Azobenzene-substituted phosphate amphiphiles
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Chapter 1

Introduction

1.1 Hydrophobic interactions

The chemistry of all life processes occurs in water and water has been called “the matrix of life”.\(^1\) But water is more: it acts as a solvent, mediator, reactant and/or catalyst. It has unique properties due to its low molecular weight and its ability to form three dimensional networks of hydrogen bonds. Each water molecule forms three to four hydrogen bonds in liquid, bulk water. Due to the hydrogen bonds, water has a high melting and boiling point.\(^2\) Although water has the potency to be a ‘green’ solvent, the low solubility of apolar substances in water generally limits the use of water as a solvent. This low solubility led to the term hydrophobicity. Hydrophobicity leads to the association of apolar groups or molecules in an aqueous environment arising from the tendency of water to exclude nonpolar molecules.\(^3\) The apolar solutes cannot compete with the strong interactions between the water molecules.

The origin of hydrophobic interactions lies in the entropy of hydration.\(^1\) Introduction of an apolar solute into water leads to preferential ordering of the water molecules around the apolar solute, accompanied by an unfavourable entropy, also called hydrophobic hydration. In 1945 Frank and Evans\(^4\) proposed the iceberg model for the hydration of apolar solutes in water. In this model, the water molecules are highly ordered in an ice-like structure. The stronger and increased number of hydrogen bonds between the waters, in comparison with bulk water, were held to be responsible for the unfavourable entropy term. Later, the theory was disputed due to new experimental results.\(^5\)\textsuperscript{-12}\) Nowadays, the hydration of apolar solutes is visualised as presented in Scheme 1.1.

\[\textbf{Scheme 1.1} \textit{Schematic representation of the preferential hydration of an apolar solute by a change in the three-dimensional hydrogen-bond network of water. Picture taken from reference 13.}\]
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The water molecules are oriented in such a way that a maximum of hydrogen bounds is retained in the system. This is accomplished by orienting one hydrogen-oxygen bond of a water molecule tangential to the hydrophobic surface. This will lead to an entropic loss. However, the strongly negative entropies of transfer of apolar molecules from the gas phase to water probably originate primarily from the excluded volume effect.

When two apolar solutes are present in water it is, above a critical concentration, thermodynamically more favourable for the system to have the two solutes bound together, less structured water is needed to hydrate the two particles (fewer people can sit around two tables when they are pushed together). This clustering happens spontaneously and is called hydrophobic interaction. These interactions are important for many biochemical processes like protein folding, substrate-enzyme binding and the formation of biomembranes.

1.2 Colloidal aggregates

Molecules which contain a polar and an apolar part are called amphiphiles or surfactants (surface active agents). These molecules tend to aggregate in water due to hydrophobic interactions. The molecules aggregate in such a way that the chains form a hydrophobic core and there are favourable interactions between the polar parts and the water.

![Figure 1.1 Some aggregate morphologies of surfactant self-assemblies in aqueous solution. (a) spherical micelle, (b) worm-like micelle, (c) bilayer fragment, (d) vesicle, (e) inverted micelle. Picture taken from reference 17.](image-url)
Depending on the structure of the molecule, a variety of colloidal aggregates can be formed (Figure 1.1). The polar part, the head group, can be uncharged, anionic, cationic or zwitterionic. The apolar part contains one or more hydrocarbon chains (tails). The morphology of the aggregate is mainly determined by a balance between the hydrophobic interactions of the hydrocarbon tails and the electrostatic repulsions and hydration of the head groups.\textsuperscript{16} Israelachvili and Ninham\textsuperscript{17} developed a model to predict the aggregate morphology of a given surfactant. The value of the packing parameter $P$ is a measure for the aggregate formed:

$$ P = \frac{V}{a_0 \cdot l_c} \quad (1) $$

In this equation is $V$ the volume of the hydrophobic part of the molecule, $a_0$ the mean cross-sectional head group surface area and $l_c$ the chain length of the fully extended all-trans alkyl tail. $V$ and $l_c$ can easily be calculated from the number of carbons in the chain. More tricky is the quantification of $a_0$, especially for cationic and anionic surfactants. The effective size of the head group area depends on the electrolyte and the surfactant concentration. Nagarajan\textsuperscript{19} proposed that the value of $a_0$ possibly also depends on the chain length. In that case are the variables in the packing parameter not completely independent of each other. Table 1.1 shows the relationship between the packing parameter (or shape factor) and the aggregate morphology. Typical inverted phases are inverted micelles, inverted hexagonal phases and cubic phases.\textsuperscript{20} This model is an approximation since, in practice, different aggregate morphologies often coexist.

