Properties of surfactants that govern their functions and applications on lipid membranes

by

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A thesis submitted in conformity with the requirements for the degree of Doctorate of Philosophy
Pharmaceutical Sciences
University of Toronto

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Abstract

This work aims at a better understanding of the parameters and properties of surfactants that govern their specific biological functions and their suitability for applications related to biological membranes. First, the hypothesis is tested that membrane lysis starts when a surfactant has built up a critical level of curvature strain, that is detected as a chain disordering. This could be confirmed for many surfactants but some, mostly biosurfactants act by a locally focused, heterogeneous perturbation, which explains their enhanced antibiotic activity and selectivity. A model and protocol were established to obtain a volumetric characterization of micelles by pressure perturbation calorimetry. For some classic detergents studied so far, their chain packing was found to agree with that of bulk hydrocarbon. Finally, the effective charge and consequent membrane permeability were assessed by zeta potential measurements.
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<tr>
<td>ASA&lt;sub&gt;ap&lt;/sub&gt;</td>
<td>Apolar accessible surface area</td>
</tr>
<tr>
<td>APLs</td>
<td>Alkylphospholipids</td>
</tr>
<tr>
<td>β-DPH-PC</td>
<td>2-(3-(Diphenylhexatrienyl)propanoyl)-1-Hexadecanoyl-sn-Glycero-3-Phosphocholine (β-DPH HPC)</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Methylene group</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl)-Dimethylammonio]-1-Propane Sulfonate</td>
</tr>
<tr>
<td>C&lt;sub&gt;10&lt;/sub&gt;EO&lt;sub&gt;5&lt;/sub&gt;</td>
<td>Pentaethylene Glycol Monodecyl Ether</td>
</tr>
<tr>
<td>C&lt;sub&gt;12&lt;/sub&gt;EO&lt;sub&gt;7&lt;/sub&gt;</td>
<td>Heptaethylene Glycol Monododecyl Ether</td>
</tr>
<tr>
<td>C&lt;sub&gt;12&lt;/sub&gt;EO&lt;sub&gt;8&lt;/sub&gt;</td>
<td>Octaethylene Glycol Monododecyl Ether</td>
</tr>
<tr>
<td>C&lt;sub&gt;m&lt;/sub&gt;EO&lt;sub&gt;n&lt;/sub&gt;</td>
<td>alkylpolyethelene glycol ether</td>
</tr>
<tr>
<td>C&lt;sub&gt;8&lt;/sub&gt;Malt</td>
<td>Alkylmaltoside</td>
</tr>
<tr>
<td>C&lt;sub&gt;8&lt;/sub&gt;Gluc</td>
<td>Octylglucoside</td>
</tr>
<tr>
<td>CL</td>
<td>Cardiolipin</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical Micelle Concentration</td>
</tr>
<tr>
<td>CsCl</td>
<td>Cesium Chloride</td>
</tr>
<tr>
<td>CYMAL-6</td>
<td>6-Cyclohexyl-1-Hexyl-β-D-Maltoside</td>
</tr>
<tr>
<td>DAS</td>
<td>Data analyzing software</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DM</td>
<td>n-Decyl-β-D-Maltopyranoside</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPH</td>
<td>1,6-diphenyl-1,3,5-hexatriene (DPH)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>DPPC</td>
<td>Dipalmitoylphosphatidylcholine</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>Fengycins</td>
<td>FE</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>GS</td>
<td>Gramicidin S</td>
</tr>
<tr>
<td>HSV-1, HSV-2</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>Iturins</td>
<td>IT</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>LED</td>
<td>Light emitting diode</td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>LM</td>
<td>Laurly maltoside</td>
</tr>
<tr>
<td>Lyso-PCs</td>
<td>Lysophosphatidylcholines</td>
</tr>
<tr>
<td>MEL-A, MEL-B, MEL-C</td>
<td>Mannosylerythritol lipids</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NG</td>
<td>n-Nonyl-β-D-Glucopyranoside</td>
</tr>
<tr>
<td>NM</td>
<td>n-Nonyl-β-D-Maltopyranoside</td>
</tr>
<tr>
<td>OG</td>
<td>Octyl glucoside</td>
</tr>
<tr>
<td>OM</td>
<td>n-Octyl-β-D-Maltopyranoside</td>
</tr>
<tr>
<td>P_{iso}</td>
<td>Isoenthalpic pressure</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td>PGLa</td>
<td>Peptidyl-glycylleucine-carboxyamide</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PLA2</td>
<td>Phospholipase A2</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>PPC</td>
<td>Pressure perturbation calorimetry</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>QACs</td>
<td>Quaternary ammonium compounds</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SLS</td>
<td>Sodium Lauryl Sulfate</td>
</tr>
<tr>
<td>SM</td>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>SOPC</td>
<td>1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>Surfactins</td>
<td>SF</td>
</tr>
<tr>
<td>$T_{iso}$</td>
<td>Isoenthalpic temperature</td>
</tr>
<tr>
<td>$T_m$</td>
<td>Phase transition temperature</td>
</tr>
<tr>
<td>TMA-DPH</td>
<td>1-(4-(trimethylamino)phenyl)-6-phenylhexa-1,3,5-triene</td>
</tr>
<tr>
<td>Tris buffer</td>
<td>(tris(hydroxymethyl)-aminomethane)</td>
</tr>
<tr>
<td>Triton X-100 (TX-100)</td>
<td>polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether</td>
</tr>
<tr>
<td>UM</td>
<td>n-Undecyl-β-D-Maltopyranoside</td>
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<tr>
<td>Val</td>
<td>Valine</td>
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CHAPTER (1) - Introduction

1.1 Lipid Membranes

1.1.1 Structures, properties and functions

A lipid membrane is a bilayer made of two layers of lipid molecules. The cell membranes of almost all living organisms and envelopes of viruses are based on a lipid bilayer, as are the membranes surrounding the cell nucleus and other sub-cellular structures such as mitochondria. Lipid bilayers are only a few nanometers in width (the bilayer thickness for pure fluid phosphatidylcholines is approximately in the range of (1.6 nm – 4 nm) but impermeable to most water-soluble (hydrophilic) molecules. Bilayers are particularly impermeable to ions, which allows cells to regulate salt concentrations and pH by pumping ions across their membranes using proteins called ion pumps.

Phospholipids are the most abundant lipids in most biological membranes. The polar head group of most phospholipids contains a phosphate group usually linked to another moiety such as choline, ethanolamine, glycerol, or serine. In glycerophospholipids, this head group is linked to two acyl chains via a glycerol backbone. Figure (1.1) shows a general structure of a phosphatidylcholine molecule.

![Diagram of Phosphatidylcholine](image)

**Figure (1.1):** A general structure of a phosphatidylcholine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC).
1.1.2 Hydrophobic effect and hydration force: Two phenomena being responsible for the formation and stabilization of lipid bilayer vesicles and cellular membranes

When phospholipids are exposed to water, they arrange themselves into a two-layered sheet with their tails pointing toward the center of the sheet. The center of this bilayer contains almost no water and excludes molecules like sugars or salts that are water soluble. This assembly process is driven by the hydrophobic effect. The hydrophobic effect is a unique organizing force, based on repulsion by the solvent (water) instead of specific attractive forces among hydrophobic tails. Water molecules form hydrogen bonds; the formation of each hydrogen bond reduces the internal energy by ≈ 5 kcal/mol. Therefore, the formation of hydrogen bonds among water molecules forces the hydrophobic parts of amphiphilic molecules to come together and form aggregates above a certain concentration. This phenomenon is responsible for assembly of membranes of cells and intracellular compartments.

![Hydration Force]

**Figure (1.2):** A schematic illustration of two lipid vesicles which are prevented from fusing by repulsion caused by the hydration force.

In addition to the hydrophobic effect, the hydration force is an important phenomenon governing membrane behaviour. It causes a strong repulsion of hydrated membrane surfaces at very small distance and, hence, prevents membranes from spontaneous fusion or aggregation. For neutral membranes with no ionic gradients across the membrane, the surface and trans-membrane potentials can be ignored and the only relevant electrostatic potential becomes the dipolar potential of the membrane. This potential is positive inside the membrane and negative towards the aqueous phase.
It has been described to contribute to the hydration force by inducing a structuring and orientational ordering of the water molecules near the membrane-surface. The water dipoles are described to show a preferential orientation with their negative ends facing towards the bulk aqueous phase.\textsuperscript{6,7} Other factors discussed to contribute to the hydration force are entropic effects of motions of head groups and lipid protrusions.\textsuperscript{6}

1.1.3 The mobility of lipid molecules within a lipid bilayer: gel phase versus fluid phase

All lipids have a characteristic temperature \( T_m \) at which they undergo a phase transition from the solid-like gel phase to the liquid crystalline (also referred to as fluid or liquid ordered) phase. For a given type of lipid, \( T_m \) decreases with decreasing length and increasing degree of unsaturation of the acyl chains. An important property of a fluid bilayer is the relatively high in-plane mobility of the individual lipid molecules. A lipid molecule exchanges locations with its neighbor on a nanosecond time scale and migrates over long distances through the process of a random walk.\textsuperscript{8} In contrast to this large in-plane mobility, it requires high activation energy for lipid molecules to flip-flop from one side of the bilayer to the other. In a phosphatidylcholine-based bilayer this process typically occurs over a timescale of weeks.\textsuperscript{9} This discrepancy can be understood in terms of the basic structure of the bilayer. For a lipid to flip from one leaflet to the other, its hydrated head-group must cross the hydrophobic core of the bilayer, an energetically unfavorable process.

Unlike liquid crystalline bilayers, the lipids in a gel phase bilayer are virtually locked in place by a hexagonal array of their chains and exhibit very little flip-flop and lateral mobility. Between clusters of quasi-crystalline chain packing there are, however, lattice defects that accumulate perturbants\textsuperscript{9}

1.1.4 Impacts of phospholipid acyl chain length and saturation on gel and fluid phases

The gel phase is stabilized by the attractive van der Waals interactions between adjacent lipid molecules. The extent of this interaction is governed by the length and packing of the acyl chains. Longer acyl chains have more contact area to interact, increasing the strength of this interaction and consequently decreasing the lipid mobility. Thus, at a given temperature, a lipid with shorter acyl chains is more fluid than an otherwise identical long-tailed lipid.\textsuperscript{10} Consequently, the fluid to gel phase transition temperature increases with increasing number of carbons in the lipid acyl chains. Saturated phosphatidylcholine lipids with tails longer than 14 carbons are solid at room temperature, while those with fewer than 14 are liquid.\textsuperscript{10}
Aside from chain length, the transition temperature is also affected by the degree of unsaturation of the lipid tails. A cis double bond produces a kink in the acyl chain, disrupting the well-packed structure of parallel chains. This disruption creates extra free space within the bilayer which allows for additional flexibility in the adjacent chains. It is this disruption of packing that leads to lower transition temperatures with increasing number of double bonds. This is a particularly powerful effect; decreasing the overall chain length by one carbon usually alters the transition temperature of a lipid by ten degrees Celsius or less, but adding a single double bond can decrease the transition temperature by seventy degrees or more.

Two lipids used here are 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC). Both lipids have phosphatidylcholine head group and share an oleoyl chain which is 18 carbons long and has a cis double bond at the C9 position. However, they differ in the number of carbons in their saturated acyl chains, which are 16 for the palmitoyl and 18 for the stearoyl chain. The transition temperature of POPC is -2 °C and it is 6 °C for SOPC. Both phospholipid bilayers are in the fluid phase at temperatures (10 °C and 20 °C) used in my project.

Most natural membranes comprise complex mixtures of different lipid molecules. Such mixtures often exhibit properties intermediate to their components, but are also capable of a phenomenon which is not seen in single component systems and that is phase separation. If some of the components are liquid at a given temperature while others are in the gel phase, two phases can coexist in spatially separated populations.

1.1.5 The role of cholesterol in cell membranes

The presence of cholesterol exerts a profound and complex influence on lipid bilayer properties because of its unique physical characteristics. Although it is a lipid, cholesterol bears little resemblance to a phospholipid. The hydrophilic domain of cholesterol is quite small, consisting of a single hydroxyl group. Adjacent to this alcohol is a rigid planar structure composed of several fused rings. At the opposite end of the ring structure is a short single chain tail. It has been known for decades that the addition of cholesterol to a fluid phase bilayer decreases its permeability for water. The mode of this interaction has more recently been shown to be due to cholesterol intercalating between lipid molecules, filling in free space and increasing the order of surrounding lipid chains. This interaction also increases the mechanical rigidity of fluid bilayers and decreases their lateral diffusion coefficient. In contrast, the addition of cholesterol to gel phase bilayers disrupts local packing order and increases the diffusion coefficient.
Membranes that are rich in cholesterol (typically about 20 mol-% or more) and saturated lipids can form a so-called liquid ordered phase, which has intermediate properties between liquid crystalline and gel phases.\textsuperscript{18} While the chains are ordered, their 2-dimensional packing and the lateral diffusion of the molecules are liquid-like. In the resulting terminology, the liquid crystalline or fluid phase is termed “liquid ordered” and the gel phase “solid ordered”. The possibility of a coexistence of fluid and liquid ordered phases in mixtures of cholesterol, saturated and unsaturated lipid has been proposed to give rise to functional domains in biological membranes referred to as “lipid rafts”.\textsuperscript{19-21}

1.1.6 Lipid compositions in eukaryotic cell membranes

A biological membrane acts as a selective barrier around a cell or organelle. The membrane has also other biological functions such as signal transduction and selective transportation. It consists of a lipid bilayer with embedded and attached proteins and carbohydrates. A mixture of various types of lipids constitutes about 50% of the mass of most animal cell membranes.\textsuperscript{22} Phospholipids with certain head groups can alter the surface chemistry of a bilayer. Phosphatidylcholines (PCs), the most abundant phospholipids, make up for almost 50% of total phospholipid content in mammalian cell membranes.\textsuperscript{23} Phosphatidylcholine (PC) is a zwitterionic headgroup with a negative charge on the phosphate group and a positive charge on the amine group.\textsuperscript{23} Most of the PC in the plasma membrane is found within the outer leaflet.

Other important phospholipids of eukaryotic cells are phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM).\textsuperscript{21} PE is the second most abundant phospholipid in mammalian membranes, contributing 20-30% of total phospholipid content.\textsuperscript{24} It is zwitterionic at physiological pH due to the protonated amino group and the dissociated phosphate group. PE is found in greater abundance in the inner leaflet of the plasma membrane, with approximately 20% of plasma membrane PE found on the outer leaflet.\textsuperscript{24} PE has a relatively small head group which accommodates the insertion of proteins within the membrane while still maintaining the integrity of the membrane. Another characteristic of PE, arising from the small head group size, is its tendency to form non-bilayer structures. This is important for the formation of structures involved in membrane fusion and budding processes.\textsuperscript{24} PE is also enriched in the membranes of mitochondria and is essential for the growth and stability of these energy-producing organelles. PE is used in the production of glycosylphosphatidylinositol which facilitates the anchoring of proteins to the membrane.\textsuperscript{24}

PS accounts for 5-10% of cellular phospholipids. It is found in greatest abundance in the inner face of the plasma membrane. The presence of PS in the outer face of the plasma membrane is a marker of cell
apoptosis.\textsuperscript{25,26} Phosphatidylglycerol, an anionic phospholipid at physiological pH, is mainly found in bacterial cell membranes. It is present at a level of 1-2\% in most animal tissues, but it can be the second most abundant phospholipid in lung surfactant at up to 11\% of the total.\textsuperscript{27,28}

Sphingomyelin has a PC headgroup but differs from glycerophospholipids by its sphingosine backbone. It is abundant in mammalian cell membranes; an SM content of 23 \% of all membrane phospholipid has been reported for the erythrocyte membrane. SM resides mainly in the outer leaflet.\textsuperscript{29} Since it typically contains saturated chains, it interacts favorably with cholesterol and the mixture can form a liquid ordered phase.

Plasma membranes of mammalian cells have high contents of cholesterol of the order of 30 mol-\% and more.\textsuperscript{29} As mentioned above (section 1.1.6), this has a crucial impact on the mechanical properties and the lateral organization of the membrane.

Lysophosphatidylcholines (Lyso-PCs), also called lysolecithins, are derived by partial hydrolysis of phosphatidylcholines by phospholipase A\textsubscript{2},\textsuperscript{30} which removes the sn-2 acyl chain. Lyso-PCs are present in trace amounts in the cell membrane (≤ 3\%) as well as in the blood plasma.\textsuperscript{30} Lyso-PCs are reported to inhibit various membrane fusion events such as microsome fusion, exocytotic fusion, and baculovirus fusion.\textsuperscript{31} It was suggested that this inhibition was due to their “inverted cone” shape, which hinders the formation of negatively curved, intermediate lipid structures required for fusion.\textsuperscript{32} Fungus, a eukaryotic cell, is enclosed by a cell wall as well as a cell membrane based on a lipid bilayer. The fungal cell membrane has similar phospholipids as those in other eukaryotic cells. For example, the major phospholipids found in the cellular membranes of \textit{Saccharomyces cerevisiae} include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS).\textsuperscript{33} Phosphatidylglycerol (PG) and cardiolipin (CL) are also major phospholipids in mitochondrial membranes. The predominant sterol in a fungal cell membrane is ergosterol.\textsuperscript{33}

\subsection*{1.1.7 Lipids in prokaryotic cell membranes}

Further to PE and PC described above, bacterial cell membranes have substantial contents of negatively charged lipids like phosphatidylglycerol (PG) or diphosphatidylglycerol (or cardiolipin, CL).\textsuperscript{34} The outer membrane of Gram-negative bacteria is principally different from the typical bilayers forming their inner membrane and the membrane of Gram-positive bacteria; its outer leaflet is primarily formed by lipopolysaccharides.\textsuperscript{34,35} Unlike eukaryotes, bacterial membranes (with some exceptions e.g. \textit{Mycoplasma} and methanotrophs) generally do not contain sterols. However, many microbes contain
structurally related compounds called hopanoids which likely fulfill the same function. The main membrane phospholipids, in *E. coli*, a Gram-negative bacterium, are phosphatidylethanolamine (PE) and the acidic phospholipids phosphatidylglycerol (PG) and cardiolipin (CL). An example of gram-positive bacteria is *Bacillus subtilis*. The cytoplasm membrane of this bacterium is composed of 75% phospholipids, and other lipids including lipo-amino acids and diglucosyl diglyceride. The major phospholipids are diphosphatidyl glycerol and phosphatidyl ethanolamine, with small amounts of phosphatidyl glycerol.

### 1.2 Surfactants

#### 1.2.1 Definition

Surfactants, in the widest sense, are “surface active agents”. That means, they are wetting agents that lower the surface tension of a liquid by adsorbing at the liquid-gas interface, and they reduce the interfacial tension between two liquids by adsorbing at the liquid-liquid interface. Surfactants are amphiphilic molecules comprising locally separate hydrophobic and hydrophilic parts and self-associate in aqueous dispersion due to the hydrophobic effect above the critical aggregation concentration. As described above, all these most general properties apply for example also to phospholipids.

Here, the term surfactant will primarily be used in a much narrower context, limited to micelle-forming amphiphiles with critical micelle concentrations in the micro- and lower millimolar range. This class shares many properties, functions and applications that do not apply to, for example, typical membrane lipids. Such surfactants possess a wealth of applications and biological functions. They are versatile agents for accomplishing tasks as different as cleaning (detergents), solubilizing integral proteins from their native membranes, dispersing poorly water-soluble active substances (e.g., for drug delivery), or exerting sterilizing or spermicidal properties in ointments and cosmetics.

#### 1.2.2 The Critical Micelle Concentration

The value of the CMC depends on the balance between the hydrophobic effects e.g., the accessible apolar surface area that can be screened from water in a micelle), the loss of motional and configurational entropy of the molecules in a micelle, and intermolecular forces such as electrostatic, dipolar, or hydration forces that repel the head groups from each other. The apparent water-accessible surface area of the apolar part of a detergent molecule, $ASA_{ap}$, is about $31 \, \text{Å}^2$ per methylene. As a consequence of enhancing the hydrophobic effect, the CMC decreases with increasing apolar accessible surface area per surfactant. The contribution to the standard free energy of micelle formation by one
water accessible methylene group is \(-3.0\) kJ/mol, which explains the rule that the CMC decreases by one order of magnitude upon addition of two CH$_2$ groups to the hydrophobic tail.$^{41}$

There are numerous techniques to determine the CMC of a detergent, including surface tension measurements, fluorescence spectroscopy of water-quenched hydrophobic dyes, conductivity (for ionic surfactants) or ultrasound measurements.$^{42}$ However, the most direct technique to determine both the CMC and the enthalpy and heat capacity changes of micellization is isothermal titration calorimetry (ITC).$^{43}$ This method will be explained in the methodology section.

### 1.2.3 Micelle Size and Structure

A micelle is an aggregate of surfactants having a hydrophobic core, where the hydrophobic parts of the surfactants are largely screened from water, and a rough hydrophilic surface covered by polar head groups.

Based on the effective molecular shape of the amphiphile (also quantified as its spontaneous curvature), micelles can be small spheres or disks, oblate or prolate ellipsoids, long cylinders, or the surfactant may form bilayers or cubic phases.$^{4,44}$ Surfactants with large positive spontaneous curvature (large head group and small tail) will aggregate into spherical or semi-spherical micelles.$^{44}$ Bile salts form very small, disk-shaped micelles. Amphiphilic molecules with about zero spontaneous curvature form bilayer vesicles. Amphiphiles with negative spontaneous curvature form inverted hexagonal, inverted micellar, or cubic structures.$^{44}$

Aggregate topology (e.g., micellar or lamellar), structure and size can be understood in terms of the packing parameter, $v/A_0 \ell_c$.$^{45}$ It reflects the molecular shape in terms of its equilibrium head-group area projected to the surface of the hydrophobic core, $A_o$, the volume of its hydrophobic part, $v$, and the critical projected length of the chain $\ell_c$.$^{45}$ In a one-component system, one chain has to reach the center so that $\ell_c \leq \ell_{max}$ where $\ell_{max}$ stands for the length of an all-trans chain. For an aggregation number $N$, the hydrophobic volume of the aggregate is $Nv$, and the surface of its hydrophobic core is $NA_0$. With the hydrophobic radius $\ell_c = R_{sph} = 3Nv/NA_0$ derived from the volume-to-surface ratio of a sphere and $R_{sph} \leq \ell_{max}$ it is found that a sphere is only possible for molecules with a “packing parameter” of $v/(A_0 \ell_{max}) \leq 1/3$.$^{46}$

Using Tanford’s approximation for alkyl chains of $n_C$ carbons:$^{47}$

$$v = 26.9 \AA^3 \cdot n_C + 27.4 \AA^3 \quad (1.1)$$
\[ \ell_{\text{max}} = 1.265 \, \text{Å} \cdot n_c + 1.5 \, \text{Å} \]  \hspace{1cm} (1.2)

one obtains that detergents with polar-head and alkyl tail with a head group area of \( A_0 > 62 \, \text{Å}^2 \) can form spherical micelles, apparently independently of the chain length.\(^{46}\) At this critical value, one detergent tail will be all-trans to reach the midpoint of the sphere. The bigger \( A_0 \), the smaller will be the micelle radius, \( \ell_{\text{sph}} = 3v/A_0 \).

Detergents with smaller head groups (for mono-alkyl detergents: \( A_0 = 41-62 \, \text{Å}^2 \)) obey \( v/(A_0 \ell_{\text{max}}) \leq 0.5 \) as required for a cylinder and form cylindrical micelles with a radius, \( \ell_{\text{cyl}} = 2v/A_0 \). Coexisting spherical and cylindrical micelles were found to share a common \( v/A_0 \) so that \( \ell_{\text{sph}}/\ell_{\text{cyl}} \approx 3/2 \).\(^{43}\)

Amphiphiles with even smaller head groups and a packing parameter of \( v/(A_0 \ell_c) \approx 1 \) form bilayers and they are not considered here. Micelles are highly disordered and dynamic; the alkyl chains adopt numerous conformations and orientations with respect to the micelle surface to accommodate the chains with a minimum of voids inside the micelle.\(^{48-50}\) Observations from molecular modeling by all-atom MD simulations, spectroscopic, and thermodynamic techniques on micelles proves that the surface of a micelle is not completely covered by the hydrophilic head groups but parts of the hydrophobic chains (or a certain number of CH\(_2\) groups) are exposed to water.\(^{41,48,51}\)

At the CMC, small spherical micelles will typically be formed. At higher detergent concentrations, the small spherical micelles may grow to worm- or disc-like micelles or vesicles either in a gradual fashion or in a rather cooperative process referred as second CMC. Thermal dehydration of the head group causes growth of the micelles. The thermotropic sphere-to-rod transition is shown to involve a coexistence of spherical and rod-like micelles rather than a gradual process from spherical micelles to prolate ellipsoids or half-spherical end caps separated by a growing cylindrical part.\(^{43}\) At the cloud point temperature, the detergents are strongly dehydrated and segregated largely from the aqueous phase. Dynamic light scattering is used to determine the hydrodynamic radius of spherical aggregates on the basis of the diffusion equation.

### 1.2.4 Dynamic structure of micelles

General considerations of topologies and geometrical constraints are helpful but fail to represent the realistic, dynamic structure of a micelle. A molecular dynamics simulation of a sodium dodecyl sulfate (SDS) micelle done by Bruce et al.\(^{52}\) shows that this micelle is not strictly spherical. In addition, the SDS monomers are not all perfectly arranged around the center of the micelle, but, instead, liquefaction of the interior of the micelle allows torsional motion to assist the tails in orienting themselves in a variety...
of directions toward and away from the hydrocarbon core while still generally associating with the other tails.

![Figure (1.3): SDS micelle cross sections after (1) t = 1 ns, (2) t = 3 ns. Atoms are represented by the following colors: Blue plus symbol for sodium ions, red for oxygen, yellow for sulfur, black for carbon. Reproduced with permission from Bruce, C. D., Berkowitz, M.L., Perera, L., Forbes, M.D.E., Molecular dynamics simulation of sodium dodecyl sulfate micelle in water: micellar structural characteristics and counterion distribution. J. Phys. Chem. B 2002, 106, 3788-3793. Copyright (2002) American Chemical Society, ISSN 0002-7863.](image)

Although the average radius of the SDS micelle is 22 Å, the micelle has a larger water-accessible surface area compared to a perfectly smooth ellipsoidal body. This implies a large amount of surface roughness. It has been found that approximately 70% of the water-to-micelle contact occurs via the headgroups, leaving a significant portion of available water to micelle contact to occur through the tails. Figure (1.3) shows the computational simulation of SDS micelle structure after 1ns and 3ns. This shows that SDS molecules are in continuous motion within the micellar assembly; the structure of micelle and the position of monomers change significantly within 2 ns. There are situations where a few hydrophobic methylene groups of the chain are exposed to water molecules.

Surfactants can be grouped into synthetic and biosurfactants. Biosurfactants are produced by living organisms, for example as self-defence weapons against their enemies, food digesting agents such as bile salts, or easing respiratory compounds as lung surfactants in mammals. Amphiphilic peptides, saponins, and lipopeptides are produced by organisms to attack others by permeabilizing their cell membranes; they are applied and studied as new types of antibiotics and pesticides. Digitonin, a saponin, is a glycoside obtained from *Digitalis purpurea*; the aglycone is digitogenin, a spirostan steroid.
Digitonin has several membrane-related applications such as solubilizing membrane proteins, precipitating cholesterol, and permeabilizing cell membranes.\textsuperscript{56}

1.2.5 Volumetric properties of micelles

Volumetric properties of micelles have been addressed by densitometry and deduced from the pressure dependence of the CMC. At ambient pressure, micelle formation was found to be is accompanied by an increase in volume of the order of $10^{–20}$ mL/mol.\textsuperscript{57-59} This may be related to the release of water from the monomers and, possibly, voids between the chains in the micellar core.

