Catalysis in membrane mimetic reaction media
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Incorporation of various additives, such as long linear alcohols, nonionic surfactants and anionic amphiphiles, into vesicles formed from dimethyldi-n-octadecylammonium chloride (C_{18}C_{18}+) leads to changes in the main phase transition temperature (T_{m}). The change strongly depends on the mole fraction and structure of the additives. Above 20 mol% of saturated (18 carbons) alcohols the T_{m} increases whereas short (ca. 10 carbons) alcohols and single-tailed surfactants lead to a decrease, or even a disappearance of the T_{m}. Addition of anionic, double-tailed amphiphiles leads in the case of asymmetry in the tails to an increase in T_{m} at the equimolar ratio, whereas in the case of symmetry this is not observed. Fluorescence and absorbance spectroscopy were used to study changes in the polarity of the vesicular surfaces, but despite the use of five different dyes no significant changes were found as a function of the concentration of the additives. Addition of single-tailed surfactants leads to vesicle solubilisation to form mixed micellar aggregates. This process was followed by turbidity measurements and light scattering experiments.

2.1 Introduction

As discussed in Chapter 1, the composition of biological membranes is important for performing their task. Additives, such as cholesterol, are a key factor in the structural integrity towards, for example, permeability of ions and hydrophilic molecules, such as glucose. Also other properties are affected by the exact composition of biological membranes. In fact, the lipids can be regarded as matrix to which other molecules can be added to give the bilayer additional functionalities. Unfortunately, due to this complexity in composition, and also the structural complexity of the phospholipids, and steroid themselves, it is difficult to identify the molecular interaction that is responsible for the properties.

The use of structurally simple molecules limits the number of possible interactions. Then, if these structures are systematically varied, information can be obtained on the importance of certain types of interactions. Upon a gradual increase in structural complexity (e.g. introduction of more functional groups) valuable information can be obtained on the molecular interactions in biological membranes.

In this chapter vesicles formed from dimethyldi-n-octadecylammonium chloride (C_{18}C_{18}+; Scheme 2.1) were studied in the absence and presence of various additives. The additives and the double-tailed amphiphile were chosen on the basis of their structural simplicity in...
order to be able to relate a change in property to a change in the structure of the additive. The properties of C₁₈C₁₈⁺ have been studied in the literature. In addition, despite the structural simplicity, the additives can be regarded as simple mimics of compounds, other than lipids, that can be found in biological membranes.

The additives can be divided into four classes. The first class consists of anionic double-tailed amphiphiles (sodium di-n-decylphosphate and sodium n-decyl-n-octadecylphosphate), the second class contains long linear alcohols (n-decanol, n-octadecanol, oleyl alcohol and batyl alcohol), the third class includes ethylene glycol surfactants (Brij 58P and a SAINT-2 derivative) and the fourth class are sugar-based surfactants (n-dodecyl-β-glucoside, n-dodecyl-β-maltoside). Finally, also a cationic phospholipid analogue has been studied. A more detailed reasoning for studying vesicles containing these specific additives can be found in the following chapters, where all classes of additives are described with respect to their influence towards a bimolecular reaction that is catalysed in the presence of cationic vesicles. This chapter will describe how properties, such as the main phase transition temperature, local polarity and vesicular size (distributions), are affected by the addition of these types of additives. Their exact structure can be found in those paragraphs where they are first used.

![Scheme 2.1. Di-n-octadecyldimethylammonium chloride (C₁₈C₁₈⁺).](image)

## 2.2 Experimental

### 2.2.1 Materials

Dimethyldi-n-octadecylammonium chloride (> 97%; Fluka), dimethylphosphate (98%; Acros), eicosyethylene glycol mono n-hexadecyl ether (Brij 58P; Fluka), n-decanol (99%; Aldrich), n-octadecanol (95%; Acros), oleyl alcohol (Aldrich), batyl alcohol (99%; Aldrich), n-dodecyl-β-glucoside (>99%; Fluka), n-dodecyl-β-maltoside (>99.5%; Glycon), sodium hydroxide (titrisol; Merck), pyrene (>99%; Aldrich), 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS; Sigma), 6-dodecanol-2-dimethylaminonaphthalene (laurdan; >99%; Molecular Probes), 9-diethylamino-5H-benzo[α]phenoxazine-5-one (Nile Red; 99%; Acros) and sodium hydroxide (titrisol) were used as received. The Et(30)-probe (2,6-diphenyl-4-(2,4,6-triphenyl-1-pyridinio)phenolate) was kindly provided by Prof. Ch. Reichardt (University of Marburg). Sodium di-n-decyl phosphate, sodium n-decyl-n-octadecyl phosphate, 2,3-bis-n-octadecyloxy-propyl)-trimethylammonium chloride and 4-(dioleymethyl)-1-(PEG₅₀₀OCH₃)-pyridinium bromide were synthesized by Mr. A. Wagenaar. Doubly distilled water was used for all solutions.

### 2.2.2 Vesicle Preparation

Stock solutions of approximately 30 mM total amphiphile concentration were prepared by weighing the needed amounts of amphiphile. Water was added to the appropriate volume and the solution was kept in a water bath at 50°C for at least 45 minutes. Then the solution was sonicated using a tip sonicator (Branson Sonifier B15-P) at 50°C for 6 min. (or longer if not all solid material was solubilised). Subsequently, the stock solution was extruded 11 times through a 400 nm filter using a mini-extruder (Avanti Polar Lipids, Alabaster, AL) at 50°C. Finally, the stock solution was diluted to
the desired concentrations and, if required, sodium hydroxide from a 1 M stock solution was added so that the total concentration of sodium hydroxide was 2.25 mM.

In an alternative procedure, stock solutions of approximately 30 mM total amphiphile concentration were prepared by weighing the needed amounts of amphiphile and additive. Chloroform was added in such an amount that the compounds were just dissolved. Chloroform was then evaporated by slowly rotating the sample vial under a stream of nitrogen. Residual amounts of chloroform were removed by storing the sample vial in vacuo for several hours. Then water was added in the appropriate amount and the solution was sonicated as described above.

Certain samples were prepared at different concentrations (e.g. some DSC samples were prepared at 2 mM).

Sometimes a larger home-built extruder was used. Up to 10 ml of sample was extruded at least five times through a 200 nm filter. Some solutions were not extruded.

No differences in behaviour between the various preparation methods were observed when various control experiments (DSC, DLS, kinetic experiments (Chapter 3) were performed.

Unless stated otherwise in the text, all samples were prepared following the above described preparation method.

2.2.3 Cryo-Electron Microscopy

A small drop of a 20 mM amphiphile solution was deposited on a glow discharged holey carbon-coated grid. After blotting away the excess of the solution under study, the grids were plunged into liquid ethane. Frozen hydrated specimen were mounted in a Gatan (model 626) cryo-stage and examined in a Philips CM 120 cryo electron microscope operating at 120 kV.

2.2.4 Differential Scanning Microcalorimetry

DSC scans were taken on a VP-DSC apparatus (Microcal, Northampton, MA) with a scan rate of 1°C min⁻¹. The total amphiphile concentration was 2 mM and the total concentration of sodium hydroxide was 2.25 mM. Five scans were performed between 5°C and 100°C. The reference cell was filled with doubly distilled water. The solutions were allowed to equilibrate at 1°C for 90 min. between successive scans. A water scan was subtracted using Microcal Origin software. The first scan was neglected due to the thermal history of the machine, but the other scans were all identical.

2.2.5 Fluorescence and Absorbance Spectroscopy

2.2.5.1 \(E_\tau(30)\) dye

4 µl of a saturated solution of the \(E_\tau(30)\)-probe in acetonitrile was added to a vesicular solution and the wavelength of maximum absorption was measured on a Perkin-Elmer spectrophotometer at least 5 min. after mixing the solutions. Vesicle concentrations were chosen such that \(\lambda_{\text{max}}\) did not change with concentration indicating that the \(E_\tau(30)\) probe was fully bound.

2.2.5.2 Pyrene

Pyrene was dissolved in water and filtered at least 1 day after dissolution. This solution was then diluted once. No excimers were present since steady-state fluorescence showed no peak near 450 nm, characteristic of pyrene excimers. Pyrene was present at concentrations lower than 10⁻⁶ M. Steady-state fluorescence spectroscopic measurements were performed using a SLM SPF-500C spectrofluorometer equipped with a thermostatted cell holder and a magnetic stirring device. Measurements were initiated at least 15 min. after mixing the vesicular and pyrene solution. The instrument settings were as follows: excitation wavelength, 335 nm; slid width 5 nm. The emission spectrum was recorded from 371 nm to 386 nm (slit width, 1 nm; step size 0.20 nm; filter 2). The intensities of the first (around 372 nm) and third peak (around 385 nm) were determined.
2.2.5.3 Nile Red, 1,8-ANS and Laurdan Fluorescence

Nile Red, 1,8-ANS and laurdan fluorescence experiments were performed similar to those for pyrene. Details are given in Table 3.

<table>
<thead>
<tr>
<th>dye</th>
<th>sol. prep. method</th>
<th>λ_exc (nm)</th>
<th>slid width exc. (nm)</th>
<th>slid width em. (nm)</th>
<th>step size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nile Red I</td>
<td>I</td>
<td>490-590</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>1,8-ANS I</td>
<td>I</td>
<td>380</td>
<td>5</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Laurdan II</td>
<td>II</td>
<td>440-490</td>
<td>7.5</td>
<td>2.5</td>
<td>4</td>
</tr>
</tbody>
</table>

*λ_em (nm) instead of λ_exc (nm).* See text below.

Solutions were prepared using two methods.
I: The fluorescent dye was added from a concentrated solution in acetonitrile. The amphiphile to dye ratio was 400 or larger. Vesicles were prepared as described in Section 2.2.2.
II: The fluorescent dye was added from a concentrated stock solution in chloroform to a solution of amphiphile and additive in chloroform. The mixture was further processed as described in Section 2.2.2 for solution prepared via the “film” procedure. The amphiphile to dye ratio was 250.

Background scans were performed with vesicles containing no fluorescent dye. The scattering at the wavelength of emission was negligible.

2.2.6 Dynamic Light Scattering

Size distributions were measured using a Malvern Zetasizer 5000 (Malvern, UK). The experiments were performed at a concentration where there is no double scattering (typically < 2.5 mM). The data was analysed using the algorithms provided with the software. A size distribution was accepted as reasonable when several analysis methods yielded similar results.

2.2.7 ζ Potentials

Mobilities were measured using a Malvern Zetasizer 5000 (Malvern, UK). ζ potentials were then calculated using the Smoluchowsky limits. All solutions used contained 5 mM total amphiphile concentration and 2.25 mM NaOH and were prepared as described (Section 2.2.2), except that 5 mM solutions were used and the solutions were not extruded. All experiments were performed around 15°C.

2.2.8 Turbidity Experiments

Turbidity was measured by measuring the absorbance at 410 nm on a Perkin-Elmer λ5 or λ12 spectrophotometer.
2.3 Results and Discussion

2.3.1 Cryo-Electron Microscopy

2.3.1.1 Sodium Di-\(n\)-decylphosphate

Vesicles, formed from \(\text{C}_{18}\text{C}_{18}^+\), are “lens-shaped” [Figure 2.1a].\(^{11,16}\) i.e. vesicles are round when looked from the top, but strongly flattened when looked from aside. The non-spherical shape suggests that packing of these synthetic amphiphiles is less efficient than in their natural analogues (phospholipids).

![Cryo-EM pictures of mixtures of \(\text{C}_{18}\text{C}_{18}^+\) and \(\text{C}_{10}\text{C}_{10}^-\).](image)

*Figure 2.1.* Cryo-EM pictures of mixtures of \(\text{C}_{18}\text{C}_{18}^+\) and \(\text{C}_{10}\text{C}_{10}^-\). The letter denotes the percentage of \(\text{C}_{10}\text{C}_{10}^-\) as a function of the total amphiphile concentration. (a) 0 mol%; (b) 10 mol%; (c) 40 mol%; (d) 50 mol%; (e) 70 mol%. Explanation of numbers: (I) top view; (II) side view; (III) side view where one “lens” has extra curvature; (IV) top view, but slightly tilted; (V) spherical vesicle; (VI) clustering of vesicles; (VII) angular vesicle. The bar represents 100 nm.
Upon the addition of 10 mol% of sodium di-\(n\)-decylphosphate (\(\text{C}_{10}\text{C}_{10}\); Scheme 2.2; Figure 2.1b) the cryo-EM picture shows more or less the same structures, except that sometimes one of the two “lenses” has extra curvature. At 40 mol% of \(\text{C}_{10}\text{C}_{10}\) (Figure 2.1c) angular spherical vesicles are observed. This angularity has been observed before\(^{11}\) and is due to the fact that the vesicles are below their main phase transition temperature (Section 2.3.4) when they are vitrified. This angularity is also observed for 50 mol% of \(\text{C}_{10}\text{C}_{10}\) (Figure 2.1d), but now also clustering occurs due to the absence of strong electrostatic repulsion between the vesicles since the vesicles are almost overall neutral. At 70 mol% of \(\text{C}_{10}\text{C}_{10}\) (Figure 2.1e) only spherical vesicles are observed.

\[\text{Scheme 2.2. Sodium di-}n\text{-decylphosphate (C}_{10}\text{C}_{10}\text{).}\]

2.3.2 Vesicle Solubilisation by Nonionic Single-Tailed Surfactants

Single-tailed nonionic surfactants are widely used to solubilise biological membranes in order to obtain cellular content or membrane proteins. But they are also used to reconstitute membrane proteins into model membranes.\(^{17,18}\) Sugar-based surfactants, oligo-ethylene glycol mono-\(n\)-alkyl ethers and derivatives of the latter, such as Triton X, are often used as solubilising agent (Scheme 2.3) and therefore their interactions with bilayers have been well studied in the literature.\(^{17}\) The most commonly used techniques to study vesicular breakdown are turbidity, fluorescence spectroscopy and isothermal titration microcalorimetry (ITC).

\[\text{Scheme 2.3. Examples of commonly used nonionic detergents used to solubilise membranes. A: oligo-ethylene glycol n-alkyl ether (C}_{n}\text{EO}_{m}; B: Triton X; C: n-Alkyl glucoside (C}_{n}\text{Glu).}\]
Membrane solubilisation proceeds via a three-step mechanism (Scheme 2.4).\textsuperscript{18,19} In the first stage, upon addition of single-tailed surfactants to a vesicular solution, the single-tailed surfactants will partially insert into the bilayer, leading to swelling of the vesicles. This proceeds until the vesicles are saturated with single-tailed surfactant.

At any time of stage I the amount of membrane-bound single-tailed surfactant can be calculated using the molar ratio of bound single-tailed surfactant to the amount of double-tailed amphiphile ($R_b$). This ratio depends on the binding constant $K$, as was derived by Schurtenberger et al.\textsuperscript{20} We will further use the words detergent (det) for single-tailed surfactant and amphiphile (amph) instead of double-tailed amphiphile.

\begin{equation}
R_b = \frac{[\text{det}]_{ves}}{[\text{amph}]} = K[\text{det}]_w
\end{equation}

In this eq. $[\text{det}]_{ves}$ and $[\text{det}]_w$ are the vesicular and aqueous concentrations of detergent with respect to the total volume, respectively.

An alternative model makes use of the mole fraction partition coefficient $P$ and assumes ideal mixing of amphiphile and detergent.\textsuperscript{21} This model makes use of the mole fraction bound detergent in the membrane, rather then the molar ratio. $K$ and $P$ are related via eq. (2.2):\textsuperscript{22}

\begin{equation}
K = \frac{P}{[\text{H}_2\text{O}]}_{w}(1 + R_b)
\end{equation}

$[\text{H}_2\text{O}]_w$ is the concentration of water in the solution and in dilute systems this is 55.6 M. This slightly different approach leads to rather different results, especially at higher detergent concentrations. Experimentally it has been found that a constant $K$ better describes the system than a constant $P$. A composition-dependent partition coefficient, $P(X_b)$, has been derived using the non-ideality parameter $\rho_b$ and this approach leads to similar concentrations of bound and unbound detergent as calculated using $K$. Therefore, we will further use the model of a constant $K$ as derived by Schurtenberger et al.\textsuperscript{20}

In stage II the breakdown of the vesicles can occur via two mechanisms. Either mixed micelles “escape” from the vesicles, or the vesicle is “eaten up” by the detergent. At the same time mixed (worm-like) micelles coexist. Due to this breakdown, compounds encapsulated
inside the vesicles are released.\textsuperscript{23-26} In the third stage all the amphiphiles are dissolved into
mixed micelles.

