Nanosized Bilayer Disks as Model Membranes for Interaction Studies

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Dissertation presented at Uppsala University to be publicly examined in B22, BMC, Husargatan 3, Uppsala, Friday, March 28, 2008 at 10:15 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract

PEG-lipid stabilized bilayer disks have been found in lipid mixtures containing polyethylene glycol (PEG)-lipids where the combination of a high bending rigidity and low PEG-lipid/lipid miscibility favours disk formation. The disks are planar and circular in shape and their long-term stability is excellent. Theoretical calculations and experimental observations suggest that the micelle forming PEG-lipid are situated at the rim of the aggregate, protecting the hydrophobic lipid chains in the bulk of the aggregate from contact with water. This thesis deals with fundamental aspects concerning the lipid distribution in the disks, as well as with development, optimization, and initial evaluation of the disks as model membranes in partition and interaction studies.

Small angle neutron scattering was used to study the partial segregation of components within the bilayer disk. The experiments verified that the PEG-lipids segregate and accumulate at the bilayer disk rim. The proof of component segregation is important from a fundamental point of view and useful, as exemplified in the below-mentioned study of melittin-lipid interaction, when interpreting partition or binding data obtained from studies based on bilayer disks.

Today liposomes are often used as model membranes in partition and interaction studies. Using liposomes to predict, e.g., drug partitioning can however have certain drawbacks. In this thesis the disks were proven to be attractive alternatives to liposomes as model membranes in partition studies. The formation of bilayer disks by a technique based on detergent depletion enabled incorporation of a transmembrane protein in the bilayer disks and opened up for the use of disks as model membranes in membrane protein studies. Further, bilayer disks were used in a comparative study focused on the effect of aggregate curvature on the binding of the peptide melittin. Various techniques were used to perform initial evaluations of the bilayer disks as model membranes. Of these, capillary electrophoresis and biosensor-based technology had not been used before in combination with bilayer disks.

Keywords: Disk, disc, lipid bilayer, PEG-lipid, model membrane, interaction, partitioning, drug, liposome, cryo-TEM, neutron scattering, capillary electrophoresis, immobilization, biosensor, membrane protein, melittin

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ISSN 1651-6214
urn:nbn:se:uu:diva-8495 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-8495)
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Melittin-lipid interaction: A comparative study using liposomes, micelles and bilayer disks
Anna Lundquist, Per Wessman, Adrian R. Rennie, Katarina Edwards
Submitted

II Nanosized bilayer disks: Attractive model membranes for drug partition studies
Emma Johansson, Anna Lundquist, Shusheng Zuo, Katarina Edwards

III Evaluation of bilayer disks as plant cell membrane models in partition studies
Elisabet Boija, Anna Lundquist, Katarina Edwards, Gunnar Johansson

IV Bilayer disk capillary electrophoresis (BDCE): a novel method to study drug partitioning into membranes
Elisabet Boija*, Anna Lundquist*, Mikael Nilsson*, Katarina Edwards, Roland Isaksson, Gunnar Johansson
(*) First authorship is shared by these authors
Accepted for publication in Electrophoresis

V Initial evaluation of biotinylated bilayer disks as model membranes for biosensor analyses
Anna Lundquist, Sören B. Hansen, Helena Nordström, U. Helena Danielson, Katarina Edwards
Manuscript

Reprints were made with permission from the publishers.
Papers not included in this thesis:

**Interactions of drugs and an oligonucleotide with charged membranes analyzed by immobilized liposome chromatography**
Anna Lundquist, Caroline Engvall, Elisabet Boija, Sanela Kurtovic, Jyoti Chattopadhyaya, Christine Lagerquist Hägglund, Per Lundahl
*Biomed Chromatogr. 2006 Jan;20(1):83-7*

**Effects of ions and detergents in drug partition chromatography on liposomes**
Elisabet Boija, Anna Lundquist, Juan José Martínez Pla, Caroline Engvall, Per Lundahl
*J Chromatogr A. 2004 Mar 19;1030(1-2):273-8*
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1. Introduction

Cell membranes are essential to life. All living organisms are built up by cells and all living cells have a cell membrane. The cell membrane turns each cell into a separate unit, a separate little factory. The membrane offers mechanical support to the cell, it restricts the movement of compounds across the membrane and also actively controls important processes within the cell. The membrane is mainly composed of polar lipids and proteins. The tendency of the polar lipids to self-associate into bilayers enables the formation of a cell membrane. The bilayer composition varies from cell to cell.

As can be imagined, there are many reasons for studying the properties of the cell membrane and the processes that takes place in, or at the surface, of the membrane. To be able to do so, artificial membranes need in many cases to be used in the laboratories as models of the biological membranes. There are many models to choose from but it is difficult, or perhaps impossible, to find a perfect model for all purposes. The work presented in this thesis deals with the fundamentals, the formation and the initial evaluation of a novel type of model membrane, the PEG-lipid stabilized bilayer disk. As will be shown in the following, the bilayer disks have properties that make them very interesting and sometimes superior to other model membranes, for use in a number of applications.

1.1 Amphiphilic molecules and self-assembly

Molecules that posses both hydrophobic and hydrophilic properties are defined as amphiphilic molecules. In aqueous solutions, amphiphilic molecules tend to self-associate since their hydrophobic and hydrophilic portions have different tendencies to mix with water. The molecules arrange so that the hydrophobic residues are screened from the surrounding water as exemplified in Figure 1.1. The aggregation behaviour is dependent on the molecular structure, the concentration of amphiphilic molecules and on environmental parameters, such as temperature. Although the amphiphilic molecules become more organized, the aggregation is actually entropy driven. Upon aggregation the hydrophobic domains are shielded from contact with the water molecules. In the absence of aggregation, the water molecules in the near proximity of the hydrophobic residues would instead have to arrange themselves into a highly ordered structure. The self-association of the amphiphilic molecules therefore leads to less ordered water structure and increases the entropy of the system as a whole [1, 2].
Polar lipids are a class of amphiphilic molecules that are abundant in nature. The amphiphilic character of these lipids enables the formation of various types of self-associated structures. Lipid bilayers represent an interesting and biologically important class of lipid aggregates. In cell membranes, for example, bilayers constitute an essential barrier that separates the inner volume of the cell from the surrounding environment. The polar lipids can, apart from forming bilayers, aggregate into a variety of micellar and other types of structures. The type of structure formed depends on the inherent properties of the lipids, such as the length and degree of saturation of potential hydrocarbon chains as well as on conditions such as lipid concentration, temperature and pH. Furthermore, when multiple lipid components participate in the aggregate, their summed characteristics determine the outcome. It is therefore difficult to predict the aggregate structure. Some direction can, however, be obtained by considering the geometrical shape and calculating the so-called packing parameter for the individual components [3, 4].

The packing parameter gives a guideline for the ability of the molecules to pack together and form a certain aggregate structure (Figure 1.1). The packing parameter $N_s$ is defined as the actual volume $v$ taken up by the hydrophobic chain or chains, divided by the product of the optimal headgroup area $a_0$ and the maximum effective length $l_c$ that the hydrophobic chain or chains can assume.

$$N_s = \frac{v}{a_0 \cdot l_c}$$

Lipids with $N_s \approx 1$ (cylindrically shaped) tend to form bilayers but if the packing parameter deviates from 1, formation of a curved structure will be preferred [3]. The lysolipids for example, used in Paper I, have only one hydrocarbon chain and tend to form micellar structures. Similarly, the PEG-lipids used in Papers I–V have large headgroups and tend to self-aggregate into micellar structures [5].

