On the functional ordering of biomembranes.

Implications for drug binding.

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To Alma and Felix
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Abbreviations

CPZ         chlorpromazine
CMC         Critical micellar concentration
DOPet       1,2-dioleyl-sn-glycero-3-phosphoethanol
DOPmet      1,2-dioleyl-sn-glycero-3-phosphomethanol
DPPC        1,2-di-palmitoyl-sn-glycero-3-phosphocholine
DPPN        1,2-dipalmitoyl-sn-glycero-3-phospho[N-(4-nitrobenz-2-oxa-1,3-diazolyl)]-ethanolamine
EDTA        ethylenediaminetetraacetic acid
egg-PA       egg yolk phosphatidic acid
LUV          Large unilamellar vesicle
Lyso-PA      1-stearoyl-2-hydroxy-sn-glycero-3-phosphate
Lyso-PG      1-stearoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)]
M-1          (2S,3R)-2,3-dimethoxy-1,4-bis(N-hexadecyl-N,N-dimethylammonium)-butane dibromide
MLV          Multilamellar vesicle
NMR          Nuclear magnetic resonance spectroscopy
POPA         palmitoyl-oleyl-sn-glycero-phosphatidic acid
POPC         1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPE         1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanolamine
POPG         1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac-glycerol
POPS         1-palmitoyl-2-oleoyl-sn-glycero-3-(phospho-L-serine)
POPI         1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoinositol
PPDPC        1-Palmitoyl-2[10-(pyren-1-yl)]decanoyl-sn-glycero-3-phosphocholine
SRM          siramesine ((1’-[4-[1-(4-fluorophenyl)-1H-indol-3-yl]-1-butyl]spiro[isobenzofuran-1(3H),4’-piperidine])
T            Temperature
$T_p$        Pretransition temperature
$T_m$        Main phase transition temperature
Original Publications

This thesis is based on the following original publications referred to in the text by the Roman numerals I-IV


1 Abstract

Cells are packed with membrane structures, defining the inside and outside, and the different subcellular compartments. These membranes consisting mainly of phospholipids have a variety of functions in addition to providing a permeability barrier for various compounds. These functions involve cellular signaling, where lipids can act as second messengers, or direct regulation of membrane associating proteins.

The first part of this study focuses on relating some of the physicochemical properties of membrane lipids to the association of drug compounds to membranes. In brief, a fluorescence quenching based method is described allowing for convenient determination of the membrane association of drugs. This method was subsequently applied to a novel drug, siramesine, previously shown to have anti-cancer activity. Siramesine was found to associate with anionic lipids. Especially interesting is its strong affinity for a second messenger lipid phosphatidic acid, with a partition coefficient of an order of magnitude larger than for other anionic or uncharged lipids. This is the first example of a small molecule drug compound specifically interacting with a cellular lipid. Phosphatidic acid in cells is required for the activation of many signaling pathways mediating growth and proliferation. This provides an intriguing possibility for a simple molecular mechanism of the observed anti-cancer activity of siramesine.

In the second part the thermal behavior and self assembly of charged and uncharged membrane assemblies was studied. Strong inter-lamellar co-operativity was observed for multilamellar DPPC vesicles using fluorescence techniques together with calorimetry. The commonly used membrane models, large unilamellar vesicles (LUV) and multilamellar vesicles (MLV), were found to possess different biophysical properties as the lamellae of MLVs strongly interact with each other, also driving the segregation of the pyrene labeled lipid analogue into clusters. The effect of a counter-ion lattice on the self assembly of cationic gemini surfactant was studied. The presence of NaCl strongly influenced the thermal phase behavior of M-1 vesicles, causing the spontaneous formation of giant vesicles with 30-50 μm diameter upon exceeding a certain phase transition temperature, followed by a subsequent transition into a more homogenous solution of more uniform vesicles with a smaller diameter in the range of 15 μm.
Understanding the underlying biophysical aspects of cellular membranes is of fundamental importance as the complex picture of the structure and function of cells is evolving. Many of the cellular reactions take place on membranes and membranes are known to regulate the activity of many peripheral and integral membrane associating proteins. From the point of view of drug design and gene technology, membranes can provide an interesting target for future development of drugs, but also a vehicle sensitive for environmental changes allowing for encapsulating drugs and targeting them to the desired site of action.
2 Review of the literature

2.1 Lipids

The main focus in the research of biological molecules was for a long time on the macromolecules proteins and nucleic acids. Watson & Crick described the structure of the DNA double helix as early as 1953 (Watson and Crick 1953). The earliest studies on protein structure and function date to 1926 when the enzyme urease was recognized to be a protein (Sumner 1926). Today the structure and material properties of a vast number of proteins are well established. From the major groups of biological molecules carbohydrates and lipids (Fig. 1) have received less attention. This relates partly to the complex chemistry involved and on the other hand to the special techniques required in studying these compounds. The bilayer structure of the cellular plasma membrane of red blood cells was established already in 1925 (Gorter and Grendel 1925) and some experimental techniques such as assessing surface tension date back to 1863 (Wilhelmy 1863). When Singer and Nicholson presented their fluid mosaic model of the cellular membrane in 1972 they revolutionized the field of lipid research (Singer and Nicolson 1972). Since those times the development of e.g. sensitive fluorescence techniques (Kinnunen et al. 1993), electron microscopic techniques together with high resolution NMR (Schiller et al. 2007) and X-ray techniques (Cevc 1991; Loebbecke and Cevc 1995) has allowed for the picture of the cellular membrane to evolve into a more specific understanding of their structure and function. They are now thought not only to serve as a solvent matrix for membrane associating proteins but also as a highly versatile and dynamic structure controlling cellular behavior (Mouritsen and Kinnunen 1996; Mouritsen 2004).

In this thesis I focus on the biophysical characterization of various phospholipid model membrane assemblies together with the role of phospholipids in the binding of drugs to liposomes.

Fig. 1 An eukaryote cell is full of membranes, mainly consisting of phospholipids.
2.1 Lipids

2.1.1 Lipids as structural components of cellular membranes

Lipids are the main constituents of cellular membranes. There are more than 1000 different lipid species in the different membranes of a eukaryote cell (Kinnunen 1991). Taking into consideration chemically modified lipids such as peroxidated lipids the number increases severalfold (Seppänen-Laakso and Oresic 2009). The main classes are glycerophospholipids, which generally have 2 fatty-acyl chains covalently attached to a glycerol backbone (Fig. 2). On the third carbon there is a phosphate group, which often carries a hydrophilic head-group, thus making the molecule amphiphilic. Sphingolipids and cholesterol are the other major lipid families present in eukaryotes. In addition to lipids eukaryote membranes contain transmembrane and peripheral membrane associating proteins (Gennis 1989).

Different membranes form diffusion barriers dividing cells into compartments, such as cellular organelles e.g. mitochondria, lysosomes, the Golgi apparatus and the endoplasmic reticulum. Membranes form permeability barriers between cellular components allowing for different chemical properties on the opposing sides of a membrane. This is of fundamental importance for the proper functioning of cells. The different chemical compositions of the inside and outside of cells maintain the osmotic balance, keeping the cells from rupturing. The unequal distribution of ions over membranes is actively maintained and is one of the most energy consuming processes in the human body. On the other hand chemical gradients over membranes are crucial also in the production of energy in the form of ATP on the inner mitochondrial membrane (Nelson and Cox 2009). Also many chemical reactions involving membrane associating enzymes or lipids take place on the membrane surface. This limits the volume in which the reactions happen increasing the

Fig. 2 The chemical structure of a glycerophospholipid POPC
1. The choline head-group
2. Phosphate group
3. Glycerol backbone
4. SN-1 acyl chain
5. SN-2 acyl chain
probability of molecules meeting each other as their mobility is limited to movements in 2 dimensions.

Cellular lipids have different chemical properties. Such differences can concern the hydrophilic head-group region where parameters such as headgroup size, shape or charge affect the molecule, or they can involve the hydrophobic hydrocarbon region of the lipid with parameters such as the length and amount of double bonds within the hydrocarbon (Lehtonen and Kinnunen 1995b) and the number of acyl chains. Such parameters determine the effective shape and overall properties of a given membrane (Kinnunen 1996). Cholesterol is a special lipid, structurally very different from the previously discussed glycerophospholipids and sphingolipids. It has an iso-octyl hydrophobic tail, connected to a stiff aromatic ring structure. Cholesterol has a very small headgroup comprising of an OH-group on the head. These structural properties give cholesterol a role in regulating the stiffness and shape of biological membranes (Yeagle 2005).

2.1.2 Water as a biological solvent

Most of the chemical reactions occurring in cells take place in an aqueous environment. Water is an abundant component of all living organisms, comprising more than 70% of the weight of living organisms (Ling 2004). Water is a small molecule and has some unique solvent properties making it a suitable medium for living organisms. These properties owe to the polarity of the H$_2$O molecule, its high dielectric constant of 78.5, together with its strong tendency to form hydrogen bonds, leading to high melting and boiling temperatures. The structure of crystal water (ice) is a highly organized hydrogen bonding network. Even for liquid water hydrogen bonding remains important giving rise to its unique solvent properties as it favours interactions of water with other polar molecules. Nonpolar molecules tend to interact with each other, to minimize the free energy penalty caused by the disturbing effect on the hydrogen bond network of water, thus being poorly soluble. Substances poorly soluble in water such as lipids, waxes and oils are referred to as hydrophobic, whereas polar well soluble compounds that ‘prefer’ being dissolved in water are hydrophilic. Many small compounds and biological molecules exhibit both hydrophilic and hydrophobic properties e.g. at the different ends of a phospholipid molecule, such compounds are amphiphilic in nature. When hydrophobic compounds are introduced to an aqueous environment the regular hydrogen bonding network is disturbed and this leads to an increase in the free energy.
of the system. Minimizing this free energy gives origin to the so-called hydrophobic effect, trying to minimize the contacts of the hydrophobic molecules with water. This is the main driving force for the self assembly of lipids into bilayer structures and also to a large extent for the proper folding of proteins.

Solubility alone does not, however, describe the complex interactions of biological molecules with water in a sufficient manner. The hydration shell of a molecule consists of the H$_2$O molecules that are in close contact to the solute differing in orientation and hydrogen bonding profile as compared to the bulk 'free' water in the solution (Gawrisch et al. 1992). These molecules are, however, not tightly bound to the solute but continuous molecular exchange occurs. The level of the hydration relates to the osmotic pressure caused by the solute and is in the case of phospholipids mainly affected by the headgroup size and charge (Israelachvili and Wennerström 1996).

2.1.3 Critical micellar concentration CMC

The solubility of lipids in water is poor. However, amphiphilic molecules due to the hydrophobic effect spontaneously form ordered soluble aggregates in an aqueous solution. The critical micellar concentration is defined as the concentration above which amphiphiles added to solution participate in aggregate formation rather than existing as free monomers in solution. For the phospholipids we have used in the work this thesis is based on, the CMC is roughly of a nM range. CMC can be determined from the Gibbs free energy isotherm from a dilution series, as surface tension is dependent on the logarithm of the dissolved monomer concentration of the surface active compound and thus remains constant after reaching CMC.
2 Review of the literature

2.1.4 On the organization of lipids

In an aqueous environment lipids self-assemble into different structures depending on their chemical and biophysical properties. These structures such as micelles, vesicles or bilayers form in order to minimize the free-energy of the system, that is, to hide the hydrophobic hydrocarbon regions away from the water molecules. This self-assembly is mainly driven by the hydrophobic effect. The main factor determining the shape of the forming aggregate is the effective shape of the amphiphile. The effective shape is determined by molecular properties, such as the van der Waals volume of the molecule, the headgroup hydration shell, intra- and inter molecular hydrogen bonding and the length and grade of saturation of the acyl chains. In order to predict and describe the effect of change in molecular shape on the organization of the lipids the concept of packing parameter (p) has been defined as (Israelachvili et al. 1980)

\[ p = \frac{v}{a \times l} \]

Where \( v \) represents the effective volume of the hydrophobic part of the molecule, \( a \) stands for the limiting area of the hydrophilic headgroup, and \( l \) is the length of the hydrophilic part (Fig. 3). Lipids with \( p = 1 \) tend to form lamellar bilayer structures, whereas values for \( p > 1 \) correspond to a cone molecular shape (▲) where the hydrocarbon volume is large compared to the headgroup.