Despite the fact that the vesicle solubilisation is widely used and the general mechanism
appears clear, many of the details, such as alternative mechanisms,\textsuperscript{27-29} domain formation
upon detergent addition\textsuperscript{30,31} and vesicle fusion upon detergent addition,\textsuperscript{32,33} are still poorly
understood.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{A: Plot of turbidity versus the concentration of added detergent \([C_{16EO_{20}}]\).
Dashed lines separate the different stages. B: Schematic and theoretical
representation of a simplified phase diagram of vesicle solubilisation. The dashed
arrow is the direction of a solubilisation experiment through the different stages. The
reverse direction of the arrow is a membrane reconstitution experiment.}
\end{figure}

Measuring the turbidity of a vesicular solution upon detergent addition, initially an increase
in turbidity is shown due to the swelling of the vesicles in the first stage (and sometimes
due to fusion of vesicles as well; Figure 2.2A).\textsuperscript{34,35} A maximum in turbidity is observed when
the vesicles are saturated with detergent. The ratio of the concentrations of detergent to
amphiphile at which this occurs is called \(R_{\text{sat}}\) (compare eq. (2.1)). Then the turbidity will
decrease since the vesicles are being consumed, and scattering by micelles is negligible.
When all vesicles have disappeared, addition of more detergent will not change the turbidity anymore. The ratio of the concentrations of detergent to amphiphile at which the turbidity becomes constant is called $R_{\text{sat}}$. When this experiment is repeated at several amphiphile concentrations a plot as in Figure 2.2B can be constructed. The intercept of the straight lines through the $R_{\text{sat}}$ and $R_{\text{sol}}$ values with the y-axis is the amount of free detergent in solution at the saturation and solubilisation point, respectively. Ideally, the $R_{\text{sat}}$ and $R_{\text{sol}}$ line cross at the y-axis. This concentration is being associated with the critical micellar concentration (CMC) of the detergent. However, usually this point underestimates the CMC. The slope of the $R_{\text{sat}}$ and $R_{\text{sol}}$ line is related to the amount of bound detergent, as can be seen in eq. (2.3).

$$[\text{det}]_{\text{tot}} = [\text{det}]_{\text{w}} + [\text{det}]_{\text{mic}} = [\text{det}]_{\text{w}} + \frac{[\text{det}]_{\text{mic}}}{[\text{amph}]} = [\text{det}]_{\text{w}} + R_{\text{sat}}[\text{amph}]$$

Taking the ratio of the slope to the intercept of a plot of eq. (2.3) one obtains the value for $K$ (eq. (2.1)) at either the saturation point, or the solubilisation point. However, experimentally this is not so straightforward. Usually, CMC values are rather small ($<1 \text{ mM}$) and since the intercept is extrapolated from experimental data the error in the intercept is quite large. Calculation of $K$ requires division by this small value and therefore small fluctuations in the intercept will lead to large deviations in the value for $K$. In addition, the relationship between the concentrations of amphiphile and detergent usually deviates from linearity close to zero amphiphile concentration.

It is more convenient to use ITC to measure binding constants, since it leads to smaller errors in the binding constant and the value of the binding constant can be obtained in one single experiment. In addition, it also gives information about the enthalpy of binding. The binding constant $K$ correlates rather well with the CMC, i.e. if $K$ increases, the CMC decreases. This is reasonable since both binding to a vesicle and aggregation into micelles proceeds via a similar mechanism (transfer of the hydrophobic tail from water to an apolar pseudophase, release of water from the hydrophobic hydration shell, etc.)

Micellisation is described by the equilibrium given below, and hence is described by the micellar binding constant $K_{\text{mic}}$ (in mole fraction).

$$n \text{ S(aq)} \rightleftharpoons \text{S}_n(\text{mic})$$

$$K_{\text{mic}} = \frac{[\text{S}]_{\text{mic}}}{[\text{S}]_{\text{w}}}$$

In this equation $[\text{S}]_{\text{mic}}$ and $[\text{S}]_{\text{w}}$ are the micellised and aqueous mole fraction of detergent, respectively. The mole fraction $[\text{S}]_{\text{mic}}$ equals approximately 1, since the number of detergent molecules in the micelle is large relative to the number of water molecules. Considering that above the CMC the concentration of aqueous mole fraction detergent is more or less given by the CMC it follows that $K_{\text{mic}}$ is related to the CMC.

$$K_{\text{mic}} = \frac{1}{\text{CMC}_{\text{mole fraction}}} \triangleq \frac{55.6}{\text{CMC}_{\text{molar}}}$$

In more detail, if $K \cdot \text{CMC}$ (in molar) equals 1 the standard Gibbs energy of detergent binding to vesicles (eq. (2.6)) is equal to the standard Gibbs energy of micellisation (eq. (2.7)).

$$\Delta_{\text{binding}} G^0 = -RT \ln(55.6 K)$$

$$\Delta_{\text{mic}} G^0 = -RT \ln \left( \frac{\text{CMC}}{55.6} \right)$$

$\Delta_{\text{mic}} G^0$
The factor 55.6 is the molar concentration of water in water and has to be added since $\Delta G$ should be calculated in terms of mole fractions rather than concentrations (eq. (2.5)). For most surfactants this relationship is valid within 2 kJ/mol (factor of ca. 2 in K or CMC) and hence $K$ can be predicted rather well once the CMC is known. This approach has been validated in the literature.\textsuperscript{40}

It should be noted that turbidity experiments are a rather insensitive method. It is based on a change in measured absorbance due to the scattering of light. Therefore, anything that scatters light significantly will be detected, and hence, anything that has a small scattering ability will not be detected. This means that a small concentration of large vesicles will be readily detected by an increase in turbidity, whereas a moderate concentration of small vesicles, worm-like micelles, or bilayer fragments will not be detected. As a result, reported values for $R_{\text{sat}}$ and $R_{\text{sol}}$ do not necessarily represent the true point of detergent saturation or solubilisation of the vesicles. Nevertheless, turbidity experiments have led to useful results. More sensitive methods, but yet more elaborate techniques, include ITC\textsuperscript{22,37,38} and dynamic light scattering (Section 2.3.3).

Finally, it is important to know whether the detergent binds to both the inner as well as the outer leaflet, or only to the outer leaflet. Ionic detergents are expected to experience a large kinetic barrier for crossing the bilayer since the bilayer interior is rather apolar. However, experiments have shown that nonionic detergents, such as Triton X (Scheme 2.3B), rapidly cross the bilayer.\textsuperscript{22,41,42}

### 2.3.2.1 Eicosa-Ethylene Glycol Mono n-Hexadecyl Ether

Eicosa-ethylene glycol mono $n$-hexadecyl ether ($C_{16}EO_{20}$; Scheme 2.3A), also known as Brij 58, is not often used in membrane solubilisation. Usually detergents with a shorter alkyl tail and a shorter ethylene glycol head group are used ($C_{n}EO_{m}$; $n$=10-12, $m$=3-8). Their binding strength has been well studied in the literature.\textsuperscript{39,43} Addition of two CH$_2$ groups to the hydrophobic tail only leads on average to an increase of the binding by a factor of at most 20, whereas addition of 1 ethylene glycol unit leads to a decrease in the binding constant by a factor of 1.6. It is ambitious to derive (extrapolate) the expected binding constant for $C_{16}EO_{20}$ from these data. The binding constant cannot be measured experimentally using ITC since ITC requires titration of a vesicular solution into a solution containing the detergent below the CMC. The CMC of $C_{16}EO_{20}$ is approximately 3.9 $\mu$M.\textsuperscript{44} However, using the relationship $K \cdot \text{CMC} = 1$ (in molar concentration; eqs. (2.6) and (2.7)) the binding constant can be roughly estimated. For $C_{16}EO_{20}$ it is expected to be around 2.5·10$^5$ M$^{-1}$. This means that at any given amphiphile concentration $C_{16}EO_{20}$ is completely bound.

Figure 2.3 shows the phase diagram for various amphiphile concentrations. As can be seen, above 2 mM $C_{16}C_{18}^{+}$ there is a linear relationship between the concentrations of $C_{18}C_{18}^{+}$ and $C_{16}EO_{20}$ at the phase boundaries. The $R_{\text{sat}}$ and $R_{\text{sol}}$ values are 0.84 and 12, respectively. The bilayer composition at the saturation point is similar to what has been found when $C_{18}C_{18}^{+}$ vesicles ($R_{\text{sat}}$ ca. 1)\textsuperscript{45} or phospholipids vesicles ($R_{\text{sat}}$=0.48-0.66) were solubilised by $C_{12}EO_{8}$.\textsuperscript{46-48} However, the value of 12 we find at the solubilisation point is much higher than reported in the literature ($R_{\text{sat}}$=1.8-5). Experiments with erythrocyte membranes and a series of $C_{n}EO_{8}$ surfactants show a decrease in $R_{\text{sat}}$ and $R_{\text{sol}}$ from 2.23 to...
0.03 and from 3.45 to 0.06, respectively, for \( n=10 \) to \( n=18 \). The largest changes are found going from \( n=10 \) to \( n=12 \). Apparently, \( \text{C}_{16}\text{EO}_{20} \) saturates membranes with a \( R_b \) similar to that of \( \text{C}_{12}\text{EO}_{8} \), but is much less efficient in completely solubilising them than most other nonionic ethylene glycol detergents. In addition, it should be noted that solubilisation of membranes that are in the gel-like state is more difficult than when they are in the liquid-crystalline state.

![Figure 2.3. Phase diagram of vesicle solubilisation of \( \text{C}_{18}\text{C}_{18}^+ \) by \( \text{C}_{16}\text{EO}_{20} \). Solid lines are linear fits through the points of detergent-saturated vesicles (\( R_{\text{sat}} \); ■) and fully solubilised vesicles (\( R_{\text{sol}} \); ○). Dashed lines are anticipated phase boundaries crossing the y-axis at the CMC of \( \text{C}_{16}\text{EO}_{20} \). The dotted line represents solutions containing 35 mol% of \( \text{C}_{16}\text{EO}_{20} \). The inset shows the \( R_{\text{sat}} \) values in more detail.](image)

Extrapolation of the \( R_{\text{sat}} \) and \( R_{\text{sol}} \) lines to zero amphiphile concentration leads to negative values of the intercept. Since the intercept is related to the free detergent concentration (CMC), this is physically impossible. Usually, the intercept is slightly smaller than the CMC of the detergent. Since the CMC of \( \text{C}_{16}\text{EO}_{20} \) is small (and \( K \) high), the ratio of the slope to the intercept is large. These factors might contribute to the negative value for the intercept, but we have no clear explanation.

In general, vesicular solutions to which micelles are added require time to reach equilibrium, since reorganisation of amphiphile and detergent into different types of aggregates can be slow. Therefore, usually solubilisation experiments are done with vesicles in the liquid-crystalline state. Our experiments were performed below the phase transition temperature, which may lead to further complications. In fact, in several of the solution mixtures we observed partial precipitation after allowing the solution to equilibrate over night, indicating that our samples precipitate after extensive equilibration. However, we note that this process is observed in detergent-free solutions as well, although in those cases the process of precipitation is much slower. We contend that our experiments were done at the optimum time to allow a large extent of equilibration, but with as little precipitation as possible. Experiments involving precipitation were repeated, and all data fitted reasonably on the absorbance versus detergent concentration curves. Solutions prepared by cosonication of detergent and amphiphile should be in the equilibrium state since no re-equilibration should have to take place, besides of course the fact that most vesicles are intrinsically metastable.
2.3.2.2 \( n \)-Dodecyl-\( \beta \)Maltoside

For membrane solubilisation the disaccharide \( n \)-dodecyl-\( \beta \)maltoside (\( C_{12}\text{Mal} \); Scheme 2.5) is not a popular detergent.\(^9\) Usually, for experimental considerations \( C_{10}\text{Mal} \) is preferred. However, in practice more experiments have been performed with monosaccharide octyl glucoside (\( C_{8}\text{Glu} \)).\(^{33,50-54}\) An important observation is that sugar-derived detergent can induce vesicle fusion prior to saturation.\(^{33}\)

\textbf{Scheme 2.5.} \( n \)-Dodecyl-\( \beta \)maltoside (\( C_{12}\text{Mal} \)).

The CMC of \( C_{12}\text{Mal} \) is 150-200 \( \mu \)M,\(^{19,22,55,56} \) and hence we expected a binding constant of ca. 5000-7000 \( \text{M}^{-1} \). Using ITC the binding constant has been measured, and a value of 5000 \( \text{M}^{-1} \) has been reported.\(^{39}\)

Figure 2.4 shows the phase diagram of mixtures of \( C_{18}C_{18}^+ \) and \( C_{12}\text{Mal} \). Due to fewer data points, and somewhat unusual behaviour of these detergent/amphiphile mixtures (Section 2.3.3.4) the pattern is not as clear as that for \( C_{16}\text{EO}_{20} \). Especially, the crossing of the \( R_{\text{sat}} \) and \( R_{\text{sol}} \) line is usually not observed. However, this crossing has been observed for solubilisation experiments of phospholipid vesicles by a series of sugar-based surfactants (including \( C_{12}\text{Mal} \)).\(^{57}\)

\textbf{Figure 2.4.} Phase diagram of vesicle solubilisation of \( C_{18}C_{18}^+ \) by \( C_{12}\text{Mal} \). Solid lines are linear fits through the points of detergent saturated vesicles (■) and fully solubilised vesicles (○). Dotted lines represent solutions containing 10 (a), 25 (b) and 50 mol\% (c) of \( C_{12}\text{Mal} \).
For these reasons and “odd” behaviour (Section 2.3.3.4) we decided to refrain from a detailed interpretation. We only note that up to 3 mM of C_{18}C_{18}^{+} about 2 mM C_{12}Mal is needed to saturate the membrane. The slope in the R_{sol} line is 0.88 (lit. 2.34^{57}), which is considerable smaller than that found for C_{18}EO_{20} indicating that C_{12}Mal readily solubilises membranes of C_{18}C_{18}^{+}. In fact, less than one molecule of C_{12}Mal is needed per molecule of C_{18}C_{18}^{+} to solubilise vesicles into mixed micelles.

The remarks made for solutions containing C_{18}C_{18}^{+} and C_{16}EO_{20} about the time required to reach equilibrium are also valid for mixtures of C_{18}C_{18}^{+} and C_{12}Mal (Section 2.3.2.1).

2.3.3 Dynamic Light Scattering

2.3.3.1 Theoretical Considerations

Dynamic light scattering (DLS), also known as quasi-elastic light scattering (QELS) and photo correlation spectroscopy (PCS), is a powerful technique to measure the size distribution of aggregates present in solution. DLS is able to report a statistically meaningful size distribution by performing only a single experiment. The time required to perform a DLS experiment on a vesicular solution is less than one hour. However, there are also several drawbacks and considerations that have to be taken into account before performing a DLS experiment, particularly since DLS always reports a size distribution regardless the quality of the experiment and/or the sample under study.

Dynamic light scattering is based on the random diffusion (Brownian motion) of particles in solution. When laser light (in our case 632 nm) enters the solution, particles scatter the light. When the particles are small (<60 nm, i.e. < \lambda_{laser}/10; Rayleigh scattering) the light is randomly scattered into all directions. When the particles are larger, scattering is no longer random (Mie scattering) and the scattering becomes particle-size and angle-dependent. However, at 90 degrees the scattering intensity is usually acceptably high for most particle sizes.

In the ideal case when only spherical particles with one size are present in solution, the diffusion coefficient of the particle can be calculated from the fluctuations in the light intensity induced by the moving particle. This is done with a photorecorrelator that correlates the signal intensity with time. In case of a large particle, the correlation is lost only slowly with time since large particles move only slowly (Figure 2.5A). In case of small particles, the correlation is lost quickly (Figure 2.5B). From the diffusion coefficient the hydrodynamic radius (R_{h}) can be calculated using the Stokes-Einstein relationship:

\[ R_{h} = \frac{k_{B}T}{6\pi\eta D} \]  

(2.8)

In this equation k_{B}, T, \eta and D are the Boltzmann constant, absolute temperature, viscosity and diffusion constant, respectively.