The thermal volume expansivity of surfactants has been reported to decrease upon micelle formation at low temperature,\textsuperscript{60} but precise information about expansivity changes is not straightforward to be obtained by traditional methods. In this study, pressure perturbation calorimetry will be utilized to resolve this problem.

The compressibility of the surfactant is increased upon micelle formation. As a result, the volume change of micellization decreases with increasing pressure and becomes negative at a certain pressure, $p_{iso}$. As explained by Le Chatelier’s principle, increasing pressure opposes micelle formation and, hence, increases the CMC as long as the volume change is positive. At $p_{iso}$, the CMC shows a maximum in its pressure dependence.

This behaviour is analogous to the typical temperature of the CMC of non-ionic surfactants; the negative heat capacity change causes a decrease of the enthalpy that reaches zero at a temperature $T_{iso}$. Consequently, the CMC(T) shows a minimum at $T_{iso}$\textsuperscript{51}

1.2.6 Synthetic detergents and their applications

Many synthetic surfactants have been developed and used as cleaning agents in the widest sense, which explains the term “detergent” that is now often used as a synonym for “micelle-forming surfactant”. The most common non-ionic detergents are ethoxylates, ethylene and propylene oxide copolymers and sorbitan esters. Examples of commercially available, ionic surfactants include sodium dodycylsulfate (SDS), and sodium dodecylbenzenesulfonate (anionic) and alkylated quaternary ammonium salts (cationic). Surfactants are used for an extremely wide variety of industrial applications involving emulsification, foaming, detergency, wetting and phase dispersion or solubilization.\textsuperscript{61} For example, SDS is used as foaming and cleaning agent in toothpaste and shampoo.\textsuperscript{62,63} Polysorbates 20 and 80 are used as solubilising and emulsifying detergents, respectively, in the production of
Shampoo.

Surfactants have also widely been used in pharmaceutical excipients, for example Tween and sorbitan monolaurate. Some detergents have been used as spermicidal agents, including nonylphenoxy polyethoxy ethanol (nonoxynol-9 or N-9), methoxy polyoxyethylene glycol 550 laurate and p-methanylphenyl polyoxyethylene (8.8) ether (TS-88). Detergent-type spermicides act primarily by breaking down the sperm membrane.

Cationic detergents, like quaternary ammonium compounds (QACs), are widely used antiseptics and disinfectants. QACs have been used for a variety of clinical purposes (e.g., preoperative disinfection of unbroken skin, application to mucous membranes, and disinfection of noncritical surfaces). In addition to having antimicrobial properties, QACs are also excellent for hard-surface cleaning and deodorization. QACs are membrane active agents with a target site predominantly at the cytoplasmic (inner) membrane in bacteria or the plasma membrane in yeasts.

1.2.7 Biosurfactants

In the past few decades, biosurfactants (surfactants from living organisms) have gained attention as good alternative to chemically synthesized, conventional surfactants because they typically have the following characteristics: structural diversity (e.g. glycolipids, lipopeptides, fatty acids, etc.), biodegradability, strong potency, specificity, synergism, high efficacy at extreme temperature and pH values, and ability to be produced from renewable and cheaper substrates. These molecules can be used as emulsifiers, humectants, antimicrobials, antifungals, preservatives, and detergents in various industries. They are ecologically safe and can be applied in bioremediation and waste treatments. Biosurfactant antibiotics are believed to be less challenged by the development of resistant pathogens. Biosurfactant-producing bacteria such as Pseudomonas and Bacillus can be cultured well on renewable resources such as vegetable oils, distillery and dairy waste. Several biosurfactants such as lipopeptides have strong antibacterial, antifungal and antiviral activities. Other medically relevant uses of biosurfactants include their role as anti-adhesive agents to pathogens, making them useful for treating many diseases as therapeutic and probiotic agents. Mammalian biosurfactants such as pulmonary surfactant and bile salts play a very critical role; lung surfactants support the process of respiration and bile salts break down the lipids in mammals.

1.2.7.1 Lung Surfactants: Make respiration possible

In 1976, von Neergard et al., suggested that the lungs might lower surface tension (γ) by producing a surfactant. Subsequent research has confirmed that suggestion. However, rather than a single
compound, pulmonary surfactant is a mixture of constituents, including a distinct set of phospholipids along with small amounts of cholesterol and specific proteins. Together, they have a synergistic effect which makes breathing possible. After synthesis by the alveolar type II pneumocytes, assembly into multilamellar vesicles, and secretion into the liquid layer that lines the alveolus, the mixture of components acts as a surfactant, adsorbing to the air/liquid interface and forming a film that lowers $\gamma$ near to zero. The function of pulmonary surfactant is essential. Ventilation of lungs that lack adequate surfactant, both in premature babies and in experimental animals subjected to repeated lavage, damages the alveolocapillary barrier and leads to pulmonary edema, respiratory failure, and death.

Mammalian lung surfactant contains less than 40% disaturated phospholipids, mainly dipalmitoylphosphatidylcholine (DPPC). DPPC is the only major component that has a gel-to-fluid transition temperature higher than the core body temperature ($37^\circ C$) and hence is capable of sustaining a near-zero surface tension of $<5mN/m$, perhaps even $1mN/m$ during the exhalation process. The ability of the compressed surfactant films to reach and sustain $\gamma$ well below the values achieved during adsorption ($\sim24 mN/m$) indicates that the films must have specific physical characteristics. The surfactant film in the lung must be in a supersaturated (highly viscous) metastable state. It maintains an extraordinary metastability, thus preventing lung alveoli from collapsing at very low surface tensions.

Pulmonary surfactant and classical molecular surfactants (or detergents) adsorb by different processes. Classical surfactants adsorb as monomers, the concentration of which determines the final $\gamma$ reached at equilibrium according to the Gibbs adsorption isotherm. At the critical micelle concentration, the interface reaches its point of saturation and additional detergents form micelles. The micelles are themselves surface-inactive and have essentially no effect on surface tension. On the other hand, the CMC of phospholipids that constitute pulmonary surfactant falls into the range of $10^{-10}$ to $10^{-9}M$. In essentially all studies in vitro, and at estimated physiological concentrations, pulmonary surfactant is far above its CMC and should therefore adsorb in vesicular form.

### 1.2.7.2 Bile Salts, human food digesting surfactants

Bile is an alkaline secretion produced by hepatocytes in the liver which is stored in the gall bladder and released into the duodenum in response to the presence of food. Its primary aim is to aid the digestion of fat. The main active components are bile acids and bile salts, which are unconventional surfactants in terms of their structure and behaviour. The molecular structure of these biosurfactants is somewhat
different from conventional surfactants in that they are derived from cholesterol, and have essentially a steroid structure composed of 4 rings with a side chain terminating in a carboxylic acid. Figure (1.4) shows chemical structures of two bile acids. The main bile acids in humans are cholic acid, deoxycholic acid and chenodeoxycholic acid. Bile acids can be conjugated with taurine or glycine (e.g. taurocholic acid, glycodeoxycholate).

![Chemical structures of two bile acids](image)

**Figure (1.4):** Chemical structure of two bile acids. Figures A (Cholic acid), and B (Taurocholic acid) show the amphiphilic nature of both molecules.

The ring structure and location of hydroxyl groups result in a molecule with hydrophobic and hydrophilic faces, which is thought to orient relatively parallel along an interface as shown in Figure (1.4). This is in contrast to the largely perpendicular orientation of the long axis of typical head-tail detergents. Because of the morphology of the bile salt, it is unlikely that it will be able to pack as effectively as conventional surfactants. Wickham et al. showed the importance of mixed micelles comprising both bile salts and phospholipids for the digestion of fats; the mixture gave rise to interfacial tensions of less than 1 mN/m.

### 1.2.7.3 Bacillus Lipopeptides

Bacteria produce a wide range of lipopeptide biosurfactants that may act as host-defence compounds against attacking or competing organisms, affect motility and adsorption, and solubilize poorly soluble nutrients. These biomolecules are synthesized non-ribosomally via large multi-enzymes (non-ribosomal peptide synthetases). These biosynthetic systems lead to a remarkable heterogeneity among the lipopeptides generated, for example, by *Bacillus* strains with regard to the type and sequence of amino acid residues, the nature of the peptide cyclization and the nature, length and
branching of the fatty acid chain.\textsuperscript{78} Serenade Biofungicide is based on the naturally occurring strain \textit{Bacillus subtilis} QST-713, discovered in a California orchard by AgraQuest scientists. \textit{Bacillus subtilis} QST-713 produces three families of lipopeptides: iturins (IT), fengycins (FE), and surfactins (SF) that synergize with each other to inhibit germ tubes, mycelium, and certain bacterial cells.\textsuperscript{79,81}

Iturins are heptapeptides linked to a β-amino fatty acid chain with a length of 14 to 17 carbons. They display a strong \textit{in vitro} antifungal action against a wide variety of yeast and fungi but only limited antibacterial and no antiviral activities.\textsuperscript{55,82-84} This fungi-toxicity of iturins relies generally on their membrane permeabilization properties.\textsuperscript{78} However, whereas for example surfactins induce relatively large unspecific leaks or pores in membranes, some iturins were described to form selective, ion-conducting pores that collapse vital pH or ion gradients between the cell and its environment.\textsuperscript{85}

Iturin A has been proposed as an effective antifungal agent for profound mycosis.\textsuperscript{55} Other members of the iturin group, including bacillomycin D and bacillomycin Lc were also found to have antimicrobial activity.\textsuperscript{79,86} A new bacterial strain of the genus \textit{Paenibacillus koreensis} produces an iturin-like antifungal compound which could prove to be even more effective against pathogenic fungi.\textsuperscript{87}

Surfactins, another group of cyclic lipopeptides produced by \textit{B. subtilis} strains, have antibiotic activity against fungi and mycoplasma and, to a lesser extent, against certain bacteria, viruses, and cancer cells.\textsuperscript{55} Bacillus surfactins comprise three different types, surfactins A, B and C, which are classified according to the differences in their amino acid sequences. Surfactin A has L-leucine, surfactin B has L-valine, and surfactin C has L-isoleucine at the amino acid position involved in lactone ring formation with the C\textsubscript{14}-C\textsubscript{15} β-hydroxy fatty acid.\textsuperscript{55}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure1_5.png}
\caption{Structure of surfactin A.}
\end{figure}
It has been shown that surfactin treatment improved proliferation rates and changes in the morphology of mammalian cells that had been contaminated with mycoplasma. In addition, the low cytotoxicity of surfactins to mammalian cells permitted specific inactivation of mycoplasmas without significant deleterious effects on cell metabolism and the proliferation rate of cells in culture. Surfactins are active against several viruses, including semliki forest virus, herpes simplex virus (HSV-1 and HSV-2), suid herpes virus, vesicular stomatitis virus, simian immunodeficiency virus, feline calicivirus and murine encephalomyocarditis virus. The antiviral activity of surfactins is more efficient on enveloped viruses than that on non-enveloped viruses, suggesting that the antiviral action of surfactins is primarily due to a physiochemical interaction between the membrane active surfactant and the virus lipid membrane. Furthermore, surfactins have been reported to (i) inhibit fibrin clot formation, (ii) induce formation of ion channels in lipid bilayer membranes, (iii) inhibit cyclic adenosine monophosphate (cAMP), and (iv) inhibit platelet and spleen cytosolic phospholipase A2 (PLA2).

Fengycins are the third family of Bacillus lipopeptides, comprising fengycins A and B, which are also called plipastatins. Fengycins are lipodecapeptides with an internal lactone ring in the peptidic moiety and with a β-hydroxy fatty acid chain (C14 to C18) that can be saturated or unsaturated. Fengycins are less haemolytic than iturins and surfactins but retain a strong fungicidal activity, especially against filamentous fungi. The mode of action of fengycins is not fully mechanistically known but they were shown to interact with lipid bilayers by altering lipid membrane order and permeability in a dose-dependent way. Bacillus lipopeptides act in a synergistic manner as suggested by several studies on surfactin and iturin, surfactin and fengycin, and iturin and fengycin.

1.2.7.4 Lysolipids as potential anticancer and anti-protozoal drugs

Lysolipids are biomembrane modulators affecting fusion and membrane protein stability; they bind to specific receptors, affect the immune system, and are involved in a variety of diseases. Lysophosphatidylcholine (LysoPC) is an important intermediate in degradation and biosynthesis of phosphatidylcholine (PC). Reduced plasma LysoPC levels observed in patients with advanced cancer indicate a deregulation of LysoPC metabolism in metastasis. This indicates a potential importance of LysoPC in metastatic and cachectic processes.

Synthetic alkylphospholipids (APLs) represent a group of membrane-permeable compounds with antineoplastic properties and a broad range of clinical applications. For example, edelfosine (1-O-octadecyl-2-O-methylrac-glycero-3-phosphocholine) has been used as a purging agent in autologous bone marrow transplantation. Topical application of miltefosine (hexadecylphosphocholine) was
shown to be an effective therapy for skin metastases of breast cancer\textsuperscript{97} and cutaneous lymphomas.\textsuperscript{98,99} Oral administration of miltefosine is successfully used in the treatment of visceral leishmaniasis, a systemic protozoal infection.\textsuperscript{100} The most recent derivative, perifosine (octadecyl-(1,1-dimethylpiperidino-4-yl)-phosphate), has been evaluated as an oral anticancer drug in clinical phase I and II studies.\textsuperscript{99} APLs differ from most of the currently used cytotoxic drugs with respect to their cellular targets. APLs primarily act on cell membranes where they are believed to accumulate in sphingolipid-enriched or cholesterol-enriched microdomains.\textsuperscript{101} Moreover, these compounds interfere with the rapid and continuous phospholipid turnover that is essential for cell survival. APLs inhibit phosphatidylcholine (PC) turnover at the level of both PC degradation and PC re-synthesis.\textsuperscript{102}

### 1.2.7.5 Other biosurfactants with biological activity

Mannosylerythritol lipids (MEL-A and MEL-B) synthesized by \textit{Candida antarctica} strains exhibit antimicrobial activity, particularly against Gram-positive bacteria.\textsuperscript{103} MEL-A could be used as a component of cationic liposomes (containing a cationic cholesterol derivative) to increase the efficiency of gene transfection and to develop effective and safe non-viral vector-mediated gene therapy procedures.\textsuperscript{104} MEL-A can also be used as a safe, affinity binding ligand to human immunoglobulin G (H\textit{I}g\textit{G}).\textsuperscript{105} Refer to Figure (1.6) for chemical structure of Mannosylerythritol lipids (MEL-A, MEL-B, and MEL-C).

![Chemical structure of MEL-A, R1 and R2 = Ac (acetylated); MEL-B, R1 = Ac, R2 = H (hydrogen); MEL-C, R1 = H, R2 = Ac.\textsuperscript{106}](image)

\textbf{Figure (1.6):} Chemical structure of MEL-A, R1 and R2 = Ac (acetylated); MEL-B, R1 = Ac, R2 = H (hydrogen); MEL-C, R1 = H, R2 = Ac.\textsuperscript{106}

Saponins are naturally occurring surface-active glycosides; they are mainly produced by plants, but also by lower marine animals and some bacteria.\textsuperscript{56} Saponins derive their name from their ability to form
stable, soap-like foams in aqueous solutions. Saponins consist of a sugar moiety usually containing glucose, galactose, glucuronic acid, xylose, rhamnose, or methylpentose that is glycosidically linked to a hydrophobic aglycone which may be triterpenoid or steroid in nature. Saponins have a lytic action on erythrocyte membranes because of their ability to form pores. The hemolytic action of saponins is the result of the affinity of their aglycone moiety for membrane sterols, particularly cholesterol, with which they form insoluble complexes.\textsuperscript{56} Digitonin is a steroid glycoside from the saponin family.\textsuperscript{56} Membrane-related applications of digitonin are solubilizing membrane proteins, precipitating cholesterol, and permeabilizing cell membranes.\textsuperscript{56,107-109}

Finally, there are biomolecules with certain surfactant-like properties such as the amphiphilic, antibiotic peptides gramicidin S, and Peptidyl-glycylleucine-carboxyamide (PGLa).\textsuperscript{105,110} Gramicidin S (GS) is a cationic cyclic decapetide synthesized by \textit{Bacillus brevis}.\textsuperscript{110,111} GS is a potent antimicrobial agent and exhibits high killing activity against a broad spectrum of both Gram-positive and Gram-negative bacteria and pathogenic fungi.\textsuperscript{110,111} PGLa is an antibacterial peptide of the magainin family, isolated from the skin of the African clawed frog \textit{Xenopus laevis}.\textsuperscript{110,111} It is non-hemolytic but exhibits a broad range of antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, and protozoa.\textsuperscript{105} PGLa molecules bind to the lipid membrane and then fold into amphipathic \(\alpha\)-helical conformation.\textsuperscript{110} Membrane permeabilization could be induced by the formation of a peptide-lipid pore, where the PGLa helices are oriented perpendicular to the membrane surface.\textsuperscript{111} The low toxicity of the magainins against eukaryotic cells makes them potential candidates for the development of new antibacterial drugs.\textsuperscript{111}

1.3 Interaction of surfactants with lipid membranes

1.3.1 The three-stage model

Lipid-detergent systems have been described in terms of the three-stage model, which was introduced by Helenius and Simons.\textsuperscript{112} The model describes three stages encountered by an aqueous lipid dispersion with increasing concentration of a detergent added: 1) detergent partitioning into the lipid bilayer, 2) lamellar-micellar phase transition, and 3) size decrease of mixed micelles.\textsuperscript{46,112} This model distinguishes only three principal topologies: 1) lamellar structures, 2) large cylindrical, or disc-shaped micelles, and 3) small micelles.\textsuperscript{112} Although, this simple model provides valuable clarity and guidance, we must be aware that there are enormous complexity of phenomena and structures involved within each stage. In some cases, it is not even straightforward to assign the specific structures to the stages and to micellar versus membrane topologies.\textsuperscript{46}
1.3.2 Surfactant partitioning into the lipid bilayer

Surfactants added to a membrane-water system tend to establish a partitioning equilibrium between water and membrane phases. The literature presents a variety of suitable definitions of the partition coefficient. Here, the definition $K = R_b/c_{Saq}$ on the basis of mole ratios will be used, where $K$ denotes the (apparent) partition coefficient, $R_b$ the effective mole ratio of bound surfactant-to-lipid in the membrane, and $c_{Saq}$ the aqueous surfactant concentration.

Membrane partitioning of detergents is primarily driven by the hydrophobic effect.\textsuperscript{44,46} This implies that the partition coefficient increases with increasing length of the alkyl chain or with the hydrophobic surface area of detergent that can be buried from water on membrane insertion. Measurements of partition coefficients of detergents of homologous series have suggested a methylene group contribution to the standard Gibbs free energy of membrane partitioning of about -3.0 kJ/mol, which essentially agrees with the methylene group contributions to micelle formation or to the solubility of hydrocarbons.\textsuperscript{44,46} This means that partition coefficients can be expected to increase by one order of magnitude for every two methylenes added to the acyl chain.\textsuperscript{44,46} For those detergents that do not simply have a polar head and acyl chain, one has to consider the change in apolar accessible surface area ($\Delta ASA_{ap}$) on membrane insertion, which relates to the methylene contributions by a $\Delta ASA_{ap} = 31 \text{Å}^2$ per methylene.\textsuperscript{44,46}

The apparent partition coefficient, $K$, of ionic detergents is strongly affected by electrostatic interactions, making it sensitive to the charge of detergent and lipid, membrane composition $R_b$, and the ionic strength of the buffer. The insertion of non-ionic detergents to a charged membrane is electrostatically favoured due to the subsequent increase in membrane area and decrease in surface charge density.\textsuperscript{46}

Also for non-ionic systems, the partition coefficient of a detergent into the lipid bilayer may vary as a function of $R_b$. This may result from curvature stress within or between the lipid leaflets (on asymmetric insertion). The detergent uptake increases if it relaxes membrane stress. For example, partitioning of the detergents into membranes of negative intrinsic curvature or stretched or positively curved membrane leaflets relaxes membrane stress.\textsuperscript{44,46}

The temperature dependence of the detergent partition coefficient can be described in terms of van’t Hoff’s law:
\[
\frac{\partial \ln K}{\partial T} = \frac{\Delta H}{RT^2} = \frac{\Delta C_p(T - T_{iso})}{RT^2}
\]  
(1.3)

Where, \(T_{iso}\) is isenthalpic temperature. The heat capacity change of partitioning, \(\Delta C_p\), is typically negative and \(K\) shows a maximum at an isenthalpic temperature. A realistic enthalpy change of \(\Delta H^{aq-b} \approx +10 \text{ to } +20 \text{ kJ/mol}^{-1}\) would imply that \(K(T)\) increases by \(\approx 10\% \text{ to } 30\% \text{ per } 10 \text{ K}\), showing that the temperature dependence of \(K\) is relatively modest.\(^{44}\)

High pressure can be expected to oppose detergent binding because it tends to order and laterally compress the membrane.\(^{46}\)

High concentrations of cosolvents affect partitioning, also in nonionic systems, according to their preferential exclusion (increasing \(K\)) or preferential interactions (decreasing \(K\)) from or with the molecular surface and their effect on head-group hydration (e.g., area and thus packing parameter).\(^{113}\) Sodium chloride (NaCl) and sucrose increase the free energy of hydrophobic surfaces exposed to water and promote surfactant incorporation into membranes; urea has the opposite effect.\(^{113,114}\)

### 1.3.3 Membrane asymmetry and permeation

After insertion of a detergent into the outer leaflet of the lipid bilayer, some of the detergent has to translocate into the inner leaflet of the bilayer to establish the partitioning equilibrium. To do so, its polar head group has to cross the hydrophobic core by a flip-flop mechanism. \(\text{C}_{12}\text{EO}_8, \text{C}_{12}\text{EO}_7, \text{Triton X100, and C}_6\text{Gluc}\) equilibrate between both leaflets of the membrane within a time window from milliseconds to some tens of seconds.\(^{115-119}\) Surfactants with larger or charged head groups may, however, require hours or days to cross the membrane, as shown for SDS at room temperature, for example \(\text{C}_n\text{Malt with } n=12, 13 \text{ and } 14\), and \(\text{C}_{16}\text{lyso-PC}\).\(^{44}\) It should be kept in mind that flip-flop is not a property of the surfactant species alone; the dynamics and barrier properties of membranes may depend substantially on lipid composition, surfactant content, membrane curvature, and temperature.\(^{44}\) For example, permeation of SDS through membranes is strongly enhanced by increasing temperature and occurs within some minutes at 65 °C.\(^{120}\) The surfactant leaving the outer leaflet by flipping to the inner is partially replaced by further uptake from the aqueous phase.

Systems with fast uptake but slow translocation across the bilayer are often studied in terms of a partial equilibrium of the surfactant between aqueous phase and outer lipid monolayer. In this case, it has been assumed that the inner monolayer of the lipid vesicle is not accessible and can be disregarded upon data evaluation. It should, however, be noted that the asymmetric insertion or extraction may in
certain cases give rise to bilayer couple effects which change the thermodynamic parameters of binding.\textsuperscript{121} The asymmetric expansion of the bilayer may contribute to shape changes of vesicles\textsuperscript{122} and erythrocytes as well as budding, ex- and invaginations, and affect endo- and exocytosis.\textsuperscript{121,122} If the bilayer is unable to bend to resume its spontaneous bilayer curvature, it develops a bilayer curvature strain by compressing the molecules in the overpopulated leaflet and/or expanding those in the under-populated leaflet.\textsuperscript{44} At a threshold value, bilayer curvature strain may also induce the transient rupture of the bilayer which anneals after translocation of some molecules to the under-populated side.\textsuperscript{121} It should be noted that, in contrast to the bilayer couple effect, membrane permeant molecules can reduce the bending stiffness by moving to the stretched leaflet of a bent membrane. Please refer to Figure (1.7).

![Spontaneous Bilayer Curvature](image1)

![Bilayer Curvature Strain](image2)

\textbf{Figure (1.7):} Schematic illustration of the bilayer couple concept. Insertion of detergents into a bilayer leads to an imbalance situation between the two coupled lipid leaflets. If this cannot be relaxed via flip-flop of the detergents between the leaflets, it gives rise to a spontaneous bilayer curvature. In turn, the bilayer tends to bend locally and form budding, and shape transformations. If bending does not occur, it develops a bilayer curvature strain which causes disorder, particularly in the low-pressure leaflet, and stores elastic energy.\textsuperscript{44} Reproduced with permission from Heerklotz, H., Interactions of surfactants with lipid membranes. Quarterly Reviews of Biophysics 2008, 41 (3/4), 205-264. Copyright (2008) Cambridge University Press, License Number 2945101459197.

\subsection{1.3.4 Curvature strain}

Surfactants have a positive spontaneous curvature, e.g., they prefer to form aggregates with a positively (outward) curved interface. This results from the relatively large interfacial area requirement of their head groups and the rather small volume of their hydrophobic tail. Their insertion into a lipid
bilayer, which has virtually no average real curvature, causes a so-called curvature stress. That means, the lateral pressure profile gets distorted to show increased pressure in the headgroup region and reduced pressure between the chains.\textsuperscript{123,124} As discussed above for pure surfactant systems (section 1.2.3), the optimum lateral area of a head group depends not only on the molecular dimensions but also on order and mobility of moieties, hydration, hydrogen bonds, dipole- and electrostatic interactions. This is one reason why mixtures do not necessarily exhibit the average spontaneous curvature of their components. For example, a mixture of anionic and cationic, micelle-forming surfactants can adopt a lamellar structure since head groups with different charge pack much closer together than equally charged ones in separate micelles.\textsuperscript{125} Equi-molar mixtures of lysolipids and fatty acids (both micelle forming) can also form lamellar structures.\textsuperscript{126} The major structural consequence of the curvature strain is a disordering of the chains. As a consequence, the membrane becomes thinner and more flexible. This is seen via deuterium NMR, NMR relaxation studies, infrared spectroscopy, fluorescence spectroscopy, electron spin resonance, X-ray and neutron diffraction.\textsuperscript{44}

Let us describe the curvature strain in mixed membranes analogously to the approach used by Israelachvili’s packing parameter.\textsuperscript{45} The basic idea is very simple; the hydrophobic volume of the molecules has to fill the space defined by their interfacial area times the hydrophobic thickness of the monolayer, \(\langle V \rangle = \langle A \rangle \times \ell_b\). The brackets refer to average values per molecule in the membrane and can be replaced by explicit expressions yielding:

\[
\ell_b = \frac{X_S^b v_S + (1 - X_S^b) v_L}{X_S^b A_S + (1 - X_S^b) A_L}
\]

where \(v_S, A_S, v_L, A_L\) denote the partial hydrophobic volume and interfacial area of surfactant and lipid, respectively. As discussed before for micelles, \(A_S\) and \(A_L\) are affected by all interactions of the head groups with its neighbors, hydration, and conformational mobility. Whereas the volumes and, essentially, also the areas are rather fixed for a given mixture, the thickness \(\ell_b\) can easily adapt to the value fulfilling the equation by a disordering of the chains. Hence, the surfactant-induced curvature strain in membranes is represented by a disordering of the chains that reduces \(\ell_b\) compared to the pure lipid layer.

Importantly, \(\ell_b\) is a property of the mixed aggregate rather than the individual chains. While a shortening of the detergent chain may have little effect on \(v/\ell_c\) in a spherical micelle, because it decreases \(v\) and \(\ell_{max}\) proportionally to each other, it reduces \(v_S/A_{OS}\ell_b\) of a detergent in a bilayer
strongly as long as \( \ell_b \) is governed by the lipids.\textsuperscript{44,46} Figure (1.8) shows the packing in a spherical micelle and the curvature strain in a lipid bilayer.

Figure (1.8): Schematic illustration of the interfacial area per detergent, \( A_0 \), and the critical length (the half-thickness) of the hydrophobic core in a spherical micelle (left) and in a lipid bilayer (right).\textsuperscript{46} Reproduced by permission from Heerklotz, H., Blume, A., Comprehensive Biophysics. Elsevier Inc.: 2012; Vol. 5. Copyright (2012) Elsevier B.V., Academic Press.