1.2 Phospholipid bilayers

Phospholipids are a type of lipid abundant in the mammalian cell membrane and four major classes of phospholipids are commonly found in these membranes: phosphatidylcholine, sphingomyelin, phosphatidylserine and phosphatidylethanolamine. Other cell membranes such as plant cell membranes are also rich in phospholipids. In addition to lipids, all cell membranes include a large amount of membrane proteins [6].

Phospholipids are composed of fatty acid chains of varying length linked via ester bonds to a glycerol backbone. A phosphate group, which constitutes a part of the hydrophilic headgroup of the lipid, is also connected to the backbone. The molecular structures of two phospholipids central to this thesis are shown in Figure 1.2.
The bilayer structure is found in a variety of phases. Transitions between the phases occur at certain compositions and transition temperatures [7]. Two phases, which are central to this thesis, are the lamellar gel and the lamellar liquid crystalline phase. In the gel phase, which exists at lower temperatures, the bilayer has a high conformational order and a solid-like appearance. Increasing the temperature, eventually results in a liquid crystalline phase in which the molecular ordering of the lipids is decreased. The lipids in the liquid crystalline phase can move laterally whereas such movements are significantly slowed down in the gel phase [8].

Apart from phospholipids and proteins, the mammalian and the plant cell membranes include sterols (20-50 mol%) [6]. Sterols are lipids that have a small polar headgroup attached to a steroid ring structure. Cholesterol is the type of sterol found in mammalian cell membranes whereas stigmasterol and sitosterol are typically found in plant cell membranes together with campesterol. Molecular structures of the sterols used in this thesis are shown in Figure 1.3. When cholesterol is mixed with phospholipids, the hydroxyl group of
cholesterol positions itself closely to the ester bonds and the steroid part of cholesterol reaches into the hydrophobic core of the membrane [9].

Sterols mainly affect the fluidity of the bilayer if added in sufficient amounts. The degree of order that cholesterol brings to the bilayer depends on the fluidity of the membrane before the addition of cholesterol. In the gel phase, inclusion of cholesterol in the bilayer disturbs the lipid packing and the fluidity of the bilayer increases. On the other hand, cholesterol brings order to phospholipid bilayers in the liquid crystalline phase and the fluidity decreases [10, 11]. High concentrations of cholesterol, typically 30 mol%, may then actually induce a new phase, often called the liquid ordered phase [12, 13].

In the liquid crystalline phase, the fluid character of the lipid bilayer allows it to stretch and bend. The energy needed to bend the membrane is dependent on the bending modulus. The area expansion modulus represents the energy required to stretch an area in the directions of its plane. Bilayers in the gel phase have an increased bending modulus compared with membranes in the liquid crystalline phase. When added to membranes in a liquid crystalline phase, cholesterol increases both the area expansion and the bending modulus [14–16]. The energy cost of bending and stretching the bilayer has important effects on the type of structure formed as discussed below for the liposomes and bilayer disks.

1.2.1 Liposomes

When a slab of a lipid bilayer is dispersed in an aqueous solution, the hydrophobic hydrocarbon chains at the fringes of the slab are exposed to the surrounding water molecules. The exposure brings about an edge-tension energy which is dependent on the exposed fringe area. This energy drives the sys-
tem to reduce the exposed area by bending the bilayer. Eventually the bending results in the formation of a liposome were the bilayer is completely closed (Figure 1.4). There is however an energy cost involved in the bending of the bilayer. The bending energy is dependent on the lipid bilayer area and mechanical properties of the bilayer. The bending modulus is in this context very important [17]. Since bilayers in the gel phase state, as well as cholesterol containing bilayers, have large bending moduli, the bending of these bilayers involves a large bending energy. The balance between the edge-tension energy and the bending energy determines how energy favourable it is to close the bilayer into a liposome [18]. In the gel phase, bilayers will not close to form liposomes for this reason.

Once the bilayer has closed, the liposome can be utilized for a number of applications. The use of liposomes as drug delivery vehicles was initially investigated in the seventies [19]. The idea is to insert a drug in the bilayer or in the inner aqueous compartment of the liposome. The composition of the bilayer can be varied to optimize the drug retention in the body. Inclusion of polyethylene glycol (PEG) conjugated lipids (Figure 1.5), can further improve the stability of the drug delivery vehicles and liposomes in general[20].

The presence of a PEG layer on the liposome surface hinders the close approach of large particles, such as other liposomes or macromolecules. The protective effect originates from an entropic as well as an osmotic contribution [21–23]. Upon compression of the polymers, there is a loss in PEG conformational freedom. The osmotic pressure contribution arises from the increase in polymer concentration on compressing the layers. The protective effect increases with increasing thickness and density of the PEG layer [21] but, as
discussed below, the phase propensity of the PEG-lipids limits the amount that can be included in the liposome bilayer.

1.2.2 The PEG-lipid stabilized bilayer disk

PEG-lipids (small $N_j$) are micelle-forming lipids and have therefore limited solubility in phospholipid bilayers. When added in concentrations above the solubility limit, PEG-lipids tend to induce the formation of mixed micelles [5, 24]. In a lipid mixture, there is an entropic driving force for all of the lipid components to mix uniformly and based on this argument spherical or thread-like micelles with homogenous composition should form. Formation of such micelles does however require bending of the lipid monolayer. As a consequence lipid compositions that give rise to rigid bilayers with large bending moduli tend to form disks, rather than thread-like micelles [24, 25]. The free energy cost of bending a monolayer composed of these lipids into a highly cylindrical micelle is too large. Instead, the PEG-lipids segregate and form a highly curved monolayer at the rim of the disk-shaped micelles. Since the disks typically have a diameter corresponding to 30 nm or more, they are best described as PEG-lipid stabilized bilayer disks. Disk-shaped aggregates have also been predicted and observed in other lipid systems [26–28] as well as systems involving peptides and proteins [29–31]. A schematic figure of the PEG-lipid stabilized bilayer disks is shown in Figure 1.6.
1.3 Partitioning of molecules

In the living cell, the cell membrane constitutes an essential barrier which controls the passage of molecules into as well as out of the cell. Lipid bilayers also form compartments within the cell which enables the cell to manage vital processes. For a drug to be potent it needs, in most cases, to pass at least one cell membrane to reach the designated target. Transport across the cell membrane can be either active or passive. Active transport is mediated by membrane proteins at a cost in energy. Passive transport can either occur by simple diffusion through the membrane or by facilitated diffusion involving proteins. Drug uptake is often governed by simple diffusion and this passive transport is therefore studied to predict drug uptake.

Passive transport is driven by the diffusion occurring when the chemical potential (molar Gibbs energy) of a substance outside the cell differs from the chemical potential inside the cell. Equilibrium is obtained when the chemical potential on the two sides equals one another. The diffusion flux or mass transport per area $J$ (mol m$^{-2}$ s$^{-1}$) from a high concentration region to a low concentration region is defined in Fick’s first law as the product between the diffusion coefficient $D$ (m$^2$ s$^{-1}$) and the concentration gradient $\frac{\partial c}{\partial x}$.
\[ J = -D \frac{\partial c}{\partial x} \]

The diffusion in a membrane is assumed to be linear and the following expression can be derived, where \( l \) is the thickness of the membrane.

\[ J = -\frac{D}{l} \Delta c \]

Hence, the flux of a substance in the membrane is dependent on the concentration difference in the membrane, the thickness of the membrane and the diffusion coefficient within the membrane. The diffusion coefficient varies depending on the temperature, as well as on the size, shape and charge of the substance.