In non-ideal cases, i.e. when there are particles present with different sizes, there is not one diffusion coefficient and hence the measured diffusion constant has to be split up mathematically. This leads to a size distribution. This splitting up can be carried out using several different algorithms, such as, for example, CONTIN, cumulant analysis, nonnegative least-squares analysis (NNLS), or the “automatic” analysis.^{58,59} In most of these fitting
procedures the upper and lower size limit can be set to a certain value. When the particle size distribution is relatively narrow and monodisperse, these algorithms will give more or less the same size distribution. However, when the size distribution is polydisperse and/or multimodal, the different analysis methods will report different results. In this case analysis of the data can become too complicated to ensure reliable values for the size distribution. Consequently, reports in the literature about measured particle sizes are rather meaningless unless the width of the distribution is reported.

In the case of non-spherical particles (Figure 2.5B) diffusion is not equal in all directions, and hence the reported size distribution becomes broader, as if there are particles in solution with the minimum and maximum width of the particle, and all sizes in between. In order to obtain an idea of the extent to which the particles are non-spherical, the angular dependence of the intensity of the scattered light can be measured. Using the Guinier approximation, the angular dependence can be described by eq. (2.9):

\[
I(\theta) \propto \exp \left( -q^2 R_g^3 / 3 \right) (qR_g \ll 1)
\]

In this equation \( R_g \) is the radius of gyration and \( q \) the scattering factor:

\[
q = \frac{4\pi n_s}{\lambda_0} \sin(\theta / 2)
\]

In this equation \( n_s \) is the refractive index of the solvent and \( \lambda_0 \) is the wavelength of the laser beam. The ratio of \( R_g / R_h \) yields then information on the shape of the particle. For example, cylindrical micelles have a value of \( R_g / R_h \) of ca. 2.60,62

We decided to refrain from such an analysis since the results are not required for an understanding of our experimental results described in the other chapters in this thesis.

\[\text{Figure 2.5.} \quad A: \text{Example of random diffusion of a large (left) and a small (right) particle through a solution.}\]
\[B: \text{Diffusion of a non-spherical particle. The arrows denote the relative size of the diffusion constant in that specific direction.}\]
\[C: \text{Example of double scattering due to a high concentration of particles in solution.}\]

Another drawback of DLS, besides the inability to handle polydisperse and multimodal size distributions, is the strong dependence of the scattering ability on particle size. For a given concentration increasing the particle size ten-fold the scattered intensity will be \( 10^6 \) times larger. This makes it difficult to observe the presence of micelles if in the same solution vesicles are present as well, except when the micelles are present in much larger
concentrations. We also note that the presence of dust particles will strongly influence the results.

As a result of the strong dependence of the scattered intensity on particle size, only an intensity-weighted size distribution can be calculated. This means that when there are two different sized particles in solution that differ by a factor of ten in size, and their concentrations are equal, that the peak of the larger particle will be $10^6$ times higher than the peak of the smaller particle. Hence, if the two peaks would be equal, this would mean that the concentration of the smaller particle is $10^6$ times higher than the concentration of the larger particle.

In concentrated samples scattered light can be scattered again (double scattering), leading to misleading results, since the doubly scattered light will not reach the detector. Therefore, experiments should be performed under conditions that the amount of scattered light is linear with the concentration of the particles.

Large particles (> 1 µm) usually have a tendency to precipitate. Since precipitation is fast relative to the Brownian motion of similar-sized particles, the reported diffusion coefficient will be largely determined by the rate of precipitation. Hence, since the size distribution is calculated from the diffusion coefficient, the size distribution will report a size that is smaller than the actual size distribution.

In Figure 2.6 an example of the results of a DLS experiment is given. The solution contains 0.5 mM $\text{C}_{18}\text{C}_{18}^{+}$ cosonicated with 10 mol% of the micelle-forming $\text{C}_{12}\text{Mal}$. Small micelles have a low scattering intensity (typically 0.1-1 kCounts s$^{-1}$), whereas that of vesicles is usually much higher (> 50 kCounts s$^{-1}$). Considering that this solution has a scattered intensity of around 130 kCounts s$^{-1}$, there are large particles in solution. All the data was analysed using the CONTIN and automatic algorithm. In the automatic algorithm the upper and lower size limits are chosen by the software, in the CONTIN algorithm the upper and lower size limit can both be chosen by the software or manually chosen. The experiment shown in Figure 2.6 was repeated five times on the same solution and two different size distributions were found (dashed and dashed-dotted line) using the CONTIN algorithm,
where the upper and lower limits were automatically chosen such that the upper limit is 100 times larger than the lower limit. One size distribution is monomodal, and the other bimodal. A closer look reveals that the maximum in the monomodal distribution is in between the two maxima of the bimodal distribution. The total width of the distribution is similar for both distributions. Considering that the overlap between the two distributions is rather large, we anticipate that the monomodal distribution is the more likely distribution, since we cannot rationalise why two different vesicle populations should exist. Therefore, we believe that these different distributions are probably due to small variations in the experimental data.

We consider a size distribution to be likely, if under the different fitting conditions the results are similar. For solutions containing 10 mol\% of C_{12}Mal we therefore suggest that they possess a distribution with a maximum around 100 nm, and a width that goes up to 200 nm.

2.3.3.2 Di-n-Octadecyldimethylammonium Chloride

In Figure 2.7A the scattered intensity and \( Z_{\text{ave}} \) as a function of the concentration of C_{18}C_{18}^+ are shown. \( Z_{\text{ave}} \) is the average particle size, assuming that there is no size distribution, i.e. there are only particles with a single size. This makes \( Z_{\text{ave}} \) independent of the size distribution algorithm. Of course, this is highly unrealistic, however, it gives a good idea of the dynamics of the system. Below a concentration of 2.1 mM of C_{18}C_{18}^+ the scattered intensity is linearly related to the concentration. In addition, \( Z_{\text{ave}} \) is independent of the concentration of C_{18}C_{18}^+, except that at higher concentrations the scattering in \( Z_{\text{ave}} \) is smaller. This proves that in this concentration range there is no double scattering. This is also supported by the independence of the size distribution with concentration.

The scattered intensity and the \( Z_{\text{ave}} \) value of a 0.5 mM solution of C_{18}C_{18}^+ were followed with time (Figure 2.8A). \( Z_{\text{ave}} \) scatters between 85 nm and 115 nm. In the first 10 hours the scattered intensity fluctuates up and down by about 6%. Then, it steadily decreases. This might indicate precipitation of larger particles. Once precipitated, the apparatus can no longer detect them, and hence a lower scattered intensity is reported. Therefore, after 19 h the solution was stirred, leading to a small increase in scattered intensity. However, neither \( Z_{\text{ave}} \) nor the size distribution changes after stirring. Then, the scattered intensity gradually increases, but again, \( Z_{\text{ave}} \) and the size distribution do not change. However, the increase in scattered intensity is rather small, making it difficult to detect subtle changes in the size distribution. Finally, the solution was stirred again after 90 hours, as can be seen in the jump in scattered intensity.

Vesicles formed from C_{18}C_{18}^+ are not spherical as was shown by cryo-electron microscopy (Figure 2.1a) and reported in the literature for C_{18}C_{18}^+ with bromide counterions.\textsuperscript{11,16} As a result, the reported size distribution will be broader relative to spherical vesicles with the same volume. The size reported by DLS is in agreement with the size indicated by cryo-electron microscopy.
Figure 2.7. Plots of the stability and size (A) and size distribution (B) of vesicles formed from $\text{C}_{18}\text{C}_{18}^+$ at various concentrations (same concentrations as in A). A: scattered intensity ($\bullet$) and $Z_{\text{ave}}$ ($\times$). $A$: The line is a linear fit of the scattered intensity (forced through zero).
2.3.3.3 Eicosa-Ethylene Glycol Mono-n-Hexadecyl Ether

Solutions containing various amounts of \( \text{C}_{16}\text{EO}_{20} \) were prepared in three different ways. In the first procedure amphiphile and detergent were dissolved and sonicated simultaneously at the appropriate ratio. The amphiphile concentration was 30 mM. DLS experiments were performed just after diluting the solution to 0.5 mM \( \text{C}_{18}\text{C}_{18}^+ \). In the second procedure vesicles containing \( \text{C}_{18}\text{C}_{18}^+ \) and 5 mol% of \( \text{C}_{16}\text{EO}_{20} \) were prepared, and then to the same sample increasing amounts of \( \text{C}_{16}\text{EO}_{20} \) were added from a concentrated stock solution (0.5 mM \( \text{C}_{18}\text{C}_{18}^+ \)). In a third procedure \( \text{C}_{18}\text{C}_{18}^+ \) vesicles were prepared, and to different samples containing 2.1 mM \( \text{C}_{18}\text{C}_{18}^+ \) various amounts of \( \text{C}_{16}\text{EO}_{20} \) micelles were added. Solutions were then allowed to equilibrate for 60 h. We decided to follow these different procedures in order to obtain information about the dynamics of the system.
Figure 2.9. Plot of the scattered intensity (A) and size distribution (B) of vesicles formed from $C_{18}C_{18}^+$ and $C_{16}EO_{20}$ at various mole fractions. Experiments were performed directly after dilution from a 30 mM stock solution, unless stated otherwise. 

B: $C_{16}EO_{20}$: 0 mol% (solid line); 5 mol% (dashed line); 10 mol% (dotted line; thick line after 14 h); 35 mol% (dash-dotted line; thick line after 5 h).

Figure 2.9 shows the results of the first procedure. Initially, upon increasing the concentration of $C_{16}EO_{20}$ the scattered intensity increases. However, already at 10 mol% of $C_{16}EO_{20}$ the scattered intensity decreases again. The increase is in line with an increase in vesicle size. At 35 mol% it can be seen that micelle formation plays a significant role, since the size distribution shows two peaks (not visible at 10 mol%). One around the original size of the vesicles, and one at about a ten-fold smaller size (7 nm), which agrees with the size of a spherical micelle. Because the scattering strongly depends on the size of the aggregate, the fact that we observe micelles, indicates that their concentration is significantly higher than that of the vesicles. Roughly, we can estimate the concentration of
micelles to be about $10^6$ times higher, since the peaks are almost equal in intensity (section 2.3.3.1).

When the solutions containing 10 mol% and 35 mol% of $\text{C}_{16}\text{EO}_{20}$ are allowed to equilibrate after dilution to 0.5 mM, neither the scattered intensity nor $Z_{\text{ave}}$ changes significantly with time (Figure 2.10). In addition, also the size distributions do not change substantially (Figure 2.9B).

![Figure 2.10. Plot of the scattered intensity and $Z_{\text{ave}}$ of vesicles formed from $\text{C}_{18}\text{C}_{18}^+$ and $\text{C}_{16}\text{EO}_{20}$ at 10 mol% and 35 mol%. Scattered intensity: 10 mol% (■); 35 mol% (□). $Z_{\text{ave}}$: 10 mol% (×); 35 mol% (+).]

It can be concluded that vesicles prepared using the first procedure are stable with time upon dilution (at least 14 h). This effect is not often observed, since upon dilution of a detergent/amphiphile mixture the detergent redistributes over the vesicular/micellar and aqueous phase. Therefore, dilution of these systems leads to reconstitution of vesicles (i.e. when the system is in stage II of the solubilisation process; Section 2.3.2), since the amount of detergent bound to the membrane decreases upon dilution. However, due to the large binding constant of $\text{C}_{16}\text{EO}_{20}$ (Section 2.3.2.1) redistribution between the two phases does not take place, since all detergent remains vesicular bound.

The second procedure was used to study the dynamics of the mixed detergent/amphiphile system. It can be seen in Figure 2.11 that the scattered intensity increases up to the addition of 65 mol% of $\text{C}_{16}\text{EO}_{20}$ that was added within 4 hours. At the same time $Z_{\text{ave}}$ increases from 100 nm to 110 nm, as does the maximum of the size distribution (Figure 2.12A). However, at the same time, it can be seen that the scattered intensity decreases in the hour after the addition of a small volume from a concentrated micellar solution of $\text{C}_{16}\text{EO}_{20}$. This indicates that the system does not reach equilibrium, and (substantial and) immediate breakdown of the vesicles only occurs above 65 mol% of $\text{C}_{16}\text{EO}_{20}$. By contrast, when the system is allowed to equilibrate (Figure 2.9A), vesicular breakdown is already seen at 10 mol% of $\text{C}_{16}\text{EO}_{20}$ (by a decrease in the scattered intensity).
**Figure 2.11.** Plots of the scattered intensity (□) and $Z_{ave}$ (×) of vesicles formed from $\text{C}_{18}\text{C}_{16}^+$ and $\text{C}_{16}\text{EO}_{20}$ at various mole fractions as a function of time. Experiments were performed by consecutive addition of $\text{C}_{16}\text{EO}_{20}$ micelles from a concentrated solution. Samples were allowed to equilibrate for 15 min. Dotted lines represent the next addition of $\text{C}_{16}\text{EO}_{20}$ micelles. The dashed line represents the time at which the solution was stirred for a few seconds.

The dynamics of vesicle solubilisation become more clear when the solution to which 94 mol% of $\text{C}_{16}\text{EO}_{20}$ was added in 6 h was allowed to equilibrate over night (Figure 2.11). Both the scattered intensity and $Z_{ave}$ decrease until they reach a stable level. At this level there are still vesicles present in solution, as indicated by the still relatively large value of the scattered intensity and $Z_{ave}$. This assumption is confirmed by the size distributions shown in Figure 2.12B. Directly after addition of 94 mol% of $\text{C}_{16}\text{EO}_{20}$ the height of both peaks is more or less similar. After 14 h the height of the peak around 100 nm has significantly decreased.

It can be seen that binding of $\text{C}_{16}\text{EO}_{20}$ leads to an increase in size as is shown by the size distribution. Not only the size of the vesicles increases by an increase of the hydrophobic volume, but also because the oligo-ethylene glycol head group increases the hydrodynamic radius. Especially at higher mole fractions of $\text{C}_{16}\text{EO}_{20}$ this is probably the result of the oligo-ethylene glycol head group going from a random coil at low molar fraction to a more extended structure at higher molar fraction as a result of steric interactions with each other.
Figure 2.12. Plot of the size distribution (A+B) of vesicles formed from $C_{16}C_{18^+}$ and $C_{16}EO_{20}$ at various mole fractions. Size distributions correspond to the experiments shown in Figure 2.11. A: $C_{16}EO_{20}$: 5 mol% (solid line); 31 mol% (dotted line); 46 mol% (dashed line); 65 mol% (dash-dotted line) and 75 mol% (dash-double-dotted line). B: $C_{16}EO_{20}$: 86 mol% (solid line); 94 mol% (dotted line) directly after addition; 94 mol% (dashed line) after 14 h.; 94 mol% (dash-dotted line) after 15 h and stirring; 97 mol% (dash-double-dotted line).
Characterisation of Cationic Vesicles with Additives

Graphs and data points showing the relationship between scattered intensity, particle diameter, and time for different compositions of C$_{16}$EO$_{20}$ (mol%).

A: Scattered intensity (kCounts s$^{-1}$) vs. percentage C$_{16}$EO$_{20}$ (mol%)

B: Intensity (A.U.) vs. particle diameter (nm)

C: Scattered intensity (kCounts s$^{-1}$) and Z$_{ave}$ (nm) vs. time (h)
Figure 2.13. Plot of the scattered intensity and $Z_{\text{ave}}$ (A+C) and size distribution (B+D) of vesicles formed from $C_{18}C_{18}^+$ and $C_{16}EO_{20}$ at various mole fractions (A+B), or as a function of the time (C+D). A+C: scattered intensity (□) and $Z_{\text{ave}}$ (∗). B: $C_{16}EO_{20}$: 0 mol% (solid line); 32 mol% (dashed line); 49 mol% (dotted line); 66 mol% (dash-dotted line); 93 mol% (dash-double-dotted line). C: 93 mol% of $C_{16}EO_{20}$. The arrow indicates the time at which the solution was stirred. D: t=0 h (solid line); t=16 h (dotted line); t=17 h and after shaking (dashed line).

