable drugs. In: Volkov, A.G. (Ed.), *Liquid interfaces in chemical, biological, and pharmaceutical applications*. Marcel Dekker, New York, pp. 729-773.

Reymond, F., Chopineaux-Courtois, V., Steyaert, G., Bouchard, G., Carrupt, P.-A., Testa, B., Girault, H.H., 1999a. Ionic partition diagrams of ionisable drugs: pH-lipophilicity profiles, transfer mechanisms and charge effects on solvation. *J. Electroanal. Chem.* 462, 235-250.

Reymond, F., Carrupt, P.-A., Testa, B., Girault, H., 1999b. Charge and delocalisation effects on the lipophilicity of protonable drugs. *Chem. Eur. J.* 5, 39-47.

Reymond, F., Steyaert, G., Carrupt, P.-A., Testa, B., Girault, H., 1996. Ionic partition diagrams: a potential – pH representation. *J. Am. Chem. Soc.* 118, 11951-11957.

Rietveld, A., Simons, K., 1998. The differential miscibility of lipids as the basis for the formation of functional membrane rafts. *Biochim. Biophys. Acta* 1376, 467-479.

Rolandi, R., Ricci, D., Brandt, O., 1992. Photovoltage generation in bilayer lipid membrane-cadmium sulfide junctions. *J. Phys. Chem.* 96, 6783-6790.

Rädler, J.O., Koltover, I., Salditt, T., Safinya, C.R., 1997. Structure of DNA – cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes. *Science* 275, 810-814.

Sackmann, E., 1995a. Biological membranes. Architecture and function. In: Lipowsky, R. and Sackmann, E. (Eds.), *Structure and dynamics of membranes. From cells to vesicles.* Elsevier, Amsterdam, pp. 1-63.

Sackmann, E., 1995b. Physical basis of self-organization and function of membranes: physics of vesicles. In: Lipowsky, R. and Sackmann, E. (Eds.), *Structure and dynamics of membranes. From cells to vesicles.* Elsevier, Amsterdam, pp. 213-304.

Salafsky, J., Groves, J.T., Boxer, S.G., 1996. Architecture and function of membrane proteins in planar supported bilayers: a study with photosynthetic reaction centers. *Biochemistry* 35, 14773-14781.

Samec, Z., Trojánek, A., Langmaier, J., Samcová, E., 2003. Cyclic voltammetry of biopolymer heparin at PVC plasticized liquid membrane. *Electrochem. Commun.* 5, 867-870.

Santos, H., Kontturi, K., 2004. Interaction of glycosaminoglycans with lipid monolayers. Conference abstract presented at the 55th annual ISE meeting, Thessaloniki, Greece, September 19-24, 2004.

Saslowsky, D.E., Lawrence, J., Ren, X., Brown, D.A., Henderson, R.M., Edwardson, J.M., 2002. Placental alkaline phosphatase is efficiently targeted to rafts in supported lipid bilayers. *J. Biol. Chem.* 277, 26966-26970.

Sawada, S., Osakai, T., 1999. Hydrophobicity of oligopeptides: a voltammetric study of the transfer of dipeptides facilitated by dibenzo-18-crown-6 at the nitrobenzene/water interface. *Phys. Chem. Chem. Phys.* 1, 4819-4825.

Saxton, M.J., Jacobson, K., 1997. Single-particle tracking: applications to membrane dynamics. *Annu. Rev. Biophys. Biomol. Struct.* 26, 373-399.

Schamberger, J., Clarke, R.J., 2002. Hydrophobic ion hydration and the magnitude of the dipole potential. *Biophys. J.* 82, 3081-3088.

Schmidt, E.K., Liebermann, T., Kreiter, M., Jonczyk, A., Naumann, R., Offenhäusser, A., Neumann, E., Kukol, A., Maelicke, A., Knoll, W., 1998. Incorporation of the acetylcholine receptor dimer from *Torpedo californica* in a peptide supported lipid membrane investigated by surface plasmon and fluorescence spectroscopy. *Biosens. Bioelectr.* 13, 585-591.

Schmidt, T., Schütz, G.J., Baumgartner, W., Gruber, H.J., Schindler, H., 1996. Imaging of single molecule diffusion. *Proc. Natl. Acad. Sci. USA* 93, 2926-2929.

Schmitt, J., Danner, B., Bayerl, T.M., 2001. Polymer cushions in supported phospholipid bilayers reduce significantly the frictional drag between bilayer and solid surface. *Langmuir* 17, 244-246.

Schwarz, G., Taylor, S.E., 1999. Polymorphism and interactions of a viral fusion peptide in a compressed lipid monolayer. *Biophys. J.* 76, 3167-3175.