### 1.2.1 Micelles

Surfactants with one unbranched alkyl tail that contains 8-18 carbon atoms often form micelles.\textsuperscript{21} Surfactants with shorter tails (< 8 carbons) are usually too small to induce micelle formation and they are called hydrotropes.\textsuperscript{22,23}

Spherical micelles are formed in aqueous solution above their critical micelle concentration (cmc) which varies per surfactant and ranges typically from $10^{-6}$ to $10^{-2}$ M. Above the cmc, monomers and spherical micelles are present and the concentration of free monomer stays fairly constant. The interior or core of the micelle consists of the hydrocarbon chains and is alkane-like. Water penetrates into the micelle up to about the second methylene group. The charged interfacial region of the micelle is called the Stern region. The linear relationship between the tail length and the logarithm of the cmc can been described by the Shinoda equation.\textsuperscript{24} Elongation of the tail leads to smaller cmc values. Alteration of the counterion can also change the cmc.\textsuperscript{25} In case of ionic
surfactants the counterions bind for 70-90 % to the surface of the micelle reducing the head group repulsions. Also changes in the pressure, temperature and ionic strength can have an effect on the properties of the micelles.\textsuperscript{25,26,27}

Micelles are dynamic, the average lifetime of a micelle is in the order of milliseconds and the monomers enter and leave the micelle on a microsecond timescale.\textsuperscript{28} At high concentration of the surfactant, interactions between the micelles become important and elongated worm-like micelles are formed.

\textbf{Table 1.1} \textit{Relationship between the effective shape of the surfactant molecule, the packing parameter and the aggregate morphology according to Israelachvili.}\textsuperscript{18} Picture taken from reference 17.

<table>
<thead>
<tr>
<th>Effective shape of the surfactant molecule</th>
<th>Packing parameter</th>
<th>Aggregate morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>cone</td>
<td>&lt; ⅓</td>
<td>spherical micelles</td>
</tr>
<tr>
<td>truncated cone</td>
<td>⅓ - ½</td>
<td>worm-like micelles</td>
</tr>
<tr>
<td>cylinder</td>
<td>½ - 1</td>
<td>flat bilayers, vesicles</td>
</tr>
<tr>
<td>inverted (truncated) cone</td>
<td>&gt; 1</td>
<td>inverted phases</td>
</tr>
</tbody>
</table>

\subsection*{1.2.2 Vesicles}

Surfactants with two alkyl tails often form vesicles. Vesicles are formed above the critical vesicle concentration (cvc). The cvcs typically are smaller than the cmcs and are in the micromolar range. Vesicles consist of a double layer and an aqueous inner compartment. Vesicles with more than one double layer also exist and are called multilamellar vesicles. The size of vesicles varies from 20 nm to several micrometers. The preparation method of the vesicles determines the size and
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lamellarity. Tip sonication leads to small unilamellar vesicles (SUVs, ca. 30 nm) and freeze-thaw cycles lead to large multilamellar vesicles (LMVs). When extrusion through a membrane is applied, after freeze-thaw cycles, unilamellar vesicles can be obtained with a defined size and a small size distribution.

An important aspect of vesicles (or in general, bilayers) is the fact that the alkyl tails can exist in two different phases. At low temperatures the tails are in a highly ordered rigid phase, the gel state. Above the main phase transition temperature ($T_m$), the tails are in a more fluid, less ordered phase, the liquid-crystalline state. Upon increasing the length of the alkyl tails, the $T_m$ increases.

Vesicles are metastable, except catanionic vesicles. They have the tendency to fuse, forming larger colloidal aggregates, and eventually precipitation occurs. The rate of this fusion process varies from seconds to months. In the case of charged vesicles and a low electrolyte concentration, fusion is effectively prevented by the electrostatic repulsion between the charged vesicles. Also hydration shell overlap between the head groups of the two vesicles and effects due to undulation forces counteract the fusion process. If the electrolyte concentration is increased, the repulsion between the vesicles is diminished, and fusion can occur easily. Other factors which govern fusion are the internal strength of the bilayer, ease of forming nonbilayer structures and mechanical tensions in the membrane. Ca$^{2+}$ and Mg$^{2+}$ promote fusion of anionic vesicles and are called fusogens.

1.3 Biological membranes

A single cell membrane has the same morphology as a vesicle, a closed bilayer of amphiphiles with an inner aqueous compartment. The cell membrane of a living cell is a complex system of lipids (biological amphiphiles) and membrane proteins (Figure 1.2).

1.3.1 Lipids

There are three types of lipids present in the membrane: phospholipids, steroids (e.g. cholesterol) and glycolipids. Phospholipids have a similar basic structure consisting of two apolar hydrophobic tails and a head group composed of a glycerol part (or ceramide), a phosphate part and a variable part that can e.g. be choline or serine.

The length of the hydrocarbon tails varies normally from 16 to 24 carbon atoms and more than half of the tails contain 1 to 6 double bonds. Nearly all phospholipids have tails with an even number of carbon atoms. Most of the double bonds have the cis (Z) configuration. Biological membranes are normally in the fluid phase and cis unsaturations in the tails promote the fluid phase. The distribution of the
different tails varies strongly per cell. Although most hydrocarbon tails are linear, also some exotic tails are found in natural membranes. Examples include branched lipids in archaea and ladderane lipids which are found in anammox bacteria. Anammox bacteria are found in the Black Sea, which has a high ammonium concentration, and they are able to oxidize anaerobically ammonium to N\textsubscript{2}. These bacteria have lipids which contain up to five cis-fused cyclobutane rings (Figure 1.3). These ladderane lipids were found in the membrane of an intracytoplasmic compartment of anammox wherein the oxidation takes place.