Eq. 1.4 explains another reason why the spontaneous curvature or packing parameter of the mixture is not the average of the respective values of its components. Adding a substance with zero spontaneous curvature and “cylindrical effective shape”, \( v_S/A_0 s/\ell c \approx 1 \), but a short hydrophobic part into a lipid layer with a hydrophobic thickness of \( \ell_b > \ell_c \) (so that also \( v_S/A_0 s/\ell b < 1 \)) induces curvature stress.

It has been hypothesized that there is a critical extent of curvature stress that can no longer be accommodated by the membrane and gives rise to the onset of membrane solubilization to micelles.\textsuperscript{127} This issue will be addressed in detail in Chapter 3.

Detergent induced curvature strains have also been found to induce membrane leakage already at concentrations below the onset of solubilization to mixed micelles. This can proceed via a number of mechanisms: 1) temporary rupture of the membrane to relax asymmetric membrane expansion by a detergent undergoing no spontaneous flip-flop, 2) thinning and disordering by curvature strain increases the probability of a mechanical failure particularly in the presence of shear, osmotic stress, or
other external stresses, 3) stabilization of a pore by covering its highly curved edges with a detergent-rich rim.46

1.3.5 Membrane solubilization

The detergent induced transition from bilayers to micellar structures is often referred to as membrane solubilization. The lipid bilayer loses its integrity and the extended lipid bilayer structure is destroyed. Membrane solubilization may occur via different kinetic pathways. In order to group these pathways, three intermediates are considered such as rod-like micelles, discs, and perforated vesicles. Please see Figure (1.9) for more clarity. These pathways are explained as follows:

1) Solubilization pathways which are completed through rod-like (cylindrical) micellization:128-130

Rod-like micelles show intermediate (one-dimensional) interfacial curvature between bilayers (none) and spherical micelles (curved in two dimensions). This renders them logical intermediates of the bilayer-to-micelle transition. Below the threshold detergent concentration for the appearance of micelles, detergent incorporating into vesicles may render them larger, leaky, cup-shaped, or may open them completely to yield lamellar sheets. Depending on the system and conditions, the transition to rods may occur from any of those structures. At the limiting detergent content $R_{e}^{Sat}$, mixed rod-like micelles appear that have a detergent-to-lipid mole ratio of $R_{e}^{Sol}$. With increasing detergent concentration, the internal compositions of the bilayers and micelles remain unchanged, $R_{e}^{Sat}$ and $R_{e}^{Sol}$, but the number of micelles increases. When the overall detergent-to-lipid mole ratio in aggregates, $R_{e}$, reaches $R_{e}^{Sol}$, only mixed micelles are left in the system.

2) Solubilization pathway via discoidal micellization:131

In this pathway, surfactant molecules partition into the lipid bilayer and form pores by covering their highly curved edge. With increasing surfactant content, the edges grow; vesicles open to lamellar sheets, and the sheets decrease in size to bicelles and discoidal micelles as the edge-to-core ratio increases. Note that this scenario maintains a coexistence of flat and highly curved regions throughout the solubilization process whereas all molecules mix to assume a common, intermediate curvature in cylindrical micelles. This implies that the pathway involving discoidal micelles should be favored for surfactants that mix poorly with the lipid.
It is now known that rod-like micelles are found in, at least, most lipid-detergent systems, but discs play a role in some cases both as equilibrium states (e.g., bicleles formed by DMPC/DHPC or DMPC/CHAPSO) or as kinetic intermediates on reconstitution of vesicles.\textsuperscript{46,130,132,133}

3) Solubilization pathway involving perforated vesicles: \textsuperscript{134}

A third pathway involving networks of cylindrical micelles arising from perforations was described particularly for charged surfactants. Addition of surfactant gives rise to perforated vesicles. The detergent-rich rims covering the edges of the perforations resemble ‘half cylindrical micelles’.

Increasing detergent concentration increases the number of pores until the pores touch each other and only the rims are left as a network of entangled, branched, interconnected cylindrical micelles. Addition of further surfactant leads to rather linear cylindrical, then (potentially) oblate discoidal and finally to spherical micelles.\textsuperscript{134,135}

Micelles can be formed by a fractionation of membranes (transbilayer mechanism) or by what has been termed micellar mechanism.\textsuperscript{136} This mechanism is based on the spontaneous formation of micelles in the aqueous phase that extract lipid from the membrane surface upon collisions. This seems at variance with the fact that solubilization proceeds at an aqueous detergent concentration ($c_{s}^{aq,sat}$) below the $CMC$, where the detergent alone forms no micelles. A possible explanation might be that detergents can form unstable, probably small and short-lived, pre-micellar aggregates already somewhat below their $CMC$. Such aggregates would become stabilized by incorporating lipid. The slow, micellar mechanism may play a role particularly for non-permeant detergents where the trans-bilayer mechanism is hindered and the protrusion of lipids may be promoted from the overpopulated outer leaflet.\textsuperscript{136}

For many detergents, their “strength” to solubilize a membrane has been found to depend on their activity to induce curvature stress and has been quantified by product of the CMC and the partition coefficient, $K$. For a pure detergent, the lower the product of $K \cdot CMC$, the stronger is the preference of a detergent for micelles and its impact to induce the onset of membrane solubilization or $R_b^{sat} \approx K \cdot CMC$.\textsuperscript{137}

As described above, the curvature strain induced by a surfactant increases with increasing head-group size, decreasing volume of the hydrophobic part, less negative/more positive spontaneous curvature of the lipid (e.g., PC compared to PE lipids), decreasing temperature (which tends to increase spontaneous curvature of lipid and detergent by stronger head-group hydration or increased order of the chain), or addition of urea as a co-solute promoting head-group hydration (glycerol has the opposite effect).\textsuperscript{113,114}

The classical detergents with a polar-head group and an alkyl-tail such as Triton, $C_nEO_n$, alkyl maltosides and glucosides show values of $R_b^{sat} \geq 0.4$, that is, their impact on the membrane seems to be limited. In contrast, some detergents with more bulky hydrophobic parts such as CHAPS, cholate, diheptanoyl
PC, or surfactin show considerably lower $R_{D}^{sat}$ values. This may have two possible reasons: 1) The impact of a detergent on membrane stability (e.g., free energy) by curvature stress cannot be higher than the free energy of anchoring the detergent in the stress-inducing position with the bulky polar group intercalated between the lipid head groups. 2) These surfactants may act by a different mechanism that does not require building up a critical curvature strain in the membrane. This problem will be addressed in detail in Chapter 3.

1.4 Objectives

1.4.1 Critical curvature strain in membranes and the mode of membrane solubilization

Preliminary data suggest a critical curvature strain in membranes to be the trigger of lysis in many cases. This scenario, however, hardly explains the enhanced activity and selectivity of some surfactants, mainly biosurfactants. The objective is to test the hypothesis of critical curvature stress and screen a range of (bio)surfactants for possible outliers from this scenario.

Techniques used are time-resolved fluorescence spectroscopy and anisotropy (revealing lipid bilayer order and dynamics), isothermal titration calorimetry (studying solubilization, and reconstitution), and dynamic light scattering (following the path of solubilization).

1.4.2 Developing a new method to measure volume and expansivity changes of detergent micelle formation

Pressure perturbation calorimetry (PPC) has become a powerful technique to study the volume changes accompanying thermotropic transitions. Micelle formation is not primarily a thermotropic transition and does not give rise to a PPC peak. The objective is to derive a protocol and model to render PPC applicable by making use of the temperature dependence of the $CMC$, which will be measured independently by isothermal titration calorimetry.

1.4.3 Improving our interpretation of volumetric parameters to characterize the internal packing and hydration properties of micelles, macromolecules, and solutes

Internal packing and hydration are key properties for the stability of macromolecules and colloidal aggregates in solution and might also be related to the suitability of micelles for a given application. Volumetric properties depend sensitively on packing and hydration, but it is not trivial to separate the two contributions from each other. The strategy pursued here is to consider not one but two
observables, volume \((V)\) and thermal expansivity \((\partial V / \partial T)\) that depend on packing and hydration to different proportions, respectively. To this end, systematic measurements are performed for homologous series of surfactants and these results are compared with \(V\) and \(\partial V / \partial T\) characteristics of other types of transitions and interactions.

1.4.4 Establishing zeta potential measurements for the study of the effective charge of membrane-bound molecules and their trans-bilayer distribution

Zeta potential measurements have been employed successfully for the determination of membrane binding of charged molecules with known effective charge number. However, due to local \(pH\) and \(pK_A\) shifts in a membrane environment, the effective charge is often an interesting, unknown parameter itself. The objective is to derive an assay that eliminates partitioning as an unknown parameter and hence yields the effective charge directly. Furthermore, it will be explored whether the fact that zeta potential measurements represent the conditions in the outer leaflet of vesicles can be utilized to characterize membrane asymmetry and transbilayer equilibration of molecules.
CHAPTER (2) - Basic Concepts of the Applied Methods

2.1 Fluorescent Probes

Fluorescence spectroscopy offers a wealth of information about the dynamics and molecular environment of intrinsic fluorophores or fluorescent probes localized in specific membrane regions. Fluorophores are molecules that can be excited by UV or visible light and emit fluorescence because these molecules contain systems of double bonds or benzene rings that have suitable electrons in π orbitals. The lipopeptides IT and FE contain intrinsic tyrosine fluorophores. Additionally, we use synthetic probes monitoring different regions of the membrane. These are 1,6-diphenyl-1,3,5-hexatriene (DPH), and DPH analogs such as 1-(4-(trimethylamino)phenyl)-6-phenylhexa-1,3,5-triene (TMA-DPH) and 2-(3-(diphenylhexatrienyl)propanoyl)-1-Hexadecanoyl-sn-Glycero-3-Phosphocholine (β-DPH HPC). DPH is a hydrophobic molecule and locates in the hydrophobic chain area of a lipid bilayer, either parallel or perpendicular to the acyl chains. TMA-DPH is an amphiphilic molecule with a polar amine group that anchors the molecule to the head-group region of the membrane. Its DPH moiety is preferably located parallel to the acyl chains of the bilayer, but a parallel orientation within the interfacial region cannot be excluded. β-DPH HPC is a phosphatidylcholine molecule in which one of its acyl chains is replaced by a DPH. Its DPH fluorophore is parallel to the acyl chains of the lipid bilayer and located in the hydrophobic part of the bilayer. Figure (2.1) shows a schematic illustration of the possible locations of these probes in a lipid bilayer.

![Figure (2.1)](image)

Figure (2.1): A schematic illustration of possible locations of DPH, TMA-DPH, and β-DPH-HPC in a lipid bilayer.
2.2 Time-resolved Fluorescence

Time-resolved measurements provide the decay of the fluorescence emission as a function of time after excitation. This yields direct information about the dynamics of the system and may help distinguishing different components of a spectrum arising from different dyes or different pools of a dye distributing between different states or localizations.

For instance, consider a protein that contains two tryptophan residues in its structure; each may have a distinct lifetime. The time-resolved data may reveal two decay times which can be used to resolve the emission spectra and relative intensities of the two tryptophan residues. The time-resolved measurements can reveal how each of the tryptophan residues in the protein is affected by the intramolecular or/and intermolecular interactions. Finally, lifetimes are not affected by sample turbidity or other error sources that may perturb fluorescence intensities.

2.3 Anisotropy

Excitation by polarized light selects dyes with a parallel orientation of their transition dipole and results in a partially polarized (anisotropic) emission. The anisotropy depends on the properties of the fluorophore and its angular motion after excitation which, in turn, is affected by the order and dynamics of the environment of the probe.

\[ \text{Figure (2.2): A schematic illustration of DPH wobble-in-cone motion.} \]
DPH is a standard dye for studying membrane order and dynamics. Structurally, it can be considered an ellipsoid of revolution that has two equal, short axes and one long axis.\textsuperscript{138} In an aqueous environment, DPH is able to rotate around its different axes randomly. However, DPH is not free to randomly rotate when it is within the membrane, where it has a preferential orientation imposed by the neighboring lipid chains. We should mention that the detailed interpretation of DPH fluorescence and anisotropy decays remains controversial in spite of extensive studies. For example, DPH was modeled to show a characteristic tilt with respect to the membrane normal (the classical wobble-in-cone model) or to tumble around the normal direction (Brownian rotational diffusion model).\textsuperscript{139,140} DPH was argued to partition between two typical localizations in the membrane: 1) essentially parallel to the chains within a given leaflet, and 2) perpendicular to the chains between the two lipid leaflets. Figure (2.2) shows a schematic illustration of wobble-in-cone motion of DPH in a lipid bilayer.

The angular motion of the fluorophore causes an exponential decay of the anisotropy from an initial value of $r_0 \approx 0.4$ to a final value referred to as $r_{\infty}$. The rotational correlation time describes the effective speed of the angular motion and, hence, the dynamics of the membrane. The limiting anisotropy, $r_{\infty}$, results from the constraints on the rotation to a limited range in angles and quantifies membrane order.

### 2.4 Isothermal Titration Calorimetry (ITC)

Isothermal titration calorimetry (ITC) is a physical technique that is used to determine the thermodynamic parameters of interactions in solution. It is most often used to study the binding of small molecules (such as active agents or surfactants) to larger macromolecules (proteins, DNA) or lipid vesicles. ITC measures the heat response of a solution or dispersion to a series of small injections of a titrant.

ITC is a quantitative technique that can directly measure the binding affinity ($K_a$), enthalpy changes ($\Delta H$), and binding stoichiometry ($n$) of the interaction between two or more molecules in solution. From these initial measurements, standard Gibbs energy changes ($\Delta G^0$), and entropy changes ($\Delta S^0$), can be determined using the relationship:

$$\Delta G^0 = -RT \ln K = \Delta H^0 - T\Delta S^0 \quad (2.1)$$

where $R$ is the gas constant and $T$ is the absolute temperature.
A series of ITC protocols has been established to characterize the interactions of surfactants with membranes. The demicellization protocol\textsuperscript{141} characterizes the self-association of the surfactant (\textit{CMC}, etc.).

The uptake-and-release protocol\textsuperscript{116,142} reveals the partition coefficient and establishes whether the surfactant can translocate across a membrane spontaneously.

The solubilization and reconstitution protocol\textsuperscript{143,144} determines the saturating local surfactant content in the membrane that causes the onset of lysis (specified as the surfactant-to-lipid mole ratio in the membrane, $R^\text{sat}_o$) and the maximum lipid content of mixed micelles (determining the amount of surfactant needed to complete membrane solubilization).

A schematic diagram of a standard ITC instrument is shown in Figure (2.3). The instrument consists of two identical cells (volume ~ 1.5 mL) housed in an adiabatic jacket. The outer shield is cooled, so heat energy is required to keep the cells and their contents at the experimental temperature.\textsuperscript{145} The two cells are kept at the same temperature ($\Delta T = 0$) throughout the experiment. One cell is filled with water (for experiments performed in aqueous solvents) or buffer solution, and acts purely as a reference. The other (the sample cell) is filled with one component of the interaction to be investigated. The basis of the experiment is to measure the heat energy per unit time ($\mu$cal sec$^{-1}$) that must be added to the sample cell to maintain zero temperature difference between the two cells at the designated temperature for an experiment. An enthalpy change results when the second component of the interaction is introduced in a series of aliquots (typically injections of 3-15 $\mu$L to give a total added volume of up to 300 $\mu$L) to the sample cell.\textsuperscript{145}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{schematic_diagram.png}
\caption{Schematic Diagram of an ITC instrument.}
\end{figure}
If the interaction is exothermic, less heat per unit time will be required from the sample cell heater to keep the two cells at the same temperature; if the interaction is endothermic, the opposite effect will be observed. The cells are in contact with thermopile/thermocouple circuits regulated by an interactive feedback control system; so the highly sensitive response to changes in temperature between the cells and between the jacket and the cells can be regulated. Mixing of the interacting species, by stirring, is very rapid and response times of instruments to incremental heats are typically less than 10 seconds.\textsuperscript{145}

### 2.5 Pressure Perturbation Calorimetry (PPC)

PPC has mainly been used to characterize volume changes of thermotropic transitions of polymers,\textsuperscript{146-148} lipids,\textsuperscript{149-152} proteins,\textsuperscript{153-157} and DNA,\textsuperscript{158,159} and for temperature-induced, irreversible protein aggregation.\textsuperscript{160,161} Here we demonstrate the use of PPC to describe and interpret the expansivity and volume changes of surfactants (monomers/micelles) in water as temperature is changing.

Measurements were carried out on a VP DSC calorimeter from MicroCal (Northampton, MA) equipped with a MicroCal PPC accessory. Two or more small pressure jumps of 5 bars were automatically carried out isothermally at a series of specified temperatures. Typically, we used 2 $K$ intervals. The system measures the differential heat responses of the cells in terms of the energy needed to maintain temperature matching. This heat response on a pressure jump is related to the volume change with temperature, the actual output parameter of the experiment.

The data evaluation to derive apparent expansivity coefficient ($\alpha_S$) is realized by the instrument software, Origin for PPC. Perfectly bubble-free filling of the cells, which is not trivial with surfactant solutions, was confirmed in terms of matching absolute heat responses to up- and down-jumps in pressure. We have characterized the non-ionic surfactants in pure water, which simplifies the equation for PPC data analysis as follows:

$$\alpha_S (T) = - \frac{\Delta Q_{SW} - \Delta Q_{WW} + c_S \bar{v}_S Q_W}{T \nu_{cell} c_S \bar{v}_S \Delta p}$$

were $\Delta Q_{SW}$ denotes the heat response with an aqueous surfactant solution or dispersion in the sample cell and water in the reference cell, $\Delta Q_{WW}$ is a blank run with water in both cells, and $Q_W$ is the heat response for pure water as taken from the literature in form of a polynomial. $T$ stands for the temperature, $\nu_{cell} \approx 0.5$ mL is the cell volume, $c_S$ is the molar concentration of surfactant, $\bar{v}_S$ (mL/g) is the partial specific volume of each surfactant, and $\Delta p \approx 5$ bar is the pressure difference.
The apparent molar expansivity of the surfactant is defined as \( E_S = \frac{\partial V_S}{\partial T} \rvert_p \). It can be obtained by multiplication of \( \alpha_S \) with the molar mass (g/mole) of the surfactant, and surfactant’s specific volume (mL/g).

### 2.6 Dynamic Light Scattering (DLS)

Dynamic Light Scattering (also known as Photon Correlation Spectroscopy or Quasi-Elastic Light Scattering) is a technique for measuring the size of particles typically in the sub-micron region. DLS measures Brownian motion and relates this to the size of the particles. Brownian motion is the random movement of particles due to the bombardment by the solvent molecules that surround them. Normally, DLS is concerned with measurement of particles suspended within a liquid.\(^{162}\)

The larger the particle, the slower will be the Brownian motion. An accurate control of the temperature is necessary for DLS because knowledge of the viscosity is required (the viscosity of a liquid is related to its temperature). The temperature also needs to be stable to avoid convection currents that would cause non-random movements that compromise the correct interpretation of the particle size.\(^{163}\)

The velocity of the Brownian motion is defined by a property known as the translational diffusion coefficient (usually given the symbol, \( D \)). The size of a particle is calculated from the translational diffusion coefficient by using the Stokes-Einstein equation:\(^{163}\)

\[
\frac{d_H}{3 \pi \eta D} = \frac{kT}{3 \pi \eta D} \quad (2.3)
\]

where \( d_H \) stands for the hydrodynamic diameter, \( D \) for translational diffusion coefficient, \( k \) for Boltzmann’s constant, \( T \) for the absolute temperature, and \( \eta \) for viscosity. Note that the experiment does not provide information about the shape of the particle. The hydrodynamic diameter is defined as the parameter of a spherical particle that shows the same translational diffusion as the particle being characterized.\(^{163,164}\) Factors that affect the diffusion speed of particles are ionic strength of the medium, surface structure and hydration, and shape of the particles (non-spherical).\(^{163,164}\)

### 2.7 Zeta Potential

The liquid layer surrounding a negatively charged particle comprises an inner region (Stern layer) where the ions are strongly bound, and an outer region (diffuse layer) where they are less firmly associated. Within the diffuse layer there is a notional boundary inside which the ions and particles form a stable
entity. When the particle moves (e.g., due to the voltage gradient in an electric field), ions within the boundary will move together with the particle. Those ions beyond the boundary stay with the bulk dispersant. The potential at this boundary (surface of hydrodynamic shear) is the zeta potential (refer to Figure 2.4).

When an electric field is applied across an electrolyte, charged particles suspended in the electrolyte are attracted towards the electrode of opposite charge. Viscous forces acting on the particles tend to oppose this movement. When equilibrium is reached between these two opposing forces, the particles move with constant velocity. The velocity is dependent on the strength of electric field or voltage gradient, the dielectric constant of the medium, the viscosity of the medium and the zeta potential. The velocity of a particle in a unit electric field is referred to as its electrophoretic mobility. Zeta potential is related to the electrophoretic mobility by the Henry equation:

$$U_E = \frac{2\varepsilon \zeta f(\kappa \cdot a)}{3\eta} \quad (2.4)$$

Here, $U_E$ denotes the electrophoretic mobility, $\zeta$ the zeta potential, $\varepsilon$ the dielectric constant, $\eta$ the viscosity, and $f (\kappa \cdot a)$ represents Henry’s function.

Figure (2.4): Schematic representation of zeta potential ($\zeta$).
The Debye length, $\kappa^{-1}$, is a measure of the thickness of the electrical double layer. The parameter $a$ refers to the radius of the particle and therefore $\kappa \cdot a$ measures the ratio of the particle radius to electrical double layer thickness (Figure 2.5). Vesicles with $a \approx 100$ nm in 100 mM salt, where $\kappa^{-1} \approx 1$ nm, are much larger than the reciprocal Debye length so that the product $\kappa \cdot a$ is $>>1$; in this case, Henry’s function $f(\kappa \cdot a)$ amounts to 1.5. This scenario is referred to as the Smoluchowski approximation. Therefore, calculation of zeta potential from the mobility is straightforward for systems that fit the Smoluchowski model, e.g., particles larger than about 0.2 microns dispersed in electrolytes containing more than 1 mM salt. For small particles in low dielectric constant media $f(\kappa \cdot a)$ becomes 1.0 and allows an equally simple calculation. This is referred to as the Hückel approximation.

![Hückel and Smoluchowski approximations](image)

**Figure (2.5):** Schematic illustration of Hückel’s and Smoluchowski’s approximations used for the conversion of electrophoretic mobility into zeta potential.
CHAPTER (3) - Classifying Surfactants with Respect to Their Effect on Lipid Membrane Order


I acknowledge the following contributions from coauthors:

Mustafa Kurdi has contributed to the data presented here in the frame of an undergraduate research project, PHC489, supervised by H. Heerklotz and myself.
Heiko Heerklotz has contributed to the design and final write-up of the study.

3.1 Abstract

We propose classifying surfactants with respect to their effect on membrane order, which is derived from the time resolved fluorescence anisotropy of DPH. This may help in understanding why certain surfactants, including biosurfactants such as antimicrobial lipopeptides and saponins, often show a superior performance to permeabilize and lyse membranes and/or a better suitability for membrane protein solubilization. Micelle-forming surfactants induce curvature stress in membranes that causes disordering and, finally, lysis. Typical detergents such as C12EO8, octyl glucoside, SDS, and lauryl maltoside initiate membrane lysis after reaching a substantial, apparently critical extent of disordering.
In contrast, the fungicidal lipopeptides surfactin, fengycin, and iturin from Bacillus subtilis QST713 as well as digitonin, CHAPS, and lysophosphatidylcholine solubilize membranes without substantial, overall disordering. We hypothesize they disrupt the membrane locally due to a spontaneous segregation from the lipid and/or packing defects and refer to them as heterogeneously perturbing. This may account for enhanced activity, selectivity, and mutual synergism of antimicrobial biosurfactants and reduced destabilization of membrane proteins by CHAPS or digitonin. Triton shows the pattern of a segregating surfactant in the presence of cholesterol.
3.2 Introduction

Membrane lysis and solubilization by detergents and surfactant-like biomolecules is a key phenomenon in many biological functions and technical applications. The most prominent examples are the leakage and lysis of cell membranes by antibiotic (lipo)peptides or antiseptic or spermicidal agents and the isolation and study of membrane proteins.

The key property of a micelle-forming amphiphile inserting into a lipid bilayer is its preference for a locally curved interface (its spontaneous curvature) that is in conflict with the, on average, planar topology of a bilayer. This misfit causes a curvature stress, that is, a penalty to membrane excess enthalpy and free energy and a strain disordering and thinning the hydrophobic core of the membrane. Depending on the size and shape of their polar and apolar parts, detergents can be weak or strong in terms of inducing curvature stress but nevertheless share the same principal mode of membrane solubilization. Similar excess enthalpies and deuterium order parameters at the onset of solubilization have led to the hypothesis of a critical curvature strain in the membrane that needs little (typically, some 20 – 40 mol %) of a strong or much (e.g., 60 mol %) of a weak detergent to be reached.

However, the fact that a variety of structural and kinetic pathways has been described for different lipid-detergent systems indicates that the state of such a system is not sufficiently determined by its average spontaneous curvature alone. Structural intermediates have been identified as wormlike micelles, perforated vesicles, or bilayer sheets and disks. Kragh-Hansen et al. described two kinetic pathways—a transbilayer mechanism proceeding via membrane destruction by inserted detergents, and a micellar mechanism based on the appearance or persistence of micelles in solution that extract lipid from the membrane upon collisions. The usually slow, micellar mechanism may in particular apply to surfactants that cannot translocate quickly to the inner membrane leaflet; they give rise to a bilayer asymmetry stress that opposes further uptake and favors the release of molecules from the overpopulated outer leaflet.

Unspecific, detergent-like, or carpet-like action has also been discussed to account for the membrane-permeabilizing activity of certain antimicrobial peptides. These molecules differ usually from the head-and-tail structure of detergents but they share with detergents their amphiphilic nature and the induction of curvature strain in membranes. This is revealed, for example, by their ability to inhibit the transition to inverse hexagonal or induce a micellar cubic (Pm3n) phase of a lipid. However, whereas such peptides form membrane defects and toroidal pores that are similar to those induced by detergents, they do so at much lower concentration and with superior selectivity.
This raises the question whether there is a principal difference between the effects of surfactants on membranes that is independent of spontaneous curvature and membrane permeability. We have addressed this question by testing the correlation between the lytic activity of surfactants with their impact on overall membrane order, which is directly related to curvature stress (see also Lafleur et al.\textsuperscript{169}). After obtaining first hints from deuterium NMR\textsuperscript{180}, we have employed time-resolved fluorescence anisotropy measurements of DPH and two of its derivatives here. Briefly, the limiting anisotropy, $r_{\infty}$, and rotational correlation time, $\theta$, of the probes represent the order (constraints to molecular orientation) and dynamics (speed of rotational motion) in the membrane. In some interesting studies, particularly $r_{\infty}$ was shown to quantify the disordering effect of detergents.\textsuperscript{165,181} Sýkora et al.,\textsuperscript{182} showed that digitonin retained a higher order as well as a better activity of a G-protein than synthetic detergents.

