Amphiphiles containing aromatic groups in the hydrophobic part
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Chapter 1
Introduction

1.1 Role of aggregates in life

Aggregation processes are essential for life. The membranes of all living cells are bilayered aggregates, consisting of phospholipids, sphingolipids, proteins and steroids. According to the Singer-Nicolson fluid mosaic membrane model, the diffusion of these membrane components was supposed to be unrestricted, but recent research supports a non-random distribution and limited diffusion of membrane components. Also in bodily functions aggregates play an important role. In lungs, a mixture of lipids and surfactant-specific proteins can be found at the air-water interface of the aqueous film at the inner surface of the alveoli, where it functions to lower the surface tension of that aqueous layer, facilitating breathing and preventing collapse of the alveoli during expiration. The micelle forming bile acids are crucial for the digestion of fat. It has even been postulated that amphiphile assemblies played an important role in biogenesis.

In everyday life micelles help us wash and clean. Also surfactants are present in e.g. cosmetics, food and paints. In industry, surfactants are used for the production of oil, paper, pesticides and the manufacture of textiles and fibres. Furthermore, surfactants can be used, inter alia, as drug delivery agents and as transfection agents in gene therapy.

1.2 Aggregation in water

The main driving force for the aggregation of amphiphiles in water is hydrophobic interaction. Both micellar and bilayer aggregates have the polar or hydrophilic groups of the amphiphiles situated on the outside of the aggregate, where they are in contact with water, whereas the apolar or hydrophobic tails avoid contact with water and reside in the (almost) anhydrous core.

Water has a high melting and boiling point, despite its small size. This is the result of the stability of the three-dimensional hydrogen-bonded network formed by water molecules. The network is highly dynamic; hydrogen bonds are continuously formed and broken. Apolar solutes do not dissolve well in water and are hence called hydrophobic. At room temperature, the change in standard Gibbs energy of dissolution is unfavourable, mainly due to a large negative entropy change, whereas the enthalpy change is negative, mainly due to favourable
London dispersion interactions between water and apolar solutes.\textsuperscript{13,14} Upon increasing the temperature the enthalpy change upon dissolution becomes gradually positive, when the favourable interactions between water and solute diminish and the H–bond network is disrupted. The entropy change upon dissolution becomes less negative at higher temperatures.\textsuperscript{12,13}

At room temperature the hydrogen–bond network is kept intact as much as possible even when an apolar molecule is dissolved, resulting in a preferred parallel (tangential) orientation of the O–H bond of water with respect to the solute surface.\textsuperscript{15} This parallel orientation causes that the water looses orientational and translational degrees of freedom and as a result entropy is lost upon dissolving an apolar molecule in water. The typical hydration of an apolar solute is referred to as hydrophobic hydration.

As mentioned above, an important driving force for aggregation in water is hydrophobic interaction. Upon interaction of two apolar solutes, above a critical concentration, their separate hydrophobic hydration shells merge into one and in that process water molecules are released. Hereby the system gains in entropy. However, this does not explain the sudden appearance of micelles or other aggregates above a critical concentration of amphiphiles in solution. The cooperativity of many aggregation processes can be explained by assuming that above a certain critical concentration, an insufficient number of water molecules is present to provide all the molecules with individual hydration shells, resulting in aggregation. The low critical aggregation concentrations of many amphiphiles imply that these hydration shells must be extensive. However, neutron scattering studies contradict this, their outcome is that hydrophobic hydration shells are relatively small.\textsuperscript{15,16} However, definite conclusions about the size of the hydration shells are difficult because the different techniques provide conflicting results.