It should be noted that if the solubility of the diffusing substance is different in the aqueous compared with the membrane phase, a factor \( K \), called the partition coefficient, must be added to the equation. \( K \) is simply the ratio of the concentration in the membrane and the concentration in the aqueous phase, given by the difference in standard chemical potential for the substance in the membrane versus aqueous environment.

\[ K = \frac{c_{mem}}{c_{aq}} \]

Introducing \( K \) in the flux equation results in the following expression:

\[ J = \frac{D K}{l} \Delta c_{over} \]

where \( \Delta c_{over} \) is the concentration difference over, instead of in, the membrane. In the above equation, the interior of the membranes is assumed to be homogeneous and the movement in and out of the membrane is assumed not to be a rate-limiting factor [32].

A permeability coefficient \( P \) (m s\(^{-1}\)) is defined as:

\[ P = \frac{D K}{l} \]
If \( l \) is kept constant, the permeability will depend on the diffusion coefficient and partition coefficient. Since the diffusion coefficient is largely governed by the size and shape of the diffusing species, the partition coefficient will reflect the permeability for molecules of comparable size and structure. As a consequence, determination of the partition coefficient gives a measure of the mass transport and can be used to predict the cellular uptake of a substance [32]. A variety of model systems, some of which are discussed below, are used in studies designed to determine the partition coefficient.

1.3.1 Model systems mimicking the cell membrane

The octanol/water system is a crude but still widely used model system for determination of the partitioning of molecules into lipid bilayers [32, 33]. The partitioning of a substance is measured by its solubility in a hydrophobic octanol phase versus its solubility in a water phase. A \( \log P_{oct} \) value is obtained by this method and is defined as the relationship between the drug concentration in the octanol phase and the drug concentration in the water phase. \( \log P_{oct} \) measurements are performed at the pH where the drug has zero net charge. If the solubility is determined at another pH of choice the coefficient is denoted \( \log D_{oct} \). The crudeness of the octanol/water methods originates from the fact that octanol only offers a reasonable model for the hydrophobic region of the membrane. The characteristics of the hydrophilic headgroup region of the membrane are completely disregarded.

It is possible to employ model systems that are more similar to cell membranes. Partition studies can be performed using a lipid monolayer coupled to a surface [34–36]. However, the lipids need to interact with the surface which can affect the packing of the lipids in the monolayer. Lipid bilayers have been used in various techniques for the determination of partitioning coefficients. Liposomes have for example been used in capillary electrophoresis, biosensor-based studies and chromatographic techniques such as drug partition chromatography (DPC) [37–41]. However, as discussed in Section 3.3 there are certain drawbacks associated with the use of liposomes as model membranes which can be overcome by the use of bilayer disks. The use of bilayer disks as model membranes was first investigated by Johansson et al. [42] and partition measurements with bilayer disks as model membranes are key experiments in Papers II, III and IV.
2. Experimental Techniques

2.1 Cryo-TEM imaging

Cryo-transmission electron microscopy (cryo-TEM) is a valuable technique for characterizing lipid aggregates in aqueous solutions [43] and the technique is used in Papers I–V. Cryo-TEM involves direct visualization of a lipid sample of dilute lipid concentration. There is no need to label or stain the aggregates. Instead, the sample is vitrified and kept at low temperatures during the investigation in the electron microscope.

Electron dense material scatters the electron beam to a large extent. The difference in electron density of the lipid aggregates and the vitrified water enables the visualization of the lipid aggregate structures. Lipid aggregates between 4 nm and 500 nm in size can be viewed by cryo-TEM. The lower limit of 4 nm is due to contrast limitations whereas the upper limit of 500 nm is due to the intensified scattering from water when the sample is thick.

When a sample is prepared for cryo-TEM imaging, it is applied as a thin film on a polymer-coated copper mesh. The procedure is performed in a temperature and humidity controlled chamber, and the sample-covered mesh is rapidly cooled in liquid ethane. The rapid cooling prevents the formation of ice crystals, which would otherwise disrupt the aggregate structures. The vitrified sample spanning over the holes in the polymer film is 10–500 nm thick; the thinnest regions are naturally found at the centre of the holes.

The frozen grid is inserted into the microscope and exposed to the electron beam. During cryo-TEM imaging, 2D images or micrographs are obtained (Figure 2.1). Interpretation of cryo-TEM images requires knowledge of possible artefacts which can appear on the micrograph. Variation in the film thickness can for example result in a sorting of the aggregates by size. By viewing a large number of micrographs, it is still possible to determine the types of aggregate structures present in the sample and estimate the average aggregate size.

A good example of the usefulness of cryo-TEM is given in experiments underlying the study in Paper I, where the peptide melittin was found to affect the structure of PEG-ylated liposomes. Melittin disrupted the liposomes and bilayer sheets were formed. Cryo-TEM imaging provided a simple characterization of these structural changes (Figure 2.2).
2.2 Dynamic light scattering

Dynamic light scattering was performed in Papers I and II to determine lipid aggregate sizes. Particles in a solution scatter light and during dynamic light scattering the intensity of the scattered light is measured over time. The intensity fluctuates as the lipid aggregates move randomly in the solution. The change in intensity as a function of time is expressed in an autocorrelation function. The autocorrelation function can be numerically fitted to an exponential expression or series of exponential expressions and used to determine the diffusion coefficient [44]. The diffusion coefficient can then be employed to calculate the hydrodynamic radius $R_h$ of the particle through the Stokes-Einstein equation:

$$D = \frac{k_B T}{6\pi \eta R_h}$$

where $\eta$ is the viscosity of the liquid, $k_B$ the Boltzmann constant and $T$ the temperature. The size, shape and number of the particles affect the scattering. The Stokes-Einstein equation assumes that the particles are spherical. In Papers I–V the aggregates were, as verified by cryo-TEM imaging, disk shaped.
Figure 2.2: Cryo-TEM micrographs showing the lipid aggregate structure (POPC/cholesterol/ceramide-PEG5000, 56:40:4 mol%) a) before and b) after addition of melittin. Scale bars indicate 100 nm.

The model proposed by Mazer et al. was therefore used to determine the radii of disk shaped aggregates, $R_{\text{disk}}$ [45].

$$R_h = \frac{3R_{\text{disk}}}{2} \left\{ \left[1 + \alpha^2\right]^{1/2} + \frac{1}{\alpha} \ln \left[ \alpha + \left[1 + \alpha^2\right]^{1/2} \right] - \alpha \right\}^{-1}$$

$$\alpha = \frac{L_{\text{disk}}}{2R_{\text{disk}}}$$

$L_{\text{disk}}$ is the thickness of the disk.

2.3 Small angle neutron scattering

Small angle neutron scattering experiments were carried out in Paper I to obtain information regarding component distribution within the PEG-lipid stabilized bilayer disks. Since the wavelength of the neutron beam (6 Å) is comparable to the molecule and aggregate size, it can be utilized to determine molecular dimensions and hence the distribution of various lipids within the PEG-lipid stabilized bilayer disks.

Neutrons are scattered by atomic nuclei. Hydrogen and deuterium for example have dissimilar scattering densities and scatter neutrons to different extents. When the incoming neutron beam is scattered, its scattered intensity will vary with the scattering angle in a way that depends on the distribution of different scattering nuclei. This angle dependence on intensity is expressed as a momentum transfer vector $Q$, i.e. the magnitude of a vector which describes the position at which neutrons (scattered at an angle $\theta$) reach the detector in relation to non-scattered neutrons.

$$Q = \frac{4\pi}{\lambda} \sin \left( \frac{\theta}{2} \right)$$
Scattering from different scattering centres will cause interference and may be constructive or not depending on their separation. Information regarding aggregate structure or internal structure in this case can therefore be obtained from plotting the total scattering cross section per unit volume \( I(Q) \) (cm\(^{-1}\)) versus \( Q \) (Å\(^{-1}\)). The total scattering cross section is defined as the total number of neutrons scattered per second and per unit solid angle, normalized by the number of incident neutrons per unit area.