We should mention that the detailed interpretation of DPH fluorescence and anisotropy decays remains partially unclear or controversial in spite of extensive, sophisticated studies. For example, DPH was modeled to show a characteristic tilt with respect to the membrane normal (the classical wobble-in-cone model) or to tumble around the normal direction (Brownian rotational diffusion model).\textsuperscript{139,140} DPH was argued to partition between two typical localizations in the membrane: 1), essentially parallel to the chains within a given leaflet, and 2), perpendicular to the chains between the two lipid leaflets.\textsuperscript{183} TMA-DPH, an analog with a cationic group at one end, cannot localize in the center of the membrane but might distribute between an inserted and interfacial orientation of the fluorophore.\textsuperscript{184} The fluorescence lifetime(s) of DPH are affected by the exposure to water and DPH self-quenching\textsuperscript{185,186}, but it is not straightforward to assign the two lifetimes to two specific localizations because the decay is biexponential even in an isotropic solvent.\textsuperscript{185} In membranes with fluid and gel-state domains, DPH partitions essentially equally between the phases but TMA-DPH and DPH-PC show a preference for the fluid phase.\textsuperscript{187}

In our case, the coexistence of (potentially heterogeneous) membranes and different types of micelles renders the physics of the fluorophore even more complex and a detailed fit is not warranted. However, it turns out that the comparison of the average (mono-exponential) decay behavior of DPH, TMA-DPH, and DPH-labeled phospholipid (DPH-PC) in different systems and under different conditions provides much insight even on a partially empirical level. Figure (3.1) shows the molecular dimensions of some of the molecules used in our study.
3.3 Materials and Methods

3.3.1 Materials

Synthetic detergents $C_{12}EO_8$ (octaethylene glycol mono-dodecyl ether), OG (n-octyl b-D-glucopyranoside), TX (triton X-100), LM (lauryl maltoside, n-dodecyl-a-D-maltopyranoside), SDS (sodium dodecyl sulfate), CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate), and lysoPC (1-lauroyl-2-hydroxy-3-phosphatidylcholine) were obtained from Anatrace (Maumee, OH) in Anagrade purity (99% high-performance liquid chromatography). Surfactins (SF), fengycins (FE), and iturins (IT) are classes of closely related lipopeptides (see Ongena and Jacques\textsuperscript{78} for an overview) produced by Bacillus subtilis QST713 and were kindly provided by AgraQuest (Davis, CA). Digitonin was purchased from Wako Chemicals (Richmond, VA).

The lipid POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) was purchased from Avanti Polar Lipids (Alabaster, AL). The probes, DPH (1,6-diphenylhexatriene), TMA-DPH (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate), and DPH-PC (2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-sn-glycero-3-phos-phocholine) were from Molecular Probes Invitrogen (Eugene, OR). NaCl (sodium chloride), Tris (tris(hydroxymethyl)aminomethane), and EDTA (ethylenediaminetetraacetic acid) were from Sigma-Aldrich (St. Louis, MO) at the highest available purity. All samples were prepared in buffer containing 100 mM NaCl, 10 mM Tris, and 0.5 mM EDTA in Milli-Q water (Millipore, Billerica, MA), adjusted to pH 8.5.
3.3.2 Samples

Lipid vesicles were prepared as described elsewhere. For anisotropy experiments, the probe (DPH, TMA-DPH, or DPH-PC) was added to a solution of the lipid (POPC) in chloroform to establish a lipid/probe mole ratio of 1:600. The solution was dried by a gentle stream of nitrogen, followed by exposure to vacuum overnight. Then, the lipid was quantified gravimetrically assuming an effective molar weight including one bound water molecule per lipid (778 g/mol for POPC) as validated earlier by phosphorus assays. Multilamellar suspensions of lipid were prepared by adding buffer to each sample, vortexing, and six freeze-thaw cycles. Then, large unilamellar vesicles were prepared by extrusion through Nuclepore filters of ~100-nm pore size in a Lipex extruder (Northern Lipids, Burnaby, British Columbia, Canada). The large unilamellar vesicles had a size of ~100 nm as confirmed by dynamic light scattering. For isothermal titration calorimetry (ITC) experiments, the same protocol was followed without adding probe.

3.3.3 Fluorescence Spectroscopy

Typically, experimental series were carried out as titrations. Small amounts of a stock solution of detergent micelles were added (keeping lipid dilution small) sequentially to a starting sample of 2 mL, 2 mM lipid. For detergents known to undergo a fast flip-flop between the leaflets of the bilayer (C\textsubscript{12}EO\textsubscript{8}, TX, OG), the sample was stirred for 15 min after each injection for equilibration. In series studying the other surfactants, the equilibration was realized for at least 15 min at 65 °C followed by readjustment of the experimental temperature, 20 °C. This procedure was based on the finding that this temperature renders POPC membranes permeable even for charged surfactants such as SDS (unpublished data for alkyl maltosides).\textsuperscript{120} Series with individual samples and 1 h heat treatments as well as spot checks with lipid and surfactant premixed in chloroform before extrusion yielded no significant deviations.

Time-resolved anisotropy decays were recorded at 20 °C on a FL3 system (Horiba Scientific, Edison, NJ) utilizing time-correlated single photon counting. The source of excitation was 340 nm LED pulsed at 1 MHz, and emission was detected at 425 nm (slit 5 nm, double-grated monochromator) by a TBX detector (Horiba Scientific). Fluorescence decays were recorded with horizontal and perpendicular orientations of polarizers in the excitation and emission path, with automatic cycling between the emission polarizer orientations every 60 s. The instrument response function was recorded with a dilute Ludox scattering dispersion.
The data evaluation utilized in DAS 6 software (Horiba Scientific) follows a similar strategy to the one explained by Lakowicz.

Briefly, the fluorescence decays were analyzed by deconvoluting the sum of curves obtained with the vertical and horizontal polarizer, $I_{VV}$ and $I_{VH}$, with the instrument response function using a biexponential decay model, considering the G-factor. Typical values of $\chi^2$ were 1.5. Then, the difference signal was deconvoluted with the exponential decay function obtained for the sum signal to obtain the parameters of a monoexponential anisotropy decay function,

$$r(t) = [r_0 - r_\infty] \cdot \exp \left\{ -\frac{t}{\theta} \right\} + r_\infty \quad (3.1)$$

where the rotational correlation time, $\theta$, reflects the dynamics of the reorientation of the probe. The limiting anisotropy, $r_\infty$, reflects constraints to the motion of the probe and is related to membrane order. As discussed above, the monoexponential model was chosen for the sake of stability, even if non-random residuals were observed. The time window of the fit was $\approx 2400$ channels (67 ns).

### 3.3.4 Other Techniques

Isothermal titration calorimetry (ITC) was performed as described elsewhere using a VP ITC (MicroCal, Northampton MA). For the solubilization experiment, the syringe is loaded with a relatively highly concentrated, micellar solution of the surfactant (150 mM of OG in the example shown) and a series of injections is carried out into the calorimeter cell ($\approx 1.5$ mL) originally filled with a 2 mM dispersion of POPC vesicles. The onset and completion of solubilization become visible as break points of the normalized heat of titration. Injection volumes were varied between 2 and 15 mL and waiting times after each injection were set to 40 min to ensure complete equilibration.

Dynamic light scattering as shown for SF-POPC mixtures was done in a Nano ZS system (Malvern Instruments, Malvern, UK), utilizing non-invasive backscattering at 173° at a wavelength of 633 nm. The measurements were done with the samples used for the DPH experiments before.

### 3.4 Results

Figure (3.2) illustrates the effects of the detergent C$_{12}$EO$_8$ on the order and dynamics of POPC membranes in terms of the decay parameters of the three probes as a function of the mole fraction of the detergent in mixed aggregates (membranes and/or micelles), $X_\phi$.

$X_\phi$ was calculated according to the following equation:
with $c_D$ denoting the total detergent concentration, $c_D^b$ the concentration of aggregate-bound detergent, $c_L$ the lipid concentration (assumed to be completely aggregate-bound), and the mole-ratio partition coefficient $K$. For $C_{12}EO_8$, we used $K = 6/mM$; variations of $K(X_e)$, particularly between bilayers and micelles, have no significant effect on the plot. This system forms mixed membranes at $X_e$ up to $X_e^{sat} = 0.31$, a co-existence of mixed membranes with wormlike micelles in the range between $X_e^{sat}$ and $X_e^{sol} = 0.62$, only wormlike micelles at $X_e^{sol}$, and a gradual transition between wormlike and small quasi-spherical micelles centered at $X_e \approx 0.85$ (see vertical grid lines in Figure (3.2)).

Figure (3.2): Fluorescence properties of DPH and its derivatives in mixtures of POPC and $C_{12}EO_8$ at 20 °C as a function of the mole fraction of detergent in mixed aggregates $X_e$ (see text). Measurements of limiting anisotropy ($A$, $r_\infty$), effective rotational correlation time ($B$, $\theta$), and average lifetime ($C$, $\tau$) of DPH (spheres), TMA-DPH (down-triangles), and DPH-PC (squares). (Vertical grid lines) Pseudophase boundaries of solubilization (dashed) and the midpoint of a broad transition from cylindrical (cyl) to spherical (sph) micelles (dotted).
Inspection of Figure (3.2A) reveals immediately that the phase boundaries correlate with break points in the \( r_\infty(X_e) \) curves of all three probes. Addition of \( \text{C}_{12}\text{EO}_8 \) into membranes causes a disordering represented by decreasing \( r_\infty \). A local minimum of \( r_\infty \) is obtained at the maximal detergent content in membranes, at \( X_e^\text{sat} \). The progressive formation of wormlike micelles between \( X_e^\text{sat} \) and \( X_e^\text{sol} \) increases \( r_\infty \) of all DPH probes. After a local maximum at \( X_e^\text{sol} \), the micelles are gradually converted into small, quasi-spherical micelles and \( r_\infty \) decreases concomitantly.

The rotational correlation times of the three probes show no break points and only minor trends. The average fluorescence lifetimes (Figure 3.2C) decrease weakly (DPH, DPH-PC) or significantly (TMA-DPH) with increasing detergent content. Resulting from biexponential fits of the sum of decays, these trends mainly reflect a decrease in the prefactor of the longer lifetime, \( \tau_2 \approx 9 \text{ ns} \). We hypothesize that absolutely lower and more strongly decreasing \( \langle \tau \rangle \) of TMA-DPH reflects its higher water exposure due to a more interfacial average location and a rougher and more dynamic interface of micelles compared to membranes.

Figure (3.3) shows the analogous data for mixtures of POPC with OG. An ITC solubilization experiment (Figure 3.3A) injecting 150 mM OG into 2 mM POPC was performed to establish the phase boundaries at 20 °C, \( X_e^\text{sat} \approx 0.61 \) and \( X_e^\text{sol} \approx 0.65 \) in line with comparable references. Figure 3.3, B–D, shows the analogous pattern as seen in Figure 3.2 for \( \text{C}_{12}\text{EO}_8 \): \( r_\infty(X_e) \) shows a minimum at the onset and maximum at the completion of solubilization. The average lifetime, \( \langle \tau \rangle \), of TMA-DPH is shorter and drops upon solubilization. As for \( \text{C}_{12}\text{EO}_8 \), DPH-PC shows a moderate increase of \( \theta \) with increasing detergent content in the membrane.
Figure (3.3): ITC solubilization experiment (A) and fluorescence properties of DPH and its derivatives (B–D) in mixtures of POPC with OG at 20 °C as a function of the mole fraction of detergent in mixed aggregates, $X_e$. A: 150mM micellar OG was titrated into 2 mM POPC to establish $X_e^{sat}$ and $X_e^{mol}$ (see vertical grid lines). B–D: Measurements of limiting anisotropy ($r_{\infty}$), effective rotational correlation time ($\theta$), and average lifetime ($\langle \tau \rangle$) of DPH (spheres), TMA-DPH (down triangles) and DPH-PC (stars).

Figure (3.4): Dynamic light scattering (A) and DPH-fluorescence (B–D) data of mixtures of POPC and surfactins from *Bacillus subtilis* QST713 as a function of the effective mole fraction of the surfactin. The z average hydrodynamic size (A) confirms the onset and completion of solubilization published for a closely related system (illustrated by gray, dashed, and vertical grid lines). Panels B–D show fluorescence parameters of DPH derivatives (conditions and symbols are analogous to those in Figs. 3.2 and 3.3).
The results for surfactin lipopeptides differ qualitatively from those for typical detergents described above, particularly in the mixed membrane range at $X_e < X_e^{sat}$ (Figure 3.4). The grid lines indicating $X_e^{sat}$ and $X_e^{sol}$ are derived from comparable literature data$^{44,189}$ and supported by the steep decrease of the average particle size (Figure 3.4A). SF induces only very little disordering as revealed by $r_\infty$ of all three probes. In contrast to the detergents in Figures 3.2 and 3.3, it causes a slight increase in $\theta$ (slower reorientation) and a slight increase of $\langle \tau \rangle$ of DPH, suggesting a slower dynamics and better screening from water in SF-containing membranes and micelles. As seen for detergents, $r_\infty$ increases upon membrane-micelle conversion and decreases again with decreasing lipid content of the micelles.

Figure (3.5A) compares the effects of C$_{12}$EO$_8$ (from Figure 3.2A), OG (from Figure 3.3B), and Triton TX-100, respectively, on $r_\infty$ of DPH as a function of detergent concentration (log scale). The stars mark the values of $r_\infty$ at the onset of solubilization as estimated from literature data$^{44}$ and ITC measurements (not shown) for OG/POPC, OG/POPC + Cholesterol, and TX/POPC + Cholesterol. For C$_{12}$EO$_8$ at 20 °C and 10 °C and TX at 20 °C, $r_\infty$ shows a local minimum at $X_e^{sat}$ (at the star); the minimum is much less pronounced for OG, which has an extremely narrow coexistence range.

The curves for C$_{12}$EO$_8$ and OG in Figure (3.5) show the following general features:

1. Membranes with higher original order may also break (start becoming solubilized) at higher order.
2. More ordered membranes are more susceptible to the disordering effect of these detergents as indicated by a steeper decrease of $r_\infty(c_D)$.
3. The lytic concentration, $X_e^{sat}$, is hardly affected: higher initial order of a membrane does not protect a membrane against solubilization by a disordering detergent.
4. For each individual membrane condition, the critical order ($r_\infty$ at the minimum) is essentially the same for C$_{12}$EO$_8$ and OG, respectively. It seems to be a property of the membrane primarily, not of the detergent.

For Triton, these apparent rules (which will be discussed below to apply to homogeneously disordering surfactants) do not apply, particularly in the presence of cholesterol. Then, $X_e^{sat}$ is significantly reduced and the minimum $r_\infty$ is substantially higher than those caused by OG or C$_{12}$EO$_8$.

Figure (3.6) compiles the curves of $r_\infty$ of DPH versus $c_D$ for a number of additional surfactants. SDS and LM share the principal behavior of the detergents C$_{12}$EO$_8$, OG, and TX (in the absence of cholesterol), as illustrated in Figure (3.5). They disorder the membrane to a minimum value of $r_\infty \approx 0.005 - 0.01$. In
contrast, CHAPS and the biosurfactants iturin (IT), fengycin (FE), digitonin (digi), and lysoPC agree with SF in causing no or little membrane disordering so that $r_{\infty}$ of DPH in POPC at 20 °C remains above 0.02. Reference data about the onset of solubilization (see stars in Figure 3.6) are available only for FE, CHAPS$^{190,191}$, and SDS (from dynamic light scattering of 65 °C-equilibrated samples, not shown). Membrane solubilization by FE causes a modest increase in order. CHAPS seems to cause a slight membrane disordering that is relaxed before the onset of solubilization, which does not cause any significant further increase in $r_{\infty}$.

![Graph](image)

Figure (3.5): Effects of $C_{12}EO_8$ (up-triangles), OG (hexa- or pentagons), and in two cases TX (circles) on the limiting anisotropy, $r_{\infty}$, as a function of the total surfactant concentration, $c_D$ (the lipid concentration is $\approx 2$ mM). The panels represent different membrane conditions, such as POPC at 20 °C (A), POPC at 10 °C (B), and POPC plus 25 mol% of cholesterol at 20 °C (C). (Dotted grid lines) $r_{\infty} = 0.01$, considered critical disordering for POPC at 20 °C (see Figs. 3.2 and 3.3); (stars) position of a curve that corresponds to the onset of solubilization for systems where this is known (see text).
Figure (3.6): Concentration-dependent effect of surfactants on membrane order as represented by the limiting anisotropy of DPH, $r_{\infty}$, as a function of the total concentration of surfactants, $c_D$, including dodecyl maltoside (LM, open circles), iturin (IT, hexagons), and lauroyl lysophosphatidylcholine (lysoPC, squares) in the top panel and sodium dodecyl sulfate (SDS, down-triangles), CHAPS (pentagons), fengycin (FE, circles), and digitonin (digi, diamonds) in the bottom panel. For LM, CHAPS, and FE, the point on the curve that corresponds to the onset of solubilization is marked by a star, respectively. (Dotted grid line) Value of $r_{\infty} = 0.01$ representing critical disorder in Figs. 3.2 and 3.3: it is reached by LM and SDS but not by CHAPS, FE, IT, lysoPC, and digi. All curves were recorded at $\approx 2$ mM POPC, DPH/POPC = 1:600 mol/mol, 20 °C. Note that the threshold concentrations observed here apply to $\approx 2$ mM lipid only and are not directly comparable to the minimum inhibitory concentrations, for example.

3.5 Discussion

3.5.1 Homogeneous membrane disordering by typical detergents

Let us first consider the detergents (C$_{12}$EO$_9$, OG, LM, SDS, and TX-100 in POPC) that initiate solubilization after disordered the membrane to a substantial, common degree (reflected by $r_{\infty} < 0.01$ in POPC at 20 °C ). This is, in fact, what one should expect for detergents disrupting the membrane by curvature stress. A straightforward, quantitative criterion for curvature strain$^{46}$ has been derived from Israelachvili’s packing parameter concept$^{45}$, expressing the half-thickness of the hydrophobic core of the membrane, $\ell_b$, as
\( \ell_b = \frac{X_b^b \nu_S + (1-X_b^b)\nu_L}{X_b^b A_S + (1-X_b^b)A_L} \)  \hspace{1cm} (3.3)

\( X_b^b \) denotes the mole fraction of surfactant in the membrane (= \( X_c \) in the absence of micelles), \( \nu_S \) and \( \nu_L \) resent the partial volumes of the hydrophobic parts of surfactant and lipid, respectively, and \( A_S \) and \( A_L \) their partial areas at the interface. This equation assumes a planar bilayer and a smooth interface and represents the trivial fact that the hydrophobic parts have to fill the volume determined by the interfacial area multiplied by \( \ell_b \). A positive curvature strain is represented by a large \( A_L \) and/or small \( \nu_S \) so that \( \ell_b < \nu_L/A_L \).

Let us use Eq. 3.3 to estimate the impact of \( C_{12}EO_8 \) and OG on \( \ell \) of a POPC membrane. We use \( \nu_L = 946 \text{ Å}^3 \) and \( A_L = 65 \text{ Å}^2 \) for POPC, Tanford’s \(^{47} \) formula \( \nu_S = n_C \times 26.9 \text{ Å}^3 + 27.4 \text{ Å}^3 \) yielding \( \nu_S \) of 296 \( \text{ Å}^3 \) and 243 \( \text{ Å}^3 \) for \( C_{12}EO_8 \) (\( n_C = 12 \)) and OG (\( n_C = 8 \)), respectively. For interfacial areas we use the published values of 116 \( \text{ Å}^2 \) for \( C_{12}EO_8 \) and 51 \( \text{ Å}^2 \) for OG. \(^{119,192} \) The predicted hydrophobic monolayer thickness, \( \ell_b \) (\( X_S^b \)), is plotted in Figure (3.7). It is intriguing that both curves reach the same value of \( \ell_b^{sat} \approx 9 \text{ Å} \) at their respective \( X_S^b = X_S^{sat} \) of 0.31 (\( C_{12}EO_8 \)) and 0.61 (OG, see Figure 3.2), when the membranes become disintegrated. \(^{193,194} \) It appears that at this point, the free energy of the curvature stress suffices to initiate micelle formation. Kinetically, this may proceed via trans-membrane or micellar mechanisms, \(^{136} \) illustrating that the latter classification is independent of the one proposed here. Mixtures of two or more homogeneously disordering detergents should have additive effects on membrane order as modeled elsewhere, \(^{190} \) whenever all interfacial areas (and hydrophobic volumes) are additive.
Figure (3.7): Schematic representation of the effect of detergents, C_{12}EO_8 and OG, on the half-thickness of the hydrophobic core of the membrane, $\ell_b$, as predicted by Eq. 3.3 using parameters mentioned in the text. $X^b_d$ denotes the mole fraction of detergent in the bilayer ($= X_e$ in the absence of micelles); the curves end at the onset of solubilization (stars). The agreement of $\ell_b$ at the onset of solubilization by the two detergents implies a common, critical degree of membrane disordering.

Summarizing, the critical disordering found here (Figures 3.2, 3.3, LM in Figure 3.6) agrees with the theoretical prediction for a homogeneous membrane (Figure 3.7) and with results of probe-free NMR and calorimetric experiments. An inhomogeneous distribution of the probes is very unlikely to substantially perturb the results given that all three probes, which should have different domain preferences^{187}, report the same behavior. This provides strong support for the hypothesis that these detergents initiate solubilization of a homogeneously mixed membrane at a critical curvature stress and that the latter is properly reported by $r_{\infty}$ of DPH analogs. DPH anisotropy studies seem to be a new, valuable tool to detect the onset and completion of membrane solubilization by these detergents (as local minima and maxima of $r_{\infty}$).

3.5.2 Heterogeneous perturbation and solubilization

The Bacillus subtilis QST713 lipopeptides surfactin, fengycin, and iturin as well as digitonin, lysolecithin, and CHAPS are all known to form micelles (e.g., to prefer aggregates with positively curved interface) and, thus, solubilize lipid membranes. In fact, where the lytic concentration, $X^e_{sat}$, is known, it is unusually low (0.05 for FE^{191}, 0.1 for CHAPS^{190}, 0.18 for SF^{189}). This requires a particularly strong
perturbation of the membrane, but this is not detected in terms of a substantially decreasing DPH order.

We propose that this lack of a substantial change in DPH order provides evidence for the system showing what we refer to as “heterogeneous perturbation”: the disruption of the membrane is localized to specific membrane defects whereas the order in the bulk of the membrane is little affected.

A straightforward, possible explanation is that the detergent mixes poorly with the lipid and segregates within the membrane into detergent-rich clusters that disrupt the membrane locally whereas most of the membrane area (where also most of the DPH is located) is little affected. Failure of the detergent to mix with the lipid can also drive a segregation of the detergent from the membrane so that micelles form spontaneously in the aqueous phase.136

Finally, one should be aware of the fact that membrane thinning by chain disordering is not the only possible way to accommodate curvature stress. A gel phase with its perfectly ordered chains cannot be disordered but matches the lateral areas of chains and headgroups by a collective tilt (Lβ phase) or interdigitation of the chains. This seems uncommon for fluid membranes but NMR measurements have provided evidence for some collective chain tilt of lipid chains caused by surfactin.180 Thinner membranes with tilted chains are likely to break at packing defects between more ordered clusters, which may also accumulate surfactant. To which extent each of these effects contributes to the phenomenon of solubilization without prior, substantial reduction of \( r_\infty \) remains to be elucidated. However, it is noteworthy that all these effects assume solubilization to be initiated in local membrane defects and we, therefore, refer to them as “solubilization by heterogeneous perturbation”.

3.5.3 Triton mixes with POPC but gets segregated in the presence of cholesterol

The behavior of Triton illustrates that the homogeneous versus heterogeneous mode of solubilization depends on both surfactant and lipid(s). Figure (3.5) shows that TX acts on POPC as expected for a homogeneously disordering detergent. However, in a membrane of POPC with 25mol % of cholesterol, it behaves as a heterogeneously perturbing, segregating surfactant instead. It induces solubilization at an average \( r_\infty \) of DPH that is well above that for OG and \( C_{12}EO_8 \) in the same membrane and it initiates solubilization at lower \( c_D^{sat} \) than in the absence of cholesterol. The downshift in \( X_e^{sat} \) is probably even more pronounced than the one in \( c_D^{sat} \) given that cholesterol also typically decreases \( K \).44

This finding is in line with the fact that Triton shows unfavorable mixed pair interactions with cholesterol118 and can, typically in contrast to OG, produce detergent-resistant membrane fragments.
from cholesterol-containing membranes by inducing, stabilizing, and/or coalescing ordered domains.\textsuperscript{44,195}

\subsection*{3.5.4 Functional consequences of heterogeneous perturbation}

Heterogeneous perturbation as described here may account for a number of peculiar properties, such as: 1) Very low active concentration for the onset of membrane lysis (\(\text{low}X_e^{\text{sat}}\)), because the local concentration in a defect may be much higher than the average \(X_e\), 2) Enhanced membrane selectivity (see, e.g., shift in \(c_d^{\text{sat}}\) of TX, Figure 3.5) because miscibility depends on specific molecular properties whereas curvature stress is less specific, 3) Synergistic action in mixtures, if one component induces co-segregation of another, 4) Relatively high order in mixed micelles. This can be expected if the surfactant fails to disorder neighboring lipids (so that membrane curvature strain requires a tilt instead). Poor miscibility in a micelle may give rise to a bicelle or small, ellipsoidal micelle with a lipid-rich core and surfactant-rich perimeter. This may explain why antibiotic biosurfactants with a major function to kill other cells (without a need to completely dissolve them) are found to be heterogeneously perturbing, whereas classic detergents used for solubilizing other molecules in their micelles (governed by \(X_e^{\text{sol}}\), not \(X_e^{\text{sat}}\)) are well miscible with lipids and cause homogeneous disordering.

The concept of heterogeneous perturbation supports the hypothesis of Sykora et al.,\textsuperscript{182} that the good tolerance of digitonin by membrane proteins is related to high order (quantified by \(r_{\text{an}}\) of DPH) in the aggregates. It is also likely to apply to the triple-detergent mixture comprising dodecyl maltoside, CHAPS, and cholesteryl hemisuccinate.\textsuperscript{196} The high order retained in the membrane upon solubilization and even in the micelles may avoid a destabilization and conformational change of the protein. Enhanced contact with lipids in the core of a micelle may stabilize a protein as well.\textsuperscript{197} The two solubilization scenarios may also explain the variety of structural pathways of solubilization.

Homogeneous disordering as described here is quantified in terms of a single parameter and systems sharing the same average spontaneous curvature (or \(\ell_d\)) should therefore also share the same structure. It typically leads to cylindrical micelles, an aggregate of largely homogeneous, one-dimensional curvature that is intermediate between those of bilayers and spherical micelles. Heterogeneous perturbation may favor structures with different local curvatures such as perforated vesicles.\textsuperscript{170} In the case of bicelles, the limited miscibility of short-chain diacyl lipids with fluid membrane lipids gives rise to segregation into surfactant-depleted bilayer and surfactant-rich, defect regions with pseudomicellar topologies that constitute rims of pores, bilayer fragments, or disk micelles.\textsuperscript{198}
3.6 Conclusions

Describing the activity of surfactants to permeabilize and solubilize lipid membranes, it is very useful to distinguish between homogeneously and heterogeneously membrane-perturbing surfactants.

Homogeneously disordering surfactants destroy the membrane when a critical curvature stress is reached. Their threshold concentration to lyse membranes can be estimated simply on the basis of their partition coefficient, headgroup size, and hydrophobic group volume. Our data suggest that typical synthetic detergents act by homogeneous disordering. This appears to be in line with their function to solubilize a maximum of cargo molecules in their micelles with minimum specificity.

We propose that surfactants initiating membrane lysis without a prior, substantial decrease in the limiting anisotropy, $r_{\infty}$, of DPH and its analogs can be classified as heterogeneously membrane-perturbing surfactants. They disrupt membranes locally in surfactant-rich defect structures. Heterogeneous perturbation may account for the superior activity, selectivity, and mutual synergism of antimicrobial biosurfactants, such as lipopeptides and saponins, to kill target cells by permeabilizing their membrane. It may also be favorable for membrane protein isolation because it avoids strong disordering and thinning of the protein’s environment and may give rise to heterogeneous micelles with a core that is relatively rich in lipid and, possibly, sterol.