When solutions containing 2.1 mM of $C_{18}C_{18}^+$ with varying amounts of $C_{16}EO_{20}$ are allowed to equilibrate for 60 h, vesicle saturation is observed below 30 mol% as is indicated by a decrease in scattered intensity (Figure 2.13A). This is in agreement with the turbidity experiments, where samples were prepared following the same procedure as here (Figure 2.3). However, $Z_{\text{ave}}$ continues to increase up to 60 mol% of $C_{16}EO_{20}$ due to incorporation of $C_{16}EO_{20}$ into the remaining vesicles. Micelles are only detected when their relative concentration is rather high and hence they are only observed above 93 mol% of $C_{16}EO_{20}$ (Figure 2.13B). This latter solution was examined as a function of time after 60 h of equilibration. Figure 2.13C shows that the scattered intensity is still rather high, and only drops by about 5% in the course of 16 h. Strangely enough, the $Z_{\text{ave}}$ values scatter either around 52 nm or around 75 nm, but not in between. They do not vary with time. We have no explanation for this observation, but we can exclude that this effect is a result of micelle formation or changes fluctuations in micelle and vesicle concentrations. This is mainly exemplified by similar size distributions for these different reported $Z_{\text{ave}}$ values (data not shown).

In principle, solutions prepared using the first and third procedure should yield similar results, since both solutions are allowed to equilibrate. However, they do not. The mole fraction of saturation and the mole fraction at which micelles are detected are different for both procedures (ca. 5 mol% and ca. 30 mol% for the first and third procedure, respectively). In addition, scattered intensities and $Z_{\text{ave}}$ do not change significantly with time. These complications probably arise from the fact that these systems are metastable, leading potentially to different metastable states, depending on the preparation method. Based on these observations and the results from solutions prepared via the second
procedure (Figure 2.11), we propose that trafficking of $\text{C}_{16}\text{EO}_{20}$ across the bilayer is rather slow, since otherwise there would be more consistency between the different procedures for vesicle preparation.

### 2.3.3.4 n-Dodecyl-β-Maltoside

Stock solutions of about 30 mM $\text{C}_{18}^{+}$ were prepared by cosonicating $\text{C}_{16}^{+}$ and $\text{C}_{12}\text{Mal}$, followed by extrusion. These solutions behaved somewhat unusually in their macroscopic behaviour. The solutions with 25 mol% of $\text{C}_{12}\text{Mal}$ became slightly turbid upon standing, but gentle shaking made the solutions transparent with a bluish colour. After about 5 min. they became turbid again. This process was repeatable. After addition of 2.25 mM NaOH from a 1 M stock solution a white precipitate was observed, which disappeared upon vigorous shaking, leading to a transparent bluish solution that was stable overnight (as observed by eye). Precipitation after NaOH addition was also observed for solutions with 50 mol% of $\text{C}_{12}\text{Mal}$. However, this solution was also rather viscous, which is indicative of the presence of worm-like micelles.\cite{61,62}

When the scattered intensity of these various solutions is considered (Figure 2.14A), it can be seen that above 25 mol% of $\text{C}_{12}\text{Mal}$ the scattered intensity decreases.
The figure caption can be found on the next page.
Figure 2.14. A: Plot of scattered intensity versus the mole percentage $\text{C}_{12}\text{Mal}$. No $\text{NaOH}$ added (■); 2.25 mM $\text{NaOH}$ added (●); diluted from a 30 mM stock solution after visual precipitation upon $\text{NaOH}$ addition (▲). B-D: Size distributions for 10 mol% of $\text{C}_{12}\text{Mal}$ (B), 25 mol% of $\text{C}_{12}\text{Mal}$ (C) and 50 mol% of $\text{C}_{12}\text{Mal}$ (D). B: 2.25 mM $\text{NaOH}$. C+D: Solid line no $\text{NaOH}$ added; dashed line 2.25 mM $\text{NaOH}$ added; dotted line represents the visually precipitated solution. D: thin lines represent data directly after preparation; thick lines after 15 h equilibration.

DLS experiments were performed using solutions prepared in three different ways. In all three cases the vesicles were prepared as described in the beginning of this paragraph, but then $\text{NaOH}$ was either not added, or added to a dilute vesicular solution, or to a concentrated vesicular solution, that was then shaken and subsequently diluted to the same concentration as the other solutions (0.5 mM $\text{C}_{18}\text{C}_{18}$).

The trend in the scattered intensity going from 25 mol% of $\text{C}_{12}\text{Mal}$ to 50 mol% of $\text{C}_{12}\text{Mal}$ is the same for all three preparation methods. Upon equilibration overnight the scattered intensity of the solution containing 50 mol% of $\text{C}_{12}\text{Mal}$ is increased for all three preparation methods. However, the absolute values are not the same.

The solution containing 10 mol% of $\text{C}_{12}\text{Mal}$ (Figure 2.14B) shows a monomodal distribution comparable to a solution without $\text{C}_{12}\text{Mal}$ (Figure 2.8B), except that the distribution is narrower. At 25 mol% of $\text{C}_{12}\text{Mal}$, in the absence of $\text{NaOH}$, the distribution is bimodal (maxima at 10 nm and 125 nm), similar as in the presence of 2.25 mM $\text{NaOH}$. However, in the presence of $\text{NaOH}$ the distribution is narrower. The small size peak is rather small, but this does not necessarily mean that the concentration of small particles (micelles) is small, since their scattering ability is rather poor. Vesicles from the precipitated solution show an even narrower distribution. At 50 mol% of $\text{C}_{12}\text{Mal}$, in the absence of $\text{NaOH}$, there is a peak around 100 nm, but a large peak is showing up at sizes smaller than 5 nm. This observation is in agreement with the low scattering intensity and indicates that the solution mainly consists of (worm-like) micelles and a few larger aggregates. Upon the addition of 2.25 mM $\text{NaOH}$ smaller sized vesicles are formed (30 nm), but the distribution is rather broad (starting at 4 nm, and ending at 300 nm). Therefore we suggest that this solution contains a broad mixture of large vesicles, worm-like micelles, and perhaps also spherical micelles. For all three solutions containing 50 mol% of $\text{C}_{12}\text{Mal}$ slightly larger vesicles are formed overnight, consistent with large increases in the scattering intensity. At the small-particle side of the graph there is still a small peak indicating that there is still a large number of small aggregates. However, at the large particle-side of the distribution small peaks are appearing indicating that also some large aggregates are formed. In fact, we visually observed on the bottom of the cuvet some precipitated vesicles, which could not be solubilised upon shaking. Growth of vesicles has also been observed in other solubilisation studies.\textsuperscript{27,28,29}

It is difficult to be fully convinced of the presence of small micelles in the previous solutions, since their scattering is usually negligible compared to the scattering from large aggregates. However, if, due to the transformation of vesicles into micelles, the scattered intensity decreases, this must be due to micelle formation. This decrease can be seen by the decrease in scattered intensity going from 25 mol% to 50 mol% of $\text{C}_{12}\text{Mal}$ and therefore we anticipate that significant micelle formation occurs above 25 mol% of $\text{C}_{12}\text{Mal}$. 
In order to study the dynamics of the systems, five solutions containing a fixed concentration of \( \text{C}_{18}\text{C}_{18}^+ \) and various amounts of \( \text{C}_{12}\text{Mal} \) were prepared. A certain amount of a concentrated stock solution of \( \text{C}_{12}\text{Mal} \) was added to a solution containing 2.1 mM \( \text{C}_{18}\text{C}_{18}^+ \) and 2.25 mM NaOH. Then they were left for 60 h to equilibrate.

In the turbidity experiments (Figure 2.4) it can be seen that at 2.1 mM \( \text{C}_{18}\text{C}_{18}^+ \), saturation and solubilisation is supposed to occur around 50 mol\% and 65 mol\% of \( \text{C}_{12}\text{Mal} \), respectively. In fact, at 50 mol\% of \( \text{C}_{12}\text{Mal} \), \( Z_{\text{ave}} \) and the scattered intensity reach a maximum. However, at 65 mol\% still not all vesicles have been solubilised into mixed micelles considering the scattered intensity. Even at 80 mol\% of \( \text{C}_{12}\text{Mal} \) complete solubilisation is not achieved. Apparently, turbidity experiments are not able to detect the remaining vesicles, or bilayer fragments.

![Figure 2.15. Plot of the scattered intensity and \( Z_{\text{ave}} \) (A) and size distribution (B) of vesicles formed from \( \text{C}_{18}\text{C}_{18}^+ \) and \( \text{C}_{12}\text{Mal} \) at various mole fractions. A: scattered intensity (■) and \( Z_{\text{ave}} \) (×). B: \( \text{C}_{12}\text{Mal} \): 0 mol\% (solid line); 32 mol\% (dashed line); 48 mol\% (dotted line); 65 mol\% (dash-dotted line) and 79 mol\% (dash-double-dotted line).](image-url)
The size distributions shown in Figure 2.15B are in agreement with those shown in Figure 2.14. The most surprising feature is the absence of any indication of micelle formation. The maximum in the size distribution decreases from around 80 nm to 20 nm, but there appears no peak in the spherical micellar region as was observed for \( \text{C}_{16}\text{EO}_{20} \).

A shift in the maximum, rather than the appearance of a peak around 5-6 nm has also been observed in the transition from vesicles to worm-like micelles to spherical micelles.\(^{61,62} \)

Therefore, we propose that solubilisation by \( \text{C}_{12}\text{Mal} \) proceeds via worm-like micelles, whereas solubilisation by \( \text{C}_{16}\text{EO}_{20} \) proceeds directly (mainly) to spherical micelles.

We do not fully understand all the processes going on in solutions containing \( \text{C}_{18}\text{C}_{18}^+ \) and \( \text{C}_{12}\text{Mal} \). Especially the addition of NaOH leads to substantial changes in the behaviour of the aggregates. It appears that upon addition of NaOH the vesicles tend to grow, but the extent and rate depend on the exact procedure.

### 2.3.3.5 (2,3-Bis-n-Octadecyloxy-Propyl)-Trimethylammonium Chloride

In order to study a molecule more closely related to phospholipids, but without the structural complexities, such as the ester linkage and dipole moment orientation, we decided to study (2,3-bis-n-octadecyloxy-propyl)-trimethylammonium chloride (\( \text{C}_{18}\text{C}_{18}^+ \); Scheme 2.6). This molecule has the same structure as \( \text{C}_{18}\text{C}_{18}^+ \), except that the hydrophobic tails are not directly connected to the head group, but they are connected via a glycerol linker. Contrary to most phospholipids, the tails are connected to the glycerol unit via an ether bond, rather than an ester bond, thereby avoiding complications arising from hydrolysis.

![Scheme 2.6. (2,3-Bis-n-octadecyloxy-propyl)-trimethylammonium chloride \( \text{C}_{18}\text{C}_{18}^+ \)](image)

The solubility of \( \text{C}_{18}\text{C}_{18}^+ \) is rather poor compared to that of \( \text{C}_{18}\text{C}_{18}^+ \). The upper limit of solubilisation is around 25 mM. Figure 2.16 shows the size distribution of a 0.5 mM solution. Rather unexpectedly, besides vesicles with a size of around 100 nm, also micelles are formed (maximum 5-6 nm). This is probably the result of an unfavourable bilayer packing.
2.3.4 Differential Scanning Microcalorimetry

2.3.4.1 General Considerations

In Chapter 1 it was discussed that an important feature of vesicles is that alkyl tails of the amphiphiles in membranes usually can reside in two states. At low temperatures the tails are in a highly ordered, rigid (gel-like) state, and at high temperatures the tails are transferred into a more fluid (liquid-crystalline) state. The temperature for this morphological change is typical for each amphiphile and is called the main phase transition temperature ($T_m$).

Differential scanning microcalorimetry (DSC) is a powerful non-invasive technique for measuring the main phase transition temperature, since it requires no fluorescent or spectroscopic probe. Instead it measures the excess heat that has to be added to the sample cell containing the vesicular solution relative to a reference cell that solely contains water, or buffer, while monotonically increasing the temperature of the cells at a constant rate. This increase is typically 1 degree per minute. Usually, the temperature at which the scan of excess heat versus temperature shows a maximum is taken as the main phase transition temperature. This is, however, in principle incorrect. It is more correct to take the main phase transition temperature at the point where the scan starts to deviate from the base line, since it is at that point that the tails start to go from one phase to the other. In the case of phospholipids the transition is usually rather narrow, indicating that all the phospholipid molecules in the membrane go from the gel-like state into the liquid-crystalline in a cooperative way. This is described by the “patch number”, which is the number of molecules that go from one phase to the other at the same time. In our system transitions are generally broader, making it more difficult to determine the temperature at which the measured excess heat starts to deviate from the base line. Therefore, we decided to take the maximum of the scans as an indication of the main phase transition temperature.
In addition to the main phase transition, other phase transitions can occur as well. Most of them are transitions to non-lamellar structures, such as inverted hexagonal phases, cubic phases, etc.\textsuperscript{76} In membranes the most common additional transition beside the gel-like to liquid-crystalline transition is the transition to the "ripple-phase" (also often referred to as the "pre-transition"),\textsuperscript{66,68,70,77-79} which is found in between the gel-like and liquid-crystalline phases. In this phase the membrane adopts a ripple-like structure, where the tails are still in a gel-like conformation. Since this phase is structurally not significantly different from the gel-like state, the enthalpy of this change is usually small leading only to a small peak relative to the main phase transition.

### 2.3.4.2 Sodium Di-n-Decylphosphate

The $T_m$ for $C_{18}C_{18}^+$ vesicles is 40°C (Figure 2.17) which agrees with the literature (37-40°C)\textsuperscript{80} and for $C_{10}C_{10}^-$ vesicles 8°C.\textsuperscript{12} In Figure 2.17 the scans for pure $C_{18}C_{18}^+$ and mixtures of $C_{18}C_{18}^+$ with $C_{10}C_{10}^-$ are shown. At 10 mol% of $C_{10}C_{10}^-$ a second peak arises around 27°C and this peak becomes more prominent until it reaches a maximum at 50 mol% of $C_{10}C_{10}^-$. At the same time the peak at 40°C decreases in size and slowly moves towards 30°C, disappearing at 30 mol% of $C_{10}C_{10}^-$. Above 50 mol% of $C_{10}C_{10}^-$ there is only one peak and upon increasing the amount of anionic amphiphile the peak shifts towards 8°C. Above 75 mol% of $C_{10}C_{10}^-$ it was impossible to perform DSC scans since precipitation took place a few minutes after preparation of the vesicles.

These results can be explained in terms of the presence of neutral microdomains between 10 and 30 mol% of $C_{10}C_{10}^-$. The peak arising in the range 23-27°C must have a 1:1 cationic:anionic amphiphile ratio since it is the only peak that is observed at 50 mol% of $C_{10}C_{10}^-$. Therefore we contend that at 10 mol% of $C_{10}C_{10}^-$ neutral microdomains are formed besides a mainly cationic phase. At 30 mol% of $C_{10}C_{10}^-$ these microdomains resolve into a single homogeneous phase again. At the same time as the microdomains are formed, the cationic domains possess an increasing amount of $C_{10}C_{10}^-$ randomly mixed-in since the peak in the DSC scans progressively moves towards lower temperatures.

In principle the appearance of two peaks in the DSC scans could also be due to the presence of two types of vesicles in solution, but this option was ruled out since all cryo-EM pictures show only one type of vesicle present. If there would be two types of vesicles in solution one would expect to see both cationic ("lens"-type vesicles; Figure 2.1a) and catanionic vesicles (aggregates of spherical vesicles; Figure 2.1d) since only the peaks corresponding to these type of bilayers are observed in the DSC scans. Since this is not the case, the peak in the DSC scan at 27°C should belong to neutral microdomains.
Figure 2.17. Heating scans for mixtures of $C_{18}C_{18}^+$ and $C_{10}C_{10}^-$ vesicles. The number denotes the mole percentage of $C_{10}C_{10}^-$ as a function of the total amphiphile concentration. Lines have been displaced vertically for clarity.