\section*{Figure 1.2} Schematic presentation of the biological cell membrane. Reprinted by permission of Pearson Education, Inc.

\section*{Figure 1.3} Stick model of a ladderane lipid. The carbon atoms are colored grey, the oxygen atoms red and the hydrogen atoms white.

In Figure 1.4 the main classes of phospholipids are shown; PC and PE are the most abundant phospholipids. PC is the major phospholipid found in membranes of animal cells, while PE is abundant in many bacteria. In Table 1.2 the lipid compositions of a few membranes are given. It is clear that there is a wide variation in membrane composition. In the biological membrane, the lipids are, normally, asymmetrically distributed. The (loss of) asymmetry of membrane phospholipids is important for several biological processes.
Figure 1.4 The structure of the main classes of phospholipids: phosphatidylcholine (PC); phosphatidylethanolamine (PE); phosphatidylserine (PS); sphingomyelin (SM). R is a variable hydrocarbon chain.

Table 1.2 Lipid composition of a few membranes (in mol%). Abbreviations, PG= phosphatidylglycerol, others, see Figure 1.4.

<table>
<thead>
<tr>
<th>Lipid/ Steroid</th>
<th>Rat liver lysosome</th>
<th>Rat liver cytoplasmic membrane</th>
<th>E. Coli cytoplasmic membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>14</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>PC</td>
<td>25</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>SM</td>
<td>24</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>PE</td>
<td>13</td>
<td>11</td>
<td>80</td>
</tr>
<tr>
<td>PS</td>
<td>-</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>PG</td>
<td>-</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>Others</td>
<td>24</td>
<td>18</td>
<td>5</td>
</tr>
</tbody>
</table>

Red blood cells have in the outer leaflet the phospholipids PC and SM and in the inner leaflet PE and PS. When the negatively charged phospholipid PS is exposed to the exterior of the cell, blood coagulation is promoted. The most common steroid is cholesterol. Besides cholesterol, desmosterol, stigmasterol and ergosterol are commonly...
found in biological membranes.\textsuperscript{38} Cholesterol is only found in animal cells and steroid-like molecules can be present in microorganisms. Biological membranes usually contain large amounts of cholesterol (up to 67 mol%) and the cholesterol is normally almost equally distributed over the two leaflets. Cholesterol stiffens the membrane and makes it less fluid and permeable.

Glycolipids are mainly found in the outer membrane leaflet, where they are exposed to the exterior of the cell. Although the function of glycoproteins is not fully understood, it is clear that they are important for the interactions of the cell with the environment, e.g. recognition processes and signal transduction.\textsuperscript{39,40}

A research field which is still in its infancy is the study of the formation of rafts.\textsuperscript{41} Rafts are lipid domains in the membrane, so the membrane is not completely homogeneous anymore. There is growing evidence that rafts exist in biological membranes. The rafts in the outer leaflet are thought to consist mainly of sphingolipids and cholesterol. The composition of rafts of the inner leaflet is still unknown. There is a growing support that rafts are important in regulating numerous cellular processes, including cell polarity, protein trafficking and signal transduction. A lot of the properties of lipid rafts are still under debate, e.g. size, lifetime and mechanism of formation.

1.3.2 Membrane transport

Only a few molecules diffuse easily across the biological membrane, examples are O\textsubscript{2}, CO\textsubscript{2}, H\textsubscript{2}O and small uncharged polar molecules (M\textsubscript{w}<100). For the transport of other molecules like ions, sugars and nucleotides, membrane proteins are used. Besides membrane proteins for transport, there are also membrane proteins which function as a linker, receptor or enzyme. In this paragraph the emphasis will be placed on the membrane transport proteins.

There are two classes of membrane transport proteins, the carrier proteins and the channel proteins. A carrier protein undergoes a series of conformational changes to transfer molecules across the membrane. In contrast, a protein channel forms a pore. The transport rate via a channel is much larger than that via the carrier. Both carrier and channel proteins have polypeptide chains which span the membrane. Normally, the polypeptide chains transverse the membrane several times.

The basic difference between the channel and the carrier is the fact that the channel proteins discriminate molecules on the basis of their size and charge. Carrier proteins only transport molecules which fit into the binding site.
Transport across the membrane in the same direction as the concentration gradient is called passive transport; charge against the concentration the gradient is termed active transport. Active transport can only occur via carrier proteins and is driven by light, ATP or a electrochemical (ion) gradients. Passive transport can occur via both types of transport proteins.