An alternative is that bulk hydrophobic interactions can be viewed as a particular kind of phase separation. Dissolving a surfactant increases the mixing entropy, but decreases the water entropy. The balance between mixing and water entropy will determine the concentration at which demixing occurs. As soon as the total entropy tends to become positive, the surfactant molecules will aggregate.\textsuperscript{17,18}

\subsection*{1.3 Aggregate morphology}

Surfactants can form a wide variety of aggregate morphologies. The morphology of the aggregate is determined by the molecular structure of the individual surfactant molecule and is the result of the interplay between the attractive hydrophobic interactions between the tails and the repulsive electrostatic and hydration shell overlap interactions between the head groups.\textsuperscript{11}
A useful concept to account for the relation between molecular structure and aggregate morphology involves the packing parameter $P$.\textsuperscript{19} This parameter is calculated by dividing the volume of the hydrocarbon chains ($V$) by the cross-sectional surface area ($a_0$) of the head groups and the length of the all-trans alkyl chain ($l$) (Equation 1).

$$P = \frac{V}{a_0l}$$

As shown in Table 1.1, micelles are formed when $P$ is lower than 1/3. If the head group area decreases relative to the hydrocarbon part, 1/3 < $P$ < 1/2, worm-like micelles are formed. An even larger packing parameter, 1/2 < $P$ < 1 predicts the formation of vesicles or bilayers. Inverted micelles or hexagonal phases are formed when the volume of the hydrocarbon part is high relative to the head group area ($P > 1$).

Table 1.1 Relation between effective molecular shape and aggregate morphology as given by the packing parameter.\textsuperscript{19}

<table>
<thead>
<tr>
<th>Surfactant shape</th>
<th>Packing parameter</th>
<th>Aggregate morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Micelle" /></td>
<td>&lt; 1/3</td>
<td>spherical micelles</td>
</tr>
<tr>
<td><img src="image" alt="Micelle" /></td>
<td>1/3 – 1/2</td>
<td>wormlike micelles</td>
</tr>
<tr>
<td><img src="image" alt="Micelle" /></td>
<td>1/2 – 1</td>
<td>bilayers, vesicles</td>
</tr>
<tr>
<td><img src="image" alt="Micelle" /></td>
<td>&gt; 1</td>
<td>inverted micelles, hexagonal phase</td>
</tr>
</tbody>
</table>
1.4 Spherical micelles

A micelle is a highly dynamic aggregate of surfactant molecules. The average residence time of a surfactant molecule in a micelle is around $10^{-5}$ to $10^{-6}$ seconds, the lifetime of a micelle is $10^{-2}$–$10^{-3}$ seconds. The shape and size of the micelle is also subjected to variations. A typical micelle generally consists of 40–100 amphiphile molecules. The size of a micelle is about 5 nm in diameter. The hydrophobic chains of the amphiphiles are conformationally disordered, which makes the hydrophobic core comparable to a liquid hydrocarbon. The hydrophobic chains can bend towards the micellar interface, so that some of the terminal methyl groups can be located near the head groups and be exposed to water. The hydrated micellar interface of ionic amphiphiles contains the head groups, a small part of the hydrocarbon tails and part of the counterions.

![Figure 1.1 Representation of a sodium n-dodecyl sulfate (SDS) micelle. Taken from ref. 23.](image)

The two methylene groups nearest to the head groups have considerable contact with the water phase, but almost no water penetrates deeper into the hydrocarbon core. The chain-water interface is smooth and has a width of a few Ångströms.
1.5 Bilayers and vesicles

In 1925 a bilayered structure was proposed for cell membranes. Later it was found that phospholipids extracted from cell membranes can solely form closed bilayer aggregates. These structures are called liposomes and have been frequently used as membrane models. Kunitake showed that synthetic amphiphiles can also form closed bilayer aggregates (vesicles). This set the stage for a new research area called membrane mimetic chemistry. Numerous different synthetic amphiphiles have been synthesised since then and the properties of the vesicles or other bilayer aggregates formed from these amphiphiles have been extensively investigated.