In a multi-component aggregate, a hydrogenous component can be exchanged for its deuterated analogue as in Paper I. The contrast of the solvent can be matched with the contrast of regions rich in deuterated material, often done by addition of D\(_2\)O in the buffer (solvent can of course also be mixed to match the hydrogenous rich regions). The \( I(Q) \) versus \( Q \) curve will then display scattering only from regions composed of hydrogenous species, since these give rise to a contrast. Fitting these curves to models gives an estimate of the size and structure of the hydrogenous region of the aggregate.

### 2.4 Peptide binding to lipid aggregates determined by fluorescence measurements

In proteins and peptides, aromatic amino acids function as intrinsic fluorophores. In a pure aqueous solution, the aromatic groups of tryptophan, tyrosine and phenylalanine absorb light at 280 nm and emit light with an emission maximum of 350 nm. The emission spectra is however sensitive to solvent polarity. If the aromatic residue is buried in a hydrophobic environment, the emission spectra will be shifted towards shorter wavelengths. This shift in emission spectra was measured in Paper I to analyze the binding of the peptide melittin, which contains only one aromatic amino acid, to lipid aggregates. During the experiment, small amounts of lipid aggregates were added to a melittin solution. The shift was quantified by the ratio 325/360 nm in fluorescence intensity for the emission spectra. Thereafter the shift was used to determine the fraction of melittin associated to the lipid aggregates and the binding isotherm was obtained [46].

### 2.5 Drug partition chromatography

Drug partition measurements were performed by drug partitioning chromatography (DPC) [41, 47] in Papers II and III. Bilayer disk or liposome samples, with lipid concentrations of 30-70 mM, were added to dry Sephadex 200 pg beads. The lipid sample is drawn into the many cavities of these beads as the gel swells and the lipid aggregates are sterically immobilized. The rehydrated beads are thereafter packed into a column and buffer is continuously pumped through the system (flow rate 0.5–1 ml/min). To determine the partition co-
efficient, the analyte is dissolved in buffer, injected into the buffer flow and allowed to pass through the column. The volume of buffer needed to pass the system in order to elute the analyte from the column $V_e$ is measured by the UV detector mounted at the column outlet. This elution volume is compared with the void volume $V_0$ needed for elution of dichromate ($\text{Cr}_2\text{O}_7^{2-}$) since dichromate is not considered to interact with the lipid material. The capacity factor $K_s$ ($\text{M}^{-1}$), related to the unitless partition coefficient $K$, is defined as:

$$K_s = \frac{V_e - V_0}{A}$$

$$K = \frac{K_s}{V_{app}}$$

$A$ is the amount of immobilized lipid. $V_{app}$ (M$^{-1}$) is the apparent molar volume [48]. $V_{app}$ is estimated to be 0.6 M$^{-1}$ for the bilayer disks used in Papers II and III.

### 2.6 Bilayer disk capillary electrophoresis

In capillary electrophoresis an electric potential is applied over a thin capillary. Ions and charged molecules within the capillary will migrate due to the applied electrical field. The migration velocity is proportional to the electrical field strength and the charge of the ions as well as inversely proportional to frictional factors. The frictional factors are determined by the size and shape of the ion and the viscosity of the medium.

The bulk flow of liquid through the capillary is called the electroosmotic flow (EOF). The EOF is enabled due to the formation of an electrical double layer, composed of one rigid layer of ions in close contact with the capillary surface and one more diffuse outer-layer. The potential of the electrical double layer decreases exponentially with increasing distance from the surface. The ions in the diffuse layer will be able to migrate in the capillary and since the ions are hydrated, a net flow of solution will pass through the capillary. Thanks to the EOF, uncharged substances will migrate through the capillary.

In Paper IV, the use of PEG-stabilized bilayer disks in capillary electrophoresis was explored. Bilayer disks were introduced as a pseudo-stationary phase in polyvinylpyrrolidine (PVP) coated fused silica capillaries. Liposomes have previously been used as pseudo-stationary phases in capillary electrophoresis [49]. Further, Lindén et al. recently published results where PEG-stabilized lipid aggregates were used as coatings and carriers in capillary electrophoresis [50].

The PVP coating is performed to mask the negative charges of the silanol groups (SiO$^-$) of the capillary wall. The analyte interaction with the capillary wall is then minimized and the sensitivity and reproducibility of the system are enhanced [51]. The coating also reduces the migration of the pseudo-stationary phase.
The capillary electrophoresis system from Agilent Technologies was used for the capillary electrophoresis study. Disks were hydrodynamically introduced by applying pressure to the sample vial at the injection end of the capillary. The introduction of disks in the capillary was stopped before the disks reached the detection window. The sample solution was then introduced and the electrical potential was applied. The migration time of the drug is affected by its partitioning into the bilayer disks and an expression for the partitioning coefficient $K$ can hence be derived [52]:

$$K = \frac{\pi r^2 l}{t_0} \frac{d\Delta t}{dV_{lip}}$$

where $r$ is the inner radius of the capillary, $l$ the capillary length measured from the inlet to the detection window and $t_0$ the retention time spent in a capillary in which the pseudo-stationary phase is excluded. $d\Delta t/dV_{lip}$ is the change in retention time related to the change in total volume of the lipid phase ($V_{lip}$) in the capillary. $V_{lip}$ is calculated by the equation:

$$V_{lip} = C_{lip} V_{inj} V_{app}$$

where the $C_{lip}$ is the molar lipid concentration, $V_{inj}$ the injected lipid sample volume and $V_{app}$ the apparent molar lipid volume.

### 2.7 Surface plasmon resonance based biosensors, immobilization of bilayer disks

Surface plasmon resonance (SPR) is an optical phenomenon that can be used for biosensing purposes [53, 54]. Valuable kinetic constants and equilibrium constants for an interaction taking place at the chip surface can be obtained without the need for labelling of the interacting molecules. A wide range of molecules, ranging from drugs with relatively small molecular weights to larger macromolecules such as proteins can be analyzed [55]. SPR-based analysis can be used in studies involving lipid monolayers, continuous bilayers or liposomes [56, 57]. The potential use of bilayer disks in SPR-based biosensor analyses was initially evaluated in experiments in Paper V.

Lipid bilayers have been immobilized on the sensor surface via streptavidin-biotin linkage [58–60]. In Paper V, disks containing DSPE-PEG$_{2000}$-biotin were produced and allowed to immobilize on sensor surfaces via streptavidin-biotin linkage. Streptavidin was covalently bonded via amide bonding to the carboxymethylated (CM) dextran matrix [61, 62] on the Biacore CM series sensor chips prior to disk immobilization. The Biacore 2000 (GE Healthcare/Biacore) was used for the biosensor analyses and the temperature was maintained at 25.0°C. Buffer is passed continuously over the sensor surface with the immobilized bilayer disks and analytes are...
injected for analysis. If the analyte interacts with the macromolecules on the sensor surface, a signal change in resonance units (RU) is registered.
3. Results and discussion

The Papers included in this thesis focus on the development and initial evaluation of PEG-lipid stabilized bilayer disks as model membranes in partition and interaction studies. A second important aim has been to obtain a more detailed understanding of the structure and component distribution in the disks. The latter is important not only from a fundamental point of view but also essential in order to exploit the full potential of the disks for analytical and pharmaceutical applications.

3.1 Component distribution in PEG-lipid stabilized bilayer disks

Bilayer disks can be considered as a type of intermediate structure between micelles and liposomes. Characterization of the component distribution in bilayer disks is important to fully understand the fundamentals of disk formation as well as to understand features that promote or inhibit interactions between proteins, peptides or drug molecules and the disks. Previous studies indicate that bilayer disk formation is accompanied by a partial segregation of the PEG-lipids from the remaining components [63, 64]. Direct proof of this behaviour has so far been lacking and the neutron scattering experiments reported in this section were performed with the aim of obtaining experimental evidence of component segregation in the disks.