Time-resolved fluorescence anisotropy of DPH and its analogs serves to distinguish between homogeneously and heterogeneously membrane-perturbing surfactants.

Furthermore, our data suggest that the onset and completion of solubilization by membrane-disordering surfactants can be identified as local minimum and maximum of the composition-dependent, limiting anisotropy.

3.7 Acknowledgement

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CHAPTER (4) - Volume and Expansivity Changes of Micelle Formation Measured by Pressure Perturbation Calorimetry


I did the experiments shown here and wrote the first draft of the paper. I wish to acknowledge contributions from the coauthors, which are:

Helen Fan established a much improved version of the Excel fitting script used to evaluate the data; this was of crucial importance for this study.

Saria Chowdhury contributed to the experimental work in the frame of an undergraduate research project, PHC489, supervised by H. Heerklotz and myself.

Heiko Heerklotz contributed to the design of the study and the final write-up of the paper.

4.1 Abstract

We present the application of pressure perturbation calorimetry (PPC) as a new method for the volumetric characterization of the micelle formation of surfactants. The evaluation is realized by a global fit of PPC curves at different surfactant concentration ranging, if possible, from below to far above the CMC. It is based on the knowledge of the temperature dependence of the CMC, which can for example be characterized by isothermal titration calorimetry. We demonstrate the new approach for decyl-\(\beta\)-maltopyranoside (DM). It shows a strong volume increase upon micelle formation of 16\(\pm\)2.5 mL/mol (+4%) at 25 °C, and changes with temperature by \(-0.1\) mL/(mol K). The apparent molar expansivity \((E_s)\) decreases upon micelle formation from 0.44 to 0.31 mL/(mol K) at 25 °C. Surprisingly, the temperature dependence of the expansivity of DM in solution (as compared with that of maltose) does not agree with the principal behavior described for polar \((E_s(T)\) decreasing) and hydrophobic \((E_s(T)\) increasing) solutes or moieties before. The results are discussed in terms of changes in hydration of the molecules and internal packing of the micelles and compared with the volumetric effects of transitions of proteins, DNA, lipids, and polymers.
4.2 Introduction

Hydration governs the structure and function of molecules and molecular assemblies in aqueous solution or dispersion, including virtually all biological macromolecules and their interactions with each other. The so-called hydrophobic effect, which is based on a preferential interaction of water molecules with each other rather than with apolar, hydrophobic molecules and moieties, is a key to the native structure of proteins and the self-association of amphiphilic molecules to form membranes or micelles. The hydration of polar moieties and the resulting “hydration force” are prerequisites for the stability of dispersions, proteins, and membranes against spontaneous aggregation or fusion.

One approach to study the thermodynamics of hydration is to measure weight and enthalpy differences accompanying changes in water activity of a sample, which can be realized either by using water vapor of defined relative humidity\(^{199-201}\) or by changing the osmotic pressure in the system.\(^{202,203}\) Another classic concept is to interpret the exposure of polar and hydrophobic groups to water in terms of empirical relationships between changes in the solvent accessible surface area (ΔASA) and changes in heat capacity (Δ\(C_p\)).\(^{204,205}\) Hence, Δ\(C_p\) of micelle formation has been interpreted in terms of the reduced water accessibility of the surfactants.\(^{41,51,205}\) However, this strategy is not universally applicable since additional contributions to Δ\(C_p\) may arise, e.g., upon binding of molecules to proteins and DNA\(^{206,207}\) or to lipid membranes.\(^{208-210}\) The apparent molar volume and its thermal expansion in aqueous dispersion are very sensitive to changes in hydration as well as internal packing of colloidal particles and provide another very promising avenue to the understanding of colloidal systems, including biological macromolecules.\(^{211}\) So far, volume and expansivity changes accompanying, for example, micelle formation could only be derived from densimetric or high-pressure measurements. A volume change of micelle formation of \(C_8E_5\) of +18 mL/mol was derived from the starting slope of the pressure dependence of the CMC.\(^{59}\) This technique yields also valuable information about compressibility changes but does not provide expansivities and the extrapolation of Δ\(V\) to ambient pressure seems challenging. The temperature-dependent apparent molar volume, \(V_S\), of pluronic copolymers\(^{212}\) obtained from concentration dependent density measurements shows a step at the critical micellar temperature (CMT). Extrapolation of \(V_S(T)\) from both lower and high temperatures to the CMT suggests a volume change of micelle formation of Δ\(V_S/V_S ≈ +2%\). The slopes suggest a decrease in the apparent molecular expansivity by 30% upon micelle formation. These values can mainly be understood in terms of the release of water from the condensing influence of the molecular surfaces of the surfactants upon aggregation (contributing positively to Δ\(V_S\)) and upon increasing temperature (causing a positive contribution to \(E_S\) that scales with ΔASA). This approach to obtain Δ\(V_S\) from \(V_S(T)\) requires a
narrow CMT range. In the general case, $V_S(T)$ has to be determined below and far above the CMC. Densimetric measurements of tetraethylammonium perfluorosulfonate$^{213}$ implied an enormous micellization volume of $\Delta V_S/V_S \approx 40\%$ and around 100\% increase in expansivity, corresponding to a change in the coefficient of thermal expansion, $\Delta \alpha \approx (0.001-0.002)/K$. These properties seem to be fundamentally different from those of hydrogenated surfactants.

While densimetry has the advantage of providing absolute values of apparent molar volumes, its precision in yielding apparent expansivities and particularly subtle expansivity peaks accompanying thermotropic transitions must be limited. Note that these quantities are obtained by differentiating a noisy volume or density curve. We have previously shown that the very small volume and expansivity changes accompanying the thermotropic sphere-to-rod transition of myristoyl maltoside would be virtually invisible to densimetry, yet could very precisely and conveniently be measured by pressure perturbation calorimetry (PPC).$^{43}$ This method$^{153,214}$ yields directly the apparent expansivity of a solute with unmatched precision by measuring the isothermal heat response of the system to a small pressure change. At first glance one might expect PPC to fail in determining $\Delta V_S$ of micelle formation because integration of the expansivity yields $V_S(T)$ plus an unknown integration constant. Here we show how this problem can be solved by evaluating the contributions to expansivity that arise from the temperature dependence of the CMC globally at a series of surfactant concentrations. The model derived to fit the data follows similar principles as that for fitting heat capacity curves of micellar solutions as measured by differential scanning calorimetry.$^{215-217}$

So far, PPC has mainly been used to characterize volume changes of thermotropic transitions of polymers,$^{146-148}$ lipids,$^{149-152}$ proteins,$^{153,154,160,161,218,219}$ and DNA$^{158,159}$ and for temperature-induced, irreversible protein aggregation.$^{160,161}$ Here we demonstrate the use of PPC to describe the state of colloidal dispersions and their concentration-induced micellization in the absence of a thermotropic transition. We believe that PPC studies of surfactants comprising a very broad range of polar, hydrophobic, and charged moieties will add a great deal to our better understanding and proper and complete interpretation of expansivities of solutes$^{153}$ and colloidal particles in general. Further to the basic insight, the characterization and understanding of surfactant systems are also of utmost importance for the progress of a very big industry helping with our laundry.

### 4.3 Materials and Methods

Decyl-β-maltopyranoside was purchased from Anatrace, Maumee. It was quantified by weighing and dissolved in Millipore water to yield the desired concentration. Samples were carefully degassed before
measurement. Measurements were carried out on a VP DSC calorimeter from MicroCal (Northampton, MA) equipped with a MicroCal PPC accessory. Two or more small pressure jumps of (±5 bar are automatically carried out isothermally at a series of specified temperatures. Typically, we used 2K intervals. The data evaluation to derive $E_S(T)$ is realized by the instrument software, Origin for PPC. Perfectly bubble-free filling of the cells, which is not trivial with surfactant solutions, was confirmed in terms of matching absolute heat responses to up- and down-jumps in pressure. We have characterized the nonionic DM in pure water, which simplifies the equation for PPC data analysis in buffer\textsuperscript{153,215} to the form

$$\alpha_S(T) \equiv \frac{\partial V}{\partial T} \bigg|_p = -\frac{\Delta Q_{SW} - \Delta Q_{WW} + c_s \bar{v}_s Q_W}{TV_{cell}c_s \bar{v}_s \Delta p}$$

(4.1)

where $\Delta Q_{SW}$ denotes the heat response with an aqueous surfactant solution or dispersion in the sample cell and water in the reference cell, $\Delta Q_{WW}$ is a blank run with water in both cells, and $Q_W$ is the heat response for pure water as taken from the literature in form of a polynomial. $T$ stands for the temperature, $V_{cell} \approx 0.5$ mL is the cell volume, $c_s$ is the molar concentration of surfactant, $v_s \approx 0.80$ mL/g is the partial specific volume of DM, and $\Delta P \approx \pm 5$ bar is the pressure difference.

The apparent molar expansivity of the solute, $E_S$

$$E_S = \frac{dV_s}{dT}$$

(4.2)

was obtained by multiplication of $\alpha_S$ with the molar mass, 482.6 g/mol for DM, and its specific volume, 0.80 mL/g. It should be noted that measurements for instance with ionic surfactants should, of course, be realized at suitable salt and buffer conditions which is possible by PPC but requires additional blanks (see refs. \textsuperscript{153,214}) and increases the errors of absolute $E_S$ values. Some earlier doubts in the design of the technique could be ruled out completely.\textsuperscript{220,221}

4.4 Theory

4.4.1 $E_S$ of Surfactant Dispersions

For $c_s \ll CMC$, all $V_s$ and $E_S$ refer directly to the surfactant monomers in solution; we refer to this $V_s$ as $V_{mon}$.
\[ V_S(c_s \ll CMC) = V_{mon} \]  (4.3)

For dilute solutions, solute-solute interactions are small, and the molar volume, \( V_S \), and expansivity, \( E_S \), do not depend on concentration.

For concentrations \( c_s \gg CMC \), the partial volume is an average of two states:

\[ V_S = V_{mic}(1 - \frac{CMC}{c_s}) + V_{mon} \frac{CMC}{c_s} \]  (4.4)

where \( CMC/c_s \) is just the fraction of surfactant that is monomeric, \( 1 - (CMC/c_s) \) is the fraction that is micellar, and \( V_{mon} \) denotes the partial molar volume of the surfactant localized in micelles.

Differentiation with respect to \( T \) yields for \( c_s \geq CMC \) the general fit equation

\[ E_S(T) = E_{mon}(T) \frac{CMC(T)}{c_s} + E_{mic}(T) \left( 1 - \frac{CMC(T)}{c_s} \right) - \Delta V_S(T) \frac{dCMC(T)}{c_s dT} \]  (4.5)

where the temperature-dependent volume change of micelle formation, \( \Delta V_S(T) \), is given by a value at a chosen temperature (we chose \( \Delta V_S(25^\circ C) \)) and the integral of the difference of micellar and monomeric expansivity:

\[ \Delta V_S(T) \equiv V_{mic}(T) - V_{mon}(T) = \Delta V_S(25^\circ C) + \int_{25^\circ C}^{T} [E_{mic}(\bar{T}) - E_{mon}(\bar{T})] d\bar{T} \]  (4.6)

We should be aware of the fact that the treatment of micelle formation as a phase transition is an approximation that works well far below or above the \( CMC \). However, it fails to explain the sometimes considerable width of the “boundary” and the possible occurrence of premicellar aggregates at \( c_s \approx CMC \). We have made sure below that such deviations from the model do not perturb our results.

4.4.2 CMC as a Function of Temperature

Since the temperature-dependent \( CMC \) (and its derivative, \( dCMC/dT \)) are involved in Eq. 4.5 we need an expression giving \( CMC(T) \). There are semiempirical equations to fit \( CMC(T) \), but the fact that the heat capacity change of micelle formation, \( \Delta C_p \), is approximately constant over the temperature range considered here (detailed, precise studies have revealed and quantified small systematic variations, though\[^{51,222}\]) allows for an easier approach. Integrating van’t Hoff’s equation for constant \( \Delta C_p \) yields the expression
CMC(T) = CMC(T_0) \cdot \left(\frac{T_0}{T}\right)^{\Delta C_p/R} \cdot \exp\left\{\frac{T - T_0}{RT}\left(\frac{\Delta C_p}{T_0} - \frac{\Delta H(T_0)}{T_0}\right)\right\} \tag{4.7}

with a reference temperature T_0. The heat capacity change and isoenthalpic temperature, T_{iso} = T(\Delta H = 0), of DM were obtained by Majhi and Blume\textsuperscript{217} from \Delta H(T) measured by ITC as

\[ \Delta H(T) = \Delta H(T_0) + \Delta C_p \cdot (T - T_0) = \Delta C_p \cdot (T - T_{iso}) \tag{4.8} \]

yielding the micellization parameters listed in the legend to Figure 4.2. The first derivative, dCMC/dT (Figure 4.2B), illustrates that for c_S > CMC micelles form with increasing T at T < T_{iso} but disintegrate at T > T_{iso}. Since \Delta C_p is known to vary somewhat with temperature, we focus our study on the temperature range of 4-60 °C where experimental data on \Delta C_p are available.

4.5 Results

The expansivities measured at 1 mM DM (navy \times symbols in Figure 4.2A) and 1.5 mM DM (navy +) correspond to solutions below the CMC over the whole temperature range (see Figure 4.2) and refer to E_{mon}. As expected for purely monomeric samples, the results agree within error. This supports the assumption that premicellar aggregates play no significant role at 1.5mM, which is \approx 60-70% of the CMC. It is a peculiarity of PPC data which are evaluated using polynomial fits of blanks that their experimental error is not illustrated by random noise of the data but involves also up- and downshifts of the whole curve. We have fitted the averaged monomer data with a third-order polynomial to represent E_{mon}(T) in the fit of the other curves in Figure 4.3B (bold, navy line in Figure 4.2A).

An interesting behavior is found for the 2.5 mM sample illustrated by blue + symbols in Figures 4.2A, B. At about 2-10 °C Figure 4.1 shows that 2.5 mM < CMC, and indeed, the expansivities shown in Figure 4.2 agree with those of the monomeric samples (1 and 1.5 mM, not shown for high-T range). At 10 °C, the 2.5 mM sample reaches its lower critical micelle temperature (CMT), and analogously to the DSC curve\textsuperscript{217} the expansivity changes rather sharply to follow the model for micellar systems (Figure 4.3B). The substantial positive deviation of E_S from E_{mon} between \approx 10 and 36 °C illustrates that micelle formation must be accompanied by an increase in volume, \Delta V_S > 0. Above T_{iso}, the measured E_S is smaller than E_{mon} because the micelles formed earlier are now disintegrating with increasing temperature, until the last micelles vanish at \approx 60 °C.
Since \( d \text{CMC}/dT \) does not depend on concentration, the relative contribution to the average \( E_S \) of the \( \Delta V_S \) term in Eq. 4.5 is largest slightly above the \( \text{CMC} \), where the heat signal due to the expansion of the micelles is still small. However, we could not exclude a priori that micelles in samples only slightly above the \( \text{CMC} \) might differ from those at high concentration. We have therefore excluded the data for 2.5 mM from the fit but simulated the curve predicted by the model for 2.5 mM DM based on the parameters obtained at higher concentration. The good agreement shows that the micelles at 2.5 mM and 20-50 °C show no substantial deviation from those at higher concentration in terms of \( E \) and \( V_S \).

**Figure 4.1:** (A) Temperature dependence of the critical micelle concentration of DM as measured by Blume and Majhi, 2001, Langmuir. The shape of the fit line is determined by \( \Delta H(T) \) measured by ITC (not shown), yielding \( T_{iss} = 36.1^\circ\text{C} \) and \( \Delta C_p = -0.386 \text{ kJ/(mol K)} \). The only parameter fitted in (A) is the \( \text{CMC} \) at \( T_{iss} \) (vertical position of the curve), \( \text{CMC}_{\text{min}} = 2.13 \text{mM} \). (B) Derivative \( d\text{CMC}/dT \) which governs the micellization term in the model equation for \( E_S(T) \), Eq. 4.5.

**Figure 4.2:** Apparent molar expansivities of DM, \( E_S \), as a function of temperature in aqueous dispersions at concentrations noted in the plot. The samples in panel A were mostly below the \( \text{CMC} \) (except for 2.5 mM between the two \( CMT \)'s); those in panel B comprise monomers and micelles. Data series for 1 and 1.5 mM were fitted by a polynomial to give \( E_{\text{mon}} \). Curves for 5-250 mM were fitted according to Eq. 4.5. Fit parameters are polynomial coefficients for \( E_{\text{mic}} \) (illustrated by the bold, solid, wine-colored line in panel B) and the volume of micellization, \( \Delta V_S(25 \text{ °C}) = 16 \text{ mL/mol} \).
At 5 mM, the sample is well above the CMC in the temperature range addressed here, and the
demicellization effect on \( E(T) \) is already slightly weaker than at 2.5 mM. The magnitude of the \( \Delta V \) term
depends on \( dCMC/dT \), which is known, and on \( \Delta V_s(T) \), and that is why the concentration dependence
of the PPC curves somewhat above the CMC will permit us to determine \( \Delta V_s \). At concentrations far
above the CMC, virtually all surfactant is and remains in micelles so that the relative contribution of the
\( \Delta V_s \) term and the expansivity of the monomers to the average \( E_s \) become negligible and \( E_s \to E_{mic} \).

Our model describes all the curves in Figure 4.2 in terms of \( CMC(T) \) (known), \( E_{mon}(T) \) (represented by
a third-order polynomial), \( E_{mic}(T) \) (another third-order polynomial), and the apparent molar volume change of micelle formation, \( \Delta V_s(25 \, ^\circ C) \). We have determined these parameters by using the solver
tool in an Excel spreadsheet as follows. As mentioned above, \( E_{mon} \) was obtained by fitting the
monomer curves independently (Figure 4.2A). A good set of starting values for the \( E_{mic}(T) \) polynomial
was obtained by fitting the data measured for 250 mM DM. Next, \( \Delta V_s(25 \, ^\circ C) \) was fitted as a shared
global parameter to all curves measured at 5-250 mM shown in Figure 4.2B, using \( E_{mic} \) and \( E_{mon} \) as
described. Finally, the polynomial coefficients representing \( E_{mic} \) were varied globally along with \( \Delta V_s(25 \, ^\circ C) \),
causing a minute downshift of \( E_{mic} \) compared to the 250 mM curve. The corresponding fit lines are
shown as thin dashed lines in Figure 4.2. Given the errors of \( CMC(T) \) and \( E_{mon}(T) \) and the fact that
this is almost a one-parameter fit of \( \Delta V_s(25 \, ^\circ C) \) (\( E_{mic} \) is largely determined by \( E_s(250 \, mM) \)), the
agreement of the fit curves with the data is highly satisfactory. That means that our model provides a
good description of the expansivity of micellar surfactant systems. A PPC curve for maltose in aqueous
solution was recorded as shown in Figure 4.4; the curves obtained for solutions of 10 and 100 mM
maltose show no significant difference.

4.6 Discussion

4.6.1 Volume and Expansivity Changes upon Micelle Formation

Using Eq. 4.6 and \( \Delta V_s(25 \, ^\circ C) \) as fitted, we have calculated \( \Delta V_s(T) \) as shown in Figure 4.3. The volume
change of micelle formation is large and positive and decreases with increasing temperature from 4 to
60 \( ^\circ C \) almost linearly by -0.13 ml/(mol K). The estimated error for \( \Delta V_s \) is 2.5 mL/mol. Two phenomena
account for this relatively large increase in volume (see below for \( \Delta V_s/V_s \) of other transitions): the
decrease in water exposure of the detergent molecules (releasing condensed water to the bulk) and the
formation of the hydrophobic core with voids between the disordered, loosely packed alkyl chains.
Increasing thermal energy tends to free adjacent water molecules from the influence of the detergent
surface; this reduces the contribution of (de)hydration to \( \Delta V_s \) and can explain the decreasing \( \Delta V_s(T) \)
(Figure 4.3). The influence of increased proportions of gauche bonds in the chains, another effect of increasing temperature, on volume is not straightforward to be predicted. On one hand, increased thermal motion might cause an expansion of the micellar core. On the other hand, it may improve packing. While all-trans chains can be most tightly packed in a monolayer arrangement, effective filling of the spherical micelle core by quasi-cylindrical chains requires a high flexibility. It might therefore be improved by an increasing number of gauche conformers, yielding a negative contribution to $E_{mic}(T)$ and $V_S(T)$.

Table (4.1): Volumetric Data for DM and Maltose and Estimates for the Decyl Chain$^a$

<table>
<thead>
<tr>
<th></th>
<th>4°C</th>
<th>25°C</th>
<th>40°C</th>
<th>$\Delta S_{40}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{mon}$ mL/(mol K)</td>
<td>0.53 ± 0.02</td>
<td>0.44 ± 0.02</td>
<td>0.435 ± 0.02</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>$E_{mic}$ mL/(mol K)</td>
<td>0.343 ± 0.003</td>
<td>0.313 ± 0.003</td>
<td>0.303 ± 0.003</td>
<td>0.04 ± 0.004</td>
</tr>
<tr>
<td>$\Delta E$ mL/(mol K)</td>
<td>-0.19 ± 0.03</td>
<td>-0.13 ± 0.02</td>
<td>-0.13 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>$\alpha_{mon}$ $10^{-3}$/K</td>
<td>1.4</td>
<td>1.1</td>
<td>1.1</td>
<td>0.3</td>
</tr>
<tr>
<td>$\alpha_{mic}$ $10^{-3}$/K</td>
<td>0.85</td>
<td>0.78</td>
<td>0.75</td>
<td>0.10</td>
</tr>
<tr>
<td>$\Delta \alpha$ $10^{-3}$/K</td>
<td>-0.6</td>
<td>-0.3</td>
<td>-0.3</td>
<td>-0.2</td>
</tr>
<tr>
<td>$\Delta V$ mL/mol</td>
<td>19</td>
<td>16 ± 2.5</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>$\Delta V/V$ %</td>
<td>4.9</td>
<td>4.1</td>
<td>3.6</td>
<td>1.3</td>
</tr>
</tbody>
</table>

measured for maltose:

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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_S$ mL/(mol K)</td>
<td>0.26 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.11</td>
</tr>
<tr>
<td>$\alpha_S$ $10^{-3}$/K</td>
<td>1.2</td>
<td>0.8</td>
<td>0.7</td>
<td>0.5</td>
</tr>
</tbody>
</table>

estimated for decyl chain from values for DM minus those for maltose:

<p>| | | | | |</p>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_S$ mL/(mol K)</td>
<td>0.27</td>
<td>0.26</td>
<td>0.29</td>
<td>-0.02</td>
</tr>
<tr>
<td>$\alpha_S$ $10^{-3}$/K</td>
<td>1.5</td>
<td>1.5</td>
<td>1.6</td>
<td>0.1</td>
</tr>
</tbody>
</table>

$^a$ Errors, where given, are estimated maximum errors.

In any case, the finding that the expansivity of monomeric DM is substantially larger than that of micellar DM is in accord with the larger accessible surface area per molecule of a monomer in solution, but the decrease is not proportional to decreased water exposure.
Temperature Dependence of the Expansivity of Polar, Apolar, and Amphiphilic Moieties

PPC is the most precise and convenient method to measure the apparent molar expansivity of a solute, $E_S$, in water but has been available for a few years only. As a consequence, our knowledge about $E_S(T)$ and its interpretation is still limited to a few pioneering studies. Lin et al. measured the group contributions of amino acid side chains and found nonlinearly decreasing $E_S(T)$ for polar and increasing...
$E_S(T)$ for hydrophobic side chains. Most curves shared a similar, quasi-exponential shape with continuously decreasing absolute values of their slope with increasing $T$. Hence, they could essentially be characterized by the difference $\Delta \alpha_{5-40} = \alpha (5 \, ^{\circ}C) - \alpha (40 \, ^{\circ}C)$. $\Delta \alpha_{5-40}$ would then be an empirical measure of hydrophilicity. Tsamaloukas et al. used these group contributions to successfully predict the expansivity curves of unstructured proteins.\textsuperscript{219}

$E_S(T)$ of maltose in aqueous dispersion shows such a curve shape with a positive $\Delta \alpha_{5-40}$ of $+ 0.53 \times 10^{-3} /K$ that is typical for polar solutes and quantitatively comparable with the values of serine or glutamine side chains\textsuperscript{153} or for apocytochrome c.\textsuperscript{219} Taking into account the negative $\Delta \alpha_{5-40}$ for hydrophobic side chains (e.g., $\approx -1 \times 10^{-3} /K$ for valine)\textsuperscript{151} and the additivity of group contributions seen for unstructured proteins,\textsuperscript{219} one would expect the decyl chain to render $\Delta \alpha_{5-40}$ of DM in solution negative or at least substantially less positive than that of maltose. However, this is not the case. The experimental values of $\Delta \alpha_{5-40}$ of maltose and DM are in fact very similar; the curve shapes differ only somewhat in the high temperature range.

The difference $E_S$ (maltose) $- E_{mon}$ (DM), which provides a crude estimate for the contribution of the decyl chain, increases only slightly in a quasi-linear fashion, very much unlike the curves for apolar amino acid side chains. Resolving this apparent paradox by establishing a more detailed, quantitative model for $E_S(T)$ of solutes will prove very valuable for obtaining insight into the subtle hydration properties of groups and molecules. Only then, the full power of PPC will become accessible.

Following the idea that the maltoside headgroup remains hydrated in a micelle but the decyl chain is essentially hidden from water, one might have guessed micellar DM to show a similar $E_S(T)$ as maltose. Instead, the data reveal a much weaker temperature dependence of the micellar curve. This might result from a partial dehydration also of the maltoside group in a micelle and/or an expansivity of the hydrophobic core of the micelle that increases with $T$ (compensating for the positive $\Delta \alpha_{5-40}$ of the headgroup).

### 4.6.3 Comparing the Volumetric Behavior of Different Thermotropic Transitions

The volumetric behavior of a transition as expressed, for instance, in terms of relative volume and expansivity changes, $\Delta V_S/V_S$ and $\Delta \alpha_S$, can provide a framework to distinguish and understand fundamental volumetric properties of these phases. Figure 4.5 collects examples for a variety of thermotropic transitions chosen in a direction of increasing structure and rigidity and/or decreasing hydration.
Blue symbols denote transitions occurring with decreasing temperature, such as folding of proteins, DNA into helices, and the freezing of lipid membranes into a gel phase. Red symbols denote transitions induced with increasing temperature, such as from spherical to rod-like micelles or the coil-to-globule transition of polymers. Black symbols indicate transitions that are not primarily thermotropic, such as micelle formation as studied here or the irreversible association of insulin to form amyloid-like fibrils. For comparison, the freezing of water causes a $\Delta V/V = +9.1\%$ and $\Delta \alpha = +2.2 \times 10^{-4}/K$.

Figure (4.5): Overview of relative volume ($\Delta V/V_S$) and expansivity ($\Delta \alpha_S$) changes accompanying different examples for folding, freezing, or association transitions occurring upon decreasing temperature (solid, blue symbols) or increasing temperature (red, open symbols). Examples are taken from literature for the liquid crystalline-to-rippled gel transition (freezing) of the lipid DMPC, folding of a number of globular proteins (ref. 154 and Lee, S., unpublished), DNA helix formation, the coil-to-globule transition of NIPAM and analogues, the sphere-to-rod transition of myristyl maltoside, formation of insulin globules and fibrils, and the micelle formation of DM (this paper). Values are approximate (in some cases estimated from figures), and ranges are to guide the eye only; the available examples might not be representative for a given transition in general.