![Figure 1.2](image)

**Figure 1.2** Representation of a vesicle (left), a fluid bilayer (middle) and a bilayer in the gel state (right).

Vesicles consist of an aqueous compartment enclosed by a bilayer composed of amphiphiles (Figure 1.2, right). The hydrophobic part of the amphiphiles forms the inner core of the bilayer, whereas the hydrophilic head groups are located at the bilayer–water interface. Bilayers can exist in various phases, dependent on the amphiphile structure and the temperature. Two important phases are the gel phase and the liquid–crystalline phase. In the gel phase the hydrocarbon chains are ordered in an all-trans conformation and the bilayer is well packed (Figure 1.2, left). In the liquid–crystalline phase (also referred to as fluid phase), the chains are more disordered, gauche rotamers occur and the head groups are more loosely packed (Figure 1.2 middle). The hydrophobic chains are more mobile in the fluid phase. The fluid phase is biologically most relevant.
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At a certain temperature the gel phase cooperatively transforms into the liquid-crystalline phase. This temperature is defined as the main phase transition temperature ($T_m$). The structure of the amphiphile has a large influence on $T_m$. Increasing the length of the hydrocarbon chain of the amphiphile increases $T_m$. Introduction of unsaturated bonds and branching decreases $T_m$. Also the nature and hydration properties of the head group influences the $T_m$.34

Other relevant membrane properties, e.g. permeability, are also influenced by the molecular structure of the membrane lipids. This can be illustrated by reviewing the enormous diversity of membrane lipids found in several organisms, each living in various habitats with different properties. Achaeae, micro organisms originally discovered in hot springs, have peculiar membrane lipids, they are bolaform, which means that the lipid has two head groups connected by one or two membrane-spanning spacers, and the hydrophobic part consists of branched alkyl chains. The exact composition of the archaeal membrane and structure of the archaeal lipids depends on the living environment.35 These branched alkyl chains can also possess cyclopentane and cyclohexane rings.36,37 Cyclopropane rings also occur in membrane lipids and can be found in several bacteria and sporadically in eukaryotes. Unsaturated fatty acids are converted into cyclopropane containing fatty acids when diverse bacteria are subjected to starvation.38

An extreme example of adaptation of lipid structure to function are the ladderane lipids.39 They can be found in anaerobic ammonium-oxidizing (anammox) bacteria and consist of up to five cis-fused cyclobutane rings and form a compact membrane that is impermeable for hydrazine and hydroxylamine, that are toxic intermediates in the metabolism of anammox bacteria.39

The residence time of an amphiphile or lipid molecule in a vesicle ($ca. 10^4$ s) is much longer than that in a micelle (Section 1.4). Also the lifetime of a vesicle is larger than that of a micelle.29 That does not mean that a vesicle is not dynamic. Amphiphiles in a bilayer diffuse freely along the bilayer plane (lateral diffusion) In phospholipid bilayers the diffusion coefficient is in the order of $2 \cdot 10^{-8}$ cm$^2$/s at 20°C.40,41 The transverse diffusion or flip-flop, the movement of a lipid or amphiphile from the inner leaflet to the outer leaflet of the vesicle or the other way around, is much slower, because the hydrophilic head group has to move through the hydrophobic interior of the bilayer. The half-time of translocation across the bilayer (flip-flop) of a labelled lipid is often between 6 and 8 hours.
Aggregation is a process governed by noncovalent interactions. Noncovalent interactions also play a role in a variety of other processes in nature. Examples are enzymatic binding of a substrate, protein folding, base stacking and hydrogen bonding in the DNA double helix. Often, aromatic rings are involved.

1.6 Noncovalent interactions involving aromatic rings

Aromatic rings display various types of noncovalent interactions. Hunter et al. wrote a review concerning aromatic interactions, in which both theoretical aspects and applications of aromatic interactions are treated. A recent extensive review by Diederich et al. deals with theoretical aspects of many noncovalent interactions involving aromatic rings and gives many examples with biological relevance. The best known are \( \pi-\pi \) interactions, but aromatic rings can also form hydrogen bonds and interact with cations. \( \pi-\pi \) interactions are very important in biological processes, e.g. they affect protein structure and stabilize the double helical structure of DNA. Also host-guest systems, rotaxanes and other supramolecular architectures may rely on \( \pi-\pi \) interactions. The geometry of benzene in the gas, liquid and crystal phase provides insights into the arrangement.
of interacting aromatic rings. In all three physical states benzene prefers an edge to face orientation. The three most stable minima geometries are shown in Figure 1.4.