Fig. 3 The packing parameter \( p \) describes the relation of the volumes of the hydrophobic and hydrophilic parts of the lipid molecule, thus defining the effective molecular shape. The figure was kindly provided by MD, PhD Samppa Ryhänen.
leading to the tendency of forming inverted phases. Values for $p < 1$ mean that the molecule is more inverted cone shaped ($\nabla$) leading to the formation of micellar structures (Kinnunen 1996; Mouritsen 2004).

In a biological membrane consisting of different lipids and lipid species the shape of the individual lipid molecules contribute to the overall shape of the membrane. In other words the membrane is curved due to the spontaneous curvature of its constituents. Membrane spontaneous curvature can be negative or positive depending on the magnitude of $p$ of the individual components. Cholesterol, having a small headgroup and a large, stiff hydrophobic part with a high negative spontaneous curvature of its own, introduces also negative curvature into the membrane (Churchward et al. 2008). This also allows for a regulational mechanism. If the lipids in a membrane are chemically modified, for example if the head-group or a hydrocarbon tail (Fuller and Rand 2001) of a lipid is cleaved, the $p$ value of the given lipid is modified, thus also altering the overall curvature in the membrane. Typical examples involve formation of ceramide from sphingomyelin (Holopainen et al. 2000) or the formation of diacylglycerol from glycerophospholipids (Szule 2002) as discussed in more detail below.

Biologically the most important structures formed by lipids are lamellar bilayer structures in which two lipid leaflets form a double layer in which the hydrophobic parts are facing each other and the polar hydrophilic head groups point outwards to the aqueous solvent. Given the fact that bilayers are kept together by non-covalent bonding of molecules, they are highly dynamic structures. From a physical point of view lipid membranes are referred to as soft matter. In addition to the constant intramolecular movements of the hydrophobic acyl chains and the headgroup happening on a picosecond ($10^{12}$ s) scale, a lipid molecule in a bilayer can move around in several ways, on different time and length

Fig. 4 A schematic representation of the cellular plasma membrane with laterally organized lipid domains together with membrane associated proteins and carbohydrates. Image kindly provided by prof. Kinnunen
scales (Yeagle 2005). Lipids can normally freely rotate around their own axis, typically requiring a few nanoseconds to occur. In the same time lipids can laterally diffuse over a range of their own diameter. On a larger scale bilayer lipids can diffuse laterally within their respective membrane leaflet, typically requiring a time of ~30 seconds for traveling across a typical cell membrane. Also trans-bilayer movements called flip-flop occur (McConnell and Kornberg 1971; Nakano et al. 2009). These however are much slower processes as compared to lateral diffusion taking hours or even days to occur for a given lipid molecule. This timescale is such that in a biological system for it to be under control a specific enzyme is required to catalyze flip-flop reactions. (Dolis et al. 1997) Singer and Nicholson presented their fundamental fluid mosaic model of the cellular membrane in 1972 (Singer and Nicolson 1972). This model is more a description of the organization of the lipids as bilayers than an actual physical model, being able to predict the behavior lipid assemblies. The fluid mosaic model assumes random distribution of the lipids in the plane of the membrane. Membrane associated proteins float in an unorganized ‘sea’ of lipids. Later this model has evolved in several aspects (Fig. 4). It has become evident that the lateral organization of lipids is not random but lipids rather form clusters or microdomains. These microdomains introduce localized areas differing from the bulk membrane not only in lipid composition but in properties such as charge density or curvature. Such local curvature changes are balanced by introducing lipids with opposite curvature effects to balance out the free energy stress (Sheetz et al. 1976). A special case of micro domains are sphingolipid and cholesterol rich domains, described in the early 1980’s (Luan et al. 1995; Pessin and Glaser 1980) and recently commonly referred to as lipid rafts (Simons and Ikonen 1997). Also the lipid composition of the membrane leaflets is strictly regulated. The best known example is the localization of phosphatidyserine in the inner leaflet of the plasma membrane. Even the surroundings of a given membrane protein are regulated. It has been shown that membrane proteins can have specific binding sites for certain phospholipids whereas other proteins require lipids as cofactors for their activity.

2.1.5 Lateral pressure, membrane electrostatics

Because of the fact that lipids spontaneously form bilayers to minimize the exposure of the hydrophobic core to water, tension exists within a membrane. This is referred to as lateral pressure. (Isrealeachvili 1991) The overall pressure profile over the membrane sums zero, since the
2.1 Lipids

membrane does not expand or shrink due to these forces. However, within the membrane considerable lateral pressure exists. It is mainly constituted by two opposite forces, namely the repulsion forces between the nearby head-group and that between the acyl chains, defined by the packing density of the membrane, opposed by the interfacial tension deriving from minimizing the contacts of the acyl chains with water and keeping the structure together (Fig. 5). For biological membranes the packing density leads to the repulsive component of the pressure profile corresponding to a monolayer equivalent lateral pressure of approx. 30-35 mN/m (Kinnunen 2000; Marsh 1996). Further the lateral pressure profile gets varying contributions from the different parts in the membrane depending on the effective shape of the lipids and other constituents of the membrane (Cantor 1999a).

The role of lateral pressure on the function of membrane embedded proteins has been discussed (Cantor 1997; Cantor 1998; Cantor 1999b; Cantor 1999c). It has been proposed that by altering the pressure within the membrane proteins such as ion channels may adopt a different conformation thus regulating the flux of ions and other solutes (Marsh 2007). This would be a mechanism not directly involving binding of substances to specific binding sites on proteins. The problem, however, is that there are no means to measure the lateral pressure, or changes in it.

Recently, the problem has been addressed by molecular dynamics (MD) simulations allowing for mathematical modeling of the lateral pressure profile and solute effects to it (Griepernau and Boeckmann 2008;
2 Review of the literature

Terämä et al. 2008). Drugs binding to lipid bilayers have a certain size and shape and based on the information gathered on the localization of a drug in the membrane it is possible to anticipate how the lateral pressure is likely to be affected in different parts of the membrane. In a lipid monolayer interfacial tension can be directly monitored using microbalances and data from such experiments can aid the interpretation of the events taking place in a bilayer (Brockman 1999).

Over a lipid bilayer there is an electric potential caused by the different chemical composition of the medium on the opposing sides (Brockman 1994). This is especially true for charged lipids, where the head-group charges and the associated counter ion lattice affect the electric charge distribution in the vicinity of the membrane. If considered more carefully the electric potential of a membrane can be subdivided into surface potential, due to the surface charge of the head-group and the closely associated counter-ions, dipole potential, related to the orientation of the head-group dipole of the phospholipids and trans-membrane potential corresponding to the potential differences in the bulk solutions on the opposing sides of the membrane (Cafiso 1998).

2.1.6 Phases and transitions

Matter exists in different forms or phases. Water for example commonly exists as a liquid, as a solid and as gas. Changes in the state of matter are referred to as phase transitions (Papon et al. 2002). Liquid crystals, such as phospholipids undergo a variety of temperature dependent transitions (Kinnunen et al. 2003; Kinnunen et al. 1991). Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) has been commonly employed as a model phospholipid in phase transition studies (Mouritsen 1991). Upon heating a sequence of three transitions can be observed for this lipid: a subtransition from the crystalline (Lc) to the gel (Lβ') phase, a pretransition from gel (Lβ') to the ripple (Pβ') phase, and the main transition from the ripple (Pβ') to the liquid-crystalline (Lα) phase (Alakoskela and Kinnunen 2004; Rappolt et al. 2000). For a given transition the temperature at which half the transition is completed is defined as the corresponding transition temperature e.g. Tp for the pretransition and Tm for the main phase transition. The subtransition corresponds to loss of order of the headgroup lattice, and the two latter correspond to phenomena closely connected to acyl chain melting (Heimburg 2000). The pretransition at Tp typically precedes the main transition at Tm by a few degrees, this difference decreasing with increasing length of acyl chains and decreasing hydration of phospholipids (Cevc 1991). Substitutional
impurities added to membranes (Prenner et al. 1999) affect the pretransition, commonly decreasing its amplitude and broadening the peak in heat capacity traces. However, also under these conditions the pretransition may remain distinguishable by other methods (Mavromoustakos et al. 1997).

The phase transition temperatures depend on a variety of factors and get contribution from the lipid headgroup, grade of saturation, charge, possible hydrogen bonding etc. (Cevc et al. 1981). In humans the phase transition temperature of the cellular plasma membranes lies close to 37 degrees, so that the plasma membrane stays fluid at most occasions. However, it has been suggested that phase transitions are involved the conduction of nerve impulses in axons, as a propagating fluid-gel-fluid phase transition wave (Kinnunen and Virtanen 1986).

2.1.7 Charged lipids

Phospholipids generally carry one or multiple charges on their head-groups. A negative charge is provided by the phosphate group linking the head group and the glycerol backbone. The remaining head group often carries a positive charge leading to the phospholipid to become zwitterionic. At pH 7 such zwitterionic lipids are usually neutral. In the literature the discussion on charged lipids usually refers to lipid carrying a net charge at neutral pH, i.e. zwitterionic lipids are considered uncharged. Having a net charge in the head group affects multiple properties of the lipid molecule, such as the hydration and thermodynamic behavior of the membrane as well as the counter-ion lattice in the immediate surroundings of the membrane (Cevc 1990).

The anionic lipids used in this study lack the positive charge of the head group. The most common anionic lipids appearing in eukaryotes carry one negative charge i.e. are monoanionic. The most important anionic lipids are phosphatidylserines, phosphatidylglycerols, phosphatidylinositols and phosphatidic acid. Incorporating anionic lipids into phospholipid vesicles attracts counter-ions towards the membrane surface in a manner rather accurately described by the Gouy-Chapman theory (Gouy, 1910; Chapman 1913; for recent review see Henderson and Boda 2009). In addition, anionic lipids also attract protons creating a local low pH environment in the vicinity of the membrane, even though the buffered aqueous bulk pH remains unaltered. For a membrane
Phosphatidylserine (PS) has been of special interest since it is the most abundant charged lipid in eukaryote cells covering up to 20% of the total phospholipid content of the plasma membrane. It generally resides on the inner leaflet of the plasma membrane, as there is a specific carrier protein aminophospholipid translocase (APTL) actively maintaining the asymmetrical distribution of this lipid (Dolis et al. 1997). Loss of the asymmetric distribution of PS is a key feature of early apoptotic cells (Fadok et al. 1992) and can be used in staining techniques to distinguish apoptotic cells from healthy cells by Annexin V (Vermes et al. 1995). This concept was developed further and also synthetic cationic lipids (Bose et al. 2004) can be used to identify apoptotic cells. Cancer cells also present phosphatidylserine on their outer membrane leaflet (Zwaal et al. 2005), and it has been proposed that the interaction of cancer cells with a natural antimicrobial peptide endostatin is due to its binding to PS (Zhao et al. 2005). Furthermore, PS has a special role in the activation of the thrombotic pathway, possibly also involved in the thrombophilia associated with various types of cancer. PS also promotes the binding of several cationic amphiphilic drugs (CADs) to membranes (Takegami et al. 2005) and has also been linked with the formation of fibrillous protein aggregates (Alakoskela et al. 2006).