3.1.1 Characterizing the rim of the bilayer disk

In Paper I, small angle neutron scattering (SANS) was used to characterize disks containing deuterated DSPC and hydrogenous PEG-lipids. The lipid distribution within the disk can be investigated since the deuterated and hydrogenous components scatter neutrons to different extents. SANS data was collected for bilayer disks (DSPC/DSPE-PEG$_{5000}$) at different D$_2$O contrasts (0, 50, 75 and 95% D$_2$O) (Figure 3.1). Peaks in the scattering intensity at approximately 0.035 Å$^{-1}$ were clearly observed at the D$_2$O contrasts closest to the contrast match (50 and 75% D$_2$O). Such peaks can only be caused by a segregation of material with different scattering densities. The scattering data could be well fitted to a model comprised of a cylindrical core (with a height corresponding to the thickness of the bilayer part of a disk) surrounded by a cylindrical corona of material with a lower scattering density (Figure 3.2).
Figure 3.1: The intensity of the scattered neutron beam as a function of the momentum transfer vector $Q$ for DSPC/DSPE-PEG$_{5000}$ (80:20), 0% (■), 50% (○), 75% (▲) and 95% (▲) D$_2$O (to the left). Cryo-TEM image of DSPC/DSPE-PEG$_{5000}$ sample (to the right). Scale bar indicates 100 nm.

Figure 3.2: The scattering patterns for DSPC/DSPE-PEG$_{5000}$ fitted to a discoidal core/shell model (75 % D$_2$O).

Although not as clearly distinct as for the DSPC/DSPE-PEG$_{5000}$ sample, peaks in the scattering intensity were also observed for the DSPC/ceramide-PEG$_{5000}$, DSPC/cholesterol/DSPE-PEG$_{5000}$ and DSPC/cholesterol/ceramide-PEG$_{5000}$ disks (see Paper I). The reason for the less distinct peaks is likely to be a result of size polydispersity in the aggregates and liposome contamination. Inclusion of cholesterol also adds an extra non-deuterated component to the lipid mixture that complicates the interpretation of the scattering data. Cryo-TEM imaging, dynamic light scattering and neutron scattering experiments show that the DSPC/DSPE-PEG$_{5000}$ sample was clearly the most homogeneous sample. It should be noted that the neutron scattering experiments verified that component segregation is present in all of the four disk types.
3.1.2 Disk/melittin interactions

Melittin has been extensively used as a model peptide in lipid/peptide studies and numerous articles deal with its interaction with lipid bilayers. The physical state of the bilayer is known to affect the melittin binding [65, 66]. Previous studies suggest that melittin has a strong tendency to induce the formation of structures with high positive curvature when added to bilayers in the gel phase and bilayers containing cholesterol [30, 67, 68]. Furthermore, melittin is known to interact not only with lipid bilayers but also with bicelles and surfactant micelles [69–71]. It can be speculated that melittin possesses high affinity for curved surfaces. Bilayer disks were used in Paper I in combination with liposomes and micelles to investigate how the aggregate mean curvature affects the level of melittin binding in POPC/cholesterol/PEG-lipid systems.

Typical cryo-TEM micrographs of POPC/cholesterol samples with varying content of ceramide-PEG_5000 are shown in Figure 3.3. An increased amount of PEG-lipid increased the mean curvature of the lipid aggregate and also increased the binding of melittin as shown by the isotherms. Furthermore, melittin was shown to bind readily to PEG-lipid micelles (Figure 3.3 inset).
The binding of melittin to POPC/cholesterol liposomes supplemented with 0.25 mol% ceramide-PEG$_{5000}$ was, as expected, modest [72]. Cholesterol condenses the area per lipid molecule and the POPC/cholesterol bilayer cannot efficiently accommodate a large amount of melittin [73, 74]. When comparing melittin binding to bilayers of varying compressibility moduli, it was found that melittin partitioning was reduced to bilayers with large bending modulus [66].

The addition of DSPE-PEG to POPC has, on the other hand, been shown to enhance melittin binding [66, 75]. One contributing factor is believed to be favourable electrostatic interactions between the negatively charged DSPE-PEG and the positively charged melittin. As shown in Figure 3.3, melittin binding also increases with the addition of the uncharged ceramide-PEG$_{5000}$. Thus, although the binding of melittin is clearly affected by the presence of charged lipids, the electrostatic interaction cannot alone explain why PEG-lipids promote melittin binding.

The binding of melittin to PEG-lipid micelles, as well as lysolipid micelles, was found to be significantly increased compared with its binding to liposomes and bilayer disks (Figure 3.3). The fact that melittin binds readily to lysolipid micelles suggests that the micellar structure, rather than the presence of PEG, has a positive effect on the melittin binding. Since neutron scattering provided evidence of PEG-lipid segregation in the bilayer disk (see Section 3.1.1), it is reasonable to assume that the rim of the bilayer disk is structurally similar to the surface of a PEG-lipid micelle. The observation that binding to the different types of aggregates increases in the order liposomes < disks < micelles, i.e. in the same order as the aggregate mean curvature increases, suggests that melittin preferentially binds to curved lipid surfaces. The results presented in Paper I stress the importance of characterizing the lipid aggregate structure in order to interpret data correctly from melittin/lipid interaction studies. This is important not least since the addition of micelle-forming PEG-lipid has also been found to affect the binding of other peptides [66, 75]. The fact that large amounts of melittin can be bound to the bilayer disks may in the future prove important for drug delivery purposes.

### 3.2 Development and optimization of bilayer disks for partitioning and interaction studies

The PEG-lipid stabilized bilayer disks were produced by simple hydration of a lipid film in previous investigations at our laboratory [42, 63]. In Paper II, a bilayer disk formation based on a detergent depletion method was introduced. Methods based on detergent removal from lipid/detergent mixtures are often utilized for insertion of transmembrane proteins into lipid bilayers [76]. The formation and insertion of membrane proteins into the disks would enable the use of disks as model membranes in studies involving membrane proteins.
The applicability of the bilayer disks would be improved further when the disk composition can be altered and optimized to mimic that of biological membranes. The possibility of varying the disk composition without introducing negative effects on lipid aggregate structure or stability was investigated in Papers II, III and V. As is discussed later, the potential of the improved bilayer disks as model membranes was evaluated by the use of chromatographic and surface-based techniques.

3.2.1 Disk formation by detergent depletion

In Papers I, II and V bilayer disks were prepared by removing octyl glucoside from a detergent/lipid mixture. A cryo-TEM micrograph of a detergent-depleted sample can be seen in Figure 3.4a. The average disk radius of the detergent-depleted disks is smaller than that of disks produced by hydration (Figure 3.4b). The effect of the preparation method on the disk size has recently been evaluated by Sandström et al. [77]. It should be noted that the preparation path not only affected the size of the bilayer disks but also that the disks produced by detergent depletion were more homogeneous in size. The improved size homogeneity constituted an important reason for the use of detergent-depleted disks (DSPC/cholesterol/PEG-lipid) in the neutron scattering experiments in Paper I.

In Paper II, bacteriorhodopsin, a transmembrane protein, was inserted into the bilayer disks by the detergent depletion method. Amino acid analysis confirmed that bacteriorhodopsin was present in the disk. The use of the detergent depletion method therefore enabled incorporation of a transmembrane protein.