2.3.4.3 Sodium n-Decyl-n-Octadecylphosphate

In nature phospholipids with two tails differing significantly in length can be a result of auto-oxidation of unsaturations in one of the tails. The $T_m$ for vesicles formed from the synthetic asymmetric amphiphile sodium n-decyl-n-octadecylphosphate ($C_{10}C_{18}$) is 21°C. 

Scheme 2.7. Sodium n-decyl-n-octadecylphosphate ($C_{10}C_{18}$).
This is considerably lower than the $T_m$ of vesicles formed from sodium di-$n$-octadecylphosphate (85°C), but close to that of $\text{C}_{10}\text{C}_{10}^-$ (11°C). In Figure 2.18 the scans for pure $\text{C}_{18}\text{C}_{18}^+$ and mixtures of $\text{C}_{18}\text{C}_{18}^+$ with $\text{C}_{10}\text{C}_{18}^-$ are shown. Already at 5 mol% of $\text{C}_{10}\text{C}_{18}^-$ a second peak arises around 34°C and this peak becomes more prominent until it reaches a maximum at 20 mol% of $\text{C}_{10}\text{C}_{18}^-$. At the same time the peak at 40°C decreases in size and slowly moves towards 37°C, disappearing at 20 mol% of $\text{C}_{10}\text{C}_{18}^-$. This behaviour is similar to what is observed for addition of $\text{C}_{10}\text{C}_{10}^-$, except that the peaks appear and disappear at much lower molar ratios. Above 20 mol% of $\text{C}_{10}\text{C}_{18}^-$ a new peak arises at 36°C that subsequently shifts towards 42°C (at 50 mol% of $\text{C}_{10}\text{C}_{18}^-$) being the only peak above 40 mol% of $\text{C}_{10}\text{C}_{18}^-$. Above 50 mol% the peak decreases towards the phase transition temperature of 100 mol% of $\text{C}_{10}\text{C}_{18}^-$.

![Figure 2.18](image-url)

**Figure 2.18.** Heating scans for mixtures of $\text{C}_{18}\text{C}_{18}^+$ and $\text{C}_{10}\text{C}_{10}^-$ vesicles. The number denotes the mole percentage of $\text{C}_{10}\text{C}_{10}^-$ as a function of the total amphiphile concentration. Lines have been displaced vertically for clarity.
It is tempting to conclude that for mixtures of $\text{C}_{18}\text{C}_{18}^+$ and $\text{C}_{10}\text{C}_{18}^-$ just as for $\text{C}_{10}\text{C}_{10}^-$ neutral microdomains are formed. However, mixtures of $\text{C}_{18}\text{C}_{18}^+$ and $\text{C}_{10}\text{C}_{18}^-$ possess a more complex behaviour (Figure 2.20). For mixtures of $\text{C}_{18}\text{C}_{18}^+$ and $\text{C}_{10}\text{C}_{10}^-$ the upcoming peak that is related to neutral microdomains has its maximum roughly at the average of the maxima of 100 mol% of $\text{C}_{10}\text{C}_{10}^-$ and 100 mol% of $\text{C}_{18}\text{C}_{18}^+$. However, in mixtures of $\text{C}_{18}\text{C}_{18}^+$ and $\text{C}_{10}\text{C}_{18}^-$ the peak that has its maximum at this average disappears above 30 mol%. In addition, at 50 mol% the maximum in the DSC scan is at a higher temperature than for 100 mol% of $\text{C}_{18}\text{C}_{18}^+$. Finally, pre-transitions are observed for solutions containing between 5 and 45 mol% of $\text{C}_{10}\text{C}_{18}^-$. These observations make it difficult to assign the peaks to particular phases.

2.3.4.4 Sodium Dimethylphosphate

Sodium dimethylphosphate ($\text{C}_{01}\text{C}_{01}^-$; Scheme 2.8) is not an amphiphile since the tails are too short. However, we decided to study mixtures of $\text{C}_{18}\text{C}_{18}^+$ and $\text{C}_{01}\text{C}_{01}^-$ in order to be able to distinguish between the hydrophobic (tails) and electrostatic (head group) interactions with the cationic amphiphile $\text{C}_{18}\text{C}_{18}^+$. The lack of hydrophobic interactions is clear from the near independence of the main phase transition temperature of the concentration $\text{C}_{01}\text{C}_{01}^-$ (Figure 2.19). It has been reported that salt can increase the main phase transition with by few degrees, this is usually found only at higher salt concentrations. However, the addition of $\text{C}_{01}\text{C}_{01}^-$ seems to induce a pre-transition.

Scheme 2.8. Sodium dimethylphosphate ($\text{C}_{01}\text{C}_{01}^-$).

Figure 2.19. Heating scans for mixtures of $\text{C}_{18}\text{C}_{18}^+$ and $\text{C}_{01}\text{C}_{01}^-$. The number denotes the mole percentage of $\text{C}_{01}\text{C}_{01}^-$ as a function of the total amphiphile concentration. Lines have been displaced vertically for clarity.
2.3.4.5 Effects of Anionic Double-Tailed Amphiphiles. A Summary

Figure 2.20 shows an overview of the temperatures at which the peaks are observed in the thermograms of mixtures of C\textsubscript{18}C\textsubscript{18}+ with C\textsubscript{10}C\textsubscript{10}-, C\textsubscript{10}C\textsubscript{18}- and C\textsubscript{01}C\textsubscript{01}-. The different effects induced by the three different anionic amphiphiles are clear. The anionic “amphiphile” C\textsubscript{01}C\textsubscript{01}- has no influence on the phase transition temperature, as anticipated for an inert ion.

**Figure 2.20.** Maxima in the DSC scans as a function of the bilayer composition. Closed symbols indicate the major peak; open symbols are for the minor peaks. C\textsubscript{10}C\textsubscript{10} (▲); C\textsubscript{10}C\textsubscript{18} (■); C\textsubscript{01}C\textsubscript{01} (×; +). Solid lines follow the trend of the initial major peak, dotted lines follow the trend of upcoming peaks and dashed lines follow the trend of pre-transitions.

In the literature only a few studies concerning mixtures of double-tailed cationic and double-tailed anionic amphiphiles with respect to the influence on the phase transition temperature are known.\textsuperscript{70,87} This is not unexpected since these types of mixtures readily form non-lamellar phases.\textsuperscript{88-90} We made an endeavour to form stable vesicles from mixtures of C\textsubscript{18}C\textsubscript{18}+ with sodium di-n-octadecylphosphate (C\textsubscript{18}C\textsubscript{18}-) and sodium dioleylphosphate (C\textsubscript{18:1}C\textsubscript{18:1}-; Scheme 2.9). However, already at 5 mol% of anionic amphiphile precipitation was observed.

**Scheme 2.9.** A: Sodium di-n-octadecylphosphate (C\textsubscript{18}C\textsubscript{18}-). B: Sodium dioleylphosphate (C\textsubscript{18:1}C\textsubscript{18:1}-).
In a 65:35 molar ratio mixture of several cationic amphiphiles and an anionic amphiphile (Scheme 2.10) the main phase transition temperature was significantly higher for the mixture than for the pure components. Depending on the exact structure the peaks are either narrow, or broad showing several maxima. The increase in the main phase transitions temperature was mainly attributed to the electrostatic interaction between the head groups, since the phase behaviour of mixtures of the anionic amphiphile with the fully \( N \)-methylated amphiphile is similar to that for the tertiary amine amphiphile (\( C_{16}C_{16}^+ \)). Other work suggests that hydrogen bonding plays a more important role.

![Scheme 2.10. Cationic and anionic synthetic phospholipid analogues (three compounds in the top), sphingosine (SP) and stearylamine (SA)](image)

More is known about the phase behaviour with respect to the phase transition temperatures of mixtures of cationic and anionic amphiphiles, where one of the amphiphiles has only one tail. Mixtures of a negatively charged phospholipid (DPPS) with sphingosine (the only natural occurring cationic surfactant\(^93\)) and with stearylamine (Scheme 2.10) were studied at different molar ratios and pH values. The DSC scans show at low pH (when all SP and SA is protonated) that only thermograms with a single peak are observed in the (near) single-component region or when both components are present in (nearly) equal amounts. In addition, at any given molar ratio the \( T_m \) is higher than the \( T_m \) of the single components, reaching a maximum at the equimolar ratio. The latter results were also found for synthetic analogues. As for mixtures of double-tailed cationic and anionic amphiphile mixtures the effect is attributed to the electrostatic attraction resulting in a tighter packing of the amphiphiles. However, mixtures of SA and the zwitterionic phospholipid DPPC also show that at low pH the \( T_m \) increases. The maximum increase only amounts to ca. 5 degrees, whereas in the case of negatively charged amphiphile this effect is about 12 degrees, suggesting that hydrogen bonding plays only a partial role. In the case of double-tailed cationic ammonium amphiphiles and an anionic single-tailed surfactant that are not capable of forming hydrogen bonds, a large (ca. 20°C) increase in \( T_m \) relative to the \( T_m \) of the ammonium amphiphiles is also observed. The mixture reaches its maximum at the equimolar ratio. However, the thermograms show often multiple
and/or broad maxima that are not always reproducible for the subsequent heating scans, indicating a dynamic metastable system.\(^\text{25}\n\)

Despite the large structural differences between the amphiphiles it can be concluded that in most cases, described in the current literature of mixtures of cationic and anionic amphiphiles, the phase transition temperature increases, when vesicles are formed from oppositely charged amphiphiles, reaching a maximum at the equimolar ratio. The thermograms often show multiple and/or broad peaks, indicating that there are different domains or various transitions in the bilayer and that those can be highly dynamic and metastable. The increase in \(T_m\) is due to electrostatic and, if possible, hydrogen-bond interactions, that leads to a tighter packing of the alkyl tails. With this in mind, it is remarkable that mixtures of \(\text{C}_{18}\text{C}_{18}^+\) and \(\text{C}_{10}\text{C}_{18}^-\) do not show a maximum in the phase transition temperature at the equimolar ratio. We speculate that this is due to the large hydrophobic mismatch in the alkyl tails inhibiting an efficient packing of the tails. This mismatch even leads to worse packing relative to pure \(\text{C}_{18}\text{C}_{18}^+\). Similar behaviour was found when negatively charged phospholipids (with \(n\)-dodecyl tails) were mixed with a series of \(n\)-alkyltrimethylammonium bromides.\(^\text{96,97}\) The \(T_m\) was depressed for an \(n\)-octyl tail, but increased for a \(n\)-tetradecyl tail. Both \(n\)-decy- and \(n\)-dodecyl-tailed surfactants showed biphasic behaviour, i.e. at low mole fraction an increase in \(T_m\) and at high mole fraction a decrease in \(T_m\). Probably, for these mixtures, initially due to favourable charge interactions lead to more efficient chain packing, whereas at higher concentration the large number of single-tailed surfactants in the bilayer counteracts this favourable interaction. We anticipate that similar behaviour is to be expected when amphiphiles with opposite charge are mixed and at least one of the tails contains an unsaturation. However, no such an example is present in the literature. Mixtures of \(\text{C}_{18}\text{C}_{18}^+\) and \(\text{C}_{10}\text{C}_{18}^-\) show at low mole fraction similar behaviour as mixtures with \(\text{C}_{10}\text{C}_{10}^-\), but at higher mole fractions the main phase transition temperatures increase again. Apparently, the mismatch is not pronounced enough to inhibit efficient packing in mixtures with \(\text{C}_{10}\text{C}_{18}^-\). This can also be seen in the phase transition temperature of pure \(\text{C}_{10}\text{C}_{10}^-\) that is also lower than that of \(\text{C}_{10}\text{C}_{18}^-\).

**2.3.4.6 Long Linear Alcohols**

Addition of small amounts (<20 mol%) of saturated \(n\)-octadecyl alcohols to cationic vesicles of \(\text{C}_{18}\text{C}_{18}^+\) leads to a decrease of maximum 5 degrees in the main phase transition temperature. At higher mole fractions the main phase transition temperature increases, but much more rapidly and to a higher temperature for \(n\)-octadecanol (\(\text{C}_{18}\text{OH}\); Scheme 2.11C) than for butyl alcohol (\(\text{C}_{18}\text{GOH}\); Scheme 2.11D). At 50 mol% the main phase transition temperature is 60°C and 45°C for \(\text{C}_{18}\text{OH}\) and \(\text{C}_{18}\text{GOH}\), respectively. Addition of \(n\)-decanol (\(\text{C}_{10}\text{OH}\); Scheme 2.11A) leads to a decrease to 30°C at 30 mol% and it only increases again above 50 mol% to reach a temperature of 33°C at 66 mol%.\(^\text{65}\) Finally, addition of oleyl alcohol (\(\text{C}_{181}\text{OH}\); Scheme 2.11B) leads to a similar pattern as for \(\text{C}_{10}\text{OH}\), except that the peaks are becoming much broader, indicating that the transition becomes much less cooperative.

These results are in agreement with literature reports where \(n\)-alcohols behave like amphiphiles leading to a decrease in the phase transition temperature for alcohols with
short (< C\textsubscript{10}) chains and an increase for alcohols with a long (> C\textsubscript{10}) chain.\textsuperscript{66-69,98-100} Also broadening of peaks has been observed.\textsuperscript{66,67,69} Experiments with linear carboxylic acids show similar trends.\textsuperscript{66,68}

\begin{center}
\textbf{Scheme 2.11.} A. \textit{n-Decanol (C\textsubscript{10}OH)}; B. \textit{Oleylalcohol (C\textsubscript{18:1}OH)}; C. \textit{n-Octadecanol (C\textsubscript{18}OH)}; A. \textit{Batyl alcohol (C\textsubscript{18}GOH)}.
\end{center}

In fact, the DSC scans are more complex than described above. Several control experiments were performed to check the reproducibility. Unfortunately, the reproducibility in certain mixtures is not too high (e.g. 20 mol\% of C\textsubscript{18}OH, Figure 2.21B), indicating that the vesicles are metastable and that their exact structure depends on the time between preparation and experiment, the procedure that was followed to make the vesicles, and probably some more variables. Also, the fact that many scans show more than one peak indicates that there is either more than one transition or that the alcohol and amphiphile are not homogeneously mixed, or both. Similar effects were seen for alcohol/phospholipids mixtures.\textsuperscript{98} This is especially the case for the mixtures with C\textsubscript{18}OH and C\textsubscript{18:1}OH, and to a much lesser extent for C\textsubscript{10}OH and C\textsubscript{18}GOH.
Figure 2.21. Heating scans for mixtures of $\text{C}_{18}\text{C}_{16}^+$ with $\text{C}_{18}\text{OH}$ (A), $\text{C}_{18}\text{OH}$ (B), $\text{C}_{18}\text{GOH}$ (C) and $\text{C}_{18:1}\text{OH}$ (D). The number denotes the mole percentage of alcohol as a function of the total amphiphile concentration. In C the heating scan for 26 mol% is represented by the dashed line. Lines have been displaced vertically for clarity.
The reason for this multiple phase formation might come from the high melting point of \( C_{18}OH \) and \( C_{18}GOH \). In combination with the unfavourable packing parameter of \( C_{18}OH \) (>1), this leads to a situation where \( C_{18}OH \) dissolution in membranes of \( C_{18}C_{18}^+ \) is rather unfavourable. Crystallisation of small domains rich in \( C_{18}OH \) within the membrane might occur. \( C_{18}GOH \) has a larger hydrophilic moiety, leading to a more favourable packing parameter and therefore it might lead to a better dissolution in the membrane making crystallisation in the membrane less favourable. The large increase in the phase transition temperature in the presence of \( C_{18}OH \) is in agreement with the observation that there is an increase in ordering in the bilayer. Phospholipids with unsaturation in the tails have usually a phase transition temperature below 0°C, since these unsaturations disrupt the bilayer and make efficient packing difficult. Likewise, in the dry state phospholipids with unsaturation are usually more wax-like than their saturated analogues. Therefore it is a bit surprising to notice that addition of \( C_{18:1}OH \) does not lead to a larger decrease in phase transition temperature than observed here. The broadening of the peaks is anticipated on the basis of the literature.