Both the edge to face and the offset stacked geometry have been found in proteins and various complexes. The preferred geometries for interactions between aromatic molecules have been explained by using a model for the charge distribution in a π-system. The electrostatic component of the π–π interaction has its origin in the quadrupole moment of aromatic rings and is an important factor in the arrangement of the interacting aromatic rings. The main attractive interactions between aromatic molecules are London dispersion forces; to maximize these interactions, the surfaces of both aromatic molecules must overlap. If we take only the dispersive forces into account, the stacked conformation (Figure 1.4, right) would be the most favourable conformation. However, the interactions of the already mentioned quadrupole moments of both aromatic rings in this geometry are unfavourable. Substituents can change the quadrupole moment and make the stacked arrangement favourable, as can be seen in the molecular complex of benzene and hexafluorobenzene. Charge transfer provides a low contribution to the ground state stability of the aromatic complex.

Figure 1.4 Geometry of π–π interactions. Edge to face (left), offset stacked (middle) and face to face stacked (right).

Calorimetric studies on apolar complexation, e.g. an enzyme binding an aromatic substrate, showed that this process often is enthalpy-driven. The enthalpic driving force for apolar complexation has been named nonclassical hydrophobic interactions. The enthalpy gain upon complexation has its origin in the large polarisability of the apolar surfaces involved in the complexation process and in favourable changes in solvent cohesive interactions. The large polarisability makes dispersion interactions between the apolar surfaces involved in the complexation process enthalpically favourable compared to the interactions of water and the apolar surfaces. Also hydration of large hydrophobic solutes and hydrophobic
Cavities\textsuperscript{64} results in a loss of hydrogen bonds of the water molecules in the first hydration–shell, due to geometrical constraints. Upon complexation the water molecules hydrating the hydrophobic surfaces are released and can form the maximum number of hydrogen bonds, which results in an enthalpy gain.

The hydrogen bonding interactions between aromatic $\pi$–systems and hydrogen bond donors have been studied extensively.\textsuperscript{65,66} Especially the hydration of benzene has been a subject of many investigations.\textsuperscript{66–69} Benzene in water has hydrogen bonds to two water molecules, one on each benzene plane.\textsuperscript{70} According to a study by Graziano and Lee\textsuperscript{71} the formation of these hydrogen bonds does not contribute significantly to the Gibbs energy, because the enthalpy gain is compensated by the enthalpy loss due to a diminished number of solvent–solvent hydrogen bonds and the restriction of the rotational degree of freedom of the solute molecule. This means that aromatics can have favourable interactions both in aqueous and in nonpolar media, in contrast to aliphatics and charged and strongly hydrogen–binding groups. This also might explain the occurrence of aromatic residues at the protein–protein interface and the presence of aromatic residues in membrane proteins at the lipid–water interface. We also note that hydrogen bonds can be found in proteins between aromatic residues and amino groups in proteins.\textsuperscript{72,73}

Cation–$\pi$ interactions are important in proteins that bind cationic substrates.\textsuperscript{74,75} In proteases many cation–$\pi$ interactions are observed.\textsuperscript{76} Calix[$n$]arenes, important building blocks in supramolecular chemistry, are also able to interact with various cations via cation–$\pi$ interactions.\textsuperscript{77} A large fraction of the cation–$\pi$ binding energy is electrostatic in origin. The origin of the remaining interaction energy is found in the interaction of the ion with the induced dipole in the aromatic molecule, donor–acceptor and charge–transfer interactions and dispersion forces.\textsuperscript{74}

Aromatic interactions are complicated due to the rather large surface area involved in the various interactions. This increases the contribution of van der Waals interactions and desolvation to the overall energy of aromatic interaction processes. The large surface area also complicates the analysis of the interactions, because the number of sites involved in electrostatic interactions is increased.