3.2.2 Optimization of disk composition

Disks stabilized by the negatively charged DSPE-PEG$_{5000}$ were used by Johansson et al. in a first study on drug partitioning [42]. The presence of DSPE-PEG$_{5000}$ can however result in unwanted electrostatic interactions with charged
drugs, which affects the interpretation of the partitioning data. In Paper II it was shown that the uncharged ceramide-PEG5000 can be used to stabilize bilayer disks. The samples were, in contrast to samples containing DSPE-PEG5000, quite polydisperse in size and a significant number of liposomes was present in the samples, as exemplified in Figure 3.5. To circumvent this problem, the use of a sonication and centrifugation procedure was applied in Papers II, III and IV. The sonication procedure improved the homogeneity of the samples, probably due to improved mixing of the lipid components. For lipid compositions where large liposomes (diameter > 50 nm) still remained after sonication, dextran containing hydration buffer was added to the lipid film. The sample was then sonicated, diluted with pure buffer and centrifuged in order to sediment large dextran-containing liposomes.

In Paper V, the biotinylated PEG-lipid (DSPE-PEG2000-biotin) was included in the lipid mixtures and disks were produced by hydration or detergent depletion. DSPE-PEG2000-biotin (0.5, 1 or 4 mol%) was included in disks composed of DSPC/cholesterol/DSPE-PEG2000 or DSPC/cholesterol/ceramide-PEG5000 in order to immobilize the disks on streptavidin-treated biosensor surfaces. A typical micrograph of bilayer disks containing biotinylated PEG-lipids is seen in Figure 3.6.

A refinement of the bilayer disk composition with the intent to mimic that of biological membranes was performed in Papers II and III. Disks with a bulk lipid composition similar to that found in porcine brush border membranes were produced (Paper II) as well as disks mimicking plant cell membranes (Paper III). A modification of the bilayer disk composition was also performed in Paper V for the interaction with cyclooxygenase 1 and 2 (Figure 3.7).
3.3 Evaluation of the bilayer disks as model membranes

Partitioning measurements can give an estimate of the likelihood of a component passively passing over a cell membrane. Liposomes have been used extensively for this purpose, as mentioned earlier, but using liposomes can have certain drawbacks. Liposomes consist of a closed bilayer and the amount of lipid initially accessible for an analyte is limited to the outer half of the bilayer. The other half of the lipid leaflet is turned towards the buffer volume enclosed inside the liposome. Moreover, if liposomes are not stabilized they tend to fuse and form multilamellar structures and the available amount of lipid is decreased even further. In the bilayer disks, all of the lipids are directly accessible for interaction. The bilayer disk thus constitutes an attractive alternative to liposomes in partition studies where analytes need to equilibrate quickly in the lipid phases [42].

Throughout this thesis, the use of bilayer disks as model membranes was evaluated. In Papers II, III and IV, bilayer disks of optimized compositions (Section 3.2.2) were used for partition studies. The partition studies were per-
formed by either drug partition chromatography or capillary electrophoresis. Drug partition chromatography previously used by Johansson et al. [42] was employed in Papers II and III in order to study the effect of disk composition on drug and lignin precursor partitioning. In Paper IV, the partitioning of drugs was investigated for the first time by means of capillary electrophoresis. The possibility of using disks as model membranes in studies involving immobilization to sensor surfaces was investigated by a SPR-based sensor study in Paper V.

Neither capillary electrophoresis nor SPR-based studies have been used earlier for studies involving bilayer disks. It is clearly advantageous to be able to optimize the bilayer disk composition and use the disks in a variety of different techniques.

3.3.1 Partition studies carried out with disks of varying compositions

In Paper II, data obtained by drug partition chromatography using ceramide-PEG$_{5000}$ stabilized disks (DSPC/cholesterol/ceramide-PEG$_{5000}$) was compared with data determined with DSPE-PEG$_{5000}$ stabilized disks (DSPC/cholesterol/DSPE-PEG$_{5000}$). Positively charged drugs partitioned more readily into disks that included the negatively charged DSPE-PEG$_{5000}$ and the opposite was observed for negatively charged drugs (Figure 3.8a). The results showed that the partitioning of the charged drugs was clearly affected by the presence of negative charges in the polar headgroup region of the membrane. The partitioning of uncharged drugs on the other hand was not noticeably different in the two disk systems.

Upon comparison of Figure 3.8a and b, partitioning data obtained with ceramide-PEG$_{5000}$ stabilized disks mimicking brush border membranes (BBM) were very similar to data obtained with DSPE-PEG$_{5000}$ stabilized bilayer disks composed of DSPC, cholesterol and DSPE-PEG$_{5000}$. The DSPC/cholesterol/DSPE-PEG$_{5000}$ disks included 15 mol% of the negatively charged lipid, DSPE-PEG$_{5000}$, an amount fairly similar to the amount (approximately 13.5 mol%) of negatively charged lipids present in the disks which mimic BBM. The electrostatics could explain why similar log $K_s$ values were obtained for charged drugs when using the two different types of disks. The log $K_s$ for the uncharged drugs were also very similar when comparing the two disk types. This trend is somewhat surprising since the two different disk systems contain different amounts of cholesterol and increasing amounts of cholesterol have been reported to reduce drug partitioning [78, 79]. One possible explanation is that the difference in cholesterol content does not largely affect log $K_s$ for this set of drugs or that the other lipid components within the BBM disk help to reduce the partitioning. A reduced partitioning could be caused by the presence of phosphatidylethanolamine and phosphatidylserine in the BBM disks [78].

In Paper III, log $K_s$ for lignin precursor models were determined by using disks that mimicked a typical plant cell membrane. The results were com-
Figure 3.8: Comparison of log \( K_s \) values for uncharged (○), positively (▲) and negatively (■) charged drugs obtained using a) DSPC/cholesterol/ceramide-PEG\(_{5000}\) disks and DSPC/cholesterol/DSPE-PEG\(_{5000}\) disks, b) DSPC/cholesterol/ceramide-PEG\(_{5000}\) disks and disks mimicking the brush border membrane (cerebroside (19.6 mol%), cholesterol (16.8 mol%), palmitic acid (12.9 mol%), POPC (12.1 mol%), POPE (12.8 mol%), POPS (4.1 mol%), sphingomyelin (3.7 mol%) and ceramide-PEG5000 (15 mol%)), c) Comparison of log \( K \) values for uncharged lignin precursor models obtained using DSPC/cholesterol/ceramide-PEG\(_{5000}\) disks and disks mimicking a plant cell membrane (for composition, see caption for Figure 3.5).

pared with data obtained when using disks with a simplified composition (DSPC/cholesterol/ceramide-PEG\(_{5000}\)). The partitioning was observed to systematically increase slightly when employing disks with the latter composition (Figure 3.8c). Therefore, contrary to the findings discussed above in Paper II (Figure 3.8a, 3.8b), the uncharged precursor models were slightly sensitive to differences in the hydrophobic region of the bilayer. One possible explanation could be that the plant cell membrane disks included sitosterol and stigmasterol which influence the packing of the phospholipids differently compared with cholesterol. Sitosterol has for example been observed to order the acyl chains more efficiently than cholesterol at sterol contents of 20 mol\% [80]. Furthermore, differences such as degree of saturation and length of the fatty acid chain might play a more important role for the uncharged lignin precursor models than for the uncharged drugs investigated in Paper II.
3.3.2 Bilayer disks as pseudo-stationary phase in capillary electrophoresis

The partitioning of drugs into bilayer disks was analyzed by capillary electrophoresis in Paper IV. Disks (DSPC/cholesterol/PEG-lipid) stabilized by either DSPE-PEG$_{5000}$ or ceramide-PEG$_{5000}$ were introduced in polyvinylpyrrolidone-coated capillaries and used as a pseudo-stationary phase. The time needed for the drugs to pass through the capillary was found to be proportional to the lipid amount in the pseudo-stationary phase (Figure 3.9). log $K$ could be determined reproducibly for a set of positively charged drugs, with relative standard deviations of $\leq 1.4\%$ for triplicate runs. An example of a set of electropherograms for verapamil obtained by using increased amounts of DSPC/cholesterol/ceramide-PEG$_{5000}$ disks is shown in Figure 3.9. Furthermore, employing the DSPE-PEG$_{5000}$ stabilized bilayer disks increased the log $K$ for the complete set of positive drugs, which was to be expected.