Phosphatidic acid (PA) is a lipid, which has a very small head-group, only the phosphate in the sn-3 position of the glycerol backbone. This gives PA a special shape, where the hydrophobic region has a very large volume as compared to the hydrophilic part. This property introduces negative curvature in the membranes and drives the formation of non-lamellar phases in PA containing vesicles (Kooijman et al. 2005). Such behavior is important as the formation of PA is involved in processes requiring membrane fusion (Holopainen et al. 1999). PA carries a net negative charge of 1 at physiological pH, although it has been proposed that upon hydrogen bond formation PA becomes deprotonated to form a divalent anion (Kooijman et al. 2007). The special role of PA in cell signaling is discussed below.

Naturally occurring cationic lipids are rare. Sphingosine, a cationic sphingolipid is present in the nuclear membranes of eukaryotes has been shown to interact with DNA (Kõiv et al. 1995). Sphingosine also affects the head-group dipole orientation of POPC in mixed monolayers (Säily et al. 2001). The other naturally occurring cationic lipid is the so called ‘sleep inducing lipid’ oleamide originally isolated from the spinal fluid of sleep deprived cats (Cravatt et al. 1995).
2.1 Lipids

Synthetic cationic lipids, however, are widely used as detergents and soaps even to the extent that on an industrial scale their waste has caused problems for river navigation (Yapijakis and Wang 2006). Lately, cationic lipids have been a promising vector for gene transfer (Ryhänen et al. 2003), due to their strong interactions with DNA together with their ability to penetrate cells without killing them. This has lead to also compounds being commercially available for this purpose. Gemini surfactants are a special group of synthetic lipids consisting of two usually identical surfactant molecules connected by a hydrocarbon spacer (Menger and Littau 1991).
2 Review of the literature

2.2 Lipids in health and disease

In addition to the more biophysical properties of lipids addressed so far, lipids also serve in a variety of physiological and pathological functions. The traditional approach to studying and treating pathological conditions has been to try to identify the malfunctioning proteins and to subsequently pharmacologically alter their functions to get the desired effect. Lipids are known to be involved in a variety of pathological conditions. Recent technological advances in the field of mass spectroscopy has allowed for the lipidomic characterization of tissues in various pathological conditions (Bougnoux et al. 2008; Graessler et al. 2009; Postle 2009). Below some of these aspects are more carefully discussed.

2.2.1 Lipids as signaling molecules

Cells utilize multiple pathways of signal transduction in translating the response to external stimuli to changes in cellular functions. One mode of action is that binding of a ligand to a receptor on the cell surface activates phosphorylation cascades which turn on or off different cellular functions. Lipids are also involved in these processes. In order to amplify and transmit the signal from the external source among the first steps after activation of a cell surface receptor is the production of a second messenger. Such second messengers are for example cyclic AMP or cAMP, but also the lipids phosphatidic acid (PA), diacylglycerol (DAG) (Goni and Alonso 1999), phosphatidylinositol and sphingolipid derivatives such as ceramide, sphingosine, and sphingosine-1-phosphate. This makes sense, since on the membrane lipids are readily available and they can be activated as signaling molecules by means of simple chemical modifications.

PA is produced in a variety of cells in response to growth stimuli. A number of proteins promoting cell survival such as Hsp (Chowdary 2007), mTOR (Hornberger et al. 2006), Raf (Ghosh et al. 1996), Ras (Andresen 2002), and Sos (Zhao 2007), require PA for their activity. Yet, it is crucial also in the activation of RAS and TNF-α mediated apoptosis (Hancock 2007). PA may further be involved in the pathogenesis of systemic vasculitis (Williams 2007). The major pathway producing PA \textit{in vitro} involves phospholipase D, which cleaves the headgroup of either PE or PC to yield PA. Other pathways producing PA in cells involve acylating lyso-PA by lyso-PA acyl transferase (La
2.2 Lipids in health and disease

Rosee et al. 2006) and phosphorylation of diglyceride by diglyceride kinase (Sakane et al. 2007). Different cancer cell lines utilize distinct pathways to generate PA allowing them to escape apoptosis (Steed and Chow 2001), and also exogenous PA has been shown to act as an anti-apoptotic signal. Consequently, there is an intense search for PLD inhibitors that could be used as anticancer drugs to decrease the levels of cellular PA (Foster 2006). Rapamycin is a chemotherapeutic compound targeting the mTOR protein. Rapamycin is a competitive inhibitor of PA binding to this protein (Foster 2007; Toschi et al. 2009).

Ceramide is a lipid also produced by chemical modifications of the membrane. Sphingomyelinases cleave the headgroup of sphingomyelin to yield ceramide. The role of ceramide on the membrane differs from PA in rather than being growth promoting, it induces apoptosis, programmed cell death, in cells. Another role of ceramide is to induce negative curvature to the membrane (Holopainen et al. 2000; Nurminen et al. 2002). This might be involved in endocytosis and vesicle formation within cells. Interestingly, sphingosine-1-phosphate, readily interconvertable with ceramide acts as a growth promoting signal. Thus the balance between these is carefully maintained.

In addition to intracellular signaling, organisms utilize lipids in a variety of other signaling functions. Steroid hormones are an example of endocrine lipid signaling, eicosanoids such as prostaglandins are important paracrine regulators of cellular functions as well as mediators for inflammation.

Oxidative stress introduces reactive oxygen species especially in mitochondria. This among other things leads to the peroxidation of lipids. The role of oxidized lipids in various pathological conditions is under intensive study. Until now lipid oxidation has been especially considered in the context of atherosclerosis, where the oxidization of LDL particles in the arterial endothelium causes a series of events finally leading to plaque formation, and calcification (Berliner et al. 2001). Recently, however, oxidized phospholipids have been identified as potential binding sites for some drugs (Mattila et al. 2008b; Mattila et al. 2007), as well as promoting the association of peripheral membrane proteins (Mattila et al. 2008a).

The development of powerful, high throughput mass spectroscopy techniques has given insight to the lipid composition of various tissues in different states and under various pathological conditions (Postle 2009).
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2.3 Why study drug lipid interactions?

2.3.1 Drugs in the context of lipids

The pharmaceutical industry is in continuous search for new biologically active compounds to be used as drugs. The traditional approach has been isolating natural compounds and testing them on cell based models. The development of molecular biology techniques has allowed for the identification of the drug targets and specifically screening large compound libraries for favourable interactions. So far these drug targets have been almost exclusively limited to proteins (Rang et al. 1999). After a suitable compound has been identified it still needs to go through a vast screen of testing to become a commercially available drug. The main concerns are the drug’s absorption, distribution, metabolism and excretion, together with the compound’s possible toxicity commonly referred to as the ADME/tox profile. A compound effectively binding its target can have such biophysical properties, that it is not absorbed from the intestine, or that it otherwise never reaches its target in the organism (Lipinski 2000; Lipinski and Hopkins 2004). Also many drugs that bind to the same receptor protein differ in their side effects. Such undesired side effects are often poorly understood and the underlying causes are complex. Predicting prior to clinical testing would be of fundamental importance for the development of novel drug compounds.

Many drugs are amphiphilic and thus readily bind to phospholipid membranes. A widely used prediction for the membrane association of a given drug has been defining the logP value for a compound. That is the logarithm of the bulk octanol/water partitioning coefficient of an uncharged drug compound (Lipinski 1997). These values can be relatively easily predicted by computation, which makes the approach convenient. Phospholipid membranes, being a highly anisotropic environment, however, differ in several critical aspects from bulk octanol as discussed in more detail above, making log P a relatively poor predictor of the membrane association of drugs. The interactions of drugs with membranes are often of nonspecific nature, as drugs rarely form an 1:1 stoichiometric complex with phospholipids. Thus the binding is dependent of the concentrations of the lipid and the drug. More specifically, the amount of membrane associated drugs can be determined by a molar partition coefficient defined as

\[ K_p = \frac{[D_L]}{[L]} \times \frac{[H_2O]}{[D_W]} \]
2.3 Why study drug lipid interactions?

where \([D_L], [L], [H_2O],\) and \([D_W]\) stand for the concentrations of drug in the lipid phase, lipid, water, and drug in water phase, respectively. The molar fraction of bound drug \(X_D\) is defined as

\[
X_D = \frac{[D_L]}{[L]}
\]

we can thus derive the equation linking the molar fraction of the bound drug with the molar partition coefficient:

\[
X_D = \frac{K_p[D_T]}{[H_2O] + K_p + [L]}
\]

where \(D_T\) stands for total the total drug concentration (\([D_T]=[D_W]+[D_L]\)). Even this approach has some problems associated with it. \(K_p\) well describes the association of drugs to lipids at low mole fractions, however drugs, especially drugs carrying a charge, tend to affect their own binding, usually decreasing the apparent \(K_p\) as a function of \(X_D\). This can usually be neglected if just comparing the binding of a drug to different lipids.

Moreover, discussion about drug lipid interactions often focuses on the drug concentrations utilized in the experiments, whereas it would be more appropriate to discuss the amount of membrane associated drug or the molar fraction of the drug within a given membrane. This approach not only takes into account the drug concentration but also associates it with the lipid concentration of the system under study. It is important to emphasize that drugs are generally not equally distributed in an organism. Even the intracellular distribution is unequal, partly due to the chemical properties of drugs and partly due to the metabolism and detoxification routes of cells leading to up to 1000-fold concentration differences in drug concentration in cellular compartments even within a single cell (De Duve et al. 1974; Trapp and Horobin 2005). This can cause accumulation of drugs in certain cellular compartments, such as lysosomes, where the effective drug concentrations can become high enough to interfere with lipid-protein interactions in cells. Taking this into consideration corroborates the importance of understanding how membrane associated drugs affect the function of these organelles.

Several techniques have been used to define the membrane partitioning of drugs. Equilibrium dialysis assays with radiolabeled drugs, in which the drug is left to partition into a liposomal solution of preferably LUVs with subsequent determination of the respective drug concentrations in the lipid containing and lipid free solution compartments have been used. This method, however, is tedious with equilibration times up to weeks, and requires the use of radioactive
Review of the literature

compounds (Kwon et al. 2007). Second derivative absorption spectroscopy can be used if the signal is affected by lipid binding (Kitamura et al. 1995; Santos et al. 2003; Takegami et al. 2005). Recently a more common approach has been use of fluorescence spectroscopy utilizing the changes in the fluorescence of the compound studied if applicable or the use of fluorescently labeled lipids (Luxnat and Galla 1986; I). The advantage of spectroscopic techniques is the lower consumption of drugs together with the relative simplicity of the measurements, allowing for screening of large groups of compounds. Surface plasmon resonance (SPR) can give good results and is not biased by the use of labeled compounds. There are, however, problems related to membrane preparation for SPR. Namely, it is argued whether immobilized vesicles fuse into a bilayer, or remain intact. This can further cause problems in the interpretation of the data, if for example the added compound causes the vesicles to fuse (Abdiche and Myszka 2004). Recently even techniques based on the use of supported phospholipid bilayers (Tammi and McConnell 1985) have been developed for high-throughput screening of compound libraries (Loidl-Stahlhofen et al. 2001a; Loidl-Stahlhofen et al. 2001b).