1.7 Changing the interactions between the tails of amphiphiles?

In the previous paragraph it was discussed that non-covalent interactions play an important role in both biological systems and synthetic molecular and supramolecular architectures. This fact led to the idea to incorporate aromatic units into the hydrophobic part of amphiphiles and to study the effect of this
incorporation on the aggregation behaviour and aggregate properties of these amphiphiles. The main issue is whether $\pi-\pi$ interactions are present, how their presence influences aggregate properties and whether it is possible to increase the interactions between the hydrophobic tails via $\pi-\pi$ interactions. Studies of amphiphiles containing aromatic moieties have been performed before. Many of them dealt with mesogenic aromatic moieties, both in single-tailed and double-tailed surfactants.\textsuperscript{78-97} The spectral properties of the aggregates formed from these surfactants have been emphasized.\textsuperscript{89-94} Some studies have examined the properties of phenyl-containing double-tailed surfactants, but mainly the phase behaviour was studied \textsuperscript{96,97} or the photo-physical behaviour.\textsuperscript{94}

1.8 Survey of the contents

In the previous paragraphs a brief overview of aggregation, properties of micelles and bilayer aggregates and aromatic noncovalent interactions was presented. In Chapter 2 the synthesis of disodium mono-\textit{n}-alkyl phosphates functionalised with a phenoxy, naphthoxy and biphenoxy group at the terminus of the hydrophobic chain is described. The aggregation properties were investigated and compared to those of disodium \textit{n}-tetradecyl phosphate.

The synthesis of sodium di-\textit{n}-alkyl phosphates containing aromatic moieties in both hydrophobic chains, the aggregation behaviour and the phase behaviour of the aggregates in water is described in Chapter 3.

In Chapter 4 the morphology of the aggregates formed from the di-\textit{n}-alkyl phosphates was studied using cryo-electron microscopy, the monolayer behaviour and the properties of the bilayer at the bilayer-water interphase. The conformations of the alkyl chain in the bilayer were also investigated.

A synthesis route for a phospholipid derivative containing aromatic units in the hydrophobic part and an oligomerisable head group is presented in Chapter 5. The possible use of this system as a tool to study membrane fusion is discussed.

An overview and discussion of the main conclusions from this thesis is presented in Chapter 6. This chapter also suggests new research projects on the basis of the presented results.
1.9 Experimental techniques

This paragraph provides a brief introduction to the main physical techniques employed in the present study.

1.9.1 Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) is a standard technique to characterise the phase behaviour of bilayers. DSC measures the isobaric heat capacity as a function of temperature ($C_p(T)$). In the DSC apparatus a sample cell and a reference cell are heated at a constant rate. Heat exchange to the environment is prevented by an adiabatic shield, so that the heat uptake of the sample corresponds to the electrical power of the heater. The difference in heat capacity between the sample and the reference is recorded. The integral of the resulting DSC curve represents the enthalpy change of the transition.

1.9.2 Fluorescence techniques

Pyrene fluorescence

Pyrene is often used to determine the critical micelle concentration (CMC) of surfactants.\textsuperscript{100} The fluorescence spectrum of pyrene is sensitive to the polarity of the environment and this can be quantified by measuring the relative intensities of the vibronic sub-peaks at 372 ($I_1$) and at 385 nm ($I_3$). In polar solvents, the $I_1/I_3$ ratio is high, in media with decreasing polarity the $I_1/I_3$ ratio also decreases.\textsuperscript{101} Upon adding surfactant to water, the $I_1/I_3$ ratio will exhibit a sudden decrease at the CMC.