As one might expect for transitions of increasing rigidity and/or decreasing hydration, almost all exhibit negative expansivity changes. However, the volume changes are vastly different and range from...
strongly negative (lipid freezing) via virtual volume conservation (proteins) to large and positive (micelle formation). This behavior can be explained in terms of the contributions to volume and expansivity. Decreasing water exposure of molecular and aggregate surfaces causes a negative contribution to $\Delta \alpha_S$ and a positive one to $\Delta V_S$. This results from water in contact with the surface being condensed compared to bulk water at lower temperature, and thermotropic freeing of this water from the influence of the surface causes an enhanced expansivity of the apparent volume of the solute. Well-defined, rigid structures such as lipid membranes in a gel phase, protein fibrils, and, to some extent, native proteins and DNA double helices show relatively small (but nonzero) void volume and negative or close-to-zero (proteins, DNA helices) volume changes upon formation. However, soft, unstructured aggregates such as micelles and partially dehydrated globules of polymers may be much less tightly packed than solutions. This may explain why formation of soft aggregates or globular structures can give rise to positive volume changes and even positive expansivity changes. The latter applies to the coil-to-globule transition of PNIPAM polymers and the transition of insulin to form a partially hydrated, partially secondary structure-retaining globule. The positive $\Delta \alpha_S$ of polymer globule formation might result from unusually large expansion of the soft core of the globules.

4.7 Conclusions

The temperature-dependent expansivities of monomeric and micellar surfactant as well as the volume of micelle formation can be conveniently measured by PPC. Here we test the model with a surfactant that allows, essentially, for an independent fit of $E_{\text{nic}}$ and $E_{\text{mon}}$. For surfactants with much lower or higher $CMC$, one of the two will be needed to be obtained by the global fit.

Micelle formation of DM is accompanied by a strong increase in volume and a decrease in expansivity, which can qualitatively be explained in terms of the release of condensed water from the molecular surface of the surfactant and a considerable void volume between the disordered alkyl chains in the core of the micelle.

The temperature dependence of the apparent molar expansivity of an aqueous solute depends on the type and strength of the interactions of the different moieties with water in a complex manner; it does not reflect an average hydrophobicity or polarity that could be quantified by a single empirical parameter. This suggests that $E_{\text{mon}}(T)$ could provide detailed insight into hydration phenomena, and further efforts are warranted to establish a quantitative model for its interpretation.
4.8 Acknowledgement

We thank Halina Szadkowska for her assistance in measuring preliminary data for this study. This work was realized on the basis of a grant from the National Science and Engineering Council of Canada, NSERC, to Heiko Heerklotz.
CHAPTER (5) - The effect of hydrophobic interactions on volume and thermal expansivity

This study by: 
**Mozhgan Nazari, Helen Y Fan, Heiko Heerklotz (2012)** is currently accepted for publication in Langmuir.

I wish to acknowledge the following contributions:

Helen Fan did the fits of most data sets using an Excel script she had designed before.

Heiko Heerklotz contributed to the design of the study and the final write-up of the paper.

5.1 Abstract

Thermodynamic and volumetric parameters have long been used to elucidate the phenomena governing the stability of protein structures, ligand binding, or transitions in macromolecular or colloidal systems. In spite of much success, many problems remain controversial. For example, hydrophobic groups have been discussed to condense adjacent water, causing a negative contribution to the volume change of unfolding. However, expansivity data were interpreted in terms of a structure-making, water-expanding effect. We have studied volume and expansivity effects of transfer of alkyl chains into micelles by pressure perturbation calorimetry and isothermal titration calorimetry. For a series of alkyl maltosides and glucosides, the methylene group contribution to expansivity was obtained as $5 \mu L/(mol \ K)$ in a micelle (mimicking bulk hydrocarbon) but $27 \mu L/(mol \ K)$ in water (20 °C). The latter value is virtually independent of temperature and similar to that obtained from hydrophobic amino acids. Methylene contributions of micellization are about $-60 \ kJ/(mol \ K)$ to heat capacity and 2.7 $mL/mol$ to volume. Our data oppose the widely accepted assumption that water-exposed hydrophobic groups yield a negative contribution to expansivity at low temperature that would imply a structure-making, water-expanding effect.
5.2 Introduction

The detailed nature of the hydrophobic effect and its importance for the structure and function of proteins and other macromolecules have been a subject of intense studies for many decades.\(^{47,228,229}\) Classically, the exposure of hydrophobic groups to water has been characterized in terms of a large, negative entropy change (at room temperature)\(^{47}\) or a positive heat capacity change.\(^{204,205}\) The parameters mentioned above are, however, not universally applicable and not necessarily attributable to hydrophobic interactions alone. Entropy gains may also arise from the release of water that had been bound to polar groups or the release of ions from the double layer adjacent to charged surfaces.\(^{204}\) Heat capacity changes may also result from changes in order and mobility of groups or molecules induced by ligand binding\(^{206}\) or incorporation into, for example, a membrane.\(^{151}\)

Volumetric properties such as the volume and thermal expansivity changes of protein unfolding depend strongly on the packing of adjacent water molecules and are, therefore, excellent parameters to characterize hydrophobic interactions.\(^{211,230-233}\) However, also this field remains partially unclear and controversial. For decades, the exposure of hydrophobic groups to water, which increases upon protein unfolding, has been stated to condense adjacent water compared to the bulk, thus contributing a negative volume change of unfolding.\(^{211,230,231,234}\) Given that also the opening of voids and the exposure of charged groups from the interior of the native structure cause a reduction in volume, a missing contribution was postulated to explain the overall very small, in some cases even positive volume of protein unfolding.\(^{230}\) More recently, pressure perturbation calorimetry (PPC) has allowed for a much more precise and convenient measurement of thermal expansivity of solutes.\(^{146,149,153,157,218}\) Apparently at variance with the traditional idea of water condensation, hydrophobic side chains of amino acids in water were discussed to show negative or anomalously low thermal expansivity at low temperature, because they would have a structure-making, expanding effect on water which vanishes gradually with increasing temperature.\(^{153,235}\)

The goal of this study is to help resolving this paradox and contribute to a better understanding of the general volumetric effects of hydrophobic association which play a key role, for example, in protein folding and ligand binding, the swelling of polymers, and micelle formation. We are tackling this problem by a comprehensive volumetric and thermodynamic characterization of the micelle formation of series of nonionic surfactants. Furthermore, some experiments with amino acids are presented to illustrate the application of the group contributions established using surfactants to amino acids and
proteins. Our study utilizes a recent protocol that is based on a combined application of pressure perturbation calorimetry and isothermal titration calorimetry.\textsuperscript{236}

This approach had been inspired by similar studies using differential scanning calorimetry,\textsuperscript{216,217} making use of the temperature dependence of the critical micelle concentration to deal with micellization as a thermotropic transition.

Volume changes of hydrocarbon dissolution or micelle formation have been described before using densimetry or based on the effect of pressure on the critical micelle concentration (CMC). Positive volume changes of micelle formation were reported at ambient pressure, for example 16.5 mL/mol for sodium dodecanoate\textsuperscript{57}, 11 mL/mol for SDS\textsuperscript{58}, and 18 mL/mol was found for C\textsubscript{8}E\textsubscript{5}.\textsuperscript{59} Values for ionic surfactants had to be corrected for counter ion effects. Generally, the compressibility of micellar surfactant is higher than that of monomers in solution so that $\Delta V$ decreases with increasing pressure and ultimately changes its sign. This corresponds to a maximum of $CMC(P)$. Studies of homologous series revealed $\Delta V = 4.9 - 11.3$ mL/mol for sodium octyl to tridecyl sulphate.\textsuperscript{237}

Before the introduction of PPC, however, information about temperature-dependent expansivity changes has been scarce and of limited precision. Masterton\textsuperscript{60} suggested that the expansivity of aliphatic solutes (but not of benzene) in water decreases with increasing temperature and explained this phenomenon with the breakdown of cage-like water structures surrounding the aliphates.

### 5.3 Materials and Methods

#### 5.3.1 Materials

Detergents used in this project are n-Hexyl-\(\beta\)-D-Glucopyranoside (6G), n-Heptyl-\(\beta\)-D-Glucopyranoside (7G), n- Octyl-\(\beta\)-D-Glucopyraonoside (OG), n-Nonyl-\(\beta\)-D-Glucopyranoside (NG), n-Hexyl-\(\beta\)-D- Maltopyranoside (6M), n-Octyl-\(\beta\)-D-Maltopyranoside (OM), n-Nonyl-\(\beta\)-D-Maltopyranoside (NM), n- Undecyl-\(\beta\)-D-Maltopyranoside (UM), and 6-Cyclohexyl-1-Hexyl-\(\beta\)-D-Maltoside (Cymal). These detergents were purchased at the highest available purity from Anatrace, Maumee. Each detergent was quantified by weighing and dissolved in Millipore water to yield the desired concentration. Samples were degassed before measurement.
5.3.2 Isothermal Titration Calorimetry

ITC demicellization experiments were done using a VP ITC from Microcal, Northampton, as described. Briefly, the about 1.5 mL cell was filled with water and a series of injections was done automatically from a 0.3 mL syringe, typically one every 10 minutes. The injectant was a micellar solution of detergent, typically at about 15 times the CMC. The compensation heat power as recorded was evaluated using instrument software, including manual baseline adjustment, peak integration, and normalization with respect to the mole number injected to give the observed heat, \( Q_{\text{obs}} \), as a function of the detergent concentration in the cell.

5.3.3 Pressure Perturbation Calorimetry

PPC measurements were carried out on a VP DSC calorimeter from MicroCal (Northampton, MA) equipped with a MicroCal PPC accessory. Two or more small pressure jumps of ±5 bar were automatically carried out isothermally at a series of specified temperatures. Typically, 2K intervals from 2 to 60°C were used. The instrument software, Origin for PPC\textsuperscript{TM} was used to analyze the data to derive the coefficient of expansion of the apparent volume of the solute, \( \alpha \).

Perfectly bubble-free filling of the cells was confirmed in terms of matching absolute heat responses to up and down jumps in pressure. All the detergents used in this project are nonionic and have been characterized in pure water. This simplifies the equation for PPC data analysis into the following form:\textsuperscript{153,214,236}

\[
\dot{\alpha}(T) \equiv \frac{\partial V}{V \partial T} \bigg|_p = -\frac{\Delta Q_{\text{SW}} - \Delta Q_{\text{WW}} + c_s \bar{v}_s Q_W}{T V_{\text{cell}} c_s \bar{v}_s \Delta p} \tag{5.1}
\]

where \( \Delta Q_{\text{SW}} \) stands for the heat response with an aqueous surfactant solution or dispersion in the sample cell and water in the reference cell, \( \Delta Q_{\text{WW}} \) is a blank run with water in both cells, \( Q_W \) is the heat response for pure water as taken from the literature in form of a polynomial. \( T \) stands for the temperature, \( V_{\text{cell}} \approx 0.5 \) mL is the cell volume, \( c \) is the molar concentration of the detergent, \( \bar{v} \) is the partial specific volume of each detergent, and \( \Delta p \approx 5 \) bar the pressure difference. Note that both \( \bar{v} \) and \( c \) are inversely temperature dependent due to the expansion of the solute and the solvent, respectively, so that the product depends only little on temperature.\textsuperscript{153}

The apparent molar expansivity of the solute, \( E \), is written as:
\[ E = \left. \frac{\partial V}{\partial T} \right|_p \quad (5.2) \]

\(E\) can be computed by multiplying \(\alpha\) with the molar mass and partial specific volume. Note that all volume (except for \(V_{cell}\)) and expansivity data discussed here refer to apparent molar quantities of the detergent; we have therefore omitted specific symbols or subscripts to emphasize this fact and use simply \(V\) and \(E\), respectively. The derivation of partial quantities from bulk measurements is explained elsewhere.\(^{214}\)

5.4 Results

5.4.1 ITC demicellization experiments

Figure 5.1 illustrates the results of ITC demicellization experiments with different surfactants as a function of temperature. Typical demicellization curves for NM are shown in Fig. 5.1A. In the beginning of the titration, the concentration in the cell is still well below the CMC and added micelles dissolve. This gives rise to a heat of demicellization. Well above the CMC, injected micelles persist and this heat is not observed. As usual, there is no sudden step between these ranges as suggested by the phase separation model. Instead, the onset of micelle formation occurs over a concentration range rendering the curve sigmoidal. The CMC is defined as the point of inflection of the curve, and the molar enthalpy of micelle formation, \(\Delta H\), is obtained from the step between linearly extrapolated pre- and post-CMC base lines at the CMC \((\Delta H_{obs})\) (see demonstration in Fig. 5.1A) after correction for injected monomers using Eq. 5.3:

\[ \Delta H = -\Delta H_{obs} \cdot \frac{c_{syrr\text{-}CMC}}{c_{syrr}} \quad (5.3) \]

Note that \(\Delta H\) refers to the formation of micelles and differs in sign from the heat of demicellization.

The results of this evaluation for NM (as shown in Fig. 5.1A) and other surfactants are compiled in Fig. 5.1B, C.

The enthalpies of micelle formation show a virtually linear decrease with temperature, \(T\), that can be described according to

\[ \Delta H(T) = \Delta H(T_0) + \Delta C_p \cdot (T - T_0) = \Delta C_p \cdot (T - T_{iso}) \quad (5.4) \]
in terms of an essentially constant heat capacity change, $\Delta C_p$, and an isoenthalpic temperature, $T_{iso}$, where $\Delta H$ changes its sign (Fig. 5.1B). $T_0$ refers to any arbitrarily chosen reference temperature. As is typical for the association of hydrophobic molecules or moieties in water, we observe negative values of $\Delta C_p$ (see Tab. 5.1).

Considering the CMC being analogous to dissociation constant and pasting Eq. 5.4 into van’t Hoff’s equation, one obtains for the temperature dependence of the CMC:

$$CMC(T) = CMC(T_0) \cdot \left( \frac{T_0}{T} \right)^{\frac{\Delta C_p}{R}} \cdot \exp \left\{ \frac{T - T_0}{R T} \left[ \Delta C_p - \frac{\Delta H(T_0)}{T_0} \right] \right\} \quad (5.5)$$

Eq. 5.5 predicts a shallow minimum for $CMC(T)$ at $T_{iso}$. Fig. 5.1C shows experimental CMC values along with fit lines according to Eq. 5.5. They were obtained by fitting $\Delta C_p$ and $\Delta H(T_0)$ to the data in Fig. 5.1B and fitting only $CMC(T_0)$ to the corresponding data in Fig. 5.1C. All fit parameters are compiled in Tab. 5.1, along with the respective data for a series of alkyl glucosides (experimental data not shown).
Figure (5.1): Results of ITC experiments. Panel A shows raw heat curves of ITC demicellization experiments with NM at different temperatures and illustrates how $\Delta H_{\text{obs}}$ was read from the curve. Panels B, C show the corrected enthalpy of micelle formation, $\Delta H$, and the $CMC$ as a function of temperature for the alkyl maltosides OM, NM, UM, and cymal studied here and for DM as published.\textsuperscript{153,168} Fit curves in panel C use $\Delta C_p$ obtained from the slope of the corresponding $\Delta H(T)$ in panel B, using Eq. (5.5).

5.4.2 PPC experiments with surfactants

Figure 5.2A illustrates the nature and evaluation of the data for OM. Points represent average values of $E(T)$ obtained at different concentrations. The bold blue line, $E_{\text{mon}}(T)$, represents a fit to a curve at 10 mM OM, i.e., well below the $CMC$, by a third-order polynomial. The sample with 30 mM follows this line up to about 5 °C, where $CMC(T) > 30 \text{ mM}$. Then, it deviates because the appearance of micelles causes a substantial positive contribution to expansivity.
Samples at 40 mM and higher are above the CMC over the whole T-range investigated. They are described by the model derived before:\(^{236}\)

\[
E(T) = E_{\text{mon}}(T) \cdot \frac{\text{CMC}(T)}{C} + E_{\text{mic}}(T) \cdot \left(1 - \frac{\text{CMC}(T)}{C}\right) - \Delta V(T) \frac{d\text{CMC}(T)}{dT}
\]  

(5.6)

with the apparent molar expansivity of the detergent located in micelles, \(E_{\text{mic}}(T)\), and apparent molar volume change of micelle formation, \(\Delta V(T)\). All these samples show the same, absolute contribution from \(T\)-induced micelle formation but since the values given in Fig. 5.2A are normalized with respect to the total surfactant concentration, we observe a decreasing relative contribution from demicellization with increasing surfactant concentration. For \(c >> \text{CMC}\), monomer and demicellization effects become negligible and \(E\) tends toward the expansivity of exclusively micellar surfactant, \(E_{\text{mic}}\). This condition could essentially be met for DM at \(250 \text{ mM} \approx 100 \times \text{CMC}\),\(^{236}\) rendering the evaluation of these data largely a one-parameter fit of \(\Delta V(25 ^\circ \text{C})\). All fits were carried out as described,\(^{236}\) using the solver tool in Microsoft Excel.\(^{223}\) For OM with its \(\text{CMC} \approx 25 \text{ mM}\), this is not feasible. Instead, the parameters of a polynomial representing \(E_{\text{mic}}(T)\) are varied in the global fit and the resulting curve (bold red curve in Fig. 5.2A) deviates strongly from the experimental data obtained at \(250 \text{ mM} (\approx 10 \times \text{CMC})\). The volume change at \(20 ^\circ \text{C}\) is fitted to \(\Delta V = 10 \text{ mL/mol}\).
Figure (5.2): Results of PPC experiments; all given as expansivities as a function of temperature. A: PPC curves for OM at different concentrations (see plot) and global fit curves yielding $E_{\text{mon}}(T)$ and $E_{\text{mic}}(T)$ as presented. B and C show $E_{\text{mon}}$ and $E_{\text{mic}}$, respectively, for a series of alkyl maltosides as indicated. In the acronyms, the first letters O, N, D, U, L stand for octyl, nonyl, decyl, undecyl, and lauryl chains, respectively; CYM denotes Cymal-6.

The fit curves for $E_{\text{mon}}$ and $E_{\text{mic}}$ are reproduced in Figs. 5.2B, C, respectively, along with the analogous data for other alkyl maltosides and cymal. Fig. 5.2B also shows $E(T)$ for a maltose solution as published.
As one might expect for $E$ given per mole, the values increase virtually continuously with increasing size of the molecule, e.g., increasing alkyl chain length. However, whereas the increase of $E$ with increasing chain length is quite pronounced for monomers, the changes are only minute if the chains are buried in the core of a micelle. Most of the $E_{mon}(T)$ curves show a decrease with a shape resembling this of maltose alone. $E_{mic}(T)$ is found very little dependent on temperature.

Table (5.1): Summary of experimental parameters at 20 °C (except for $T_{iso}$), including the critical micelle concentration (CMC), molar isobaric heat capacity change on micelle formation ($\Delta C_p$), isenthalpic temperature ($T_{iso}$, where $\Delta H(T)=0$), the specific volume used ($\bar{v}$), the expansivity change upon micelle formation ($\Delta \alpha = E_{mic} - E_{mon}$), the change in the expansion coefficient upon micelle formation ($\Delta \alpha$), and the absolute ($\Delta V$) and relative ($\Delta V/V$) change in apparent molar volume upon micelle formation. *ITC data for OG, NG, and DM are taken from Majhi and Blume.

<table>
<thead>
<tr>
<th></th>
<th>CMC</th>
<th>$\Delta C_p$</th>
<th>$T_{iso}$</th>
<th>$\bar{v}$</th>
<th>$\Delta E$</th>
<th>$\Delta \alpha$</th>
<th>$\Delta V$</th>
<th>$\Delta V/V$</th>
</tr>
</thead>
<tbody>
<tr>
<td>OM</td>
<td>24.6</td>
<td>320±20</td>
<td>45.9</td>
<td>0.78</td>
<td>-72</td>
<td>-0.22</td>
<td>10.4±1.5</td>
<td>2.9%</td>
</tr>
<tr>
<td>NM</td>
<td>7.3</td>
<td>335</td>
<td>40.4</td>
<td>0.79</td>
<td>-127</td>
<td>-0.34</td>
<td>17±2.5</td>
<td>4.6%</td>
</tr>
<tr>
<td>DM</td>
<td>2.3*</td>
<td>386*</td>
<td>35.6*</td>
<td>0.80</td>
<td>-136</td>
<td>-0.35</td>
<td>16±2.5</td>
<td>4.1%</td>
</tr>
<tr>
<td>UM</td>
<td>0.66</td>
<td>-468±11</td>
<td>31.8</td>
<td>0.81</td>
<td>-171</td>
<td>-0.42</td>
<td>20±3.5</td>
<td>5.2%</td>
</tr>
<tr>
<td>cymal-6</td>
<td>0.65</td>
<td>-466±30</td>
<td>28.5</td>
<td>0.77</td>
<td>-184</td>
<td>-0.47</td>
<td>13±2.5</td>
<td>3.3%</td>
</tr>
<tr>
<td>OG</td>
<td>24.6*</td>
<td>-383±20*</td>
<td>46.9*</td>
<td>0.80</td>
<td>-39</td>
<td>-0.17</td>
<td>9.5±2</td>
<td>4.1%</td>
</tr>
<tr>
<td>NG</td>
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<td>-380±20*</td>
<td>40.8*</td>
<td>0.81</td>
<td>-110</td>
<td>-0.44</td>
<td>11±2.5</td>
<td>4.4%</td>
</tr>
</tbody>
</table>
5.4.3 Expansivity of amino acids in solution

The aim of this paper is to provide insight into the interpretation of volumetric effects of hydrophobic interactions in general. For several reasons discussed in the introduction, we have chosen micelle formation as the main object to tackle this issue. A few measurements were, however, done with amino acids to illustrate that and how the data obtained here apply to other systems as well.

![Figure 5.3](image)

**Figure (5.3):** PPC curves for Gly, Leu, and Val (15 mg/mL in water) presented as coefficients of expansion ($\alpha$, left) and molar expansivities ($E$, right). The arrows illustrate that Leu has a lower $\alpha$ but higher $E$ than Gly at 20 °C (see text).

Expansivities and expansivity coefficients of Gly, Val, and Leu are shown in Fig. 5.3. The differences between $\alpha$ of Leu and Gly and of Val and Gly, respectively (see black arrow for difference at 20 °C) are at least qualitatively in line with the behavior described for hydrophobic amino acids. They are negative at low temperature but become positive at about 50 °C.

For a detailed, quantitative comparison, we should for example have explored possible concentration effects, but this is beyond the scope of this study. Importantly, the molar expansivities, $E$, of Leu and Val above 20 °C are larger than that of Gly. Furthermore, the almost constant offset of the $E$(Leu) compared to $E$(Val) suggests a largely temperature-independent methylene group contribution, it
amounts to $\approx 19 \mu L/(mol \ K)$ at 20 ℃. Similar absolute volume changes for Gly and Leu (Fig 5.3, right) correspond to larger relative volume changes of Gly (Fig. 5.3 left) because Gly is much smaller than the other amino acids.

5.5 Discussion

5.5.1 Methylene group contributions to volumetric parameters

Figure 5.4 summarizes the effects of the size of the hydrophobic part of the surfactants on thermodynamic and volumetric parameters of micelles formation. The CMC decreases with increasing chain length in line with the usual standard free energy increment per CH$_2$; see Tab. 5.2. Cymal-6 includes 12 carbons in the hydrophobic part but shows a higher CMC than lauryl maltoside because the carbons in the cyclohexyl moiety are less accessible to water than those in a straight chain. Fig. 5.4B illustrates the heat capacity changes of micelle formation, which have been interpreted in terms of changes water accessible apolar surface.\textsuperscript{41,51,204,205} We emphasize that the dash-dot line is not a fit to the data obtained here but represents the fit published for a series of lysophosphatidylcholines.\textsuperscript{41} The values measured here for alkyl maltosides agree with this line. The slope of $\approx -57$ kJ/(mol K) per CH$_2$ (see also Tab. 5.2) had been discussed as a hallmark of the hydrophobic effect and the n$_C$-intercept at $\approx 3$ had been interpreted in terms of an average value of $\approx 3$ methylene groups per chain that remain exposed to water in a micelle.\textsuperscript{41,51} Molecular dynamics simulations have revealed and illustrated the highly dynamical and disordered structure of micelles,\textsuperscript{48} making this interpretation very plausible. One may expect the average exposure per chain to depend somewhat on the size of the head group, which might explain the moderate deviations of $\Delta C_p$ values from the dash-dotted line as found for OG (Fig. 5.4B) and a number of other detergents, such as -0.4 kJ mol$^{-1}$ K$^{-1}$ for CTAB (n$_C$ = 10)\textsuperscript{238}, -0.57 kJ mol$^{-1}$ K$^{-1}$ for C$_{10}$EO$_5$ (own unpublished data), and -0.62 kJ mol$^{-1}$ K$^{-1}$ for C$_{12}$EO$_8$\textsuperscript{188}.

The concept of the surface area of $\approx 3$ methylenes per chain remaining exposed to water in a micelle is impressively supported by the expansivity data shown in Fig. 5.4C. Linear extrapolation of the data for E in micelles (open circles) and in aqueous solution (solid spheres) predicts the difference between the two (e.g., $\Delta E$ of micelle formation) to vanish at n$_C$ $\approx 3$. The volume changes of micelle formation (Fig. 5.4D) are in agreement with an n$_C$-intercept of 3 as well (but the error is larger).

The expansivity, Emic, of alkyl chains in micelles shows essentially the same methylene group contribution (slope of open spheres in Fig. 5.4C; value in Tab. 5.2) as that of liquid hydrocarbon (crosses
in 5.4C). This suggests that the packing of the chains in a micelle and its changes with temperature resemble that of bulk hydrocarbon.

An offset of $\approx +0.1$ mL mol$^{-1}$ K$^{-1}$ between expansivities of alkanes and micellar surfactants represents the effect of the headgroup and interfacial water exposure. The offset of the data for alkyl glucoside micelles (open squares) is about half that of the maltosides, in line with the smaller headgroup.

We should mention that the assignment of slope and intercept of $E(n_C)$ to chain and headgroup, respectively, is not unequivocal. We cannot strictly exclude specific effects in micelles that fortuitously compensate each other to allow for the matching of methylene group contributions in micelles and liquid alkanes. Chains in a micelle are subject to positional constraints. Micellar size and, possibly, shape change with $n_C$ and could systematically vary the contributions from headgroups and interface, thus affecting not only the intercept but also the slope of $E_{\text{mic}}(n_C)$. However, such effects are unlikely to play a substantial role for a number of reasons. First, they would probably compromise the linearity of $E_{\text{mic}}(n_C)$ and the $n_C$-intercept at $\approx 3$. Second, essential agreement between methylene group contributions in micelles and bulk hydrocarbon has also been found for heat capacities (see above and Tab.5.2) and adiabatic compressibility. In any case, the equivalence of the micellar core with bulk hydrocarbon does not seem trivial and warrants further attention.
Figure (5.4): Thermodynamic and volumetric parameters of surfactants as a function of the size of the hydrophobic part, given as the number of carbons there, $n_c$. The properties are the critical micelle concentration (CMC, A), heat capacity change upon micelle formation ($\Delta C_p$, B), molar expansivity at 20°C (E, C), and the apparent molar volume change of micelle formation ($\Delta V$, D). The symbols denote alkyl maltosides (circles), alkyl glucosides (squares), and Cymal (hexagons); open symbols refer to micelles or micelle formation, solid ones to monomers in solution. The slope of the line in A corresponds to a standard Gibbs free energy increment of $dG^0/dn_c = -3.0$ kJ/mol. The dash-dot line in panel B represents the fit obtained for lysophosphatidylcholines in Ref. 41. Crosses in C denote values for bulk hydrocarbons.
Table (5.2): Apparent methylene group contributions to thermodynamic and volumetric parameters of micelle formation (or desolvation) at 20 °C as obtained from their slope as a function of the carbon-number, nC, in the alkyl chain of different homologous series of detergents. References 231 for amino carboxylic acids, 237 for sodium alkyl sulfates, 188 for lyso-PC at 25 °C, 240,241 for hydrocarbons, 239 for CnTAB, 242 for alcohols.