The activity and membrane association of peripheral and integral membrane proteins is affected by a number of parameters (Subramanian et al. 1998). Direct effects of a ligand on protein activity caused by ligand binding to a specific binding site are the easiest to understand. However, protein activity is also affected by e.g surface potential, lateral pressure, membrane fluidity, curvature, dipole potential, and bilayer elasticity, many of which are properties also affected by the interaction of drug compounds with phospholipid membranes. Given that equations commonly employed to fit binding such as the Hill equation (Cantor 1999b) describing allosteric effects on proteins can give good quality fits even in this case. However, it is almost impossible to distinguish between the direct drug effects on proteins and the indirect effects caused by changes in the membrane properties in the vicinity of the receptor.

The electrostatics of phospholipid membranes allow for another possible factor to affect the binding of drugs. First of all anionic lipids attract cationic drugs simply due to electrostatics. However, even more avid interactions can form as a result of correct matching of the shape and charge distribution of a drug. If the drug forms a suitable dipole compatible with the dipole of the phospholipid head group, it will favor drug association. This has been recently suggested to underlie the enhanced partitioning of many halogenated compounds to membranes even though logP is not necessarily increasing due to this chemical modification (Alakoskela et al. 2009).
2.3 Why study drug lipid interactions?

2.3.2 Lipids as drug targets

Few examples exist, where it has been shown that a drug or compound actually exerts its action via direct interactions with phospholipid bilayers. Polyene antifungal agents such as Amphotericin B were originally extracted from *Streptomyces nodosus*. They are widely used in clinical work to treat invasive fungal infections mainly in immunocompromised patients (Ablordeppey et al. 1999). Amphotericin B acts via interacting with ergosterol, the main steroid component of fungal membranes, forming a pore allowing the exchange of ions across the membrane. Even though this hypothesis has been proposed more than 30 years ago (Andreoli 1974; de Kruijff and Demel 1974), direct evidence of the interaction between amphotericin B and ergosterol was only recently first demonstrated by NMR techniques (Matsumori et al. 2009).

Antimicrobial peptides have lately been under intensive study, and several modes of interaction with various types of membranes have been shown. Lately a lot of effort and funds have been put in understanding diseases involving amyloid formation. Amyloids are insoluble alternatively folded (Dobson 2003) protein aggregates accumulating in e.g. Alzheimer’s disease. It has been established that anionic lipids promote the formation of such aggregates in vitro. Amyloid formation involves several steps from reversible oligomerisation of proteins into functional units to formation of the inactive, insoluble mature amyloid seen in various pathological conditions (Gorbenko and Kinnunen 2006). It has been suggested that oligomerisation of proteins acts as a way to conveniently regulate their activity (Code et al. 2008). Peptides such as LL37 (Sood et al. 2008), temporins (Code et al. 2009; Mahalka and Kinnunen 2009), endostatin (Alakoskela et al. 2006; Zhao et al. 2005) and indolicidin (Zhao et al. 2001), have been shown to associate with phospholipid vesicles.
2.3.3 Chlorpromazine (CPZ)

In this study we used chlorpromazine (CPZ, Fig. 6), a widely used classical antipsychotic drug as a model compound. As an amphiphile with an appropriate surface activity profile it readily crosses the blood-brain barrier (Suomalainen et al. 2004), partitions into cellular membranes and elicits its effects on the central nervous system by influencing various transmitter systems in the human brain (Mycek 1997). Conventionally specific receptor-mediated interactions have been thought to account for most of the observed actions of CPZ in humans and that binding to dopaminergic receptors is mostly responsible for the beneficial effects of CPZ seen in patients suffering from schizophrenia. It remains uncertain, however, if binding to different receptors and the effects mediated through them are alone enough to describe all the diverse actions of this compound. CPZ has been shown to avidly bind phospholipid membranes (Conrad and Singer 1981; Luxnat et al. 1984) with direct effects on their structure, organization, and dynamics (Jutila et al. 2001). CPZ has a high affinity to acidic phospholipids, such as phosphatidylserines (PS) and phosphatidylinositols (PI), which are commonly found in the inner leaflet of the plasma membrane (Bretscher 1972;
2.3 Why study drug lipid interactions?

Dolis et al. 1997). Interestingly, in mouse synaptosomal membranes up to 20% of the total PS is located in the outer leaflet (Devaux 1991). Considering the affinity of CPZ to negatively charged lipids the availability of PS on the cellular surface could thus provide an additional receptor for this drug, for instance when considering potential interference with lipid-protein interactions of membrane associating proteins by CPZ (Jutila et al. 1998). Nuclear magnetic resonance and electron spin resonance studies on model membranes have shown that CPZ binds to the head group region as well as intercalates into the membrane between the acyl chains (Nerdal et al. 2000; Underhaug Gjerde et al. 2004). This is supported also by monolayer studies showing that CPZ increases the surface area/molecule for certain phospholipid species (Agasosler et al. 2001; Varnier Agasoster and Holmsen 2001).

2.3.4 Siramesine

Siramesine \(((1'\,[4\{-1-(4-fluorophenyl)-1H-indol-3-yl\}-1-butyl]spiro[isobenzofuran-1(3H),4'-piperidine], SRM,Fig. 6)\) was originally synthesized by H. Lundbeck A/S for the treatment of anxiety. Phase II trials showed this compound to be non-toxic and well tolerated in humans. However, the clinical efficacy was not satisfactory and its development was discontinued in 2002 (Heading 2001). The primary target of SRM has been suggested to be the σ-2 receptor (Soby et al. 2002), an orphan receptor with no endogenous ligand known. These receptors have unique drug interaction profiles binding for instance haloperidol and sertraline and their activity has been associated with various psychiatric disorders (Guitart et al. 2004). The physiological functions of σ-receptors remain unknown. The σ-1 receptor has been cloned, whereas the σ-2 receptor still needs to be isolated (Colabufo et al. 2006). The latter have been found to be associated with the so-called lipid rafts (Gebreselassie and Bowen 2004). Interestingly, σ-receptors are abundant in many malignant cells and there is evidence suggesting their crucial role in cell proliferation (Vilner et al. 1995). Along these lines, SRM has been recently demonstrated to suppress the growth of several cell lines in vitro and it has also been shown to inhibit the growth of solid tumors in mice (Ostenfeld et al. 2005).
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2.3.5 Anesthetics and lipids

Anesthesia is needed on a daily basis in clinical medicine, allowing surgical procedures. It is commonly divided into three parts: hypnosis, relaxation and analgesia. These three can be induced by one compound or a combination of different drugs can be used. Surprisingly, despite the fact that the first public demonstration of ether anesthesia by Dr. William T. Morton dates back as early as 1846, the molecular level mechanism underlying general anesthesia remains elusive. According to the Mayer-Overton rule, anesthetic potency of a given substance correlates well with the mineral oil/water partitioning of the substance irrespective of molecular size or chemical structure. This rule applies well to volatile anesthetics over a wide range of molecules, the simplest being the noble gas xenon up to more complex compounds, and stands over several orders of magnitude of molecular size. This lead to the lipid mediated anesthesia hypothesis which has been widely studied over the past decades (Seeman 1972). Factors such as solubilization of the nerve cell membrane and changes in the phase transition temperature together with curvature and dipole potential changes have been proposed to underlie these effects (Cafiso 1998; Cantor 2001; Heimburg and Jackson 2007). In recent years, however, increasing evidence suggesting the importance of especially the complex and extensive GABA receptor family with a multitude of various combinations of receptor subunits, with different drug binding properties etc. has been gathered. Maybe the most important finding opposing the idea of anesthesia induced by direct, nonspecific actions of compounds on the lipid bilayers is the lack of specific membrane effects of the stereo-isomers of steroid anesthetics (Antkowiak 2001). A range of compounds exist of which one stereoisomer is anesthetically active, where as the other is not (Covey 2009). Studies on *C. elegans* have been conducted, as well as fluorescence studies (Alakoskela et al. 2007). The mineral oil/water partitioning is not affected by the stereochemistry, nor has it been possible to distinguish any other property in lipid bilayers that would explain the difference in potency of these substances. One plausible explanation could be that the binding site for the anesthetic molecule resides in the transmembrane part of the receptor protein. This would require the compound to first partition into the membrane prior to being able to interact with its target (Grasshoff et al. 2006). It can of course be argued, that the stereo specificity lies somewhere else in the organism than in the membrane, e.g. differences in detoxification leading to different effective concentrations of a given substance.
2.3 Why study drug lipid interactions?

2.3.6 Phospholipidosis

Phospholipidosis is a pathological condition characterized by phospholipid accumulation in lysosomes (Reasor et al. 2006). Under electron microscopy the affected lysosomes have a typical pattern of membrane stacking (Kodavanti and Mehendale 1990). The earliest descriptions of the condition date back to the 1970s (Lullmann-Rauch 1979). Lysosomes have an important role in lipid degradation, since as a side product of organelle autophagy and endocytosis lipids, including sphingolipids, cholesterol esters and glycerophospholipids, end up in lysosomes for degradation by phospholipases (Johnson et al. 1996).

Interestingly, phospholipidosis is induced by a variety of different drug compounds, mainly cationic amphiphilic drugs. It is hypothesized that phospholipidosis is caused by drug induced inhibition of the phospholipid degradation in lysosomes. Three prerequisites have been identified for a drug to be able to induce phospholipidosis, 1 it must enter the cell, 2 it must be enriched in lysosomes, and 3 it must inhibit the activity of lysosomal phospholipases A and C (Hostetler 1984).

Many of the compounds inducing phospholipidosis differ from each other significantly both structurally and by their receptor binding profiles, thus suggesting a more general mechanism of action behind the observed phospholipid accumulation. Since lysosomes are rich in anionic phospholipid species it has been suggested, that the underlying cause of phospholipidosis is the interference of the accumulated drugs with the normally membrane associated proteins requiring anionic lipids for their activity (Alakoskela et al. 2009). The significance of phospholipidosis remains somewhat unclear, since this disorder of a cellular organelle seems quite isolated. There is a correlation that drugs efficiently inducing phospholipidosis are often toxic, however even nontoxic drugs may cause this disorder. Pharmaceutical companies screen for phospholipidosis using cell based assays, this however, is tedious and expensive (Kasahara et al. 2006). This has lead to the development of another approach in screening for phospholipidosis, namely screening for the interaction of drugs with anionic phospholipids, by observing the change in the apparent CMC of a short chained phosphatidylserine due to interaction of drugs (Vitovic et al. 2008).
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2.3.7 Proteins requiring a special lipid environment

Together with the recent advances in protein and lipid research technologies the detailed crystal structure of many transmembrane proteins has been solved. This has revealed some intriguing details about proteins having specific binding pockets for lipids, not only on the surface of the membrane spanning parts but also inside the protein molecules. It has been shown that cholesterol is needed to stabilize the structure of the nicotinic acetylcholine receptor (Brannigan et al. 2008). Further, the lipid composition of the immediate surroundings of this receptor is important in regulating the activity of the nicotinic acetylcholine receptor (Barrantes 2004; Bhushan and McNamee 1993; Dickey and Faller 2008). The presence of PA in the membrane was required to activate the protein, in the absence the protein is stabilized in a resting conformation. Such binding sites within the membrane open interesting prospects for further projects. Other membrane properties shown to affect protein activity involve surface dipole potential (Poolman et al. 2004), membrane thickness (Xu et al. 2008), lateral pressure (Kung 2005), membrane curvature (Lehtonen and Kinnunen 1995a; Nylund et al. 2007), and membrane elasticity (Sogaard et al. 2006).

2.4 Lipids in drug delivery & transfection

10 years ago a lot of time and money was spent in solving the genetic code. It was postulated that it would open the way to treatment of many so far untreatable or lethal diseases such as many forms of cancer, diabetes etc. In the post genomic era, that is after the human genome was published (International Human Genome Sequencing Consortium 2004) many of these fundamental goals have not been reached.