![Figure 2.22. Phase transitions of \( C_{18}C_{18}^+ \) vesicles with added alcohols. Closed symbols are major peaks and open symbols are minor peaks. \( C_{18}OH \) (\( \bullet \)); \( C_{18}OH \) (\( \bigcirc \)); \( C_{18}GOH \) (\( \blacksquare \)); \( C_{18:1}OH \) (\( \blacktriangle \)). Lines are only drawn to guide the eye.](image)

### 2.3.4.7 Nonionic Single-tailed Surfactants

As can be seen in Figure 2.23A-C the addition of additives with a larger hydrophilic part compared to that of the alcohols leads to a lowering of the main phase transition temperature. In fact, these single-tailed surfactants destabilise vesicles (disturb the packing of the bilayer, section 2.3.3.4). Linear alcohols can, in principle, destabilise vesicles as well. However, due to their small hydrophilic moiety their packing parameter is larger than 1, whereas single-tailed surfactants generally have a packing parameter around \( 1/3 \). Therefore the alcohols are not able to solubilise the vesicles into mixed micelles. With this in mind the decrease in the main phase transition temperature is not surprising, since the main phase transition temperature for a great deal reflects the packing efficiency of the tails.
which is disturbed by the addition of single-tailed surfactants. A decrease in the main phase transition temperature was also observed for phospholipid and cationic vesicles with cationic, anionic and nonionic surfactants.\textsuperscript{92,96,97,102-104}
Figure 2.23. Heating scans for mixtures of $C_{18}C_{18}^+$ and $C_{12}Mal$ (A), $C_{12}Glu$ (B), $C_{12EO_{20}}$ (C). The number denotes the mole percentage of alcohol as a function of the total amphiphile concentration. Lines have been displaced vertically for clarity. A: dotted line is a solution prepared by mixing a vesicular solution of $C_{18}C_{18}^+$ with a micellar solution of $C_{12}Mal$. D: Maxima in the DSC scans as a function of the bilayer composition. Closed symbols are the major peaks; open symbols are minor peaks. $C_{12}Mal$ (●); $C_{12}Glu$ (♦); $C_{12EO_{20}}$ (▲). The line is only drawn to guide the eye.

Figure 2.23D shows an overview of the maxima in the peaks. Surprisingly, the different additives more or less have a similar influence on the phase transition temperatures. At higher mole fractions the number of additional transitions increases, which is in line with the potential appearance of other morphologies than vesicular bilayers. This is especially the case upon the addition of $C_{16EO_{20}}$. At 35 mol% the solution contains a mixture of vesicles and mixed (worm-like) micelles, as is shown by both turbidity experiments (Section 2.3.2.1) and dynamic light scattering (Section 2.3.3.4).

2.3.4.8 4-(Dioleylethyl)-1-(PEG$_{5000}$OCH$_3$)-Pyridinium Bromide

4-(Dioleylmethyl)-1-(PEG$_{5000}$OCH$_3$)-pyridinium bromide (SAINT-44; Scheme 2.12) is similar to $C_{16EO_{20}}$ with respect to the head group. Both molecules have a large ethylene glycol head group, but the size of the head group is over two times as large for SAINT-44. In addition, SAINT-44 is cationic, has a bromide counterion and has two oleyl tails (approximately 20% trans).
In water SAINT-44 forms micelles with a CMC in the micromolar range. Therefore, its binding efficiency towards vesicles formed from $C_{18}C_{18}^+$ is high (Section 2.3.2), resulting in almost complete binding. Unfortunately, SAINT-44 has a bromide counterion, and therefore we have to compare the results with vesicles formed from 95 mol% of $C_{18}C_{18}^+$ and 5 mol% of dimethylid-n-octadecylammonium bromide ($C_{18}C_{18}^+Br^-$). In Figure 2.24 the heating scans for the mixed vesicular systems are shown. Surprisingly, upon the addition of 5 mol% of $C_{18}C_{18}^+Br^-$ the main phase transition temperature is decreased, which disagrees with the literature where the $T_m$ is a few degrees higher for 100 mol% of $C_{18}C_{18}^+Br^-$ than for $C_{18}C_{18}^+$. An even lower $T_m$ is found when 5 mol% of SAINT-44 is added. This is not unlikely since micelle-forming surfactants lower the $T_m$ (Section 2.3.4.7) and the tails have unsaturations that further disturb the packing of the bilayer. In fact, the effect is quite large considering that the other additives do not have such a large effect at 5 mol%.

![Graph showing heating scans](image-url)

**Figure 2.24.** Heating scans for $C_{18}C_{18}^+$ (solid line), 5 mol% of $C_{18}C_{18}^+Br^-$ (dashed line) and 5 mol% of SAINT-44 (dotted line).

### 2.3.4.9 (2,3-Bis-n-Octadecyloxy-Propyl)-Trimethylammonium Chloride

The heating scan for vesicles formed from $C_{18}C_{18}G^+$ (Scheme 2.6) is shown in Figure 2.25. The main phase transition temperature is $56.8^\circ$C, which is about 17 degrees higher than that for vesicles formed from $C_{18}C_{18}^+$. Apparently the packing of the tails is much more efficient. This is probably a result from the presence of the glycerol linker. This linker is also found in phospholipids.
2.3.5 Membrane Polarity

2.3.5.1 Theoretical Considerations

With the aim to understand the influence of various additives on the properties of vesicles it is necessary to study the polarity of the polar-apolar interface both as a function of the type of additive and as a function of the mole fraction of additive in the bilayer. The polarity can be probed using several different dye molecules. A few commonly used dyes are shown in Scheme 2.13. However, their way of reporting the polarity, or better, their sensitivity towards changes in the chemical structure of solvents or changes in the composition of solvent mixtures is quite different, and this has led to different polarity scales for different dyes. \(^{110}\) For example, the wavelength of maximum fluorescence of Prodan shifts from 400 nm in cyclohexane to 530 nm in water. \(^{111}\) The fluorescence spectrum of pyrene, on the contrary, has several maxima, and the wavelengths of these maxima are rather insensitive upon a change in solvent. However, the relative intensity of the first and third peak \(I_1/I_3\) is sensitive for solvent polarity, leading to a value of \(I_1/I_3\) of 0.62 and 1.80 for \(n\)-hexane and water, respectively. \(^{112}\) It becomes more interesting when different classes of solvents are considered (hydrocarbons, alcohols, amines, etc.). Within a class there is usually a trend between the structural variation and the reported polarity. However, between classes a relationship between structure and reported polarity is not necessarily present. The origin of this effect comes from the different interactions that the dyes are sensitive to. Prodan and the \(E_4(30)\) dye are, for example, sensitive to hydrogen bonding, whereas pyrene is not. In solvent mixtures preferential solvation becomes important. These properties make it difficult to design a universal polarity scale, since the intermolecular forces (hydrogen bonds, dispersion forces, etc.) depend strongly upon both probe and solvent. However, the general features are usually similar, \(i.e.\) water is at the polar side of the scale and \(n\)-hexane on the apolar side. Reichardt has reviewed the literature about a number of solvatochromic
probes and several (empirical) models to describe the interactions between these dyes and solvents.\textsuperscript{110}

It should be noted that not all polarity scales are established using fluorescence spectroscopy. The $E_T(30)$ probe, for example, reports its local polarity through a change in the maximum UV/vis absorbance.

\begin{center}
\begin{figure}
\centering
\includegraphics[width=\textwidth]{scheme2_13}
\end{figure}
\end{center}

\textbf{Scheme 2.13.} Some examples of commonly used probes for measuring solvent polarity. (A) Pyrene; (B) Pyrene-3-Carboxaldehyde; (C) $E_T(30)$; (D) Prodan; (E) Nile Red.

In order to explain the observations discussed above, intramolecular energy transfer and specific dye-solvent interactions in fluorescent dyes have to be considered.\textsuperscript{113} When a dye molecule is excited from its ground state to an excited state, it usually rapidly decays ($10^{-12}$ s) to the first singlet state ($S_1$). Often the dipole moment is increased upon excitation, leading to fast solvent relaxation ($10^{-11}$–$10^{-10}$ s) in low-viscosity media\textsuperscript{114} and in polar solvents this leads to a lowering in the excited state energy. From this state, the locally excited state (LE), a photon can be emitted ($10^{-8}$ s). The more polar the solvent, the lower the excited state energy and hence, the longer the wavelength of emission. This effect of the solvent is called the general solvent effect. If specific dye-solvent interactions can occur, for example, by hydrogen bonding, the excited state can be further stabilised, and the wavelength is red-shifted. In addition to these two interactions the dye can form an internal charge-transfer state (ICT), or a twisted internal charge-transfer state (TICT). From the ICT and TICT also radiationless decay via electron transfer can occur, which makes the fluorescence yield in polar solvents for certain dyes low. Examples of such behaviour are ANS (Scheme 2.15) and Nile Red fluorescence in water. It should be noted that emission is always from the lowest-energy excited state. This means that in a series of solvents a change in mechanism of photon emission can occur. This is the reason that there are different polarity scales, since changes in fluorescence mechanism are dye-dependent. This is best seen in Lippert plots, where the difference in wavenumber of maximum absorption and emission is plotted against the orientation polarisability ($\Delta \mu$).\textsuperscript{115} If there is no change in fluorescence mechanism this plot yields a straight line that is related to the squared difference of the dipole moment in the ground and excited state. A change in emission mechanism is usually accompanied by a change in dipole moment of the excited state and hence the slope in the Lippert plot will change. A change in mechanism can also be
observed in other ways. Specific interactions are also the reason that certain dyes are, for example, rather sensitive in the polar solvent region, whereas they are not very sensitive in the apolar solvent region.

Some fluorescent dyes exhibit several peaks in their emission spectrum. This is the case when fluorescence occurs from different vibrational levels within one excited state. This means that the path to the ground level does not proceed exclusively via solvent relaxation, but partly directly from the vibronic level to which the dye was excited. Of course, partial relaxation to lower energy states cannot be excluded in all cases. The relative intensity of the peaks depends on the vibronic level of the preferred excited state. This type of fluorescence mechanism is shown by pyrene, but also by Nile Red fluorescence in very apolar solvents.

In micellar and vesicular aggregates the interpretation of the data becomes even more troublesome. The probes are sometimes rather large and their presence can induce local structural changes in the aggregates or even aggregation. The $E_T(30)$ probe, for example, is quite large compared to a micelle. But also for large aggregates the exact binding location of the probe is uncertain. Some probes seem to have different binding sites within micelles, other probes have more well-defined binding sites. This problem has partially been overcome by designing probes that are covalently bound to surfactants fixing the location of the probe. In other cases incomplete binding of the probe has to be taken into account, so that the measured signal is sometimes an average of the fluorescence spectra of the probe in water and in the aggregate. Moreover, additional interactions between the probe and the surfactant have to be considered.

These considerations make it difficult to assign a well-defined polarity to bilayers. However, by employing different dyes that are sensitive for different interactions, and by looking at trends, rather than absolute values, still useful information can be deduced from the experiments.

In order to have some way of comparing the different polarity scales the probes that report a wavelength of maximum fluorescence or absorbance the energy of the transition ($E_T$) can be calculated and subsequently normalised. The $E_T$ transition (in kcal mol$^{-1}$) is calculated according to eq. 2.11:

$$E_T = h c \nu_{\max} N_A = \frac{28591}{\lambda_{\max}}$$  \hspace{1cm} (2.11)

In this equation $h$, $c$ and $N_A$ are Planck’s constant, the speed of light and Avogadro’s number, respectively. $\lambda_{\max}$ and $\nu_{\max}$ are the wavelength (in nm) and frequency of the maximum absorption of the dye, respectively.

Usually, the normalised $E_T$ is then calculated by taking the $E_T$ value of tetramethylsilane (TMS) and water and adjusting them to 0 and 1, respectively, by employing eq. 2.12:

$$E_T^{\text{N}} = \frac{E_T(\text{solvent})-E_T(\text{TMS})}{E_T(\text{water})-E_T(\text{TMS})} = \frac{\lambda_{\max,\text{solvent}}^{-1} - \lambda_{\max,\text{TMS}}^{-1}}{\lambda_{\max,\text{water}}^{-1} - \lambda_{\max,\text{TMS}}^{-1}}$$  \hspace{1cm} (2.12)

For our purposes we normalised against water and 1,4-dioxane, since we anticipate that differences between the vesicles with the various additives are much smaller than the change from water to TMS. The normalised polarity $P^{w/d}$ can then be calculated via eq. 2.13:

$$P^{w/d} = \frac{\lambda_{\max,\text{mixt}}^{-1} - \lambda_{\max,\text{dioxane}}^{-1}}{\lambda_{\max,\text{water}}^{-1} - \lambda_{\max,\text{dioxane}}^{-1}}$$  \hspace{1cm} (2.13)
2.3.5.2 Laurdan Fluorescence

Laurdan has a similar structure as Prodan (Scheme 2.13D), except that the ethyl group is replaced by an undecanol group (Scheme 2.14). This ensures its full binding to aggregates possessing hydrophobic binding sites. The fluorescence spectrum of Laurdan in vesicles is independent of the structure of the head group, but depends on the phase of the tails.\textsuperscript{126,127} The fluorescence spectrum has two peaks. When the tails are in the gel-like state, the peak on the blue side of the spectrum (ca. 440 nm) is larger than the peak at the red side (ca. 490 nm). When the tails are in the liquid-crystalline state the peak on the red side is the largest peak. This change in the spectrum originates from the possibility of water molecules near the fluorescent probe to reorient on the time scale of the lifetime of the excited state when the tails are in the liquid-crystalline state. This reorientation is not possible when the tails are in the more rigid gel-like state.\textsuperscript{113,126} When 360 nm is chosen as excitation wavelength laurdan molecules residing in gel-like domains are mainly excited, whereas when 390 nm is chosen mainly laurdan molecules residing in liquid-crystalline domains are excited. Hence, the excitation spectrum can also be used to probe the phase of the tails. In principle, both the excitation and emission spectra lead to similar results. However, the excitation spectrum shows two clear peaks, whereas for the same sample the second peak in the emission spectrum is much less pronounced.

\begin{center}
\begin{overprint}
\includegraphics[width=0.3\textwidth]{Scheme2.14.pdf}

\textit{Scheme 2.14. Laurdan.}
\end{overprint}
\end{center}

The excitation or emission spectra of the same sample at various temperatures show a change at the main phase transition temperature. This change can be quantified by calculating the generalized polarization ($GP$):

$$GP = \frac{I_{\text{blue}} - I_{\text{red}}}{I_{\text{blue}} + I_{\text{red}}}$$

From a mathematical point of view it makes more sense to use the excitation spectrum, since this spectrum shows two clear peaks, whereas in the emission spectrum one of the peaks is usually just a small bump in the curve. When two clear peaks are present the peaks can be deconvoluted (Figure 2.26B) and the $GP$ can be calculated using the fitted intensity at the maxima. This gives better values for $GP$ than taking the measured intensities at fixed wavelengths. Contrastingly, in the literature usually the experimental intensity at fixed wavelengths is taken.\textsuperscript{126,128-135} Unfortunately, the calculated $GP$ from the deconvoluted spectra depends on the wavelengths between which the experimental data is fitted, illustrating the general problem that is encountered when two overlapping peaks are being deconvoluted.

In our set of experiments we are interested in the (relative) change of the state of the tails as a function of the type and molar ratio of additives at a fixed temperature (15°C). Therefore the trend in the calculated $GP$ is important, rather than the absolute value.
The excitation spectra were recorded following the fluorescence at 440 nm and 490 nm (Figure 2.27) in order to study the influence of selective excitation of laurdan molecules that are present in liquid-crystalline domains and gel-like domains. The $GP$ values were calculated using fits between 318 nm and 408 nm. The $GP$ is independent of the bilayer composition and emission wavelength for the range of additives studied here, despite the large structural variation in additives. The results are in agreement with our observation using differential scanning microcalorimetry, where we concluded that all phase transition temperatures are above $15^\circ C$. In addition, the wavelength of maximum excitation for both peaks is independent of the bilayer composition (data not shown). This wavelength has been suggested to be a measure for bilayer polarity.\textsuperscript{129} The large scattering in the data of Figure 2.27A compared to Figure 2.27B comes from the lower intensity (quantum yield) in the excitation spectrum, which makes it more difficult to deconvolute the peaks.