Most channel proteins in the plasma membrane only transport small inorganic ions like Na\(^+\), K\(^+\), Cl\(^-\) and Ca\(^{2+}\). Therefore they are often called ion channels. Most ion channels are gated, they can switch between an open and a closed state. The gating of ion channels is controlled by voltage, binding of a ligand and/or mechanical forces. In §1.5 mechanosensitive channels will be discussed in more detail, but first protein-lipid interactions will be introduced.

1.4 Protein-lipid interactions

There are two types of protein-lipid interactions, specific and non-specific.\(^{42}\) In the crystal structures of a number of membrane proteins, specific lipids were found at particular positions, the deep clefts between the transmembrane \(\alpha\)-helices (non-annular sites).\(^{43-46}\) An example is the light harvesting complex of photosystem II, which contains PG (phosphatidylglycerol).\(^{43}\) PG cannot be removed by treatment with a non-ionic detergent and therefore binds firmly. Treatment with phospholipase or a protease leads to dissociation of the trimeric complex. It has been suggested that PG has an important role in trimer formation and stabilisation of the protein. These types of protein-lipid interactions are called specific.

Also nonspecific protein-lipid interactions are important for the stability and function of membrane proteins. Important properties of the lipids in this respect are the charge of the head group,\(^{47}\) phase of the bilayer,\(^{42}\) extent of hydrophobic mismatch\(^{48}\) and the effective shape of the lipid.\(^{49,50}\)

It was found that the charge of the head group has an effect on the activity of membrane proteins. The activity of membrane proteins reconstituted into bilayers of pure anionic or zwitterionic phospholipids seems, in general, to be low.\(^{42,51}\) However, addition of small quantities of anionic phospholipids to zwitterionic lipids often leads to higher activity. The presence of PS has an influence on the equilibrium of the intermediates MI and MII of rhodopsin. Rhodopsin is an example of a G protein-coupled receptor and is a part of the system responsible for vision. 11-Cis-retinal is the ligand for rhodopsin. The complex decomposes upon exposure to light into all-trans retinal and ops in via five short-lived intermediates.\(^{36,52}\) It is assumed that the intermediates are important for signal transduction. Metarhodopsin I and II are two of
these intermediates and the equilibrium of intermediates MI and MII is thought to be controlled by the membrane lipids. The presence of PS in the membrane causes an increase in the concentration of H⁺ close to the membrane surface. The equilibrium MI/MII is affected because it is pH-dependent.

1.4.1 Hydrophobic mismatch

Hydrophobic mismatch means that there is a mismatch between the hydrophobic bilayer thickness and the length of the hydrophobic membrane-spanning domain of the protein. Exposure of the hydrophobic part of either the lipids or protein to water is thermodynamically unfavourable. Therefore, the system tries to compensate for the mismatch. A simple solution is thinning or an increase in thickness of the bilayer around the protein. Another possibility is tilting of the helices of the protein to reduce their effective length. Most models assume that the lipids adjust their lengths but it is also assumed that the adjustment is not large enough to compensate for the total hydrophobic mismatch. The activities of many membrane proteins depend on the membrane thickness, e.g. Ca²⁺-ATPase, rhodopsin, and the glucose transporter from red blood cells.

1.4.2 Curvature stress

In water-oil mixtures containing a surfactant, a monolayer of the surfactant is formed at the interface of water and oil. Two types of curvature can occur: positive and negative ones (Figure 1.5).

![Figure 1.5 Schematic representation of negative (left) and positive curvature (right).](image)

When a nonbilayer-forming surfactant (e.g. DOPE) is incorporated into a membrane, the curvature is not expressed. The bilayer structure does not allow formation of curved monolayers. The curvature is stored as potential energy with a latent ability to destabilize the bilayer, this is called curvature stress (frustration). Relaxation of this energy can be accomplished by a transition of the system to an inverted hexagonal H₁₁
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structure. The curvature stress energy ($E_{cs}$, bending energy) can be calculated via the (simplified) Helfrich free energy equation:

$$\frac{E_{cs}}{A} = \frac{1}{2} k (c - c_0)^2$$

(2)

where $k$ is the bending rigidity (or splay modules), $c$ is the curvature of the monolayer or bilayer, $c_0$ is the spontaneous curvature (curvature at which the Gibbs energy is minimal) and $A$ is the area of the monolayer or bilayer. $E_{cs}/A$ is the curvature stress energy per unit area. The curvature $c$ is defined as $1/R$, where $R$ is the radius of the curvature. For a planar membrane, $c=0$.

The value for $k$ has been determined for a series of model and biological membranes and it was found that the values were similar and in the order of $10^{-19}$ J. The spontaneous curvature $c_0$ can be calculated from the isotropic radius ($r_0$) and the thickness of the monolayer.

The curvature stress energy has so far only been measured indirectly. Janes$^{57}$ studied the effect of membrane constituents on the phase transition from $L_\alpha$ to $H_{II}$. It was proposed that there is a relation between changes in the phase transition temperature and the (enthalpic) curvature stress induced by a membrane constituent. Epand and Epand$^{63}$ detected calorimetrically the relief of curvature stress by adding lysophosphatidylcholine to vesicles. It was found to be an exothermic process corresponding to a relief of curvature stress, as expected. Estimation of the magnitude of the curvature stress energy was difficult because of several other processes that take place in parallel and which have endothermic enthalpies.