Fluorescence anisotropy

1,6–Diphenyl–1,3,5–hexatriene (DPH) can be used to monitor membrane fluidity. Its fluorescence anisotropy depends on the rotational diffusion and motional freedom. In a well–packed membrane DPH exhibits a large fluorescence anisotropy, this anisotropy decreases upon increasing the temperature above $T_{m_0}$ when the membrane is more fluid and DPH experiences more motional freedom.\textsuperscript{102,103}

Laurdan fluorescence

The fluorescent membrane probe 2–dimethylamino–6–lauroylnaphtalene (Laurdan) has a charge–separated excited state. In polar solvents this excited state
can cause a reorientation of the solvent dipoles, called dipolar relaxation. This decreases the energy of the excited state, resulting in a red shift of the emission band.\textsuperscript{104} When embedded in a phospholipid bilayer, Laurdan exhibits a sensitivity to the phase state of the bilayer.

In the gel state the maximum emission is around 440 nm, whereas the maximum emission is around 490 nm in the liquid-crystalline phase.\textsuperscript{104} The cause of this phenomenon is that in the gel phase no dipolar relaxation occurs, because the bilayer is tightly packed. In the liquid-crystalline phase, the water both penetrates deeper into the bilayer and its mobility increases, so reorientation of the water molecules can occur. Interestingly, the red shift is independent of the head group structure and only depends on the phase state of the bilayer.\textsuperscript{105} The location of Laurdan in the phospholipid bilayer is in the region of the glycerol backbone.\textsuperscript{106} The emission maximum of Laurdan in bilayers is both a measure for the packing of the chains and the penetration of water into the head group region.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{laurdan.png}
\caption{Chemical structure of LAURDAN (2-dimethyl-amino-6-lauroyl-naphthalene).}
\end{figure}

\subsection{Fourier transform infrared spectroscopy}

The frequencies observed with infrared spectroscopy depend not only on the atoms and the different bonds in a molecule, but also on the conformation of molecules. The CH\textsubscript{2}-stretching frequency increases upon increasing molecular disorder and can be used to monitor the phase transition from the gel state (all-\textit{trans} conformation) to the liquid crystalline phase (more \textit{gauche} defects).\textsuperscript{107} Some bands are associated with specific conformations. In the wagging region three bands are of interest. The kink (gtg') and gtg conformer give rise to a band located near 1368 cm\textsuperscript{-1}, the double gauche near 1353 cm\textsuperscript{-1} and the end gauche band is
situated near 1341 cm\(^{-1}\).\(^{108}\) The intensity of these bands changes relative to the methyl “umbrella” deformation mode at 1378 cm\(^{-1}\).\(^{109,110}\) Relative intensities of these bands were measured for saturated alkanes.\(^{111}\) The percentage of conformers can be calculated using the rotational isomeric state (RIS) model.\(^{112}\) The intensity of the wagging bands and calculated percentages of the conformers were used to derive conversion factors\(^{111}\) and these have been used to derive the acyl-chain conformation of various phospholipids and phospholipid derivatives.\(^{109-111,113-116}\)

1.9.4 Cryo-transmission electron microscopy

Cryo-transmission electron microscopy (cryo-TEM) is a useful technique for imaging aggregates in solution. A thin film of an aqueous suspension is plunged into liquid ethane, which cools the specimen so fast that the water solidifies without forming crystals (vitrification). No staining is necessary, so no structural artefacts are induced due to the salts used for staining.\(^{117,118}\) The freezing is so fast that normally no rearrangement of the suspended structures takes place, except for micelle-forming surfactants that have a large temperature dependence on morphology.\(^{119}\) However, cryo-TEM may have its artefacts. Invaginated liposomes can be observed when osmotic stress is created due to evaporation of water during vitrification.\(^{120}\) Also liposomes or vesicles vitrified below their phase transition temperature differ in appearance from liposomes or vesicles vitrified above their phase transition temperature.\(^{121-123}\)

1.10 References


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