One key issue in replacing a malfunctioning or faulty gene is to introduce the working copy to the organism and furthermore to the organ in question. A traditional way to accomplish this so called transfection is to use nature’s own transfecting agents, viruses, to carry the genetic material to its target. This, however, poses some drawbacks. Since viruses are pathogenic it is imperative to abolish the infectious material from the virus prior to introducing the particle to the target. Even when this is successfully accomplished the immune system of complex organisms is prone to
attack this foreign material (Thomas et al. 2003). Consequently, an intensive search for a safer transfection vector is ongoing. One successful approach has been the use of cationic liposomes, which bind and form complexes with DNA to transport the genetic material to cells (Felgner et al. 1987; Ryhänen et al. 2003). This is a safer approach, since cationic lipids are not infectious. It is also possible to find non-toxic compounds, that generally do not cause strong immune responses in the target organism. So far transfection efficacy has been the major advantage of viral transfection. Therefore the properties responsible for the transfection efficacy of lipids and lipid-DNA complexes have been studied over the past years in order to find more effective liposomal compounds. Other means of increasing transfection efficacy involve increasing the circulation half-time of liposomal particles by coating them with for instance polyethyleneglycol (PEG). Another line of research deals with targeting such compounds to various tissues with the help of peptides or other molecules that find specific targets at the desired place of action. It has also been proposed that incorporating reactive groups, such as disulfide bridges in the spacer of a gemini surfactant lipid could facilitate the release of DNA from such vesicles (Säily et al. 2006).

In addition to introducing genetic material to cells liposomes can and have been employed as transport vehicles for more conventional drugs. There are several advantages in packing drugs into liposomal carriers. The circulation half-time of a drug is increased, thus providing a more stable drug concentration in circulation. Liposomes can be used to mask the drug molecule, which otherwise would cause an immunological response (Blume and Cevc 1990).

Future prospects involve constructing functional liposomes or liposomes that can be packed with a drug, targeted to a specific location and triggered to release their content as a response to some stimulus such as pH, temperature, or a chemical gradient. Methods such as focused ultrasound are being studied in order to create such functional nanoparticles. One practical approach involves triggering a phase transition in the carrier vehicle, as during the main transition a maximum in permeability of the liposome occurs, allowing it to deliver its cargo. Therefore it is important to understand the molecular events underlying lipid phase transitions and the various factors affecting those.

Specific issues to be considered upon designing new biologically active functional nanoparticles involve factors such as keeping the particles in circulation, finding the desired target, penetrating the target and subsequently delivering the cargo into the nucleus to incorporate the functioning gene into the genome.
3 Aims of the study

This thesis focuses on the relationship of the organization, function and phase behavior of model membranes, together with drug-effects on membranes.

The first aim of this study was to develop a fluorescence quenching based assay for fast and convenient quantification of the membrane association of various membrane active compounds.

In the second part the first described method was employed to assess the membrane binding properties of siramesine, a drug recently found to cause apoptosis in cancer cells and hypothesized to interact in a non-specific way with cellular membranes.

The third part focuses on the different behavior of the commonly used membrane models large unilamellar vesicles (LUVs) and multilamellar vesicles (MLVs) to I) get insight into the effect of lamellarity on membrane properties and II) to more thoroughly characterize the effects of the commonly used fluorescent probe 1-Palmitoyl-2[10-(pyren-1-yl)]decanoyl-sn-glycero-3-phosphocholine (PPDPC) on the biophysical properties of the lipid membrane, further aiding the interpretation of studies employing this fluorescent lipid analog.

In the fourth and final part the organization of cationic liposomes in the presence of salts is studied. The reasoning behind involves the use of cationic liposomes as gene transfection vectors requiring understanding of the assembly and structure of such nanoparticles.
4 Materials and methods

4.1 Chemicals

1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, (POPC), and 1-palmitoyl-2-oleoyl-sn-glycero-3-(phospho-1-serine), (POPS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac-glycerol (POPG), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoinositol (POPI), 1,2-dipalmitoyl-sn-glycero-3-phospho[N-(4-nitrobenz-2-oxa-1,3-diazolyl)]-ethanolamine (DPPN), 1,2-dioleyl-sn-glycero-3-phosphoethanol (DOPet), 1,2-diolelyl-sn-glycero-3-phospholipid (DOPmet), 1-stearoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine (Lyso-PA), and 1-stearoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)] (Lyso-PG) were from Avanti Polar Lipids (Alabaster, AL). 1-Palmitoyl-2-[10-(pyren-1-yl)]decanoyl-sn-glycero-3-phosphocholine (PPDPC) was from K&V Bioware (Espoo, Finland). (1'-[4-[1-(4-fluorophenyl)-1H-indol-3-yl]-1-butyl]spiro[isobenzofuran-1(3H),4'-piperidine] (SRM) was kindly provided by H. Lundbeck A/S (Copenhagen, Denmark). Deionized Milli-Q (Millipore, Bedford, MA) filtered water was used in all experiments. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), NaCl, ethylenediaminetetraacetic acid (EDTA), Chlorpromazine (CPZ), and egg yolk-phosphatidic acid (egg-PA) were from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of analytical purity. The lipids were analyzed by thin layer chromatography on silicic acid coated plates, developed with chloroform/methanol/water/ammonia (65/20/2/2, by volume) as the solvent. No organic impurities were detected upon examination upon UV-illumination or after iodine staining.

Lipid and drug concentrations were determined gravimetrically using a high precision microbalance (Cahn Instruments Inc., Cerritos, CA). The concentrations of PPDPC, DPPN and CPZ were determined from their absorption spectra (Cary-100 Bio, Varian Inc, Victoria, Australia) recorded using quartz cuvettes with one cm pathlength and employing 42,000 M⁻¹cm⁻¹ at 342 nm, 21,000 M⁻¹cm⁻¹ at 463 nm, and 24,000 M⁻¹cm⁻¹ at 254 nm, as their molar extinction coefficients, respectively. Stock solutions of SRM and CPZ were made in chloroform. Proper aliquots were dried under a gentle flow of N₂ and the dry residue dissolved in dimethylsulfoxide (DMSO, Merck), ethanol, or buffer as indicated. The molar absorptivity of SRM was 20,000 M⁻¹cm⁻¹ at 258 nm determined from its absorption spectra recorded for a 10 μM solution in ethanol.
4 Materials and methods

4.2 Preparation of liposomes (I-IV)

Upon hydration a dried phospholipid film forms multilamellar vesicles, with a diameter of typically 200-1500 nm and with multiple overlapping lipid bilayers forming a shape resembling the structure of an onion. Depending on the desired application or experimental conditions MLVs can further be processed e.g. extruded through 100 nm polycarbonate filters to yield large unilamellar vesicles (LUVs), commonly employed in fluorescence spectroscopic experiments.

More specifically, the lipids were dissolved in chloroform and subsequently mixed in this solvent so as to obtain the desired compositions. The solvent was removed using a gentle stream of nitrogen whereafter the dry lipid residues were maintained under reduced pressure for at least 2 h in order to remove trace amounts of chloroform. The lipids were hydrated with 20 mM Hepes, 0.1 mM EDTA, pH 7.0. During hydration the dispersing was aided by a shaking water bath at room temperature so as to yield multilamellar vesicles. Where desired, large unilamellar vesicles (LUV) were prepared by extrusion using a LiposoFast (Avestin, Ottawa, Canada) small-volume homogenizer. The lipid dispersions were passed 19 times through polycarbonate filters with an average pore diameter of 100 nm (Millipore, Bedford, MA), in order to yield LUVs with an average diameter of 80 ± 25 nm (MacDonald et al. 1991).

4.3 Fluorescence spectroscopy (I-III)

For fluorescence spectroscopy we incorporated fluorescent lipid analogs in the liposomes. PPDPC (I-III) was chosen because its pyrene moiety was covalently attached to a decanoyl chain at the $sn$-2 position. The photophysics of pyrene, namely its propensity to form excited dimers, excimers, upon collision of an excited pyrene with a ground state pyrene make it a suitable probe to reflect the lateral movement of lipids in a bilayer (Galla and Sackmann 1974; Lehtonen and Kinnunen 1994; Muller et al. 1986). The pyrene moiety resides in the hydrocarbon region of the membrane. Accordingly, for collisional quenching to occur, the quencher needs to penetrate the membrane core. DPPN (II) was chosen as a second probe, since it carries a fluorescent NBD-group covalently attached to the head-group, thus being susceptible for quenching if the quencher is enriched on the membrane surface.(Alakoskela and Kinnunen 2001) Steady-state fluorescence spectra were
measured in four window quartz cuvettes with one cm pathlength, using a fluorescence spectrophotometer (LS-50B, Perkin-Elmer, MA, USA (I) or Varian Cary Eclipse (II, III) Varian Inc.) equipped with thermostated temperature controlled cuvette holder. The excitation and emission wavelengths were selected according to the probe in question as indicated. Emission and excitation bandpasses were set at 5 or 10 nm depending on the probe. If not otherwise stated measurements were conducted at 25° C.

4.4 CMC determination (II)

The critical micelle concentration (CMC) for SRM was determined at ambient temperature (approx. 22 °C) with an 8-channel surface tension platereader (Delta-8, Kibron Inc., Espoo, Finland). For these measurements serial dilutions prepared in 96-well plates (DynePlates, Kibron) in the indicated concentration range were employed. The data were analyzed utilizing the Gibbs adsorption isotherm embedded in the dedicated software from the instrument manufacturer (Delta-8 Manager).

4.5 Binding of SRM to lipid monolayers (II)

Penetration of SRM into monomolecular lipid films was measured using a Langmuir-tensiometer (DeltaPi, Kibron Inc., Espoo, Finland) with magnetically stirred circular wells with a subphase volume of 1.2 ml (Multiwell plate, Kibron Inc.). Surface pressure \( \pi \) was monitored with a metal alloy probe hanging from a high precision microbalance (KBN129, Kibron Inc.). The indicated lipids were mixed in chloroform (c=1 mM) and spread on the air–water interface using a microsyringe. The monolayers were allowed to equilibrate for 5 to 15 min to reach the indicated initial surface pressure values (\( \pi_0 \)). 4.8 μl of 0.4 mM SRM (dissolved in DMSO) was then injected the subphase (20 mM Hepes, 0.1 mM EDTA, pH 7.0) to yield a final drug concentration of 1.6 μM. This amount of DMSO as such had no effect on the surface pressure. The difference between \( \pi_0 \) and the final surface pressure after the addition of drug was taken as the increase in surface pressure (\( \Delta \pi \)). The data are represented as \( \Delta \pi \) vs. \( \pi_0 \), thus revealing the effect of increased lateral packing on the penetration of the drug into the monolayer (Brockman 1999).
4.6 Differential scanning calorimetry (DSC) (II-III)

The indicated amounts of the drug and lipids were mixed in chloroform. These mixtures were dried under a stream of nitrogen and subsequently kept under reduced pressure for at least 2 h to remove traces of the solvent. The samples were hydrated in 20 mM Hepes, 0.1 mM EDTA, pH 7 at 60 °C for 30 min in a shaking water bath as to yield multilamellar liposomes utilized in the DSC measurements. The samples, final lipid concentration 0.4 mM, were equilibrated on an ice water bath for at least 10 h to ensure equal thermal histories. The endotherms were recorded using a microcalorimeter (VP-DSC, Microcal Inc., Northampton, MA) at a heating rate of 30 deg/h. All scans were repeated to assure their reproducibility. Deviation from the baseline was taken as the beginning of the transition and return to the baseline as its end. The endotherms were analyzed using the routines of the software provided by the instrument manufacturer.