Modulation of the activity of membrane proteins by curvature stress has been reported for the G-protein coupled receptor rhodopsin,$^{64}$ the ion channel gramicidin,$^{65,66}$ the voltage-gated ion channel alamethicin$^{67,68}$ and the enzyme phosphocholine cytidylyltransferase.$^{69}$

In recent years there is a tendency to combine all bilayer properties in one general concept: the lateral pressure profile.$^{70,71}$ Since Cantor further developed the concept in 1997,$^{72,73}$ an increasing number of research groups applies the concept to explain nonspecific protein-lipid interactions.$^{74-80}$

1.4.3 Lateral pressure profile

The fundamental idea of the lateral pressure profile is that the lateral pressure$^{81}$ varies with depth in the membrane.$^{72,73}$ In Figure 1.6 a schematic presentation of the lateral pressure as a function of the depth is given. Between the (charged) head groups there is repulsion, which leads to a positive lateral pressure. Attractive interactions between head groups are not excluded. At the interface of water and hydrocarbon
phase there is a substantial attraction, a negative lateral pressure. This attraction is due to hydrophobic interactions, often named by biologists interfacial tensions. The system tries to minimize the contact between the hydrocarbon phase and the water phase. The tails in the hydrocarbon phase are partially ordered, which is accompanied by a loss of conformational entropy. Their preference for gaining more conformational freedom leads to a repulsive, positive lateral pressure. This repulsive lateral pressure might appear somewhat strange, because hydrophobic interactions are expected between the hydrocarbons. It is important to remember that hydrophobic interactions occur due to the fact that water molecules like each other more than hydrophobic molecules.

The overall lateral pressure should be zero in a (meta)stable membrane. So, the area under the curve in Figure 1.6 should also in total be zero. The attractive hydrophobic interactions at the interface are compensated by positive lateral pressures in the remainder of the membrane.

![Diagram of lateral pressure profile](image)

**Figure 1.6** Schematic presentation of a lateral pressure profile in a bilayer. Here only half of the lateral pressure profile is given (one monolayer), the same profile applies for the other monolayer. The lateral pressure $\pi$ depends on the depth $z$ in the membrane.

Cantor\textsuperscript{82} made an estimation of the average pressure density in the hydrophobic core. For this purpose, the compensating lateral pressure for the interfacial tension was divided by the thickness of the hydrophobic core. The average was found to be 300 atm, but it is predicted that the pressure at the interfaces is considerably larger and in the center of the membrane smaller.\textsuperscript{83}

Direct and unambiguous measurements of the lateral pressure in the bilayer are up to now not possible. However, efforts have been made to get an estimate of the lateral pressure profile. Templer et al.\textsuperscript{84} used a
fluorescent probe to sense lateral pressure at several places in the hydrocarbon part of the membrane. They used a homologous series of di-pyrenyl phosphatidylcholine (dipyPC) probes with the pyrene moiety at 4 different positions in the tail. Formation of excimers of pyrene can occur when a pyrene moiety in its excited state comes into close proximity to a ground state pyrene. It was found that the aggregation of the two pyrene moieties is the rate-limiting step in the excimer formation in dipyPCs. Therefore, it was assumed that the formation of the aggregate is proportional to the lateral pressure. The excimer signal is also proportional to the lateral pressure. The ratio of the excimer to the monomer fluorescence was used as a measure of the lateral pressure. This fluorescence ratio ($\eta$) was measured for vesicles composed of DOPC and DOPE with an increasing amount of DOPC. A decrease in $\eta$ was found in the region between the head group and the cis double bond of DOPC. An increase in $\eta$ was observed for the central part of the membrane. The method had some drawbacks; firstly the large size of the pyrene unit. Secondly, there could be a disturbance of the measurements by the cis double bonds of the unsaturated phospholipids. And finally, the spread in the data points limited the ability to draw clear conclusions.

Another possible measure for the lateral pressure are the (chain) order parameters, which can be determined by $^2$H-NMR spectroscopy. For this purpose lipids are used which are (partially) deuterated. The quadrupolar splitting, the distance between the two peaks in the $^2$H NMR spectrum, is a measure of the chain order. An increasing chain order leads to an increasing quadrupolar splitting. On the basis of $^2$H NMR studies, Gawrisch and Holte suggested that the introduction of a lipid with the shape of an inverted cone (e.g. DOPE, Table 1.1) leads to a reduction in repulsions between the head groups, a reduction in the area per lipid and an increase in chain order. An increase in chain length, chain unsaturation or a rise in temperature leads to more repulsions in the hydrocarbon area, a higher lateral pressure, more area per lipid and thus an decrease in chain order. Addition of short-chain alcohols to membranes leads to a decrease in chain order. The addition of longer alcohols to membranes has almost no effect or increases the chain order slightly. More chain repulsion is expected with an increasing lateral pressure.