<table>
<thead>
<tr>
<th></th>
<th>$\Delta G^0$</th>
<th>$\Delta C_p$</th>
<th>$E_{\text{mon}}$</th>
<th>$E_{\text{mic}}$</th>
<th>$\Delta E$</th>
<th>$\Delta V$</th>
</tr>
</thead>
<tbody>
<tr>
<td>alkyl-maltosides</td>
<td>-2.9±0.1</td>
<td>-50±10</td>
<td>27±2</td>
<td>5±2</td>
<td>-22±4</td>
<td>2.7±1</td>
</tr>
<tr>
<td>alkyl-glucosides</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu-Val</td>
<td>-3.1±0.1</td>
<td>-57±341</td>
<td>20231</td>
<td>4240</td>
<td></td>
<td></td>
</tr>
<tr>
<td>literature</td>
<td>0.141</td>
<td>-66241</td>
<td>4231</td>
<td></td>
<td>0.6±0.3243</td>
<td></td>
</tr>
</tbody>
</table>

The expansivities for the surfactants in solution ($E_{\text{mon}}$, solid symbols) show a larger slope of ≈ 27μL mol⁻¹ K⁻¹, respectively. These molecules have no significant internal voids and their intrinsic expansivity in solution should not be substantially larger than that in micelles. We can therefore interpret the large positive value primarily in terms of hydration effects, e.g., changes in the volume of water molecules interacting with the chain compared to bulk water. That means that water exposed to the alkyl chain expands more strongly with temperature than bulk water. This is expected if water interacting strongly with the chain is condensed in its volume compared to bulk water. With increasing temperature, the water becomes more and more freed from this condensing effect, yielding a positive contribution to $E_{\text{mon}}$.

5.5.2 Temperature dependence of expansivities

Temperature-dependent expansivities typically reflect the thermotropic release of molecules from a more ordered, maybe “bound” state. If the bound state is more densely packed than the free state, its thermotropic “dissociation” causes a positive contribution to expansivity that decreases with increasing temperature. Particularly the relaxation of water from polar surface-induced, condensing effects to
become more similar to bulk water is strong at low temperature and vanishes gradually towards high
temperature (about 140 °C as described by ²¹⁸). This is illustrated by the curve for maltose in aqueous
solution (Fig. 5.2B). A similar phenomenon is seen for the thermotropic dissociation of condensed
cholesterol-lipid complexes. ¹⁵¹

Interestingly, the typical shape of $E$(maltose, $T$) is essentially conserved for the alkyl maltosides shown
here. This suggests that the release of water from the influence of the hydrophobic chain is essentially
independent of temperature. This decreasing or virtually temperature-independent expansivity of
hydrophobic groups is in line with the assumption that water interacting with the chain is slightly
condensed (as discussed above) and that the release of the water from this influence with increasing
temperature is more gradual than that of water molecules compacted by polar groups. As discussed
above, the rather constant $\Delta E(T)$ per CH₂ could also be a consequence of different trends compensating
each other. Further work will be needed to establish a better understanding of this phenomenon
presented here.

In this context, it is important to note that this observation is in accord with the data for amino acids
(see Fig. 5.3 and literature, e.g., ¹⁵³). One important point is that a group contribution is to be obtained
by subtracting the values for the same (mole) number of, say, Leu and Gly. That means, it is obtained
from the difference of E values (not $\alpha$) which is, as illustrated in Fig. 5.3, largely positive for Leu-Gly.
Furthermore, the choice of Gly as a reference for obtaining side chain contributions appears to be
misleading. Note that Gly does not simply lack the hydrophobic Val side chain but shows an additional
polar patch at this position instead. Hence, $E$(Val)-$E$(Gly) is still negative at low temperature. In
contrast, a more conservative determination of a hydrophobic group contribution by enlarging (not
adding) a hydrophobic group, $E$(Leu)-$E$(Val), does not show such negative or low values - it yields a
virtually temperature independent, positive difference. This is very well in line with our surfactant data,
the slightly smaller value of 0.19 μLmol⁻¹K⁻¹ may be explained by a partial screening of the methylene
group by the two terminal methyls in Val.

5.5.3 Correlation of $\Delta C_p$ and $\Delta E$

One important result of the PPC experiments presented here is that not only $\Delta C_p$ but also $\Delta E$ and $\Delta V_S$
show a linear variation with $n_C$ that extrapolates to 0 for $n_C \to 3$. This finding supports the hypothesis
that also $\Delta E$ and $\Delta V_S$ of micelle formation is governed by the hydrophobic chain.

Heimburg ²⁴⁴ has discussed a proportionality of $\Delta E$ and $\Delta C_p$:
\[ \Delta E = \gamma_{vol} \cdot \Delta C_P \quad (5.7) \]

and measured values of \( \gamma_{vol} \) for the chain melting transition of phospholipids. He obtained \( \gamma_{vol} = 0.77 \) mL/kJ for DMPC and 0.86 mL/kJ for DPPC. Our data imply a value of \( \gamma_{vol} = -0.0162 \) mL / -0.058 kJ = 0.28 mL/kJ for micelle formation of alkyl maltosides at 20 °C, independently of chain length (within \( n_c = 8 – 11 \)).

This finding raises the hypothesis that a ratio of \( \gamma_{vol} \approx 0.3 \) mL/kJ at 20 °C may be characteristic for transitions that are governed virtually exclusively by the hydrophobic effect. This would be important because the interpretation of negative \( \Delta C_P \), or even positive \( \Delta S \) alone as markers for hydrophobic interactions may be misleading as discussed above. Note that \( \gamma_{vol} \) increases somewhat with decreasing temperature when \( E_{mon} \) typically increases while \( E_{mic} \) and \( \Delta C_P \) remain largely constant. More data for \( \gamma_{vol} \) will be needed to derive a better understanding how it can be interpreted and utilized to classify the nature of thermotropic transitions.

**5.6 Conclusions**

1) We have addressed the controversy about structure-making versus water condensing effects of hydrophobic groups exposed to water. The expansivity change of exposing, for example, a methylene group to water is positive and very little dependent on temperature. It is crucial that such a group contribution is obtained by subtracting molar (or molecular) expansivities, not coefficients of expansion. There is no true indication for structure-making effects of hydrophobic groups from expansivity data.

2) \( \Delta E \) and \( \Delta V \) of micelle formation vary linearly with alkyl chain length and vanish, just as \( \Delta C_P \), for \( n_c \) extrapolated to 3. This suggests that these quantities are governed by the chain only and that 3 methylene groups, on average, remain exposed to water in a micelle.

3) The expansivity of chains buried in a micelle is close to that of pure hydrocarbons. The expansivity of water-exposed alkyl chains is considerably more positive and increases by 27 \( mLmol^{-1}K^{-1} \) per methylene (at 20 °C), suggesting adjacent water to be condensed compared to bulk water.
5.7 Acknowledgement

We thank Halina Szadkowska for preliminary PPC experiments and Andreas Beck, Lorenz Fuchs, and Gillian Higgins for contributions to the ITC experiments. We thank Tigran Chalikian and Yuen Shek for providing specific volume data of some surfactants. We are indebted to Tigran Chalikian and Sandro Keller for valuable comments on our data and manuscript. Financial support by NSERC is gratefully acknowledged.
CHAPTER (6) - Membrane binding and permeation of surfactants and lipopeptides characterized by zeta potential measurements

This study authored by:

Mozhgan Nazari, Gaurav Raval, Zubeir Khan, Hiren Patel, and Heiko Heerklotz, is being prepared for submission to a journal.

I wish to acknowledge: Gaurav Raval for providing experimental advice and running part of the experiments, Zubeir Khan for contributing to the work in the frame of an undergraduate research project supervised by H. Heerklotz and myself, Hiren Patel for establishing the assay and providing valuable advice, and H. Heerklotz for contributions to the design of the study and the final write-up.

6.1 Abstract

We present approaches of zeta potential measurements of liposomes to address membrane binding, membrane-induced protonation and counterion binding effects, membrane asymmetry and permeation, and membrane domain formation. This way, we studied the interactions of lipopeptides of the surfactin (SF) and fengycin families produced by Bacillus subtilis QST713 and of sodium dodecyl sulfate (SDS) with lipid membranes. Utilizing what we refer to as an equi-activity evaluation of potentials obtained at an array of different additive and lipid concentrations, the results were corrected for membrane partitioning effects. SDS showed an effective charge number of -1 in CsCl but substantial neutralization binding by Na⁺. Translocation of externally added SDS to the inner leaflet occurs above a threshold asymmetry of ≈20-30 mol-%. While fengycins retain their nominal charge of -1 at pH 8.5 and 7.4, SF becomes partially neutralized at pH 7.4 and, in turn, probably membrane permeant. Increasing neutralization of SF due to SF-SF interactions at as little as 3 mol-% is in line with a segregation of SF in the membrane as proposed. The collapse of membrane asymmetry at ≈3% SF is in line with its known pore forming activity. Summarizing, the charge state of membrane-bound peptides and surfactants is hard to guess but crucial for its membrane perturbation, permeation, and permeabilization. Zeta potential measurements as presented here are uniquely suited to address this behaviour.
6.2 Introduction

We are interested in the mechanisms that ensure high fungicidal activity, sensitivity, and synergistic action of lipopeptides of the surfactin, fengycin, and iturin families produced by *Bacillus subtilis* QST713. This strain is successfully used for crop protection against a variety of fungal pathogens and there is strong evidence that the mode of action involves the permeabilization of the target cell membrane. A key property governing the behaviour of these lipopeptides is their charge. Charged molecules are much more soluble and usually not spontaneously membrane-permeant. On the one hand, asymmetric insertion of an impermeant additive into the membrane causes “bilayer couple” effects that give rise to bending stress and, possibly, transient mechanical failure of the membrane. On the other hand, the initiation of membrane pores may require a lipopeptide to reside in both membrane leaflets and may thus be kinetically hindered for impermeant peptides.

We describe a new strategy to conduct and evaluate zeta potential measurements that eliminates partitioning effects and yields the effective charge and, to some extent, membrane permeability of lipopeptides and ionic surfactants. It turns out that subtle changes in pH or the nature of available counterions can have strong effects on the effective charge and consequent behaviour of ionizable surfactants, including surfactin.

Measurements of electrophoretic mobility and electrostatic models have provided a wealth of insight into the binding of proteins, peptides, and small molecules to lipid membranes. McLaughlin and coworkers showed early on that zeta potentials of vesicles containing anionic lipids can be described by the Stern equation if one allows for a specific adsorption of certain ions to the charged surface. Note that classical double-layer theory implies all monovalent cations to share the same shielding effect of a surface potential. Hence, the finding that the surface potential of liposomes containing anionic lipids is more strongly neutralized by sodium then by cesium ions can be interpreted as a specific adsorption of Na\(^+\) in the most general sense and semi-empirically be quantified by a binding constant. The detailed nature of this ion-specific effect may be direct binding (for example as a locally dehydrated, “inner-core complex”), size-dependent differences in ion accessibility of the charged groups, or size effects on double-layer properties that are not considered by classical Gouy-Chapman theory. In the same study, they established that zeta potentials of vesicles can be used to derive surface potentials if one assumes the plane of shear to be 2Å away from the surface. They obtained this crucial information by comparing measurements of electrophoretic mobility (yielding $\zeta$) with the binding of an anionic dye to the vesicle surface.
These fundamental insights have permitted studying the electrostatic binding of peptides to membranes.\textsuperscript{254} Recently, they have used zeta potential measurements for example to show that calmodulin inhibitors bind to the negatively charged, cytoplasmic side of the membrane, render its potential more positive, and hence cause the release into the cytoplasm of membrane-associated proteins that are anchored by cationic patches (often in addition to hydrophobic anchors).\textsuperscript{255} Since the surface potential affects the local concentration of an ionic surfactant or peptide at the membrane surface and, hence, the apparent partition coefficient from bulk, membrane binding data can provide information about effective charges of membrane additives as well. A good example is the fit of isothermal titration calorimetry data on the basis of the Gouy-Chapman model.\textsuperscript{120,256,257} Although this model makes a number of non-trivial assumptions, it was found to represent the binding isotherms well. However, the effective charge corresponding to the best fit may deviate from the nominal charge. Other reasons for such deviations are, of course, counterion binding and (de)protonation events due to local pK\textsubscript{A} shifts. Insights into the charge state of membrane additives have also been obtained from NMR measurements detecting their effects on the orientation of neighboring lipid head groups (molecular voltmeter concept).\textsuperscript{258}

Schaffran et al.,\textsuperscript{259} quantified the membrane binding of surfactant-like molecules from the slope of the zeta potential as a function of the square root of the concentration. This relation is linear at low concentration, where the effective charge remains largely constant and the zeta potential is proportional to the surface charge density to a good approximation. They observed this to be the case up to additive-induced zeta potentials of about -30 mV. It should be noted that this approach requires the knowledge of the effective charge number of the additive and its ability to translocate to the inner leaflet of the liposomal membrane.

Here we will show that these parameters may often be hard to estimate since they depend on local pK\textsubscript{A} shifts due to intra- and intermolecular interactions, ion-specific counterion binding, and concentration dependent changes in membrane permeability. We demonstrate that one can avoid any assumptions regarding the effective charge or partitioning model by what we refer to as an equi-activity analysis. To our knowledge, this robust and largely model-independent approach has not been used so far to interpret zeta potential data. It is, however, very well established for fluorescence data from different dyes,\textsuperscript{260,261} leakage data,\textsuperscript{262,263} and phase transitions;\textsuperscript{264} for a review see \textsuperscript{44}. The only assumption it requires is that the observable is unequivocally related to the membrane composition represented, for example, by the additive-to-lipid mole ratio in the membrane, R\textsubscript{bp}. 
As a result of this procedure, one obtains the zeta potential as a function of $R_b$ and, in turn, the effective charge numbers of additive and lipid. For additives with moderate apparent partition coefficients, the method provides also a model-free partitioning isotherm and, hence, composition-dependent apparent partition coefficient. Another very interesting feature of zeta potential measurements is that they report, virtually exclusively, the concentration of peptide or surfactant in the outer leaflet of the liposomes. Comparing the local peptide or surfactant concentration in the outer leaflet after an addition with that after artificial transmembrane homogenization provides valuable insight into its membrane asymmetry and concentration-dependent membrane permeability.

6.3 Materials and methods

6.3.1 Materials

Large unilamellar vesicles were made from 1-palmitoyl-2-oleoyl-3-sn-glycero-phosphatidylcholine (POPC) purchased from Avanti Polar Lipids, Alabaster AB. The surfactin and fengycin fractions of the lipopeptides produced by Bacillus subtilis QST713 were kindly provided by AgraQuest, Inc., Davis CA.

All samples were made using Millipore water for preparing Tris buffer, 10 mM, including 100 mM salt (NaCl or CsCl as described), and adjusted to pH 7.4 or 8.5, respectively. These materials were purchased from Sigma (highest purity available).

Large unilamellar liposomes of approximately 100 nm diameter were produced by extrusion as described. Briefly, an appropriate amount of lipid dissolved in chloroform was dried to a thin film by a gentle stream of nitrogen, followed by exposure to vacuum overnight. Then, appropriate amount of buffer was added, the lipid dispersed by vortexing, and the sample homogenized by 6 freeze-thaw cycles. Extrusion was done 15 times through Nuclepore polycarbonate filters of 100 nm pore size in a Lipex extruder (Northern Lipids, Burnaby BC) at room temperature.

The liposome size was monitored by dynamic light scattering using a Malvern Nano ZS. Previous phosphorus assays indicated that a gravimetric determination of the lipid has to take into account one water to remain bound per lipid (e.g., an effective molar weight of 778 g/mol POPC) and that the lipid concentration is essentially unchanged upon extrusion.
6.3.2 Zeta potential measurements

Measurements were made in a Malvern Nano ZS zeta sizer based on dynamic light scattering. The system works according to the PALS principle, and the data are automatically evaluated on the basis of the Smoluchowski equation (the particle size of ≈100 nm is larger than the Debye length, ≈1 nm). The sample was thermostated to 20 °C by a built-in Peltier device.

Typically, a series of samples was prepared showing a constant lipid concentration and increasing concentrations of the surfactant or lipopeptide. After adding appropriate amounts of a stock solution of surfactant or lipopeptide in buffer to the pre-established liposomes, a sample was incubated for 10 minutes at 20 °C. Then the zeta potential was measured, at least in some cases, to assess the state after addition or without additional equilibration procedures. Subsequently, transbilayer equilibration of the surfactant was realized by heat treatment, e.g., heating the sample to 65 °C for 1 hour, followed by cooling back to the experimental temperature, 20 °C. There is good evidence that the enhanced temperature stimulates the flip-flop of membrane-bound surfactants across POPC bilayers, including ionic ones. After the heat treatment, the zeta potential was measured again.

6.4 Theory

6.4.1 Relationship between zeta potential and surfactant content on the liposome surface

Charged head groups of surfactant and, possibly, lipid on the surface of a liposome give rise to a surface charge density, $\sigma$: \(^{259}\)

$$
\sigma(X_S^b) = \frac{e_0 [X_S^b z_S + (1-X_S^b)z_L]}{X_S^b A_S + (1-X_S^b) A_L} = e_0 \frac{z_L + R_b z_S}{A_L + R_b A_S} 
$$  (6.1)

Here, $z_S$ and $z_L$ represent the signed charge numbers of surfactant and lipid and $R_b$ and $X_S^b$ denote the mole ratio and mole fraction of surfactant within the membrane, respectively. Then, $1-X_S^b$ gives the mole fraction of lipid (the mole fractions of lipid and surfactant in a binary system add up to 1). Hence, the numerator of Eq. (6.1) represents the weighted average of the electrostatic charge per molecule on the surface. The denominator gives the average area per molecule, considering the interfacial areas per surfactant and lipid, $A_S$ and $A_L$, respectively.
One of the experimental observables that shed light on $\sigma$ is the zeta potential, $\zeta$. It depends on $\sigma$ as well as on the partial screening of the related potential by the ions in the layer of aqueous solution that travels with the liposome. The screening depends on the reciprocal Debye length of the solution, $\kappa$, the effective thickness of the surface-immobilized layer, $x$, and the permittivity of the medium, $\varepsilon_r \varepsilon_0$. For low absolute values of the zeta potential up to about ±25 mV, Gouy-Chapman theory implies the relationship:

$$\zeta = \sigma \frac{\exp(\kappa \cdot x)}{\kappa \varepsilon_0 \varepsilon_r} \quad (6.2)$$

The reciprocal Debye length, in turn, depends on the ionic strength, $I$, of the solution as:

$$\kappa^{-1} = \sqrt{\frac{\varepsilon_0 \varepsilon_r kT}{2N_A e^2 I}} \quad (6.3)$$

With:

$$I = \frac{1}{2} \sum_i c_i z_i^2 \quad (6.4)$$

yielding a value of $1/\kappa = 9 \text{ Å}$ for an ionic strength of 110 mM and $\varepsilon_r = 80$. The index $i$ sums over all charged components in solution, with the respective concentrations $c_i$ and charge numbers $z_i$. The effective distance of the plane of shear of the surface-immobilized layer from the surface has been reported as $x = 2 \text{ Å}$.\textsuperscript{251,259}

### 6.5 Results

#### 6.5.1 Equi-activity analysis of SDS data

Fig. 6.1A shows the zeta potential of liposomes as a function of SDS concentration for several series differing in lipid concentration. As expected, the partitioning of SDS into the membrane renders $\zeta$ increasingly negative. Since $\zeta$ is related to the surface charge density, a larger SDS concentration, $c_s$, is needed to induce a given $\zeta$ at higher lipid concentration, $c_l$. 

---

Footnotes:

\textsuperscript{251,259}
The grid line in Fig. 6.1A intersects with all curves at $\zeta = -25$ mV. The abscissa values of the interpolated intersection points of the four curves with the grid line are plotted as green pentagons in Fig. 6.1B, as a function of the lipid concentration referring to each zeta curve. The linear relationship $c_S (\zeta=25\text{mV})$ versus $c_L$ is a consequence of the mass balance for the surfactant (total concentration $c_S$), which is either bound (concentration $c_S^b$) or free in aqueous solution (concentration $c_S^{aq}$):

$$c_S = c_S^b + c_S^{aq} = R_b c_L + c_S^{aq} \quad (6.5)$$

Fig. 6.1: Equi-activity analysis of zeta potential measurements of POPC/SDS vesicles. Panel A shows the measured $\zeta$ as a function of SDS concentration, $c_S$, for 4 series of different lipid concentration (see plot). Panel B shows the SDS versus lipid concentration of hypothetical samples sharing the same $\zeta$, respectively, as indicated. The window is zoomed to low concentrations for better visibility, not all points are shown. According to Eq. (6.6), the slope of these lines yields $R_b$ and, after conversion, the corresponding membrane-bound mole fraction of SDS, $X^b_S (\zeta)$ as plotted in Fig. 6.1 C. The Y-intercepts in 1B yield estimates of the free SDS, $c_S^{aq}$, as used in the binding isotherm (panel D) and for estimating apparent partition coefficients (E). Note rather large errors of $c_S^{aq}$ yet small errors of $R_b$ (panel D).
The second equality includes the mole ratio of bound surfactant to lipid in the membrane, \( R_b = c_{Sb} / c_L \). Eq. (6.5) represents, indeed, a straight line of \( c_{S}(c_{L}) \) if \( R_b \) and \( c_{S}^{aq} \) are constant. We have fulfilled this condition by choosing conditions \( c_{S}(c_{L}) \) that all share the same \( \zeta \), because \( \zeta \) is unequivocally related to the surface charge density and, hence, to \( R_b \). If \( R_b \) is constant, the equilibrium bulk aqueous concentration is given by the apparent partition coefficient, \( K_{app} \):

\[
K_{app} \equiv \frac{R_b}{c_{S}^{aq}} \quad (6.6)
\]

and, hence, constant as well. For the green points in Fig. 6.1B derived from the -25mV-"equi-activity line" a linear fit yields a slope of \( R_b = 0.113 \pm 0.003 \) and \( c_{S}^{aq} = (10 \pm 8) \mu \text{M} \). The errors given here are standard errors of the fit, additional systematic errors (e.g., of concentrations) apply.

This result provides one point in the partitioning isotherm, Fig.6.1D, and corresponds to an apparent partition coefficient of \( K_{app}(R_b=0.11) \approx 11/\text{mM} \) as shown in Fig. 6.1E. Inspection of the error bars in Figs. 6.1D,E reveals that the data set obtained here gives only an estimate of \( c_{S}^{aq} \) and, hence, of \( K_{app} \). Naturally, the slope of the binding isotherm and \( K_{app} \) decrease with increasing \( R_b \) because electrostatic repulsion decreases the local free SDS concentration in the aqueous phase adjacent to the membrane. More zeta curves would be desirable for a more reliable partitioning study, but the result that \( K_{app} \) decreases with increasing SDS content in the membrane from \( =10/\text{mM} \) at \( R_b = 0.1 \) to \( =1/\text{mM} \) at \( R_b = 0.5 \) (Fig. 6.1E) is in good agreement with comparable, published values of \( (6 - 2)/\text{mM}^{136} \) and, modelled for \( R_b = 0 - 0.6 \), \( K_{app} \approx (50 - 0.5)/\text{mM} \). Experiments at 65°C had yielded virtually identical values, e.g., \( K_{app} \approx 1/\text{M} \) at \( R_b \approx 0.6 \).^{257}

What is crucial for our current study is that in contrast to substantial possible errors of \( c_{S}^{aq} \) and \( K_{app} \), the precision of the \( R_b \) values is generally excellent (see \( R_b \)-errors shown in Fig. 6.1D) and the data are therefore very suitable for deriving a partitioning-corrected zeta curve, \( \zeta(R_b) \). We have, in fact, not plotted \( \zeta(R_b) \) directly but converted it to a mole fraction scale, using \( X_{S}^{b} = R_b/(1+R_b) \), as shown in Fig. 6.1C. The points follow a linear behavior from \( \zeta(0) = 0 \) (the lipid is zwitterionic) up to \( \zeta = -30 \) mV reached at \( X_{S}^{b} = 0.13 \); then they level off to a lesser change in \( \zeta \) with increasing concentration. The fit of \( \zeta(X_b) \) will be discussed in the next section.
6.5.2 Effective charge and transmembrane equilibration of SDS

The previous section has derived the zeta potential as a function of the mole fraction of SDS in the membrane as shown in Fig. 6.1C. As long as the effective charge numbers of SDS and lipid are constant, this line can be fitted by Eq.

\[
\zeta(X^b_S) = b(\kappa)e_0 \frac{X^b_S z_S + (1-X^b_S)z_L}{X^b_S A_S + (1-X^b_S)A_L} \quad (6.7)
\]

derived from Eq. (6.1) with Eq. (6.2) and the constant factor of \(b(\kappa)e_0 = 1.69 \times 10^4 \text{ mVÅ}^2\) obtained for a monovalent 110 mM electrolyte (\(\kappa^{-1} = 9.3 \text{ Å}\)) using Eqs. (6.2) and (6.3). The distance between the plane of shear, where the electrostatic potential is \(\zeta\), and the surface was assumed to be \(x = 2\ Å\) as published.\(^{251}\)

The solid red line illustrates this eq. for \(A_L = 65 \text{ Å}^2\) (from \(^{224,259}\)), \(z_S = -1\) (nominal), \(z_L = -0.005\) (fitted), and \(A_S = 80 \text{ Å}^2\) (chosen for demonstration). The dotted line refers to a smaller interfacial area per SDS of 40 \(\text{Å}^2\), illustrating that this parameter has little effect; it matters somewhat only at high \(X^b_S\). In this context it should be noted that areas per surfactant obtained on Langmuir monolayers differ from partial areas in a membrane.\(^{266}\) The latter include area increases of the lipids (yet given per surfactant) due to surfactant-induced curvature strain.\(^{192}\)

Moderate deviations of the effective distance from the surface to the plane of shear, \(x\), from the value of 2 \(\text{Å}^2\) derived in the literature\(^{251}\) have rather little effect on the effective charge of the surfactant derived by the fit. The starting slopes in Fig. 6.1C (\(A_S=80 \text{ Å}^2\)) imply effective charge values of \(z_S = 1.10 - 0.94\) for \(x\) ranging from 1.5 – 3 \(\text{Å}\) (0.99 for \(x=2\ \text{Å}\)).

The agreement with the data indicates that the effective charges, \(z_L\) and \(z_S\), are in good agreement with the nominal charge numbers of lipid and SDS, respectively. At a zeta potential of \(\approx -30\text{mV}\), the slope of \(\zeta(X^b_S)\) becomes less steep. This is to be expected because the relationship between surface charge density and zeta potential used here, Eq. (6.2), applies only at low \(\zeta\) up to \(\approx \pm 25 \text{ mV}\).\(^{251}\) Additionally, there may be neutralization effects or curvature strain-induced increases in membrane area contributing to this non-linearity between \(\zeta\) and \(\sigma\).

Having obtained the effective charge numbers to agree with nominal values may lend support to the value of \(x\) and other parameters used here, but it is by no means trivial. Fig. 6.2A shows the analogous data for SDS in 100 mM NaCl, not CsCl as discussed so far. While the effects of sodium and cesium ions in the electrical double layer are the same, the smaller sodium ions bind directly to the sulfate group of
SDS. This is suggested by the fact that in NaCl, the effective charge of SDS is only $z_S = -0.4$. This finding underlines one of the advantages of the protocol proposed here: an interpretation of binding data with a guessed charge number of -1 would be false.

Fig 6.2: The effective charge of SDS is reduced in sodium chloride solution. Zeta potential of POPC vesicles as a function of the mole fraction of SDS in the membrane, in contrast to Fig. 6.1C, the data were recorded in 100 mM NaCl, 10 mM Tris, pH 7.4. Measurements were done after transmembrane equilibration by heat treatment (solid symbols) and without equilibration (impermeant surfactant limited to outer leaflet: open symbols). Lines illustrate potentials calculated using Eq. (6.7) for $z_S$ given in the plot and $A_L = 65 \text{Å}^2$, $A_S = 60 \text{Å}^2$.