Paper IV showed that bilayer disks have future potential in studies based on capillary electrophoresis. This finding is of value since capillary electrophoresis offers a fast screening of analytes and only small volumes of sample and pseudo-stationary phases are needed. Furthermore, the disks that are used as a pseudo-stationary phase need not be immobilized on a matrix or surface. The stability and good shelf-life of the disks as well as the possibility to vary the disk composition further strengthens the use of bilayer disks as pseudo-stationary phases.

It is difficult to compare the partition data for the set of drugs analysed in Paper IV with the log $K$ values obtained by drug partition chromatography.

Figure 3.9: Overlaid cutouts from electropherograms showing the increased migration time of verapamil with increasing amount of disks stabilized with ceramide-PEG$_{5000}$ (0.80 mM). Inset: a diagram of $\Delta t$ (s) of verapamil versus the lipid amount (mol), $R >0.99$. The RSD was $\leq 0.8\%$ of the migration times.
3.3.3 A comparison between bilayer disks and other model systems used for partition studies

The partitioning of drugs has repeatedly been determined by the use of the octanol-water system discussed in the introduction (Section 1.3.1). As expected, the partition coefficients for charged drugs obtained by octanol-water partitioning correlated poorly with partitioning data obtained with bilayer disks in Paper II (Figure 3.10a). For the uncharged drugs, the correlation was somewhat better. In this case, the partitioning of the uncharged drugs does not seem to be largely dependent on headgroup characteristics.

The partitioning of the uncharged lignin precursor models was enhanced in the bilayer disks compared with the octanol-water system (Figure 3.10b). The increased partitioning of lignin precursors into the disks demonstrates that the hydrophilic headgroups play an essential role in the partitioning. The comparison between a disk system and an octanol-water system shows that the interactions taking place in the lipid headgroup region are important for the partitioning.

Papers II and III include a comparison between partitioning data obtained by the use of bilayer disks and liposomes. The partitioning of drugs and lignin precursor models was systematically more pronounced into disks than into liposomes (Figure 3.11). The same trend was seen in a previous study by Johansson et al. [42]. The drug partitioning into disks versus liposomes was also determined by isothermal titration calorimetry (ITC) by Johansson et al. The
Figure 3.11: Comparison between log $K_s$ or log $K$ values obtained using multilamellar liposomes (DSPC/cholesterol) and bilayer disks (DSPC/cholesterol/ceramide-PEG) for a) drugs and b) lignin precursor models.

partitioning of drugs into the two types of lipid aggregates was then proven to be similar when examined by ITC. It should be noted that ITC does, in contrast to the comparably fast drug partition chromatography and capillary electrophoresis techniques, allow the drugs to fully equilibrate in the system. The lower log $K$ or log $K_s$ values obtained by using liposomes compared with disks most likely originate from the fact that the directly available lipid amount, i.e. $A$ in the equation for $K_s$ (Section 2.5), is overestimated. The drugs simply do not have time to efficiently equilibrate in the system.

3.3.4 Disk immobilization on sensor surfaces

In Paper V we explored the possibility to immobilize bilayer disks on sensor surfaces used in SPR-based analyses. Successful immobilization would permit the use of disks in various types of biosensor-based interaction studies. Inclusion of biotinylated PEG-lipid (DSPE-PEG2000-biotin) in the disks enabled stable immobilization to carboxymethylated dextran matrices on the sensor chips (CM 3, CM 4 and CM 5) as exemplified in Figure 3.12a. The binding of bilayer disks to the chip was dependent on the amount of streptavidin present on the chip as is shown for the CM 5 chip in Figure 3.12b.

Disk immobilization was performed on the three different chip types to investigate the chip characteristics necessary for disk immobilization. The CM 5 chip has a dextran matrix that is as thick as for the CM 4 chip and a degree of carboxylation that is equal to that on the CM 3. The maximum immobilization signal was lower for the CM 4 compared with the CM 5 but the disk/streptavidin immobilization ratio was larger for the CM 4 chip. This indicates that the streptavidin sites available on the CM 4 chip were better utilized but the low carboxylation level on the other hand led to a lower total disk immobilization. The maximum signal for disk immobilization was similar when comparing the CM 3 and CM 5 chips but the disk/streptavidin immobilization ratio was larger for the CM 3. The longer dextran chains of the CM 5 chip did not improve the disk binding. The disk size was also found to have an effect on
Figure 3.12: Left) Immobilization of disks on a CM 5 chip. DSPC/cholesterol/DSPE-PEG2000/DSPE-PEG2000-biotin (35:40:24:1 mol%) disks (---). DSPC/cholesterol/ceramide-PEG2000/DSPE-PEG2000-biotin (35:40:24:1 mol%) disks (—). Injection of disks stopped at the arrow (↑). Right) Reported signal for disk immobilization as a function of the signal obtained upon streptavidin binding to the chip.

the binding capacity and reduced disk size gave an increased lipid/streptavidin ratio as seen for the disks produced by detergent depletion (Table 3.1). It can not be excluded that the positioning and the density distribution of the disks in the matrix affects the recorded signals for the different CM-chips. A comparison of the data obtained for different sensor chips should therefore be done with care. As a next step, the actual amount of lipid-material present on the chips should be determined to resolve this matter.

The L1 chip is often used for SPR-based interaction studies involving liposomes [56, 81]. Immobilization is here facilitated by the presence of lipophilic anchors on the matrix of the L1 chip which insert into the hydrophobic region of the bilayer. An advantage with the L1 chip is the possibility to reuse it and immobilize new liposomes after washing the surface with a detergent solution. One disadvantage can be that the lipophilic anchor inserts directly and disturbs the lipid packing in the hydrophobic part of the bilayer. Immobilization on L1 chips was performed with bilayer disks (DSPC/cholesterol/DSPE-PEG, 35:40:25 mol%) and PEG-lipid containing liposomes (DSPC/cholesterol/DSPE-PEG 56:40:4 mol%) in Paper V. The presence of PEG-lipid clearly decreased the signal obtained and a maximum immobilization signal could not be reached even after prolonged immobilization times. The presence of PEG-lipid clearly hinders the close approach of lipophilic anchors and lipid aggregates by the mechanisms discussed in Section 1.2.1.

As discussed earlier, the transmembrane protein, bacteriorhodopsin, was inserted into bilayer disks by the detergent depletion method in Paper II. Membrane proteins can also bind peripherally to lipid bilayers that have already been formed. Cyclooxygenase 1 and 2 (COX 1, COX 2) are examples of such proteins [82]. SPR-based experiments involving COX 1 and 2 were performed in Paper V. COX was incubated with bilayer disks and the mixture was then
Table 3.1: *Immobilization responses for bilayer disks (DSCP/cholesterol/DSPE-PEG\textsubscript{2000}/DSPE-PEG\textsubscript{2000}-biotin, 35:40:21:4 mol%) onto sensor chips from the CM-series*

\textsuperscript{a}Corresponds to maximum streptavidin immobilization level obtained with the current immobilization technique.

\textsuperscript{b}Averaged immobilization ratio obtained from results in Figure 3.12.

\textsuperscript{c}Disks produced by detergent depletion.