Van den Brink-van der Laan et al. investigated the effect of addition of short-chain alcohols to DOPC membranes on the quadrupolar splitting using DOPC that was labelled at the C11 position. Addition of all alcohols led to a decrease in quadrupolar splitting. They suggested that a decrease in chain order is accompanied by a decrease in lateral pressure in the hydrocarbon part of the membrane. These results are in line with the conclusions of Gawrisch and Holte. At the moment, the exact relation between lateral pressure and chain order is still uncertain.
Different computational methods have been used to calculate lateral pressure profiles. These were methods based on mean field theory, Monte Carlo simulations, molecular dynamics (MD) simulations or simplified coarse-grained MD simulations. The calculations based on the mean field theory and the Monte Carlo simulations are restricted to the hydrocarbon region of the bilayer. The head group region was taken into account in MD simulations. Calculation of the lateral pressure profile in the headgroup region is quite complicated. MD simulations of lipid bilayers are most promising but still computationally demanding and therefore limited to relatively small systems and relatively short time scales.

It has been suggested by several researchers that changes in the lateral pressure can have an influence on the conformational state of membrane proteins. Besides the more general papers on modulation of protein function by changes in the lateral pressure, some papers were published which dealt with specific membrane proteins like MscL, KscA and Alamethicin.

Changes in the lateral pressure will only affect membrane proteins whose function depends on a transition between conformational states. With the assumption that the protein only exists in two states, Cantor stated that the conformational change of the protein should be accompanied by a depth-dependent change in the cross-sectional area of the protein (Figure 1.7). For the example sketched in Figure 1.7, an increase in lateral pressure near the bilayer centre and a decrease near the interface could lead to a change in conformation from state r to t.

**Figure 1.7** Schematic presentation of a membrane containing a protein in two conformational states, r and t. At the right, the cross-sectional area profile $A(z)$ of each of the two states is presented as a function of depth $z$. The membrane has thickness $h$. Picture taken from reference 73.
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It has been suggested\textsuperscript{101} that changes in the lateral pressure profile in biological membranes is accomplished by the incorporation of interfacially active solutes, altered lipid composition or turgor pressure (\textit{vide supra}). Although the concept of the lateral pressure profile becomes generally more accepted, some authors have serious objections.\textsuperscript{103} Firstly, molecules cannot feel local pressures because they can only feel concentrations. Secondly, different types of calculations of the lateral pressure give a large variety in results. Lastly, it is not easy to define unambiguously the lateral pressure in the membrane.

1.5 Mechanosensitive channels

1.5.1 Introduction

The ability to detect mechanical force, mechanosensation, is an inherent property of almost all living organisms.\textsuperscript{104} Mechanosensation is used for the senses of touch, hearing, balance and change in osmolarity. But also plants and bacteria use mechanosensation. For the majority of the mechanosensitive reactions, mechanosensitive (MS) channels play an important role. MS channels are found in bacterial, yeast, plant and animal cells.\textsuperscript{105} The opening of MS channels is induced by external mechanical forces which destabilize the closed conformation of the channel. Other types of stimuli for channels are the interaction of a ligand-binding pocket with a ligand (ligand-gated channels) or changes in the electrical potential across the cell membrane (voltage-dependent channels).\textsuperscript{106,107} There is a large diversity of MS channels\textsuperscript{106}, but still little is known about how MS channels function. Up to now, the MS channels of bacteria have been studied in most detail. Bacterial MS channels were first reported in 1987 by Martinac \textit{et al.}\textsuperscript{108} by using the patch clamp technique on giant spheroplasts of \textit{Escherichia coli} (\textit{E. coli}). Sukharev \textit{et al.}\textsuperscript{109} found in 1993 that there were two types of MS channels in the membrane of the \textit{E. coli} cell, one with a large (~3 nS) and one with a small (~1 nS) conductivity. One year later they found, for the MS channel with large conductance, the corresponding gene (\textit{mscL}) and they were able to purify and reconstitute the mechanosensitive channel of large conductance (MscL).\textsuperscript{110} After purification and reconstitution into liposomes the channel still showed activity. The nucleotide sequence of the channel gene corresponds to an open reading frame of 136 \textit{α}-amino acids, and the protein was found to be very different from other known channels. In 1998 another step forwards was made by the elucidation of the crystal structure of MscL by Chang \textit{et al.}\textsuperscript{111}
Figure 1.8 Crystal structure of the closed conformation of MscL.\textsuperscript{112} (M. tuberculosis) Left: side view, right: top view.