Seddon, J.M., Templer, R.H., 1995. Polymorphism of lipid-water systems. In: Lipowsky, R. and Sackmann, E. (Eds.), *Structure and dynamics of membranes. From cells to vesicles.* Elsevier, Amsterdam, pp. 97-160.

Seelig, A., Seelig, J., 1974. The dynamic structure of fatty acyl chains in a phospholipid bilayer measured by deuterium magnetic resonance. *Biochemistry* 13, 4839-4845.

Seelig, J., Nebel, S., Ganz, P., Bruns, C., 1993. Electrostatic and nonpolar peptide – membrane interactions. Lipid binding and functional properties of somatostatin analogues of charge z = +1 to z = +3. *Biochemistry* 32, 9714-9721.

Seitz, M., Ter-Ovanesyan, E., Hausch, M., Park, C.K., Zasadzinski, J.A., Zentel, R., Israelachvili, J.N., 2000. Formation of tethered supported bilayers by vesicle fusion onto lipopolymer monolayers promoted by osmotic stress. *Langmuir* 16, 6067-6070.

Shapovalov, V.L., Kotova, E.A., Rokitskaya, T.I., Antonenko, Y.N., 1999. Effect of gramicidin A on the dipole potential of phospholipid membranes. *Biophys. J.* 77, 299-305.

Shen, W.W., Boxer, S.G., Knoll, W., Frank, C.W., 2001. Polymer-supported lipid bilayers on benzophenone-modified substrates. *Biomacromolecules* 2, 70-79.

Shih, M.C., Bohanon, T.M., Mikrut, J.M., Zschack, P., Dutta, P., 1992. X-ray-diffraction study of the superliquid region of the phase diagram of a Langmuir monolayer. *Phys. Rev. A* 45, 5734-5737.

Shinoda, W., Shimizu, M., Okazaki, S., 1998. Molecular dynamics study on electrostatic properties of a lipid bilayer: polarization, electrostatic potential, and the effects on structure and dynamics of water near the interface. *J. Phys. Chem. B* 102, 6647-6654.

Sillerud, L.O., Barnett, R.E., 1982. Lack of transbilayer coupling in phase transitions of phosphatidylcholine vesicles. *Biochemistry* 21, 1756-1760.

Simons, K., Ikonen, E., 1997. Functional rafts in cell membranes. *Nature* 387, 569-572.

Sinensky, M., 1974. Homeoviscous adaptation – a homeostatic process that regulates the viscosity of membrane lipids in Escherichia coli. *Proc. Natl. Acad. Sci. USA* 71, 522-525.

Singer, S.J., Nicolson, G.J., 1972. The fluid mosaic model of the structure of cell membranes. *Science* 175, 720-731.

Sinner, A., Offenhäusser, A., 1998. The electrical characterization of supported phospholipid bilayers using titanium/titanium oxide microelectrodes. *Thin Solid Films* 327-329, 758-761.

Smaby, J.M., Brockman, H.L., 1990. Surface dipole moments of lipids at the argon – water interface. *Biophys. J.* 58, 195-204.

Šnejdárková, M., Rehák, M., Babincová, M., Sargent, D.F., Hianik, T., 1997. Glucose minisensor based on self-assembled biotinylated phospholipid membrane on a solid support and its physical properties. *Bioelectrochem. Bioenerg.* 42, 35-42.

Sonnleitner, A., Schütz, G.J., Schmidt, Th., 1999. Free Brownian motion of individual lipid molecules in biomembranes. *Biophys. J.* 77, 2638-2642.

Starr, T.E., Thompson, N.L., 2000. Formation and characterization of planar phospholipid bilayers supported on TiO_2 and $SrTiO_3$ single crystals. *Langmuir* 16, 10301-10308.

Steinem, C., Jahnshoff, A., von dem Bruch, K., Reihs, K., Goossens, J., Galla, H.-J., 1998. Valinomycin-mediated transport of alkali cations through solid supported membranes. *Bioelectrochem. Bioenerg.* 45, 17-26.

Stern, H.A., Feller, S.E., 2003. Calculation of the dielectric permittivity profile for a nonuniform system: application to a lipid bilayer simulation. *J. Chem. Phys.* 118, 3401-3412.

Steverding, D., Kadenbach, B., 1991. Influence of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline modification on proton translocation and membrane potential of reconstituted cytochrome-oxidase support 'proton slippage'. *J. Biol. Chem.* 266, 8097–8101.

Suarez-Isla, B.A., Wan, K., Lindstrom, J., Montal, M., 1983. Single-channel recordings from purified acetylcholine receptors reconstituted in bilayers formed at the tip of patch pipets. *Biochemistry* 22, 2319-2323.

Sugahara, M., Uragami, M., Yan, X., Regen, S.L., 2001. The structural role of cholesterol in biological membranes. *J. Am. Chem. Soc.* 123, 7939-7940.