4.7 Optical microscopy of a liposome solution (IV)

An Olympus IX-70 inverted microscope with differential interference contrast optics was used to observe the microscopic changes in the surfactant suspension upon heating from 24 to 50 °C by a Peltier element controlled thermal microscopy stage (TS-4, Physitemp, Clifton, NJ, USA). The images were captured using an attached Canon Eos 10D SLR camera. The surfactant suspension (1.5 ml) with the indicated [NaCl] was applied into a chamber with quartz glass bottom.
5 Results

5.1 Interactions of cationic amphiphilic drugs with liposomes

5.1.1 Reversible binding of CPZ to liposomes (I)

Our preliminary experiments demonstrated that CPZ quenches pyrene fluorescence emission by acting as a collisional quencher, most likely involving the aromatic ring structures of CPZ (See above, Fig. 6). Accordingly, binding of CPZ to liposomes can be monitored by observing changes in the fluorescence emission from the pyrene-labeled phospholipid analog PPDPC.

For PPDPC liposomes with \(X_{PC}=1.0\) (20 \(\mu\)M total lipid), 40 \(\mu\)M CPZ reduced the fluorescence intensity to 15% of the initial value (Fig. 7), with no further decrease at higher [CPZ]. In keeping with the high affinity of CPZ to the anionic phosphatidylserine (POPS) (Chen et al. 2003), significantly enhanced quenching was observed in the presence of this lipid in the bilayers (Fig. 7). Accordingly, only 4 \(\mu\)M CPZ was needed for similar quenching with liposomes containing \(X_{PS}=0.50\), compared to neat POPC liposomes requiring ten times higher [CPZ]. Saturation was observed already at [CPZ]=6\(\mu\)M. Upon further increase of \(X_{PS}\) up to 0.99 no further enhancement was observed. The reversibility of this interaction was studied by adding phospholipid vesicles without the fluorescent probe to allow for a redistribution of the drug between the original labeled and the unlabeled vesicles. Assuming that CPZ is evenly distributed between the liposomes, we can depict the values for RFI against the assumed mole fraction of CPZ in the labeled liposomes. The exact matching of the data (I:Fig. 3) reveals that the drug is evenly distributed between the labeled and unlabeled vesicles, reflecting their respective concentrations.

Fig. 7 Quenching of pyrene monomer fluorescence by CPZ. PC LUVs at 25°C in 20 mM Hepes, 0.1 mM EDTA, pH 7.0 with \(X_{PPDPC}=0.01\). Vesicles with \(X_{PS}=0\) (■), 0.05(●), 0.1(▲), 0.2(▼), 0.5 (●)and 0.99(▲)Figure 1:2 reproduced with permission of Springer.
5 Results

5.1.2 Calculating the molar partition coefficient for CPZ and SRM (I, II)

In order to determine the molar partition coefficient for the studied drug-liposome interactions quenching data from two series of measurements were collected. More specifically, in the first series the phospholipid concentration was maintained constant and the drug concentration was progressively increased. In the second series the concentration of the drug was constant and the phospholipid concentration was increased by adding increasing amounts of unlabeled liposomes. The fluorophore concentration was held constant throughout the titrations in order to have an indicator of drug distribution as described below in detail. These data are compiled in Table I.

The molar partition coefficient \( K_p \) is defined by

\[
K_p = \frac{[D_L]}{[L]} \cdot \frac{[H_2O]}{[D_W]},
\]

where \([D_L], [L], [H_2O], \) and \([D_W] \) stand for the concentrations of drug in the lipid phase, lipid, water, and drug in water phase, respectively. Of course, this partition coefficient can only be applied for relatively low drug concentrations when the drug does not significantly change the properties of the bulk phase (usually the lipid phase). Thus, for relatively low membrane drug concentration we have

\[
X_D = \frac{[D_L]}{[L]}, \text{ and further}
\]

\[
K_p = X_D \cdot \frac{[H_2O]}{[D_W]},
\]

in which \( X_D \) is the mole fraction of drug in the membrane. Using the relationships between the total drug concentration and the drug concentrations in the different phases we get

\[
X_D = \frac{K_p[D_T]}{[H_2O]+K_p[L]},
\]

where \( D_T \) stands for total drug concentration. Evaluation of the partition coefficient was based on the assumption that in both of the above two experimental conditions at identical mole fractions of bound drug in the bilayer (\( X_D \)) the quenching efficiency should always be identical. Accordingly, each RFI value corresponds to a unique \( X_D \), although the absolute numerical value of \( X_D \) is not known. The cubic interpolation function of Matlab (The MathWorks, Natick, MA) was used to
5.1 Interactions of cationic amphiphilic drugs with liposomes

retrieve the drug concentrations in the second series that would produce identical RFI values to those of the first series. This yielded a series of paired values (Table II), in which each unknown $X_D$ corresponds to two different combinations of the concentrations of drug and phospholipid.

We then assumed that the data could be described in terms of a single, concentration independent lipid/water partition coefficient. An initial guess of the partition coefficient was made and subsequently the mole fractions for constant drug concentration ($X_{C,i}$) and constant phospholipid concentration ($X_{L,i}$) corresponding to each RFI, were calculated. Ideally, with the correct $K_p$, for each value of $i$ the equality $X_{C,i} = X_{L,i}$ should apply. Subsequently, by varying $K_p$ we minimized the following sum:

$$\sum_{i=1}^{5} \left[ \left(1 - \frac{X_{C,i}}{X_{L,i}} \right)^2 X_{L,i} \right]$$

in which $(1 - X_{C,i}/X_{L,i})^2$ requires that the values in each pair should be equal. Accordingly, this gives identical weight to relative variations for each $X_D$, whereas larger weight is given to higher values of $X_D$ by weighing this term with $X_{L,i}$. Alternative weighing, like using $(X_{C,i}-X_{L,i})^2$ would give identical weight to differences from pairs like $X_{C,i} = 0.02, X_{L,i} = 0.04$ and $X_{C,i} = 0.28, X_{L,i} = 0.30$. On the other hand, using $(1 - X_{C,i}/X_{L,i})^2$ without weighing would give identical weights to differences from pairs like $X_{C,i} = 0.02, X_{L,i} = 0.03$ and $X_{C,i} = 0.20, X_{L,i} = 0.30$. The weighing scheme used is thus an intermediate between the two extremes.

For vesicles with $X_{PS}=1.0$ the above sum could be minimized by setting the value of the molar partition coefficient to $(2.2\pm0.2) \times 10^7$, which corresponds to $(5.1\pm0.6) \times 10^5$ on volume/volume basis. The molar partition coefficient of CPZ in DPPC bilayers has been previously determined as $(8.0\pm0.7) \times 10^4$ (Luxnat and Galla 1986). The presence of negatively charged lipids enhances the binding of CPZ to membranes. The partition coefficient of CPZ to acidic phospholipids containing membranes has been calculated in a second-derivative spectrophotometry study and reported as $(5.33\pm0.43) \times 10^5$ for vesicles containing egg yolk phosphatidylcholine (PC) and bovine brain PS, 80% and 20% respectively in the presence of 50 mM NaCl (Takegami et al. 2005). Accordingly, comparing these values to those in literature for the partitioning of CPZ reveals more than a tenfold increment in the partitioning of CPZ to pure POPS vesicles as compared to DPPC vesicles.
5 Results

5.1.3 Interactions of siramesine with phospholipids (II)

As expected from its chemical structure (See above, Fig. 6) SRM is fluorescent. In ethanol it has absorption bands at 258 nm and 297 nm, and upon excitation at 258 nm a broad emission band peaking at 356 nm is seen (II: Fig 2). The emission intensity was significantly reduced in water, with a weak band centered at 425 nm.

Further SRM is amphiphilic as demonstrated by its partitioning into the air/water interface (II: Fig. 3). The recorded isotherm yields $37 \pm 6.7 \text{ Å}^2$ as the interfacial area of SRM, with CMC observed at $32 \pm 1.2 \mu \text{M}$. As expected from the above, SRM partitions efficiently into phosphatidylcholine liposomes, with a significant increase seen in its quantum yield together with a 4 nm blue shift in the peak wavelength (II: Fig. 2), suggesting SRM to become accommodated in a non-polar environment, in the hydrocarbon region of the bilayer.

As already demonstrated for several other membrane associating cationic drugs, anionic phospholipids promote the binding of SRM to bilayers. Accordingly, increasing the content of phosphatidylserine X$_{\text{ps}}$ in liposomes caused a marked increase in the membrane binding of SRM as reflected by a pronounced increase in its fluorescence.

In order to study the localization and to quantitate the association of SRM to liposomes we used SRM as a quencher for trace amounts of different fluorescent lipid analogs. We first employed PPDPC which was found to be collisionally quenched by SRM, reflecting efficient binding of SRM to POPC vesicles and causing at a drug:phospholipid ratio of 1:2 a maximally 60 % decrement in PPDPC emission. While the hydrophobicity of the pyrene moiety of PPDPC causes this fluorophore to reside in the hydrocarbon region of the membrane, its polarizability together with cation-$\pi$ interactions should prefer a more interfacial orientation(Hoff et al. 2005; Yau et al. 1998). Interestingly, including PS (X=0.50) to the vesicles attenuated the quenching of PPDPC by SRM (Fig. 8) and increasing the content of PS further to X=0.99 augmented this effect, with only a minor decrement caused by SRM in PPDPC fluorescence. For vesicles with X$_{\text{ps}}$=0.2 the quenching was biphasic with the initial small decrement in fluorescence caused by 4 μM SRM (corresponding to an apparent drug:PS molar ratio of 1:1) being followed by a progressive and pronounced quenching upon further increase in [drug]. This was further elucidated for vesicles with X$_{\text{PA}}$=0.02, 0.05, 0.10, and 0.20. Similarly to X$_{\text{ps}}$=0.2 quenching was biphasic (Fig. 8) with the discontinuity observed at SRM concentrations varying linearly with X$_{\text{PA}}$ and corresponding to an apparent 1:1
5.1 Interactions of cationic amphiphilic drugs with liposomes

drug:PA molar ratio. The above data suggest the formation of stoichiometric complexes by SRM and PA on the membrane surface, preventing the penetration of SRM into the membrane hydrocarbon region where the pyrene moiety of PPDPC is accommodated.

This was confirmed using another probe DPPN bearing a fluorescent NBD label in the headgroup. For this probe (X=0.01) and POPC vesicles, the decrement in fluorescence caused by 4 μM SRM was 50% (II: Fig. 6) and efficient quenching was observed also for PS containing LUVs (X_{PS}=0.20) with little difference when compared to neat POPC LUVs.

The partition coefficients for SRM between the aqueous solution and vesicles containing POPC, POPS, POPG, egg-PA, Brain-PI, POPE, DOPMe, DOPet, lyso-PA, and lyso-PG using the headgroup labeled DPPN (X=0.01) as a fluorophore (II: Table 1). In keeping with the above fluorescence studies SRM preferentially binds to acidic lipids, with the highest affinity K_P=240±80 x 10^6 measured for PA.