The experiments in spheroplasts derived from E.coli cells showed that also another MS channel was present in the membrane. More detailed studies revealed that the small conductance channel could, in fact, be ascribed to two distinct proteins, MscS (S corresponds to small) and MscK (a potassium-regulated mechanosensitive channel), which belong to the same family.\textsuperscript{113} Conformational switching of MscS was found to depend on the applied pressure (mechanosensation) and on the voltage across the membrane.\textsuperscript{114} The crystal structure of MscS was published in 2002 (Figure 1.9).\textsuperscript{114}

MscK is peculiarly regulated by extracellular monovalent cations. Yet, another channel was found in the membrane of E.coli, that is MscM (M corresponds to mini), but so far, this channel has only been characterised electrophysiologically.\textsuperscript{115} Its conductance is the smallest in comparison with MscL, MscS and MscK. Little is known about MscM, MscK and MscS and their gating properties.
Figure 1.9 Crystal Structure of MscS in the open conformation (E. Coli).\textsuperscript{114} Left: side view, right: top view.

1.5.2 Osmoregulation by MS channels

It was already Martinac\textsuperscript{108} who suggested that the MS channels in bacteria act as sensors and pressure relieve valves upon osmotic changes. Under conditions of high osmolarity environments and water efflux, the cell uses several active transport systems to take up solutes (betaine, proline, K\textsuperscript{+}). In an environment of low osmolarity there is a water influx that creates a turgor pressure and ultimately the plasma membrane can be ruptured. This type of osmotic imbalance is usually relieved by the opening of MS channels.\textsuperscript{116} A fast release of solutes occurs when the internal hydrostatic pressure becomes too high, allowing the cell to survive. In Figure 1.10 a schematic representation of the release of osmolytes via MS channels is given. When the membrane tension increases due to an osmotic downshock MscM opens first, followed by MscS and finally MscL. The membrane tension necessary for opening (opening threshold) differs for the three types of channels. MscL opens at a membrane tension just before the membrane lysis. Remarkably, MscL of \textit{E. coli} has a significantly larger opening threshold than MscL of \textit{M. tuberculosis}.\textsuperscript{117} The reason could be found in the fact that MscL resides in different lipid environments in the two different bacteria. This suggests the importance of lipid-protein interactions.
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Figure 1.10 Schematic presentation of the release of osmolytes and proteins during osmotic downshock.\textsuperscript{118}

MscL has a large enough pore (\textasciitilde 30Å in diameter) to release even small proteins to the external media. In a proteoliposome system it has been shown that MscL can release insulin (a 6 kD protein).\textsuperscript{119} The pores of MscS and MscM are most likely too small to release proteins from the cytoplasm. For MscS and MscL it is clear that they play a relevant role in osmoregulation because cells lacking both channels lyse upon an osmotic downshock.\textsuperscript{116}

1.5.3 MscL structure and gating mechanism

As already shown, MscL (Figure 1.8) resides in the inner membrane of bacteria and consists of five identical subunits (pentamer). The N terminus is located in the cytoplasm and the protein monomer crosses the membrane twice via two \( \alpha \) helices (TM1 and TM2). The two helices are connected via a periplasmic loop (S2). The main hydrophobic pore is formed by the TM1 helices and the TM2 helices are situated between TM1 and the membrane. Helix TM2 is followed by a second loop that connects to the cytoplasmic helix (S3) which ends in the C terminus. Blount \textit{et al}.\textsuperscript{120} showed that the S3 helix can be deleted without loss of function of the channel. At the moment, it is assumed that the five S3 domains act as a size-exclusion filter at the cytoplasmatic entrance.\textsuperscript{121}
In subsequent studies, the first twelve N-terminal residues, not resolved in the crystal structure (Figure 1.8), were modelled as an amphiphatic $\alpha$-helix, called S1. A bundle of five S1 domains was postulated to form an additional cytoplasmic gate. It was found that the S1 domain can be mutated without a large change in the channel function. The precise conformation and function of the S1 domain is still unclear.

The gating mechanism of MscL is still a hot research topic and its details are not completely revealed. Several possibilities have been suggested. Already around the time that the crystal structure of MscL was published, several proposals where made for possible mechanisms. It was clear that TM1 had to swing away from the centre to form a gate and it was proposed that all TM domains contributed to the pore like staves in a barrel (barrel stave model).

More recently, an alternative mechanism was presented by Sukharev et al., the TM tilted model (Figure 1.11). In this model both TM1 and TM2 swing away to form a pore, which causes a gradual iris-like expansion and flattening of the channel. The pore is lined mostly by the polar parts of the TM1 helices. Although the model is found to be consistent with numerous experimental results and modelling criteria, the role of the S1 domain is still subject of discussion. In the model the S1 domain is an integral component but it was already shown that MscL still functions after severe mutations in this domain.

It is clear that the gating of MscL brings about a large change in conformation. Sukharev suggests that MscL reaches the open conformation via several ‘sub-states’, which are all in equilibrium. Under influence of tension in the membrane there is a transition from the closed resting state to the closed expanded state (Figure 1.11). Experimental results revealed the existence of at least three subconducting states. The transition from the closed expanded state to the first subconducting state appeared to be tension-dependent but the other transitions between subconducting states are not. In this model the outer wall of the transmembrane region acts as the ‘tension sensor’.