<table>
<thead>
<tr>
<th>Sensor chip</th>
<th>Streptavidin immobilization signal (RU)</th>
<th>Disk immobilization signal (RU)</th>
<th>Disk/streptavidin immobilization ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM 3</td>
<td>2318</td>
<td>1646</td>
<td>0.71</td>
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<tr>
<td></td>
<td>4160\textsuperscript{a}</td>
<td>2970</td>
<td>0.71</td>
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<tr>
<td>CM 4</td>
<td>1966</td>
<td>1055</td>
<td>0.54</td>
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<tr>
<td></td>
<td>4003\textsuperscript{a}</td>
<td>2270</td>
<td>0.57</td>
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<tr>
<td>CM 5</td>
<td>-</td>
<td>-</td>
<td>0.38\textsuperscript{b}</td>
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<td></td>
<td>1208\textsuperscript{c}</td>
<td>1237\textsuperscript{c}</td>
<td>1.02\textsuperscript{c}</td>
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<td>9875\textsuperscript{c}</td>
<td>3597\textsuperscript{c}</td>
<td>0.36\textsuperscript{c}</td>
</tr>
</tbody>
</table>

injected over the sensor surfaces. The incubation resulted in an increased signal compared with the signal obtained by injection of pure disks (Figure 3.13). The results clearly show that COX 1 and COX 2 bind to the bilayer disks.

Indomethacin is known to interact with COX 2 and was injected over the immobilized disks to which COX 2 had bound. The response obtained was greater than that obtained when indomethacin was injected over immobilized disks in the absence of COX 2. This further verifies the presence of COX 2 on the bilayer disks.
Figure 3.13: Sensorgram obtained upon immobilization of pure bilayer disks (- - -) and disks incubated with COX 2 (—). Disk composition: POPC/PE (soy)/cholesterol/ceramide-PEG_{2000}/DSPE-PEG_{2000}-biotin (30:28:17:21:4). Injection of disks stopped at the arrow (↑).
4. Conclusion

The work presented in this thesis focuses on fundamental details concerning the internal structure of PEG-lipid bilayer disks, the development of bilayer disks of optimized compositions and the use of these as model membranes.

First, a study designed to resolve fundamental details concerning the internal structure of the PEG-lipid bilayer disks was presented. The neutron scattering experiments in Paper I verified that a partial component segregation of the PEG-lipids did indeed occur and showed that the PEG-lipids form a highly curved monolayer at the rim of the disk. Coming one step closer to resolving the details of the internal aggregate structure helps to understand the factors that promote disk formation. The information was also clearly valuable when interpreting results from the binding study with melittin. The special features of the bilayer disks enabled a comparatively large number of melittin molecules to bind to these lipid aggregates. In fact, melittin was found to interact mainly with the rim of the disk. It is possible that the bilayer disk can be used as both a model membrane to resolve factors that affect peptide/lipid interactions and as a drug delivery vehicle for pharmaceutically interesting peptides.

Continuing on our path towards an understanding of the world of PEG-lipid stabilized bilayer disks, the use of bilayer disks as model membranes was next to be presented. In Papers II, III and IV, bilayer disks were used as model membranes in partition studies with encouraging results. Disks with compositions mimicking those of natural cell membranes were employed to study the partitioning of drugs and lignin model precursors. Furthermore, capillary electrophoresis was used for the first time to investigate the partitioning of drugs into bilayer disks. The unique structural features and characteristics of the bilayer disks can be exploited for other purposes than partition studies. The experiments reported in Papers II and V reveal, for example, that bilayer disks have potential as model membranes in studies focused on membrane proteins. It is very likely that disk composition and solution conditions can be tailored to allow activity measurements on specific membrane proteins. The positive results reported in Paper V open up for the future use of disks in applications that involve immobilization on biosensor surfaces.
5. Svensk Sammanfattning

Alla levande organismer är uppbyggda av celler. I våra kropper finns det ca 100 000 000 000 000 st celler, ja visst, hur många som helst. Cellerna i sin tur är uppbyggda av flera mindre delar. En för cellen livsviktig del är det cellmembran som omgärder övriga beståndsdelar. Cellmembranet gör det möjligt för cellen att fungera som en enskild enhet, som en egen liten fabrik. Membranet hjälper till att kontrollera varutransporterna in och ut ur fabriken och kan även signalera start eller stopp av viktiga processer. Om nu cellmembranet kan göra allt detta måste det vara speciellt, något värt att undersöka närmare. Detta arbete handlar just om membran, närmare bestämt en typ av modellmembran som t.ex. kan användas för att studera interaktioner mellan cellmembran och läkemedel.

Cellmembran

Cellmembranet består till en stor del av molekyler som kallas polära lipider. De polära lipiderna är uppbyggda av kol-, syre-, kväve- och fosforatomer. De polära lipidernas molekylstruktur är sådant att de har en vattengillande del och en fettgillande del (Figur 5.1). När en viss mängd lipider blandas i vatten associerar de spontant och bildar aggregat. I aggregaten ordnar lipiderna upp sig så att en vattengillande del angränsar till en annan vattengillande del och en fettgillande del angränsar till en annan fettgillande del. De vattengillande delarna vänds dessutom mot vattnet i omgivningen. (Figur 5.1)


Modellmembran

När vi sväljer en värktablett måste läkemedlet ta sig från tarmen till blodbanan för att ha effekt och lindra smärtan. Läkemedlet tar sig då först igenom cellmembranen som täcker tarnens insida och vidare in i blodbanan för att via den spridas i hela kroppen. Om läkemedlet inte når fram till det värkande området spelar det liten roll om substansen i sig är
Vattengillande del

Fettgillande del

Micell

Bilager

Figure 5.1: Schematisk bild av två polära lipider och deras aggregat.


För själva screeningsprocessen av läkemedelssubstanser behövs i det enklaste fallet bara utrustning i form av en pump, ett rör med modellmembran och en detektor. Läkemedlet löses i vatten och lösningen pumpas igenom röret med membran. Den tid som läkemedlet uppehåller sig i röret mäts och ger ett mått på hur villigt läkemedlet är att ta sig in i membranet. Ett läkemedel som lätt tar sig igenom cellmembranen sätter sig gärna i modellmembranen och fördröjs på sin väg genom röret.
Bilager-diskar


I framtiden kan diskarna eventuellt även användas för transport av läkemedel i kroppen. I delarbete I fanns ledtrådar som tyder på att diskarna
har egenskaper som gör dem intressanta för transport av läkemedel i kroppen.
Acknowledgements

Tack! Katarina för 1671 dagars fin-fin handledning och för att jag fick möjligheten att doktorera i en så bra grupp.

Tack! Per Lundahl för din omtänksamhet och för att ditt härliga forskningsengagemang smittade av sig.


Thanks! Adrian for being the perfect guide to all the nice restaurants in Grenoble.

Tack! Emma, Maria, Per för att ni är så himla bra!!! på allt från jobb till pyssel, påskäggsletning, mera pyssel och kick offs.


Tack! Göran K för hjälp med allt från mikroskopstrul till 1.04 cm långa sträck och annat bildfix.

Tack! Mats A, Göran S, Laila, Lilian, Per-Axel, Ulla för all hjälp och det som ni fixat runt i kring.

Tack! Alla på fysikalen, gamla som nya, för att ni är så bra kollegor


Tack! Busse, Jonas L, Malin M för bästa layout- och text-hjälpen någonsin.

Tack! Busse för att du får mina tår att vifta, för att du hejar på mig även kl 03.10 och för att du envisas med \LaTeX. Du är min bästa grej!!! Kramar och tack till resten av Pettersssonska klanen så klart! Svartsoppa är ju gott . . .

Stort TACK och bamsekramar till världens bästa familj!!!!
References


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