To further assess the specificity of the binding and the preference of different cationic amphiphilic drugs for anionic phospholipids we determined the partitioning of chlorpromazine, doxorubicin, clozapine, and haloperidol to vesicles containing PG, PA, or PS. The highest affinities of the above

---

**Fig. 8** Quenching by SRM of the pyrene-labeled phospholipid analog PPDPC (X=0.01) in POPC vesicles: with X_{POPS}=0 ( ), 0.20 ( ), 0.50( ), and 0.99 ( ), panel A), and with X_{PA}=0 ( ), 0.02 ( ), 0.05 ( ), 0.10 ( ), and 0.20 (•), panel B). Total phospholipid was 20 μM in 20 mM Hepes, 0.1 mM EDTA, pH 7.0, T=25 °C). Figure II:5 reproduced with permission of ACS Publications.
drugs were measured for PS, followed by PG, while the least affinity was observed for PA containing vesicles (II:Table 2)

The above quenching experiments suggest that SRM intercalates into the hydrocarbon region of neat POPC bilayers, while in the presence of anionic lipids two modes of binding are evident: a high affinity binding site in the membrane surface with SRM bound to the acidic phospholipid headgroup, and a second site with the drug intercalating into the hydrocarbon region. To verify the above conclusions from the fluorescence quenching experiments study we measured the penetration of SRM into lipid monolayers residing on the air/buffer interface (Fig. 9). In accordance with the above fluorescence data a pronounced increase in surface pressure due to SRM added into the subphase was observed for pure PC films, while $X_{ps}=0.2$ dramatically decreased the penetration. This readily complies with an interfacial location of SRM in films containing the acidic phospholipid. Intriguingly, the association of SRM to monolayers containing PA is different from those with PS (Fig. 9), with high values for $\Delta \pi$ being measured. This is most likely caused by the more extensive partitioning of SRM to PA together with a pronounced relative increase in the effective headgroup size of this lipid upon binding of SRM.

Fig. 9 Intercalation of SRM into phospholipid monolayers. SRM (1.6 μM) added into the subphase and the increase in surface pressure $\Delta \pi$ vs. initial surface pressure $\pi_0$ was recorded. The films were composed of POPC ○, POPC with POPS ($X=0.20$, ●), and POPC with egg-PA ($X=0.20$, ■). The lines represent linear fits to the data as a guide for the eye. Figure II:7 reproduced with permission of ACS Publications.
5.2 Thermal phase behavior of DPPC MLVs and LUVs (III)

Temperature scans from 10 to 50°C were recorded for DPPC LUVs and MLVs involving sequential Lc → Lβ' → Pβ' → Lα transitions, while monitoring fluorescence of PPDPC (Fig. 10, III: Fig.1). The obtained values for T_m (III: Fig.2B) agree with the DSC data (Fig. 12, see below) up to X_{PPDPC}=0.035, whereafter the fluorescence data shows slightly lower temperatures, most likely revealing a local enrichment of PPDPC during melting. The pretransition of MLVs (III: Fig.2A) shows a pronounced shift towards lower temperatures with increasing X_{PPDPC}, reaching T_p=22 °C for membranes with X_{PPDPC}=0.05, and indicating stabilization of the Pβ' phase by PPDPC, increasing its span from 11.4 to 17 degrees (III: Fig.2C). This is most likely caused by partitioning of this lipid analog into phase boundaries along the ripples together with poor partitioning into the gel phase bilayer. For LUVs the pretransition is not clearly defined. A change in the slope observed in the I_e/I_m trace preceding T_m probably represents the beginning of the main phase transition (Fig. 10B).

For MLVs in the Pβ' phase, a maximum in I_e at T_p is followed by a steep decrease and a further less pronounced decrement upon approaching T_m. The change in excimer emission may be explained by the initial partitioning of the pyrene lipids along the more fluid line defects formed in the membrane, thus limiting the collisions between molecules to take place in one dimension, followed

![Temperature scans employing PPDPC as a reporter monitoring thermal phase behavior of DPPC MLVs (panel A) and LUVs (panel B). I_e/I_m is plotted against temperature. Total phospholipid concentration was 20 μM in 20 mM Hepes, 0.1 mM EDTA, pH 7.4, with X_{PPDPC}=, 0.01(●), 0.02 (▲), 0.03 (▼),0.04(●), and 0.05 (▲). Unpublished data.](image-url)
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by gradual partitioning of PPDPC into the melting surroundings of the line defects. The decrease in \( I_e \) ends in a steep drop at the main phase transition, reflecting the melting of the chains allowing unconstrained lateral diffusion of the labeled lipids within the bilayer surface, resulting in effective dilution of the probe.

For \( I_m \) two processes are evident at \( T_m \) for both LUVs and MLVs. At low \( X_{PPDPC} \) (up to \( X_{PPDPC}=0.01 \)) a decrease of \( I_m \) is observed, together with a concomitant decrease in \( I_e \). This is likely to relate to the melting of the acyl chains, increasing the area/molecule in the bilayer, thus allowing water to partition deeper into the membrane hydrocarbon region. This increase in polarity leads to a reduced quantum yield for pyrene fluorescence, evident upon approaching \( T_m \) until \( X_{PPDPC}=0.01 \). However, further increasing \( X_{PPDPC} \) enhances excimer formation below \( T_m \), resulting in a net increase in \( I_m \) at \( T_m \) due to the diminished excimer formation becoming dominant over the quenching. These two effects cancel each other out at \( X_{PPDPC}=0.015 \), with no change in \( I_m \) observed at \( T_m \).

For LUVs \( I_e/I_m \) reaches its maximum slightly below \( T_m \), reflecting the enrichment of PPDPC in a fluid phase concomitant with fast lateral diffusion, \( I_e \) thus reaching a maximum at a point at which a sufficiently large proportion of the pyrene probe is enriched in a small region of fluid phase membrane. Above \( T_m \) a percolation threshold is exceeded, allowing the initially clustered PPDPC molecules to freely diffuse within the continuous fluid phase, reducing inter-molecular collisions. Upon further increase in temperature the collision rate increases linearly, reflecting augmented thermal motion.

The dependence of \( I_e/I_m \) at a given temperature on \( X_{PPDPC} \) was analyzed (III: Fig.3). For gel state MLVs the \( I_e/I_m \) ratio initially exceeds that for LUVs e.g. \( I_e/I_m \) has approximately the same value when \( X_{PPDPC}=0.005 \) for MLVs and \( X_{PPDPC}=0.025 \) for LUVs or when \( X_{PPDPC}=0.010 \) for MLVs or \( X_{PPDPC}=0.045 \) for LUVs. A steep increase in \( I_e/I_m \) until \( X_{PPDPC}=0.02 \) is seen, however above this point the increment is significantly less pronounced. In the \( L\beta' \) phase the excimer fluorescence from PPDPC outside the PPDPC-enriched clusters is probably negligible and the \( I_e/I_m \) ratio is determined by two factors viz. the fraction of PPDPC in the clusters and the local PPDPC concentration within these domains. The decrease in the slope of \( I_e/I_m \) vs. \( X_{PPDPC} \) thus most likely reflects reaching either the maximal fraction for the PPDPC enriched in domains or the maximal local concentration within these domains. For both LUVs and MLVs in the \( P\alpha \) or \( L\alpha \) phases a linear
5.2 Thermal phase behavior of DPPC MLVs and LUVs (III)

dependence of $L/I_m$ on $X_{\text{PPDPC}}$ was observed, in accordance with previous studies and theoretical predictions concerning diffusion reactions in 2-dimensions (Martins et al. 1996).

In order to compare the acyl chain order of DPPC MLVs and LUVs upon heating, the fluorescence anisotropy ($r$) of DPH-PC ($X_{\text{DPH-PC}=0.005}$) was determined (Fig. 11). The values for anisotropy did not reveal significant differences between LUVs and MLVs in the Lc and Pβ phases. In keeping with the pyrene fluorescence, $T_m$ reported by this probe is slightly lower for LUVs than for MLVs. In the Lα phase $r$ was consistently approx 15% lower for LUVs than for MLVs, reflecting lower chain order in the former bilayers.

The heat capacity traces for pure DPPC MLVs show an asymmetric main transition peak at 41.2 °C together with a pretransition at 33.5 °C. The fluorescent lipid analog incorporated into the bilayer represents a substitutional impurity in the membrane, and is thus expected to affect the melting process, typically decreasing $T_m$. With increasing $X_{\text{PPDPC}}$ the heat capacity traces (Fig. 12) show a broadening of the endotherms toward lower temperatures, indicating decreased co-operativity of the transition (III: Fig. 6), together with a gradual decrease in the main transition temperature $T_m$. To further correlate the above fluorescence data with the thermotropic phase behavior of DPPC MLVs and LUVs as a function of $X_{\text{PPDPC}}$ we measured their melting endotherms by DSC (Fig. 12). The lift-off temperature for the main transition peak (defined as $T$ at which the endotherm initially deviates from the baseline) shifts to lower temperatures upon increasing $X_{\text{PPDPC}}$. Variation of the lift-off temperature was observed between independent samples, suggesting the extent of PPDPC segregation to vary, depending on the less tightly controlled cooling of the samples before the measurement. To the right of the main transition endotherm of MLVs at $X_{\text{PPDPC}}>0.015$ a small
5 Results

shoulder is seen, which gradually develops into a separate minor peak for vesicles with $X_{PPDPC}>0.03$ at $T=41.2 \, ^{\circ}C$, corresponding to $T_m$ of (almost) pure DPPC, separating from the DPPC:PPDPC mixture. Estimated from the enthalpy of this peak the DPPC in the membrane represents approx. 1% of the total DPPC. The pretransition of DPPC can be observed at low $X_{PPDPC}$, and upon increasing $X_{PPDPC}$ it is dramatically shifted toward lower temperatures. This decrease in $T_p$ is accompanied by broadening and lowering of the pretransition endotherm, disappearing at $X_{PPDPC}=0.035$ (Fig. 12).
5.2 Thermal phase behavior of DPPC MLVs and LUVs (III)

Fig. 12 Excess heat capacity ($C_p$) traces of 1 mM DPPC MLVs (panel A) and LUVs (panel B) in 20 mM Hepes, 0.1 mM EDTA, pH 7.4. The calibration bar corresponds to 2 kJ/deg mol$^{-1}$. $X_{PPDPC}$ increases as moving up in the graph. For MLVs $X_{PPDPC}=0, 0.005, 0.01, 0.015, 0.0175, 0.02, 0.0225, 0.025, 0.03, 0.035, 0.04,$ and 0.05 and for LUVs $X_{PPDPC}=[0, 0.005, 0.01, 0.015, 0.02, 0.025, 0.03, 0.035, 0.04, 0.045,$ and 0.05]. Unpublished data.

For LUVs, pure DPPC produces a single asymmetric endotherm peaking at 41°C representing the main transition. As reported previously, the pretransition is poorly visible for pure DPPC LUVs by DSC (Heimburg 2000). Incorporating the fluorescent lipid analog into LUVs lowered and broadened the main endotherm, and shifted it toward lower temperatures. Similarly to MLVs, also for LUVs with $X_{PPDPC}>0.035$ phase separation is evidenced by a small, high-temperature shoulder in the main transition, centered approximately at 40.8°C. At high $X_{PPDPC}$ variation between independent samples increased, perhaps reflecting the effect of clustering of PPDPC upon sample preparation and cooling.
5.3 Counter-ion driven self-assembly of a cationic gemini surfactant (IV)

Visual inspection of the gemini surfactant suspensions of M-1 at various [NaCl] upon hydration revealed unexpected behavior of the lipid. More specifically, at [NaCl]< 1.5 M the suspensions were optically clear, whereas at higher [NaCl] the suspension became bleary, with a bluish tint. This was observed for [NaCl]=1.5 and 2 M at temperatures of approx. 60°C, and for [NaCl]=3M even at ambient T (=22°C). These phenomena were reversible, as cooling the suspensions at [NaCl]=1.5 or 2M to 20°C resulted in a clear solution again. Changes in the optical density of the solution are likely to result from a transition in particle size upon heating.