An interesting feature of the MscL channel is the function of the S2 domain, which has been called the ‘hairpin’. Upon shortening this domain, a higher tension is required to open the channel. Cleavage of the loop led to a channel that could be opened more easily. It is suggested that the loop acts as a spring that tries to keep the channel closed, a sort of fine-tuning of the mechanosensitivity.

But what is now the trigger for opening of the channel?
**Figure 1.11** Gating model of MscL (E. Coli) as proposed by Sukharev et al.\(^{122,126}\) The first structure (closed) is built by homology to the crystal structure of MscL.\(^{111}\) Picture taken from reference 106.

### 1.5.4 Possible triggers for opening of MscL

Since MscL still functions after purification and reconstitution in liposomes, the trigger should come from membrane-protein interactions.\(^{110}\)

A first explanation could be membrane thinning. Upon an osmotic downshock, a cell swells. It is assumed that then the membrane becomes thinner. A hydrophobic mismatch\(^{48}\) is induced between the membrane and the protein. Hydrophobic parts of the protein are exposed to water, which is thermodynamically unfavourable. There is also the possibility of exposure of polar parts of the protein to the lipid environment. Perozo et al.\(^{129}\) investigated the function of MscL as a function of the tail length of the lipids. Hydrophobic mismatch alone could not induce full opening of the channel but a decreased bilayer thickness led to easier opening of the channel. They presume that an intermediate closed state is stabilised by thinner membranes. Striking is the observation made by Yoshimura et al.\(^{130}\) in this context. Substitution of one of the hydrophobic residues facing the membrane lipids resulted in a situation that the MscL protein lost the ability to open. It was suggested that hydrophobic interactions between the membrane lipids and the periplasmic part of the protein are important for the functioning of MscL.

Another trigger for opening of the channel can be the curvature in the membrane. Asymmetric addition (addition to one leaflet) of 1-oleoyl-2-
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hydroxy-sn-glycero-3-phosphocholine (LPC, lyso PC) to membranes, induced opening of MscL without additional applied pressure. Symmetric addition (addition to both leaflets) of LPC did not lead to opening of MscL. Opening of MscL induced by an additive without extra pressure was also reported, earlier, by Martinac et al. Asymmetric curvature in the membrane seems to trigger the opening of MscL.

Although curvature and hydrophobic mismatch are discussed separately, they are also a part of the concept of lateral pressure. The opinion expressed in the latest publications is that an (asymmetric) change in the lateral pressure profile could induce opening of the channel.

Recently, other approaches, involving chemical modification of the proteins, have been applied for regulation of channel proteins. Banghart et al. constructed a Shaker K+ channel with a bi-functional group consisting of a blocker and a photoisomerisable group. They were able to regulate the function of the K+ channel by irradiation with suitable light. Also the opening and closing of a chemically modified MscL protein could be regulated by irradiation with the light of the appropriate wavelength.

1.6 Aim and outline of this thesis

As has become clear from the preceding sections, interactions between lipids and membrane proteins are not completely understood. Membrane properties that might have a large influence on the functioning of membrane proteins were discussed. At the moment there is a large interest in the elucidation of the mechanism of opening of MscL. The aim of this thesis is to obtain more insight into this mechanism. The activity of MscL was studied as a function of changes in the membrane properties. The goal was to induce changes in the membrane without the addition of additives and to this end novel surfactants were designed. These amphiphiles can undergo a photochemical trans-cis (E-Z) isomerisation by irradiation with light of a suitable wavelength. Subsequently, cis-trans isomerisation upon irradiation with light with another wavelength is also possible. So, the membrane properties are changed upon irradiation with light.

In Chapter 2 the synthesis of these novel light-sensitive surfactants is described. Two types of light-sensitive surfactants were prepared, single-tailed and double-tailed.

Chapter 3 describes the properties of the membranes which contain the double-tailed light-sensitive surfactants. Special features of these membranes are discussed but also the trans-cis isomerisation of the light-sensitive surfactants is studied in detail. A start has been made with the study of the effect of the isomerisation on the membrane properties.
In Chapter 4 the effect of trans-cis isomerisation of the double-tailed light-sensitive surfactants in the membrane on the activity of MscL was studied with two techniques: calcein efflux experiments and patch clamp.

Chapter 5 reports the properties of the single-tailed light-sensitive surfactants. The trans-cis isomerisation and the binding to membranes were studied in detail. Also the effect of trans-cis isomerisation on the function of MscL was examined.

In Chapter 6 the effect of trans-cis isomerisation of the light-sensitive lipids on the membrane properties is studied in greater detail. The effect on the T_m was studied using differential scanning calorimetry (DSC). Changes in the order parameters were examined by $^2$H NMR. A visualisation of the light-sensitive lipids as a part of a membrane has been obtained by MD simulations.

Chapter 7 reviews the most important conclusions from this thesis and its relevance in a broader perspective.

1.7 References

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