**Figure 2.26.** Typical plot of $GP$ versus temperature using fictional data (A) and laurdan excitation spectrum followed from the emission at 490 nm (B). Solid squares are experimental data points. Solid lines are at the wavelength of maximum fluorescence, dashed lines are the deconvoluted peaks, when fitted between 338 nm and 408 nm, dotted lines when fitted between 318 nm and 408 nm.
From eq. (2.14) it can be seen that the value of \(GP\) is 1 when all the amphiphiles are in the gel-like state, and -1 when in the liquid-crystalline state. These values are usually not reported in the literature since the peaks at 440 nm and 490 nm are rather broad. Also in this study the value of 1 or -1 is not reported suggesting that the bilayers are not fully in one type of state. The average value of \(GP\) of all the data points in Figure 2.27A and B are 0.32 and 0.25, respectively, indicating that in Figure 2.27B laurdan reports a slightly more fluid-like bilayer, which is in agreement with a better excitation of laurdan molecules present in liquid-crystalline domains. This approach assumes the presence of distinct liquid-crystalline and gel-like domains that are co-existing in bilayers. This assumption is also used for the calculation of patch numbers (Section 2.3.4.1)

![Figure 2.27](image)

**Figure 2.27.** Plot of \(GP_{exc}\) versus bilayer composition. Emission followed at (A) 440 nm and (B) 490 nm. \(C_{10}OH\) (■); \(C_{12}OH\) (▲); \(C_{12}GOH\) (▲); \(C_{12}OH\) (▲); \(C_{12}Glu\) (○); \(C_{12}Mal\) (◇); \(C_{18}EO_{20}\) (◇); \(C_{10}C_{10}r\) (×).
Chapter 2

2.3.5.3 $E_T(30)$ Absorbance

The $E_T(30)$ probe (Scheme 2.13) is an excellent solvatochromic dye to probe the polarity of solvents, and solvent mixtures. The wavelength of maximum absorbance shifts from 930 nm to 453 nm going from TMS to water, which makes it a very sensitive probe. For most dyes upon increasing the polarity of the solvent the wavelength of maximum absorbance shifts towards longer wavelength. However, for those dyes the ground state has a smaller dipole moment than the excited state, whereas for the $E_T(30)$ dye it is the other way around. This means that in polar solvents the ground state of the $E_T(30)$ dye is stabilised relative to the excited state, and in apolar solvents the $E_T(30)$ dye is destabilised. In addition, hydrogen bond donation stabilises the ground state. The absorbance is due to an intramolecular charge transfer from the negatively charged oxygen to the positively charged pyridinium ring leading to a diradical species.

Unfortunately, due to its size it is less suitable for measuring the polarity of micellar and vesicular surfaces. As can be seen in Figure 2.28 the reproducibility is rather poor. This can originate from several possibilities: 1. The probe might not be fully bound. We checked this by adding more $E_T(30)$, but this did not change the $E_T(30)$ value. 2. The structure of the aggregate might change. This is a well-known problem. Vesicles are metastable and their size, shape and stability depend on the preparation method. Hence, with time the structure of the bilayer aggregate changes. Depending on the molecular structure the rate of this may be highly variable. Addition of the large $E_T(30)$ probe might induce morphological changes, and depending on the history of the sample, this might lead to different structures.

Despite these problems, within the two series (initial and duplo experiments) there is a good correlation between the different data points, suggesting that upon the addition of the various additives the measured local polarity does not change too much. The average value of the normalised polarity ($P^{w/d}$) is 0.68, which corresponds to a polarity similar to methanol (0.72). This is consistent with an almost constant $E_T(30)$ value for several CTAB/alcohol micellar solutions.

![Figure 2.28. Plots of normalised polarity ($P^{w/d}$) as measured by $E_T(30)$ versus bilayer composition. Closed symbols are initial experiments, open symbols are duplo experiments. $C_{10}$OH (■); $C_{18}$OH (▲); $C_{18}$GOH (◆); $C_{18:1}$OH (▼); $C_{12}$Glu (○); $C_{12}$Mal (◇); $C_{10}C_{10}$- (×).](image-url)
2.3.5.4 Pyrene Fluorescence

Pyrene has been used already since the late 1970s for measuring solvent polarity and the polarity of micellar aggregates. The fluorescence spectrum shows several vibronic peaks of which the intensity depends on solvent polarity. The ratio of the first and third peak is usually taken as a measure of polarity. Hydrocarbon solvents generally have an $I_1/I_3$ value around 0.6, aromatic solvents between 1.0 and 1.3 and simple polar solvents between 1.3 and 2.0 (water: 2.0).

The wavelengths of the peaks in the fluorescence spectrum are hardly sensitive towards solvent polarity, which is reasonable since pyrene has no dipole moment in the ground state and only a small dipole moment in the locally excited state (LE). In the excited state it exhibits an LE state, with several vibronic levels. In water excitation is mainly to the first vibronic level leading to a relatively high intensity of $I_1$, and hence a large value of $I_1/I_3$. In more apolar media the energy minimum of the LE state is shifted with respect to the ground state minimum. As a result excitation is mainly to and emission is mainly from the third vibronic level, and hence $I_1/I_3$ becomes smaller.

Despite the lack of functional groups in pyrene, we anticipate that it binds in the Stern region, since arenes are known to bind near the interface of micelles.

An experimental problem in employing pyrene fluorescence in probing vesicular polarity is the background scattering. The background scattering is rather independent of the wavelength between 370 to 390 nm. This is the region of the first and third emission peak. The intensity of the background scattering depends both on the size and concentration of the vesicles. In practice the background scattering leads to a lowering of the value of $I_1/I_3$.

![Figure 2.29](image.png)

*Figure 2.29. Plots of $I_1/I_3$ versus bilayer composition. $C_{10}OH$ (■); $C_{18}OH$ (▲); $C_{18}GOH$ (▲); $C_{18:1}OH$ (▼); $C_{12}Glu$ (●); $C_{10}C_{10}$ (×).*

Plots of $I_1/I_3$ versus bilayer composition are shown in Figure 2.29. The value of $I_1/I_3$ varies between 1.2 and 1.35, which is comparable to a change from methanol to THF (1.33 and 1.20, respectively). There seems to be a trend that the addition of alcohol and pyranoside...
leads to a more apolar environment. Addition of $\text{C}_{10}\text{C}_{10}^{-}$ does not lead to a change in polarity, except that at 70 mol% there is quite some scattering. The value of 1.2-1.35 is similar to that which has been observed for cationic and nonionic micelles of DeTAB (1.28), CTAC (1.35), Triton-X100 (1.32) and $\text{C}_{12}\text{Mal}$ (1.24), but slightly higher than that for anionic micelles such as sodium n-dodecanoate (1.04) and SDS (1.14). Overall, it can be concluded that the change in polarity as sensed by pyrene does not change significantly in the presence of the additives.

### 2.3.5.5 ANS Fluorescence

The fluorescent behaviour of 1,8-ANS (Scheme 2.15) and its derivatives in solution has been studied in some detail in the literature. In water the fluorescence intensity of ANS is rather poor due to a preferred radiationless electron transfer from the lowest-energy excited state (ICT). However, bound to a hydrophobic binding site the fluorescence readily increases. In addition, the emission spectrum is sensitive to the local polarity making it a popular probe to study subtle changes in the surface polarity of liposomes or biological membranes. In water and 1,4-dioxane its wavelength of maximum emission is at 555 nm and 472 nm, respectively. This change in wavelength of maximum emission can be explained by a general solvent effect and a specific solvent-dye interaction leading to an intramolecular charge transfer mechanism, depending on the solvent, or binding site.

In solvents of higher viscosity the spectra are blue shifted.

![Scheme 2.15. 1-Anilinonaphthalene-8-sulfonate ammonium salt.](image)

In Figure 2.30 the normalised polarity ($P^{\mu/d}$) is shown for vesicular solutions containing the additives. The experiments were performed in the absence (A) and in the presence (B) of 2.25 mM NaOH. The general trend is not different in the presence of NaOH, but the absolute value varies slightly. In general there seems to be a slight trend towards higher values of $P^{\mu/d}$ upon increasing the amount of additive. It should be noted that the changes in $P^{\mu/d}$ are significantly smaller than those shown in Figure 2.28. Special attention should be drawn to $\text{C}_{16}\text{EO}_{20}$ and $\text{C}_{18}\text{OH}$. At 20 mol% of $\text{C}_{16}\text{EO}_{20}$ and 50 mol% of $\text{C}_{18}\text{OH}$, $P^{\mu/d}$ is significantly higher than for the other data points, suggesting water penetration into the bilayer. For $\text{C}_{16}\text{EO}_{20}$ this is reasonable since this surfactant is able to solubilise vesicles.
The value of $P_{w/d}$ is 0.01 and –0.01 in the absence and presence of NaOH, respectively. This value of around zero suggests that the local polarity is comparable to that of 1,4-dioxane. However, these values are not far off for the value of methanol (0.07). The value of $P_{w/d}$ in cationic vesicles of di-n-dodecyltrimethylammonium bromide is comparable to what we observe here (0.02). The bilayers formed from $C_{18}C_{18}^+$ are in general less polar compared to biological membranes (0.08-0.28) and membranes formed from natural or synthetic phospholipids (-0.06-0.27). In some of the biological membranes still some membrane protein is present, which has binding sites for ANS as well. Binding to membranes is, however, more favourable than to enzymes. The wavelength of maximum fluorescence depends on the temperature. In sarcolemma vesicles $P_{w/d}$ drops from 0.28 to 0.22 when the temperature is lowered from 25°C to 15°C. This might explain why the average value of $P_{w/d}$ that we measured for our cationic vesicles is slightly lower than what is reported for the $E_t(30)$ probe.
2.3.5.6 Nile Red Fluorescence

Both the absorption and fluorescence spectrum of Nile Red (also known as Nile Blue A oxazone) are sensitive to changes in solvent polarity. The wavelength of maximum absorbance shifts from 591 nm to 484 nm going from water to \( n \)-heptane, respectively, whereas the wavelength of maximum fluorescence shifts from 657 nm to 529 nm for the same solvents.\(^{156} \) This effect is typical for dyes that are more polar in their excited state than in their ground state, and in fact, the dipole moment of the excited state is approximately 7 D larger.\(^{157,158} \) Both the absorption and fluorescence emission spectra show a significant shift in the wavelength of maximum absorption or fluorescence upon a change in polarity. This makes it possible to preferentially excite dye molecules residing in domains of different polarity, such as is the case in microemulsions.\(^{159,160,161} \) The mechanism of fluorescence is similar to that for ANS (Section 2.3.5.5), except that the intramolecular charge-transfer state is replaced by a twisted intramolecular charge-transfer (TICT) state. Hydrogen bonding plays an important role in the emission from the TICT state,\(^{157,158} \) leading to potential electron transfer and hence only little fluorescence is observed in water. In long linear \( n \)-alkanes the fluorescence spectrum shows two peaks, probably due to emission from two vibronic levels.\(^{158,159} \)

In an initial study the fluorescence behaviour of Nile Red in several solvents and solvent mixtures was measured as a function of the excitation wavelength. The wavelength of maximum emission was in all cases well within 1 nm of each other indicating that the excitation wavelength has no influence on the emission wavelength in pure solvents and solvent mixtures. The normalised polarity as measured by Nile Red is plotted against the normalised polarity as measured by the \( E_{T}(30) \) dye (Figure 2.31A).\(^{110} \) A linear relationship between the normalised polarity as sensed by \( E_{T}(30) \) and Nile Red was found, except in the region of very apolar solvents. This provides evidence that both Nile Red and the \( E_{T}(30) \) dye sense their environment in a similar fashion in this polarity region. In fact, in the literature a more extensive set of solvents was used and a deviation from linearity was only observed below an \( E_{T}(30) \) value of 40 (less polar than \( CH_{2}Cl_{2} \)).\(^{160} \) This observation indicates that the \( E_{T}(30) \) dye is less sensitive in very apolar solvents than Nile Red.

In microemulsions the fluorescence emission spectrum is a sum (of log normal plots) of the dye located in the different phases (hydrocarbon region, micellar interface and water).\(^{160} \) Due to quenching of the fluorescence in water the dye in the aqueous phase is usually not observed. The wavelength of maximum fluorescence of the dye located at the aqueous/hydrocarbon interface is usually somewhere between the value of water and that of a liquid surfactant. In solutions with large hydrocarbon content the wavelength of maximum fluorescence depends on the excitation-wavelength, indicating that the dye is selectively excited. However, in solutions with low hydrocarbon content the excitation wavelength dependence is not present. Therefore, it is anticipated that this is due to a preference of the Nile Red dye to bind at the interface.

In Figure 2.31B the excitation-wavelength depended fluorescence in vesicles with different additives is shown. \( P^{p/3} \) increases with increasing excitation wavelength. This is in agreement with an increase in maximum absorbance and fluorescence with increasing polarity. This means that upon increasing the excitation wavelength, dye molecules that are located at more polar binding sites are relatively better excited, and, as a consequence, an
average (slightly) higher polarity is reported. We anticipate that in vesicles there is no selective excitation, but a preferred excitation, since our system lacks a true hydrocarbon phase (as in microemulsions), and, since Nile Red has a preference for the interface, the relative number of Nile Red molecules present deep in the bilayer is small. Consequently their contribution is relatively small.

Figure 2.31. A: Plot of normalised polarity measured by Nile Red fluorescence versus the normalised polarity as measured by $E_T(30)$ for several solvents and solvent mixtures. The excitation wavelength was 490 nm (□) and 590 nm (■). Mixtures of water and 1,4-dioxane or acetonitrile (590 nm: ●). Data from literature was included for comparison: Greenspan\textsuperscript{156} (×); Sackett\textsuperscript{162} (△); Sarkar\textsuperscript{157} (○); Oliveira\textsuperscript{159} (+); Boldrini\textsuperscript{163} (⊿); Hungerford\textsuperscript{160} (○). The line is the linear relationship between the normalised polarities of $E_T(30)$ and Nile Red (slope=1). B: Plot of normalised polarity as measured by Nile Red fluorescence versus excitation wavelength for vesicles of $C_{18}C_{18}^+$ with, from top to bottom, 50 mol% of $C_{10}OH$ (□), 20 mol% of $C_{16}OH$ (△), 40 mol% of $C_{10}OH$ (■), 5 mol% of $C_{18:1}OH$ (⊿), 25 mol% of $C_{18}GOH$ (○), 10 mol% of $C_{18}GOH$ (●), 10 mol% of $C_{12}OH$ (▲) and 40 mol% of $C_{12}Mal$ (●). Lines are only drawn to guide the eye.
Figure 2.32. Plot of normalised polarity ($P_{w/d}$) as measured by Nile Red fluorescence versus bilayer composition. Excitation wavelength 490 nm (A) and 590 nm (B). $\text{C}_{10}\text{OH}$ (■); $\text{C}_{18}\text{OH}$ (▲); $\text{C}_{18}\text{GOH}$ (●); $\text{C}_{18:1}\text{OH}$ $\text{C}_{12}\text{Glu}$ (○); $\text{C}_{12}\text{Mal}$ (▲); $\text{C}_{16}\text{EO}_{20}$ (△); $\text{C}_{10}\text{C}_{10}^-$ (×;:+). Dashed lines are drawn to guide the eye through vesicular solutions containing alkyl pyranosides.