Sugao, N., Sugawara, M., Minami, H., Uto, M., Umezawa, Y., 1993. Na^+/D-glucose cotransporter based bilayer lipid membrane sensor for D-Glucose. *Anal. Chem.* 85, 363-369.

Sugawara, M., Kurosawa, M., Sakai, K., Kobayashi, M., Iseki, K., Miyazaki, K., 2002. Ionic strength has a greater effect than does transmembrane electric potential difference on permeation of tryptamine and indoleacetic acid across Caco-2 cells. *Biochim. Biophys. Acta* 1564, 149-155.

Tadini Buoninsegni, F., Herrero, R., Moncelli, M.R., 1998. Alkanethiol monolayers and alkanethiol|phospholipid bilayers supported by mercury: an electrochemical characterization. *J. Electroanal. Chem.* 452, 33-42.

Tadjibaeva, G., Sabirov, R., Tomita, T., 2000. Flammutoxin, a cytolysin from the edible mushroom *Flammulina velutipes*, forms two different types of voltage-gated channels in lipid bilayer membranes. *Biochim. Biophys. Acta* 1467, 431-443.

Takagi, M., Azuma, K., Kishimoto, U., 1965. New method for the formation of bilayer membranes in aqueous solution, *Ann. Rept. Biol. Works Fac. Sci. Osaka Univ.* 13, 107-110.

Tamm, L.K., 1988. Lateral diffusion and fluorescence microscope studies on a monoclonal antibody specifically bound to supported phospholipid bilayers. *Biochemistry* 27, 1450-1457.

Tamm, L.K., McConnell, H.M., 1985. Supported phospholipid bilayers. *Biophys. J.* 47, 105-113.

Tanaka, K., Manning, P.A., Lau, V.K., Yu, H., 1999. Lipid lateral diffusion in dilauroylphosphatidylcholine/cholesterol mixed monolayers at the air/water interface. *Langmuir* 15, 600-606.

Tancrède, P., Paquin, P., Houle, A., Leblanc, R.M., 1983. Formation of asymmetrical planar lipid bilayer membranes from characterized monolayers. *J. Biochem. Biophys. Meth.* 7, 299-310.

Tatulian, S.A., 1999. Surface electrostatics of biological membranes and ion binding. In: Sørensen, T.S. (Ed.), *Surface chemistry and electrochemistry of membranes.* Marcel Dekker, Inc., New York, pp. 871-922.

Tatulian, S.A., 1993. Ionization and ion binding. In: Cevc, G. (Ed.), *Phospholipids handbook.* Marcel Dekker, Inc., New York, pp. 511-552.

Thompson, M., Lennox, R.B., McClelland, R.A., 1982. Structure and electrochemical properties of microfiltration filter-lipid membrane systems. *Anal. Chem.* 54, 76-81.

Tieleman, D.P., Marrink, S.J., Berendsen, H.J.C., 1997. A computer perspective of membranes: molecular dynamics studies of lipid bilayer systems. *Biochim. Biophys. Acta* 1331, 235–270.

Tien, H.T., Ottova, A.L., 1999. From self-assembled bilayer lipid membranes (BLMs) to supported BLMs on metal and gel substrates to practical applications. *Coll. Surf. A* 149, 217-233.

Tien, H.T., Salamon, Z., 1989. Formation of self-assembled lipid bilayers on solid substrates. *Bioelectrochem. Bioenerg.* 22, 211-218.

Tocanne, J.-F., Cézanne, L., Lopez, A., Piknova, B., Schram, V., Tournier, J.-F., Welby, M., 1994. Lipid domains and lipid/protein interactions in biological membranes. *Chem. Phys. Lipids* 73, 139-158.

Tsionsky, M., Bard, A.J., Mirkin, M.V., 1997. Long-range electron transfer through a lipid monolayer at the liquid/liquid interface. *J. Am. Chem. Soc.* 119, 10785-10792.

Tu, K., Tobias, D.J., Blasie, J.K., Klein, M.L., 1996. Molecular dynamics investigation of the structure of a fully hydrated gel-phase diplamitoylphosphatidylcholine bilayer. *Biophys. J.* 70, 595-608.

Ulmeanu, S.M., Jensen, H., Samec, Z., Bouchard, G., Carrupt, P.-A., Girault, H.H., 2002. Cyclic voltammetry of highly hydrophilic ions at a supported liquid membrane. *J. Electroanal. Chem.* 530, 10-15.

Ulrich, W.-P., Vogel, H., 1999. Polarization-modulated FTIR spectroscopy of lipid/gramicidin monolayers at the air/water interface. *Biophys. J.* 76, 1639-1647.

van Golde, L.M.G., Tomasi, V., van Deenen, L.L.M., 1967. Determination of molecular species of lecithin from erythrocytes and plasma. *Chem. Phys. Lipids* 1, 282-293.