Fig. 6.2 contains a second data set (open symbols), which has been measured after a ten minute incubation after adding SDS stock solution to POPC vesicles yet without a homogenization of the two membrane leaflets by a heat treatment. The initial slope is considerably larger because the outer leaflet contains virtually all bound SDS, about twice the total bound amount. A formal fit yields $z_S \approx -0.65$ but this is not quantitatively meaningful because the model is not set up to treat asymmetric membranes. For example, the effective $A_S$ is altered by asymmetric insertion, too.

At an average SDS content of $X_S^b = 0.15$, that means a local SDS content of $\approx 30$ mol% in the outer leaflet, the slope of this $\zeta(X_S^b)$ curve starts to match that of the heat treated sample. One may speculate that this may reflect an asymmetry threshold that stimulates the translocation of SDS to the inner leaflet.

### 6.5.3 Lipopeptides in membranes

Fig. 6.3 displays the $\zeta$-versus-$X_S^b$ plots for surfactin with and without heat-equilibration at pH 7.4 and 8.5, respectively. The equilibrated samples show starting slopes implying charge numbers of -2 (pH 8.5)
and -1.8 (pH 7.4). Deviations from the fit that suggest further protonation or counterion binding events due to SF-SF interactions in the membrane start at about -15 mV already.

Non-equilibrated probes show twice the apparent charge for pH 8.5 (SF accumulates in outer leaflet) and a spontaneous equilibration at about 5 mol% SF total. At pH 7.4, there are no substantial differences between heat treated and not heat treated samples.

Fig. 6.3: Zeta potential of POPC vesicles as a function of the added, average mole fraction of membrane-bound surfactin, $X_S^b$, at pH 8.5 (top) and 7.4 (bottom), respectively. As before, solid symbols represent equilibrated samples and open symbols possibly asymmetric additive insertion from outside. Lines are simulated according to Eq. 6.7 for selected effective charge numbers, $z_S$ (using $z_L = -0.006, A_L = A_S = 65 \text{ Å}^2$, the latter value is virtually meaningless at $X_S^b < 0.06$ shown here).
Fengycin contains three ionizable residues, one Orn and two Glu. We have assessed its charge state in membranes at pH 7.4 and pH 8.5 as illustrated by Fig. 6.4. In both cases, the effective charge agrees with the nominal charge, $z_S = -1.0 \pm 0.1$ (standard error of the fit). Note that the phase range showing exclusively mixed membranes and fengycin monomers is restricted to $X_S^b < 5\%$. For reasons that are not clear at this point, the Y-intercepts show a slightly larger value of the apparent charge of the lipid of $\approx -0.01$. Since this is still small compared to the potential arising from the lipopeptide, we assume that it does not interfere with our experiment to determine the effective charge of the lipopeptide.

Fig. 6.4: Zeta potentials of POPC vesicles as a function of the mole fraction of membrane-bound fengycin at pH 7.4 (circles) and 8.5 (squares). Samples were equilibrated by heat treatment.

### 6.6 Discussion

#### 6.6.1 Effective charge

Our data illustrate the fact that it is often not trivial to guess the effective charge number of an additive in a membrane. For example, SDS shows its nominal charge of -1 in CsCl solution but some factor possibly a counterion condensation of Na$^+$ reduces its effective charge number to -0.4 in 100 mM NaCl. Without specifying the detailed nature of the specific neutralization effect, it can empirically be described by a binding constant of Na$^+$ to the sulfate of membrane-bound SDS of the order of $10^2$ M$^{-1}$. This effect is somewhat stronger than described for binding to the phosphate of membrane lipids, about 1 M$^{-1}$ as discussed by McLaughlin and coworkers. Finally, it is much stronger than the affinity to SDS at 65 °C as deduced from modelling binding isotherms and ITC curves using the Gouy-Chapman
Intramolecular and membrane-induced pK$_A$ shifts reduce the absolute charge number of surfactin to $\approx -1.8$ at pH 7.4. One might expect the pK$_A$ of SF dispersed in a non-charged membrane to be closer to the value of $3.5 - 4$ for isolated monomers than to $5.5 - 6$ measured for SF monolayers$^{267}$ with their strong, electrostatic SF-SF interactions. Our result implies that this is not the case, which might be due to a lower dielectric environment in the membrane or a partial segregation of SF that renders the local concentration higher than the average value.$^{127,247}$

As shown for SDS/C$_s$ in Fig. 6.1C and in line with findings for other systems$^{251,259}$, $\zeta(XSb)$ is described by the model used here up to zeta potentials of $\approx -30$ mV, where Eq. 6.7 becomes no longer valid. It is virtually linear for low $X_s$ where, additionally, $X_{SAS}$ is negligible. These limiting effects do, however, not explain the deviations from the model seen for SF already at $\approx -15$ mV reached at $\approx 3$ mol-% SF in the membrane (Fig. 6.2). One plausible explanation for this finding is that SF has been discussed to segregate in a membrane, forming domains of enhanced local concentration and, consequently, membrane perturbation.$^{127,247}$ These SF-rich areas may very well show local zeta potentials of more than $-30$ mV or SF-SF interactions that induce pK$_A$ shifts and, hence, a change in effective charge. A dramatic increase in $A_S$ due to lipopeptide-induced membrane disordering, which could at least contribute to the deviation, is not expected given recent anisotropy data.$^{127}$

Summarizing this section, we stress that the effective charge number of peptides and surfactants depends sensitively on a number of environmental conditions. On the one hand, that means that it might not be justified to use the nominal charge of an additive to derive its membrane concentration and, in turn, partition coefficient from zeta potential measurements at a single lipid concentration. The equi-activity assay proposed here solves this problem by yielding, instead of assuming, an effective charge. On the other hand, our data illustrate that the effective charge number of a bound surfactant or peptide may actually provide interesting, additional information about, for example, membrane
segregation phenomena. Further studies will be required to validate the relationship between the apparent zeta potential, membrane segregation and domain formation.

### 6.6.2 Membrane permeability and asymmetry

So far, we have discussed the results of heat-treated samples that are assumed to represent liposomes with virtually equal concentrations of the additives in the outer and inner lipid leaflets. However, zeta potential measurements provide also an interesting opportunity to study membrane asymmetry because they detect the charged compounds in the outer leaflet selectively. The open symbols in Fig. 6.2 A illustrate the zeta potential obtained when SDS is titrated into the liposome dispersion and then $\zeta$ is measured without prior heat treatment. The apparent charge number per SDS is almost twice as much as for the equilibrated sample, which is to be expected if all SDS (e.g., twice the equilibrium concentration) resides in the outer leaflet of the liposomes. It has been described in detail that such an asymmetric insertion into a membrane causes a bilayer curvature stress due to the discrepancy between the optimum lateral areas of the outer (“over-populated”) and inner (relatively under-populated) leaflet. Studies with alkyl maltosides have indicated a stress-induced breakthrough of molecules from the outer to the inner leaflet to occur at an average mole fraction of 15% of titrated additive. Then, the mole-fraction asymmetry is 30 mol% outside to ≈ 0 inside. In good agreement with these data, we find the slope of $\zeta(X_b)$ for the titration (open symbols) in Fig. 6.2 to change at $X_b = 0.15$ to match that of the heat-treated samples. That means, SDS titrated to the vesicles beyond a threshold (a local content of ≈30% in the outer leaflet) translocates to the inner leaflet. It is interesting to note that a largely constant difference remains between the signals with and without heat treatment above $X_b = 0.15$, suggesting that the relaxation of bilayer stress might happen via an “overflow” mechanism, whereas an overall rupture of the membrane or pore formation would be expected to dissipate also all or part of the remaining asymmetry.

The latter seems to be the case for surfactin at pH 8.5 (Fig. 6.3) where open and closed symbols match above $X_b = 3\%$. This is in agreement with the previous finding that surfactin translocation as seen by ITC coincides with membrane leakage (calcein release) and proceeds at about 3 – 5 mol%. The area requirement of SF is likely larger than that of SDS or dodecyl maltoside but this can hardly compensate for the 3-5 fold lower, critical asymmetry for translocation. Instead, surfactin forms aqueous pores or defects in the membrane that allow for balancing the asymmetry before a critical asymmetry is reached.
Another interesting observation is that no detectable asymmetry effect builds up upon titration of surfactin at pH 7.4. It should be noted that the fit of the data for the equilibrated system implies a partial protonation of the peptide, and that all but the two acidic side chains of surfactin are hydrophobic. We may therefore speculate that a non-negligible, uncharged fraction of surfactin allows for membrane permeation at this pH.

Comparing “asymmetry-driven, transient rupture” (SDS) with “spontaneous, stable pore formation” (surfactin), one has to be aware of the fact that both are extreme, idealized scenarios that are connected in real systems. This has impressively been illustrated by MD simulations of Marrink and coworkers. Membranes with different numbers of DPPC molecules in both leaflets were unable to activate membrane rupture in the ns-to-1μs time range up to extreme asymmetries. In contrast, asymmetric insertion of 30% of a short-chain lipid, dioctanoyl phosphatidylcholine, caused spontaneous transient rupture within 70 ns; no rupture was observed within 1 μs after adding 20%. Hence, the pore-inducing properties of the short-chain lipid reduced the activation energy for transient membrane rupture, even at a concentration where it does not form pores in a symmetric membrane. The somewhat larger threshold (20-30%) in the simulations for the short-chain lipid compared to experimental values for alkyl maltosides and SDS (=15%) can be explained by the different time scales and, possibly, a stronger pore-forming activity of the detergents.

### 6.7 Conclusions

1. The effective charge of a molecule in a membrane controls crucial properties such as membrane partitioning and permeability and its interaction with other membrane components. It depends sensitively on membrane-induced protonation and counterion effects, which cannot simply be predicted on the basis of $pK_a$ values and ion binding constants in solution.

2. Zeta potential measurements of peptides and other additives binding to liposomes can be corrected for partitioning effects using an equi-activity analysis as used for other observables before. As a result, one obtains an estimate of the partition coefficient and the apparent charge number per additive.

3. Since the zeta potential represents the additive in the outer membrane leaflet, it indicates non-permeation of SDS at room temperature starts at about an average 10 - 15 mol%, e.g., $\approx$
20 - 30 mol-% SDS in the outer leaflet. For surfactin, this threshold is much lower, 5 mol% average, and causes a total match as expected for the existence of relatively stable pores.

4. Surfactin is fully charged (\(z_S = -2\)) at pH 8.5 and low membrane content but becomes partially neutralized at a membrane content above \(\approx 3\) mol% (\(\zeta \approx -15\) mV) and generally at pH 7.4.

6.8 Acknowledgement

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CHAPTER (7) - Discussion and outlook

7.1 Rationale and overview

In many respects, micelle-forming surfactants are sharing a principal behaviour of self-association and solubilization. The hydrophobic core of their micelles can solubilize small molecules or cover transmembrane segments of membrane proteins. Virtually all these surfactants partition into membranes and cause a stress there due to their positive spontaneous curvature. From this perspective it is surprising how very specific and selective many surfactants act, and how much they differ in their suitability for a certain application or function. Only a very specific surfactant or mixture of surfactants may serve to solubilize a specific membrane protein. Pore-forming and lytic activity against membranes differs dramatically between different surfactants and between different target membranes. At this point, most of these specific properties are tested on a largely empirical level and cannot be predicted on the basis of the structural properties of the surfactants or their commonly listed physico-chemical parameters. This raises the question which other key numbers and parameters govern their different behaviour. The general theme and goal of this work has been to identify more physical quantities or properties of surfactants that may allow for a better understanding and prediction of their behaviour. This involves and requires establishing new protocols and models to measure these parameters. The following sections collect the contributions of this work to parameters describing surfactants, their relevance for functions and applications, and their measurement. Innovations will be put into the context of established knowledge and procedures.

7.2 Molecular properties of surfactants that may predict their suitability for a given application molecular shape and curvature stress

Key properties of a surfactant are the dimensions of the polar “head” group and the apolar part, defining the “effective shape” (quantified by the packing parameter) and, together with the properties of the lipid, the spontaneous curvature of a surfactant in a membrane. This structural property predicts, to some extent, the size and shape of the micelles, the curvature stress in membranes, the relationship between CMC and partition coefficient, and for some “traditional” detergents the onset of membrane solubilization. It should however be emphasized that the “size”, e.g., the interfacial area requirement, of a head group is not determined by the van der Waals radii of its atoms alone but
depends also on hydration, electrostatic, dipolar, H-bond, or other forces between the molecule and its neighbors.\textsuperscript{44}

Chapter 3 has systematically and quantitatively described the relationship between curvature strain and the onset of solubilization. For the first time it could be shown that many typical detergents have to induce a common, critical strain in a given membrane in order to initiate membrane solubilization.

7.2.1 The critical micelle concentration

The CMC describes for a given concentration the amount of micelles (responsible for all solubilization phenomena) and monomers (required for surfactant exchange between aggregates and its removal). This has not been a main objective of this work but a series of micellization experiments involved in Chapter 5 has provided new, high-quality data about the CMC of alkyl maltosides and glucosides and their enthalpies and heat capacities of micellization. The results have confirmed and refined previous knowledge about, for example, the relationship between heat capacity and accessible apolar surface area of the surfactants.

7.2.2 Micelle structure

As mentioned above, the size and topology of a micelle can be predicted on the basis of the packing parameter and intermolecular interactions between the surfactants. The question has been raised whether micelles may differ in the packing of the hydrophobic chains which may, in turn, affect water accessibility, the capacity to solubilize other molecules, and the flexibility of solubilizates (including transmembrane segments of proteins).

Here, this hypothesis has been tested by developing and using a new approach, pressure perturbation calorimetry (Chapter 4). This method reveals effects of packing and hydration of molecules and particles in aqueous systems in terms of volumetric parameters. The two contributions have, to some extent, been separated by studying two homologous series of surfactants (Chapter 5). The results show very pronounced effects of hydration, but seem to rule out the packing hypothesis at least for alkyl-chain surfactants. The group contributions to expansivity of the hydrophobic tails in a micelle agree within error with those of bulk hydrocarbon, implying that there is very little specificity of the micellar packing. Further work is warranted to clarify whether a surfactant with a cyclohexyl group in its hydrophobic part might show a significantly different packing behaviour.
Interesting information about micellar packing has been obtained in Chapter 3: The order parameter of DPH is larger in cylindrical micelles than in membranes. This finding warrants further investigation.

### 7.2.3 Membrane partitioning and permeability of a surfactant

The ability of a surfactant to spontaneously flip-flop across a membrane determines whether it can induce an asymmetry stress between the two lipid leaflets. On one hand, bilayer couple effects of asymmetric insertion can cause membrane bending and transient failure. On the other hand, the formation of more stable, surfactant-induced toroidal pores in membranes may require the surfactant to reside in both layers. These effects are related to the concept that impermeable surfactants are more prone to the micellar mechanism of solubilization, as compared to the transmembrane mechanism typical for permeant ones.

The work presented here has contributed to the better understanding of these phenomena particularly with respect to charge effects. A rule of thumb is that charged surfactants are membrane impermeant whereas non-ionics may or may not flip-flop quickly depending on the size and hydration of their head group. Chapter 6 presents a new approach to determine the effective charge of membrane-bound surfactants. It reveals that local pH and pKₐ-shifts as well as specific counterion binding render the effective charge hard to predict. For example, subtle changes in pH may change the effective charge and, in turn, permeability and possibly leakage and solubilization modes of a surfactant.

### 7.2.4 Surfactant miscibility with a given lipid

It is one of the key results of the thesis (Chapter 3) that the miscibility of a surfactant with the membrane lipid or, more generally, the homogeneity of the mixed membrane is a very important factor determining the behaviour of the system. Some surfactants, primarily lipopeptides and glycolipids of biological origin, have been identified as exceptions to the rule of critical, homogeneous curvature strain described above. A heterogeneous perturbation of the membrane caused, for example, by a lateral segregation of surfactant-rich and –depleted domains in the membrane gives rise to a focused action of the perturbant that may greatly enhance pore formation and the onset of solubilization. At the same time, the completion of solubilization may be opposed by a poor ability of the micelles to incorporate lipid. The demixing effects may explain in particular enhanced selectivity of antibiotic biosurfactants.

Whereas the curvature strain in a membrane is a rather generic effect as long as the thickness and intrinsic curvature of the lipid membrane is within the typical range, demixing depends on a
considerable number of intermolecular interactions. An impressive example for such a selectivity effect that might have biological relevance is the effect of cholesterol. In most cases, cholesterol stabilizes the membrane against permeabilization and lysis by surfactants and antibiotic peptides due to its membrane-ordering effect. However, some surfactants such as Triton X-100 seem to be poorly miscible with cholesterol so that their encounter in a membrane induces segregation, high local Triton concentrations in cholesterol-depleted domains, and thus enhanced lytic activity. Hence, immiscibility with cholesterol could provide a targeting mechanism against mammalian cells.

7.3 The relevance of these parameters for surfactant functions and applications

7.3.1 Solubilization of small molecules

Synthetic detergents have typically been developed and optimized for cleaning purposes of all kinds, that is, for the solubilization of molecules. Considering the parameters in the previous section, one should favor a detergent with a rather strongly conical shape (low packing parameter) because the addition of solubilizate adds to the hydrophobic volume and increases the effective average packing parameter. The CMC should be in the high micromolar range, because monomers are inactive for solubilization but too low a CMC would require stronger rinsing for removal. Nonionic surfactants can be expected to show a better penetration into hydrophobic particles. A good miscibility with solubilizates is desirable for affecting all target solubilizate and maximizing its payload within a micelle.

Of course, there are many other parameters to be controlled such as foaming etc. as well as cheap production, safety, and environmental aspects. This section simply illustrates the approach taken with this work.

7.3.2 Antibiotic and sterilizing activity

Biosurfactants such as Bacillus lipopeptidases or saponins (e.g., digitonin) appear to be optimized, maybe among other functions, for host defense functions by perforating the membranes of attacking or competing organisms. That means, selective action against membranes of a specific lipid composition is mandatory. Cationic surfactants such as quaternary ammonium surfactants are used for sterilizing purposes in products such as toothpaste and have been proposed for spermicidal ointments. The common theme of these functions is a local membrane disruption, not a general solubilization of the target organism. Particularly biosurfactants that have to act at very low concentration need to have a
low CMC and high partition coefficient, because molecules in solution are not active. Removal is not an issue here but a precipitation of very hydrophobic molecules has to be prevented, typically by one or more charges. As described in Chapter (6), a predominantly charged molecule may still be membrane permeant as long as partial protonation on the membrane surface or specific counterion binding allow for a small neutral fraction. Micelle structure is little relevant for antibiotic activity but some biosurfactants may also be involved in solubilizing nutrients etc.

Above all, selectively segregating, heterogeneously membrane-perturbing surfactants, described in Chapter (3), seem clearly superior for these functions. Optimally, poor miscibility should apply with a specific component of the target membrane such as, possibly, ergosterol for a fungal target. This may explain why the mechanism of “heterogeneous perturbation” was first explicitly described and discussed in our study paying special attention to biosurfactants.

7.3.3 Solubilization and reconstitution of membrane proteins

One may speculate that proteins are more stable in micelles that mimic best the conditions in a membrane, including a relatively ordered environment with the matching hydrophobic thickness. We have utilized PPC to quantify the packing density in the micellar core (Chapter 4) and found no significant deviation from bulk hydrocarbon (Chapter 5); it will however be interesting to study detergents with cyclohexyl groups, sterols, or highly-branched hydrophobic chains which have become available now and which are claimed to provide improved conditions for membrane proteins. For a fast and complete removal after reconstitution into proteoliposomes, surfactants with a rather high CMC are often preferred, and a fast membrane flip-flop is favored.

It is interesting to note that two major solubilization protocols being used for G protein-coupled receptors (GPCRs), e.g., particularly demanding systems are based on detergents identified in Chapter 3 as “heterogeneously membrane perturbing”. One is digitonin, which is being used in spite of the high costs and complicated behaviour.\(^{269}\) Another is a specific mixture of CHAPS (a heterogeneous perturbant), dodecyl maltoside, and cholesteryl hemisuccinate.\(^{196,270}\) We have speculated that a poor miscibility of detergent and lipid may account for a higher detergent content along the periphery of, for example, a somewhat oblate ellipsoidal micelle, whereas the core shows a somewhat higher lipid concentration surrounding the protein. This may cause a higher order and improved interactions of the protein with potentially essential lipids or maybe sterols. In the extreme case, poor lipid detergent miscibility leads to bicelles, bilayer fragments that show a number of peculiar properties and are being formed, for example, by mixtures of DMPC with diheptanoyl PC or CHAPSO.\(^{198}\)
7.4 Methods to assess surfactant properties

7.4.1 Micelle structure

A major methodical development achieved here is the application of pressure perturbation calorimetry to characterize the volumetric properties and, hence, hydration and packing effects of micelles. The first set of systematic experiments contributed to the general debate about the nature of the hydrophobic effect but did not reveal significant condensation of the micellar core of alkyl-chain detergents compared to bulk hydrocarbon. Further studies will be required to refine the interpretation of such PPC data and address other types of detergents that might show different packing phenomena.

Measurements of the size and shape of micelles are typically carried out by light or neutron scattering techniques; they have not been considered here. The determination of the CMC and thermodynamic parameters has routinely been realized by isothermal calorimetry, alternative techniques such as fluorescence studies with hydrophobic probes might be necessary for systems with sub-micromolar CMCs which have not been of interest here.

7.4.2 Partitioning and membrane permeation

Membrane-water partition coefficients and membrane permeability of the surfactant can be established by ITC uptake and release protocols, particularly for systems reaching a stationary state within some minutes after surfactant addition. A more laborious yet robust alternative to this is called “equi-activity analysis” here (Chapter 6) and based on finding different combinations of lipid and surfactant concentration that give rise to the same surfactant content within the membrane. This is not new per se but has been applied to zeta potential data for the first time. This eliminates the need to guess the effective charge, which is a serious error source as described. Even if the precision of the composition-dependent partition coefficient is limited, the approach provides a very precise determination of the membrane composition, given as $R_b$ or $X_s^0$, at a given zeta potential and hence frees zeta data from all partitioning effects.

The innovative treatment of zeta data provides also a new avenue to permeability. Inspired by publications using ITC protocols, Chapters 3 and 6 introduce and validate a heat treatment, e.g., the transient incubation of a membrane sample at 65 °C for 10 minutes, as a very useful way to induce a transmembrane equilibration of externally added surfactants that are impermeant at room temperature. An interesting new approach to monitor the concentration-dependent membrane translocation of ionic surfactants (including lipopeptides) has been described in Chapter 6. Zeta
potential measurements are peculiar at reporting the behaviour of the outer leaflet of vesicles only. In contrast to other experiments distinguishing between outer and inner leaflet, this does not require the use of shift reagents, quenchers, or other membrane impermeable reagents that change the behavior of probes in the leaflet that is exposed to them.

The asymmetric accumulation of charged additives in the outer leaflet is revealed as an anomally large zeta potential, which tends toward the average value when the surfactant equilibrates. Hence, the asymmetry is indicated by a difference in the zeta potential of freshly titrated versus heat-treated (or otherwise equilibrated) vesicles. More work is needed to better establish this approach but first results suggest that one might even be able to distinguish different equilibration mechanisms such as stimulated flip-flop above a threshold asymmetry and pore-formation.

7.4.3 Curvature stress and strain

The quantification of curvature stress in membranes is not straightforward. Since it depends on the mutual packing of surfactant and lipid, it cannot be deduced from the packing parameter alone. The same problem applies if the spontaneous curvature of the surfactant as derived from the dimensions of micellar or inverse micellar structures using, for example, x-ray diffraction. Generally, there seem to be two possible strategies: to measure (i) the stress or (ii) the strain. Curvature stress is represented by a penalty to the free energy and, particularly, enthalpy of the system and has been quantified by calorimetric methods.$^{167,271}$ Curvature strain manifests itself in terms of a membrane lateral expansion and reduction in thinning, typically achieved by a disordering of the chains. In Chapter 3, the order of DPH (represented by the limiting anisotropy $r_\infty$) has been successfully used to quantify curvature strain.

7.4.4 Homogeneous versus heterogeneous perturbation

As described in Chapter 3, a homogeneous disordering of a membrane by a well miscible surfactant causes a curvature strain that induces solubilization when a critical, threshold value is reached. Hence, the onset of solubilization in the absence of an overall, critical disordering detectable by DPH has been taken as a clue for heterogeneous perturbation. It should be noted that heterogeneous perturbation defined this way does not necessarily describe one single mechanism. The important functional consequences of heterogeneous perturbation discussed here clearly warrant further experiments also with other methods to shed light on mechanisms. Of interest would, for example, be the detection of segregation processes by FRET measurements.
7.4.5 Onset of completion of solubilization

The onset and completion of solubilization, usually quantified as $R_{b}^{sat}$ and $R_{e}^{sol}$, can be determined by ITC very precisely and conveniently, if the sample equilibrates or reaches a stationary state within ≈10 minutes after an injection. This approach must fail for detergents with flip-flop rates of the order 1 h$^{-1}$ and one depends on scattering or spectroscopic parameters that can be recorded after an equilibration step. The work presented in Chapter 3 has revealed that the limiting anisotropy of homogeneously disordering detergents shows breakpoints at $R_{b}^{sat}$ and $R_{e}^{sol}$, suggesting DPH experiments as yet another, useful approach to detect these boundaries.

7.5 Conclusions and outlook

This study has improved our understanding of many, previously discussed aspects of surfactant-lipid interactions and pioneered a number of new techniques, models, and ideas. Some major conclusions are:

1) There is a minimum order required for a stable membrane; surfactant-induced curvature strain that disorders the membrane below this limit leads to membrane solubilization. Importantly, some surfactants induce solubilization without such critical overall disordering of the membrane. They act by heterogeneous perturbation, which may account for enhanced antibiotic activity and selectivity and good suitability for membrane protein studies.

2) Although micelle formation is not a thermotropic transition per se, the temperature dependence of the CMC can be utilized to measure volumetric parameters by pressure perturbation calorimetry. Water exposure of hydrophobic chains causes a marked decrease in partial volume and increase in expansivity that is little temperature dependent. This challenges the idea of a structure-breaking effect on the water molecules. No significant differences have been found between the volumetric properties of alkyl chains in a micelle core and bulk hydrocarbon.

3) Zeta potential measurements of surfactant-binding lipid vesicles can be evaluated according to an equi-activity approach revealing and/or eliminating membrane-water partitioning effects. The new approach quantifies protonation and counterion binding phenomena that are hard to predict but govern, among other things, the membrane permeability of the surfactants.

These results suggest and warrant a number of follow-up studies as discussed before, for example:
1) Further PPC measurements of surfactants, particularly such that differ from the traditional polar-head-and-alkyl-chain design, may reveal reasons for the alleged superiority of such surfactants for membrane protein studies.

2) Alternative methods should be used to explore the specific mechanism or mechanisms of heterogeneous membrane perturbation by surfactants.

3) Suggestions for improving existing protocols for membrane protein solubilization in specific respects (yield, homogeneity, preservation of quartary structure, etc.) should be attempted on the basis of the results obtained here and tested in collaboration with laboratories using such protocols.

Finally, the better characterization of surfactant properties and their interpretation to predict and optimize their functions and applications pursued here has been part of a large, long-term effort.
References

11. Avanti, W.
29. Lehninger Principles of Biochemistry, (Freeman, 2005).
63. Robbins, C.R. *Chemical and physical behavior of human hair*, (Springer Verlag, New York, 2002).


74. La Mesa, C., Khan, A., Fontell, K., Lindman, B. Phase diagrams and nmr studies of some ternary sodium deoxycholate-surfactant-water systems. *J Colloid Interface Sci* 103, 373-391 (1985).


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169. Lafleur, M., Bloom, M., Cullis, P.R. Correlation between lipid plane curvature and lipid chain order. *Biophys. J.* 70, 2747-2757 (1996).