As can be seen in Figure 2.32A and B $P_{w/d}$ is more or less constant. In Figure 2.32A and B $P_{w/d}$ is on average 0.68 and 0.73, respectively. This value is close to the value of methanol (0.70). We refrain from a more detailed comparison since the value of $P_{w/d}$ is only an average of the dye molecules that are preferentially excited. However, trends are still informative, assuming that the distribution of Nile Red is not affected by changes in bilayer composition. When the excitation wavelength is 490 nm the reported value for $P_{w/d}$ in vesicles with $\text{C}_{10}\text{C}_{10}^-$ is lower than that reported for vesicles with other additives. However, this difference is not apparent when excitation occurs at 590 nm. Upon the addition of alcohols $P_{w/d}$ remains constant, whereas upon the addition of the alkyl pyranosides $P_{w/d}$ decreases slightly. This effect could originate from a dehydration of the vesicle/water interface, making it less polar, as was observed for mixed micelles of SDS and $n$-dodecylmalono-bis-$N$-methylglucamide. Below a mole fraction of SDS of 0.3 the head group region is completely
dehydrated, since the sugar units have replaced all the water molecules in the interfacial region. However, at this stage the counterion binding is still about 40%. In addition, nonionic micelles containing \textbf{C}_{12}\textbf{Mal} or \textbf{C}_{12}\textbf{Glu} have an interface that is “aqueous-like” in nature, i.e. the effective dielectric constant \(\varepsilon_{\text{eff}}\) is larger than that for nonionic micelles with an oligo ethylene oxide head group (but the \(\varepsilon_{\text{eff}}\) of sugar-based surfactants is lower than the \(\varepsilon_{\text{eff}}\) of water). These two observations suggest a situation that the polarity, as sensed by Nile Red, is slightly lower in cationic vesicles with alkyl pyranosides. However, we stress that the change in \(P_{w/d}\) is not large, which complicates the interpretation of the experimental data. The values of \(P_{w/d}\) that are reported here are slightly higher than what has been measured for egg PC vesicles (0.58-0.65; \(\lambda_{\text{exc}}=550\) nm)\textsuperscript{156,166} and DPPC (0.51 (estimate); \(\lambda_{\text{exc}}=550\) nm).\textsuperscript{167}

### 2.3.5.7 Summary of Membrane Polarity Experiments

The different results for pyrene, laurdan, ANS and Nile Red fluorescence and \(E_T(30)\) absorbance are consistent with each other. The reported values of \(P_{w/d}\), \(I_1/I_3\) and the wavelength of maximum fluorescence in the spectrum of laurdan in vesicles are mostly independent of the bilayer composition. However, for certain dyes in some vesicular systems there seems to be a slight deviation from this general trend. The absolute value of \(P_{w/d}\) varies from dye to dye. The value of \(P_{w/d}\) reported by Nile Red and \(E_T(30)\) is about 0.7 (Figure 2.28 and Figure 2.32), whereas ANS reports a value around 0 (Figure 2.30). Despite these differences in absolute value they reflect a difference in interaction between dye and its environment rather than a difference in polarity. This is apparent when the values of \(P_{w/d}\) are compared with the value of methanol as sensed by these dyes (0.72, 0.70 and 0.07, respectively). This is reasonable considering that our choice of taking 1,4-dioxane as a reference is arbitrary and the sensitivity of these dyes towards 1,4-dioxane is not necessarily the same. The observation that in aqueous aggregates the polarity is similar to the polarity in methanol has been observed before.\textsuperscript{147,168} Also the polarity as sensed by pyrene is similar in vesicles as in methanol (1.20-1.35 and 1.33, respectively).

It should be noted that in order to minimise scattering from the vesicles the fluorescence experiments were done on solutions containing at most 0.5 mM \textbf{C}_{18}\textbf{C}_{18}^+. Control experiments were performed in the absence of fluorescent dye. Except for pyrene background scattering was negligible. However, in the case of vesicular solutions containing \textbf{C}_{12}\textbf{Mal} the amphiphile concentration of 0.5 mM leads to incomplete binding of \textbf{C}_{12}\textbf{Mal}, and therefore at the same mole fraction, but higher amphiphile concentration, the observed changes in polarity might be larger.

In general, it can be concluded that the local polarity of the membrane interface as reported by the dyes used in this study is only slightly affected by the addition of double-tailed anionic amphiphiles, single-tailed nonionic surfactants, and long linear alcohols.
2.3.6 $\zeta$ Potential

2.3.6.1 Theoretical Considerations

A charged particle that diffuses through a solution interacts with its counterions and other charged particles. Depending on the surface charge and the counterion concentration a certain number of counterions will be in close proximity of the charged particle. The amount of counterions that compensate for the charges on the surface is often described by the fraction of counterion binding $\beta$. This parameter is part of the pseudophase model derived by Romsted. In this model there are two pseudophases, a vesicular pseudophase and an aqueous pseudophase. The concentration of ions in the vesicular pseudophase is determined by the counterion binding $\beta$, and the aqueous ion concentration is simply given by the total ion concentration minus the concentration bound to the surface. Since the model assumes two phases, at the border of the vesicular and aqueous pseudophase there is a sharp change in ion concentration.

A more realistic, yet also more complex, description of the ability to bind counterions is given by the Poisson-Boltzmann equation. This model describes the distribution of ions as a function of the potential at a certain distance from the surface of a spherical particle and depends on the surface charge density and total ion concentration.

The Poisson-Boltzmann equation is described by eq. (2.15):

$$\frac{1}{r^2} \frac{d}{dr} \left( r^2 \frac{d \psi}{dr} \right) = -\frac{F}{e} e_R^0 e_r^0 \left( \frac{z e v}{k_b T} \right), \quad R < r \leq b$$

(2.15)

In this equation $F$, $e_0$, $\varepsilon_r$, $k_b$, $e$ and $z$ are the well-known physical constants: Faraday constant, vacuum permittivity, the relative permittivity, Boltzmann constant, elementary charge, and the sign of the charge (+1 or -1), respectively. $T$ is the absolute temperature, and the potential at a distance $r$ from the centre of a particle with radius $R$ is given by $\psi$. $b$ is the distance at which the potential approaches 0 mV. This equation is solved under the boundary conditions, i.e. $d\psi/dr|_b = 0$ and $d\psi/dr|_R = -\sigma/e_0 e_r$, where $\sigma$ is the surface charge density. These conditions specify that the potential at infinite distance from the charged surface is zero and that the potential at the surface is linearly related to the surface charge density.

Experimentally it is difficult to measure the surface potential. It is easier to measure the $\zeta$ potential (Scheme 2.16), although this parameter is not entirely free from ambiguity. The $\zeta$ potential is measured at the slipping plane of a charged particle that is moving through a solution. The location of slipping plane is difficult to measure and it depends on many variables, e.g. temperature, salt concentration, pH, etc. In a typical experiment to measure the $\zeta$ potential, a voltage is applied over a capillary containing a solution with charged particles. Due to this voltage there will be a flow in the cell. However, since the flow is circular (going into one direction in the middle of the capillary and back along the cell walls) at 14.6% and 85.4 % from the cell wall there is no voltage-induced flow. This is called the stationary phase. There, the particles move entirely due to the voltage applied and their $\zeta$ potential. From the measured electrophoretic mobility the $\zeta$ potential can be calculated via the Henry equation (eq. (2.16)), although other equations and empirical relationships exist to calculate the $\zeta$ potential. Differences between these models originate for experiments under other conditions, or just different approaches. Below a potential of ca. 150 mV differences are usually smaller than 10%.
\[ \zeta = \frac{3\eta \mu}{2\varepsilon_0 \varepsilon_r f(\kappa, R)} \]  

(2.16)

In this equation is \( \eta \) the viscosity, \( \mu \) the electrophoretic mobility and \( f(\kappa, R) \) is a function of the inverse of the Debye length and the radius of the particle.

In practice, the \( \zeta \) potential is often used as a measure for colloidal stability. Below a potential of 30 mV the electrostatic repulsion between particles becomes too small and they tend to aggregate as a result of Van der Waals interactions. However, this threshold value is rather arbitrary.

### 2.3.6.2 Dimethyldi-n-Octadecylammonium Chloride and Sodium Di-n- Decylphosphate

In Figure 2.33 the \( \zeta \) potential is shown for mixed vesicles containing C\(_{18}\)C\(_{18}\)\(^+\) and increasing amounts of C\(_{10}\)C\(_{10}\)\(^-\). The \( \zeta \) potential only slightly decreases with increasing C\(_{10}\)C\(_{10}\)\(^-\) content up to 50 mol\%. Then the sign of the \( \zeta \) potential is reversed.

The value we find for 100 mol\% of C\(_{18}\)C\(_{18}\)\(^+\) is also found in the literature,\(^{173}\) but also a value of 84.8 mV has been reported.\(^{174}\) However, several remarks have to be made. Vesicles formed from C\(_{18}\)C\(_{18}\)\(^+\) are not spherical. The preparation method has a large influence on the mobility. Not only because it might lead to a different size distribution for the vesicles, but also because residual organic solvent (used in the literature reports) might be present.\(^{175}\)
Finally, also the concentration plays a role. Therefore, comparison with the literature is difficult. In addition, we used 2.25 mM NaOH, which leads to a lowering of the $\zeta$ potential. One might anticipate that upon the addition of $\text{C}_{10}\text{C}_{10}^-$ the $\zeta$ potential should decrease more strongly since the surface potential is decreased upon the addition of more $\text{C}_{10}\text{C}_{10}^-$. However, at the same time also the salt concentration is increased and therefore the $\zeta$ potential is measured closer to the vesicular surface. Apparently, these two effects compensate each other, and the $\zeta$ potential is only slightly decreased.

Special attention should be drawn to the $\zeta$ potential for 50 mol% of $\text{C}_{10}\text{C}_{10}^-$. For this solution the $\zeta$ potential is approximately 30 mV indicating that the outer leaflet is positively charged and the aggregates are colloidally stable. This observation is in disagreement with the cryo-EM pictures (Section 2.3.1.1), indicating aggregation of vesicles. Apparently, 30 mV is in our case not large enough to prevent aggregation.

![Figure 2.33](image.png)

**Figure 2.33.** Plot of the $\zeta$ potential (■) and electrophoretic mobility (Δ) as a function of the bilayer composition. The error bars denote the widths of the $\zeta$ potential peak. Dashed lines are drawn to show the colloidally stable region ($\zeta$ potential).

### 2.3.7 General Overview

It is surprising to notice that addition of $\text{C}_{10}\text{OH}$ and $\text{C}_{18}\text{GOH}$ to vesicles formed from $\text{C}_{18}\text{C}_{18}^+$ has little influence on the general properties. The main phase transition temperature is only slightly affected up to 50 mol%, and fluorescence spectroscopy using various dyes does not reveal significant changes in measured polarity. Similar results apply when $\text{C}_{18:1}\text{OH}$ and $\text{C}_{18}\text{OH}$ are added, except that addition of $\text{C}_{18}\text{OH}$ leads to a significant increase in the main phase transition above 20 mol% of $\text{C}_{18}\text{OH}$. Addition of more than 20 mol% of $\text{C}_{18:1}\text{OH}$ leads to severe broadening of the main phase transition. Both observations are reasonable since the packing of the tails has a large influence on the main phase transition temperature. Hence, addition of $\text{C}_{18}\text{OH}$ leads to a better packing, whereas addition of $\text{C}_{18:1}\text{OH}$ leads a worse packing.
Upon increasing the size of the hydrophilic part of the additive, the packing parameter of the additives is severely changed. Whereas for the alcohols the packing parameter is larger than 1, for C_{12}Mal, C_{12}Glu and C_{16}EO_{20} the packing parameter is about $1/3$, leading to micelle formation when dissolved in pure water. C_{12}Glu does not dissolve in water, due to a high Krafft temperature, which is observed more often for these types of surfactants. C_{12}Mal and C_{16}EO_{20} are able to solubilise vesicles into micelles. The point where the surfactants saturate the membrane is readily achieved, and this is also the case for the point of complete solubilisation by C_{12}Mal. A large mole fraction is required to completely solubilise vesicles by C_{16}EO_{20}. However, to reach an equilibrium state in mixtures of C_{18}C_{18}^+ and C_{12}Mal or C_{16}EO_{20} (when micelles are added to vesicles of C_{18}C_{18}^+) requires at least 15 h. In addition, the phase behaviour of mixtures of C_{18}C_{18}^+ and C_{12}Mal in the presence of NaOH shows precipitation and reorganisation into larger aggregates. We do not fully understand this behaviour, but considering that sugar-based surfactants have anomalous behaviour with respect to other nonionic or charged surfactants, this is not surprising. This anomalous solution behaviour includes a complex phase diagram (strongly dependent with respect to the $\alpha$ or $\beta$ anomer), binding of hydroxide ions (unknown mechanism), a relatively low effective dielectric constant, dehydration of the polar shell and weak carbohydrate-carbohydrate interactions (under debate). Polarity experiments also reveal no or little changes in polarity upon the addition of C_{12}Glu, C_{12}Mal and C_{16}EO_{20}. However, measurements were performed under conditions where no or small amounts of micelles were formed.

In vesicles formed from the cationic C_{18}C_{18}^+ and the anionic C_{10}C_{10}^- amphiphile the main phase transition temperature is lowered, but the DSC scans show that between 10 mol% and 35 mol% of C_{10}C_{10}^- neutral microdomains are formed. Surprisingly, at 50 mol% of C_{10}C_{10}^- no increase in the main phase transition temperature is seen, although this is usually the case in catanionic surfactant mixtures. This latter behaviour is observed when C_{18}C_{18}^+ is mixed with the asymmetric C_{10}C_{18}^- amphiphile. We anticipated a dehydration of the Stern region due to charge compensation by the head groups, but this is not indicated by the fluorescent dyes, except perhaps Nile Red when excited at 490 nm. It should be noted that at 490 nm mainly dye molecules residing in the hydrophobic interior of the bilayer are excited.

In general, addition of a wide variety of additives leads to relatively small changes in the properties of the formed vesicles. The major effects are found in changes of the main phase transition temperature, and the (partial) solubilisation of the vesicles by C_{12}Mal and C_{16}EO_{20}.

2.4 Conclusions

Addition of linear 1-alcohols, anionic double-tailed amphiphiles and nonionic single-tailed surfactants to vesicles formed from C_{18}C_{18}^+ leads to little or no changes in measured surface polarity as shown by (fluorescent) dyes, such as 1,8-ANS, Nile Red, pyrene, the $E_T$ (30)-dye and laurdan. On the contrary, the main phase transition temperature can be increased or decreased to quite a large extent. In addition, (neutral) microdomain formation has been observed as well. An increase in the phase transition temperature (at mole
fractions over 0.2) is observed for saturated linear alcohols, where the extent of the increase is larger with increasing carbon atom content in the alcohol. Transitions are broadened extensively upon addition of C_{18:1}OH, or disappear completely at higher mole fractions due to micelle formation as observed for C_{12}Mal and C_{16}EO_{20}. In the case of the addition of C_{10}C_{10} or C_{10}C_{18} (neutral) microdomain formation is observed. C_{12}Mal and C_{16}EO_{20} solubilise the vesicles into mixed micelles, but the process is rather slow.

Vesicles containing C_{12}Mal exhibit somewhat unusual phase behaviour. In a mixed system containing C_{10}C_{10} and C_{12}Mal “reversible” precipitation is observed upon the addition of small amounts of NaOH. Upon shaking this precipitate disappears. Dynamic light scattering experiments show that size distributions are not greatly effected, upon addition of NaOH. Overnight the vesicles tend to grow, whereas they do not grow in size in the absence of NaOH. This type of behaviour is not observed in mixed systems with C_{12}Glu, since C_{12}Glu is insoluble in water at 15°C due to its high Krafft temperature.

2.5 Acknowledgements

Dr. Marc Stuart is acknowledged for taking the cryo-EM pictures and fruitful discussions on solvatochromic dyes. Marco Scarzello is also thanked for discussions on solvatochromic dyes.

2.6 References

This effect is due to a better ability to scatter light when particles increase in size.


The "automatic" analysis is provided with the Malvern Zetasizer 5000 software. Using this algorithm the software chooses the analysis algorithm itself.


The orientational polarisibility is a function of the dielectric constant, refractive index, and some other physical constants.

References:

(101) Other factors that can contribute to the temperature of the main phase transition are head group interactions, salt effects, and the presence of small organic additives (organic solvents).
(105) Johansson, M. unpublished results
(115) The orientational polarisibility is a function of the dielectric constant, refractive index, the radius of the cavity in which the dye resides and some physical constants.
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(117) Strictly speaking this is not correct. Excitation can also occur to different excited states, but this is rare.


(155) Trissié, J.; Baudras, A. Biochimie 1977, 59, 693-703.


(164) In Figure 1.3A the normalised polarity was calculated using the wavelength of maximum fluorescence of water and 1,4-dioxane as measured when excited with light of 490 nm. In Figure 1.3B it was calculated using the average of the wavelength of maximum fluorescence as measured by excitation at 490 nm and 590 nm. This slightly different approach leads to...
slightly different values of the same experimental data in Figures 1.31B and 1.32A. This is also the case in Figure 1.32B.