Vaz, W.L.C., Almeida, P.F., 1991. Microscopic versus macroscopic diffusion in one-component fluid phase lipid bilayer-membranes. *Biophys. J.* 60, 1553-1554.

Vié, V., Van Mau, N., Chaloin, L., Lesniewska, E., Le Grimellec, C., Heitz, F., 2000. Detection of peptide-lipid interactions in mixed monolayers, using isotherms, atomic force microscopy, and Fourier transform infrared analyses. *Biophys. J.* 78, 846-856.

Vitovič, P., Kresák, S., Naumann, R., Schiller, S.M., Lewis, R.N.A.H., McElhaney, R.N., Hianik, T., 2004. The study of the interaction of a model α-helical peptide with lipid bilayers and monolayers. *Bioelectrochemistry* 63, 169-176.

Vodyanoy, V., Halverson, P., Murphy, R.B., 1982. Hydrostatic stabilization of solvent-free lipid bimolecular membranes. *J. Colloid Interface Sci.* 88, 247-257.

Vogel, V., Möbius, D., 1988. Local surface potentials and electric dipole moments of lipid monolayers: contributions of the water/lipid and the lipid/air interfaces. *J. Colloid Interface Sci.* 126, 408-420.

Wagner, M.L., Tamm, L.K., 2000. Tethered polymer-supported planar lipid bilayers for reconstitution of integral membrane proteins: silane-polyethyleneglycol-lipid as a cushion and covalent linker. *Biophys. J.* 79, 1400-1414.

Wang, L., Schönhoff, M., Möhwald, H., 2004. Swelling of polyelectrolyte multilayer-supported lipid bilayers. 1. Layer stability and lateral diffusion. *J. Phys. Chem. B* 108, 4767-4774.

Watanabe, A., Fujii, A., Sakamori, Y., Higashitsuji, K., Tamai, H., 1971. Studies on the behaviour of phospholipids at oil-water interfaces. Part I. The isoelectric points of phospholipids. *Kolloid-Z. Z. Polym.* 243, 42-47.

Watanabe, A., Matsumoto, M., Tamai, H., Gotoh, R., 1968. Electrocapillary phenomena at oil-water interfaces. Part III: The behaviour of lecithin at oil-water interfaces. *Kolloid-Z. Z. Polym.* 228, 58-63.

Welby, M., Poquet, Y., Tocanne, J.-F., 1996. The spatial distribution of phospholipids and glycolipids in the membrane of the bacterium Micrococcus luteus varies during the cell cycle. *FEBS Lett.* 384, 107-111.

Welti, R., Glaser, M., 1994. Lipid domains in model and biological membranes. *Chem. Phys. Lipids* 73, 121-137.

Wiegand, G., Arribas-Layton, N., Hillebrandt, H., Sackmann, E., Wagner, P., 2002. Electrical properties of supported lipid bilayer membranes. *J. Phys. Chem. B* 106, 4245-4254.

Wong, J.Y., Majewski, J., Seitz, M., Park, C.K., Israelachvili, J.N., Smith, G.S., 1999. Polymer-cushioned bilayers. I. A structural study of various preparation methods using neutron reflectometry. *Biophys. J.* 77, 1445-1457.

Yamada, H., Shiku, H., Matsue, T., Uchida, I., 1993. Electron transfer at a planar bilayer lipid membrane incorporated with 7,7,8,8-tetracyanoquinodimethane studied by ac impedance spectroscopy. *J. Phys. Chem.* 97, 9547-9549.

Yang, J., Klejin, J.M., 1999. Order in phospholipid Langmuir-Blodgett layers and the effect of the electrical potential of the substrate. *Biophys. J.* 76, 323-332.

Yuan, H., Leitmannova-Ottova, A., Tien, H.T., 1996. An agarose-stabilized BLM: a new method for forming bilayer lipid membranes. *Mater. Sci. Eng. C* 4, 35-38.

Zhang, J., Unwin, P.R., 2002. Proton diffusion at phospholipid assemblies. *J. Am. Chem. Soc.* 124, 2379-2383.

Zhang, J., Strutwolf, J., Cannan, S., Unwin, P.R., 2003. Combined scanning electrochemical microscopy – Langmuir trough technique for investigating phase transfer kinetics across liquid/liquid interfaces modified by a molecular monolayer. *Electrochem. Commun.* 5, 105-110.

Zviman, M., Tien, H.T., 1995. Reconstituted olfactory receptors in bilayer lipid membranes. *Bioelectrochem. Bioenerg.* 36, 127-135.