This was further studied in a quartz bottomed chamber by optical microscopy. These experiments revealed the formation of giant vesicles as the chamber was heated on the stage. A tentative phase diagram (Fig. 14) was constructed to elucidate the observed relationship between [NaCl] and T. At [NaCl]=0.5M no visible structures were observed, and with [NaCl]=1 M only sporadic microscopical vesicles appeared at T>43°C. Upon heating of a solution with [NaCl] > 1M a series of transitions was observed as exemplified in the micrographs below with [NaCl]=2M (Fig. 15). More specifically at 24°C no visible structures could be identified, upon heating to 29 °C tubular structures started to arise at the air/water interface (Fig. 15a). Further heating to 33°C caused these structures to fuse to round giant vesicles, also observed below the surface in the liquid (Fig. 15b). These giant vesicles had an approximate diameter of 30-50μm, with significantly larger vesicles occasionally seen. In this temperature range even cytotoxic vesicles with morphologies resembling those of living cells, with internal ‘organelles’ were observed (Fig. 15b, inset). Above

Fig. 14 A tentative phase diagram of the relation of [NaCl] and T on the microscopic morphology of the M1 water suspension.

- a) submicroscopic structures
- b) heterogeneous giant vesicles (diameter 30-50μm)
- c) homogeneous vesicles of 10-15 μm diameter.

For further details see text.

Figure IV:3A reproduced with permission of ACS Publications.
5.3 Counter-ion driven self-assembly of a cationic gemini surfactant (IV)

T=36°C vesicle size started reducing towards T=40 °C, where after a homogenous distribution of vesicles with a diameter of 10-15 μM was seen (Fig. 15c). The observed transitions were named T₉ and T₁, referring to the formation of giant vesicles and smaller homogenous aggregates, respectively.

Interestingly, the formation of more complex vesicular structures such as tubular aggregates and cytomimetic vesicles, constituting of several embedded vesicles required the presence of the air/water interface, as these structures were absent when the experiment was conducted in a sealed chamber. However the transitions from clear solution to GVs and further to smaller homogenous vesicles remained visible even under such conditions.

Fig. 15 1 mM M-1 Surfactant solution in 2M NaCl as seen under the microscope at T=30 (a), 33 (b), 43(c) °C. The scale bar represents 100 μm. Figure IV:2 reproduced with permission of ACS Publications.
6 Discussion

The unifying theme of the original publications (I-IV) is the relationship of the function and order of biological model membranes. The motivation of the study was to further the understanding of drug-lipid interactions as potential modes of action participating in the observed clinical effects of various drug compounds. The self assembly of lipid structures and the responses to changes in the environment provide a possible tool for the controlled release of liposomal preparations of drug compounds as well as gene delivery.

6.1 A fluorescence based assay for determining the molar partitioning coefficient

Even though the use of fluorescent probes bears some problems as discussed in more detail below, there are several advantages in the approach for determining the molar partition coefficient of drug compounds to phospholipid vesicles described in this study. First of all the fluorescent probe is known to be located in the liposomes. If the fluorescence of the studied compound was to be used, the fluorescence might get contribution from water dissolved drug molecules or fluorescent drug-drug aggregates. Further, the affected fluorescent property is not of importance, as long as the bound substance causes a consistent change in the measured property. This approach can be used to quenching, irrespective whether the mode of quenching is colltional or via resonance energy transfer. Even ratiometric properties such as changes in $I_o/I_m$ could be used.

The disadvantages of this type of approach include the fact that the compound under study must affect the fluorescence of the probe in a dose dependent manner. Further the change in fluorescent properties needs to be continuous i.e. there must not be any local minima, thus each drug concentration produces a unique value. The results can be biased if the partitioning is affected by the drug or if the drug irreversibly binds to the liposomes. This will result in unequal partitioning between the labeled and non-labeled liposomes leading to poor fits for the data. Such behavior can however be noticed during the fitting of data. This type of assay can be easily expanded for high throughput screening with the necessary robotics.
6.2 Phosphatidic acid as a drug target

This method will obviously fail to give appropriate results, if the partitioning of a compound cannot be described in terms of a simple partition coefficient. However, in this case the fitting of the data will give poor results and thus reveal such behavior.

6.2 Phosphatidic acid as a drug target

Siramesine has been shown to inhibit the growth of solid tumors in mouse models as well as in cell culture (Groth-Pedersen et al. 2007; Ostenfeld et al. 2005). Siramesine binds tightly to the orphan sigma-2 receptors, with a dissociation constant in the nanomolar range. The observed anti-tumor effects, however, still have a dose dependent response at 1-5 μM concentrations of the drug at which the sigma-2 receptors should by far be saturated by the drug. This raises the question whether there is another target behind the observed actions of the drug. In study II siramesine was found to associate tightly with phospholipid membranes. It was found to form stoichiometric complexes with anionic lipids of which especially the interaction with phosphatidic acid containing membranes was avid. The partition coefficient for PA containing membranes was found to be of an order of magnitude larger for this interaction as compared to the other studied lipid compositions, roughly corresponding to a dissociation constant of 0.23 μM. The strong interaction with PA may relate to the small head-group size of the lipid, allowing for tight association in the head-group region. PA has also been suggested to be deprotonated into a divalent anion, especially when hydrogen bonded (Kooijman et al. 2005; Kooijman et al. 2007).

Considering the role of PA as a second messenger in cells the observed binding poses the question of the potential biological relevance of this interaction. Several proliferative signaling pathways such as Raf, Ras, Sos, and mTor require PA for activity, more specifically crucial proteins need PA to bind to membranes in order to gain activity. The formation of a SRM:PA complex might interfere with this binding, thus leading to detachment, and inactivity of these proteins (Fig. 16). This provides an intriguing, simple molecular mechanism, possibly underlying the observed anti-cancer activity of this drug. This is the first example in which a small molecular drug compound is suggested to exert its effects via specific drug lipid complex formation, and opens a new paradigm in the search for anti-cancer drugs. Importantly, in the case of siramesine the interaction of the drug with PA is not intentional from the point of view of the drug designer. In the future it might
6 Discussion

Fig. 16 A schematic illustration depicting the role of PA in cellular signaling on the left and on the right the proposed mechanism of action of SRM blocking PA signaling and leading to the down regulation in signaling pathways requiring PA.

It is possible to directly target PA with even more specific small molecule drugs with a further increased affinity for this target.

6.3 Material properties associated with membrane stacking

In lipid research the model system is often chosen according to the desired application. LUVs are commonly employed for fluorescence studies, whereas for DSC, X-Ray diffraction and NMR techniques MLVs are commonly used. This relates at least partly to the idea that LUVs provide a more realistic model of a cellular environment, whereas MLVs produce better quality data on the above named techniques. In biological systems or organisms unilamellar membrane assemblies are common. Some examples, however, stand in which multilamellar membranes exist also in vivo.
6.4 Fluorescent probes as impurities in model membrane systems

such as the myelin sheath of axonal nerve cells. The differences between MLVs and LUVs derive from the different vesicle size but also from the inter-bilayer interactions in a multilamellar system. The transitions for MLVs are more co-operative as seen by DSC. Furthermore DPPC MLVs have a clearly defined rippled phase, whereas in LUVs this cannot be clearly identified by DSC or PPDPC fluorescence. The fluorophore PPDPC was found to segregate into domains for both LUVs and MLVs. For MLVs however, this segregation is much more pronounced, as evidenced by the high collision rate ($I_c/I_m$ values) of the pyrene molecules in gel phase liposomes. The fluorescence data also reveals a clear pretransition for MLVs, after which the collision rate of pyrene is decreasing due to the liberty to redistribute along the formed membrane ripples upon heating. This behavior is missing from LUVs as reflected by a continuous increase in $I_c/I_m$ up to $T_m$. Interlamellar interactions are evident even in fluid phase vesicles, where MLVs have a lower collision rate of pyrene molecules as compared to LUVs. This was hypothesized to result from slower lateral diffusion within the membrane leaflets and could also be confirmed using DPH fluorescence as an indicator.

6.4 Fluorescent probes as impurities in model membrane systems

The fluorescent probes PPDPC and DPPN were used in this study to assess drug binding and the lateral organization of liposomes. Incorporating fluorescently labeled lipid analogs into an otherwise pure membrane system allows for the use of very sensitive techniques to monitor small changes in the organization and dynamics of the system. The drawback is of course that incorporating fluorophores or other substances represent a substitutional impurity in the system under study and the effects seen might be due to the probe and not due to real effects on the lipid dynamics. For example the fluorescent probe might affect drug binding, thus affecting the absolute values of $K_p$. These types of effects can usually be neglected if just comparing different membrane assemblies, keeping the probe concentration constant. When the results are compared to results obtained by other methods, where the fluorescent probes are not needed they must be accounted for by separating the probe effect on the system by systematically going through various concentrations.
6.5 Counter-ions in the self assembly of cationic lipids

In the presence of a sufficient amount of NaCl the cationic gemini surfactant M-1 was found to assemble into microscopic vesicles rather than forming micelles. Screening of the cationic charge has been previously associated with the loss of interdigitation in DHAB vesicles (Ryhänen et al. 2005). Cationic gemini surfactants have a relatively large effective head-group due to the two cationic charges at the opposing ends of the molecules. Electrostatic repulsion between these tends to expand the molecule, thus favoring the formation of micellar structures. A possible reasoning for the observed transition into low curvature giant vesicles with bilayer structure is the effective screening of the cationic charges by the chloride counter-ions. This leads to a dramatic change in the molecular shape since the charges that are responsible for the effective size are screened, thus allowing for tighter packing of the molecules. The fixed distance of the two cationic charges on the gemini surfactant also provide a nucleation center for the Cl⁻-ions followed by an adjacent layer of Na⁺-ions forming a commensurate pseudocrystalline counter-ion lattice. Such a lattice is planar in shape and thus further favors the formation of GVs as compare to membrane vesicles with larger absolute curvature.

Temperature and ionic strength induced changes in the self assembly of liposomes are especially interesting as such factors could be relatively easily modified in tissues for example when targeting nanoparticles for e.g. drug delivery to tumors. Such applications could involve the use of focused ultrasound to locally increase the temperature of the target and by such manipulation force the nanoparticle to release its cargo in a controlled manner.

6.6 Conclusions

To conclude lipid membranes are highly diverse cellular components with a number of parameters regulating cellular function as well as affecting drug binding and distribution in an organism. Understanding the role of phospholipid membranes in cells requires thorough biophysical characterization of simple model systems in order to expand our view to more complex living systems. In this thesis I address some of these properties with special focus on lipid phase behavior and drug effects on membranes.
In the first part a fluorescence based method for determining the association of various compounds to phospholipid membranes was developed. This method was employed in the original publication II where a small molecule drug compound was found to interact specifically with phosphatidic acid. This counters the original hypothesis of there being a non-specific membrane disturbing effect of this drug underlying its observed apoptosis induction in cancer cell lines. The interaction with PA offers an alternative interpretation of the anti-cancer action of the drug, namely disturbing the physiological function of PA as a growth promoting second messenger. Further studies with structurally different compounds specifically designed to interact with PA would be of great interest in furthering our understanding of lipid second messengers as potential drug targets.

In the original publications III and IV the self-assembly of lipids was addressed. In III the phase behavior of a binary lipid mixture was studied with special focus on the effect of the lamellarity of the lipid vesicles. Inter-lamellar interactions have a strong influence on the behavior of the commonly used membrane models LUVs and MLVs. This needs to be considered when interpreting future experiments using these models. Also the incorporation of a pyrene labeled lipid analog was found to affect the behavior of especially MLVs below Tm.

In the final part (IV) cationic gemini surfactant vesicles reacted strongly to changes in the ionic strength of the solvent spontaneously forming giant vesicles. Such transitions, triggered by environmental changes are of interest in e.g. the design of functional nanoparticles aiming for specific targets and being able to release their cargo in a controlled manner.
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