Azobenzene-substituted phosphate amphiphiles
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Chapter 5

Single-tailed azobenzene-substituted phosphates

5.1 Introduction

In the previous two chapters the properties of azobenzene-substituted dialkyl phosphates and the effect of trans-cis isomerisation on bilayers and membrane proteins were studied. To make a comparison, in this chapter the behaviour of single-tailed analogues is described.

5.1.1 Monoalkyl phosphates

The physical and chemical properties of amphiphilic monoalkyl phosphates in aqueous solution depend strongly on the ionisation state of the head group and therefore the pH. The structural charge of the head group can vary from neutral to double negative:

\[
\begin{align*}
\text{ROPO}_3\text{H} & \quad \xrightarrow{K_1} \quad \text{ROPO}_3\text{H}^- \\
\text{ROPO}_3\text{H}^- & \quad \xrightarrow{K_2} \quad \text{ROPO}_3^{2-}
\end{align*}
\]

The value of \( pK_1 \) is ca. 2 and of \( pK_2 \) is ca. 7.\(^1\) Also the solubility depends on the charge of the head group. Imokawa et al.\(^2\) determined the Krafft temperatures of monosodium and disodium monoalkyl phosphates. At the Krafft temperature a remarkable increase of the aqueous solubility is observed, and the solution becomes transparent. An increase in tail length (from \( n=10 \) to \( n=18 \)) led to an increase in the Krafft temperature. The disodium salts have a lower Krafft temperature than the monosodium salts. It is striking that at concentrations below 30 mM, the Krafft point increases. An increase of more than 10°C in the Krafft temperature was reported for \( \text{C}_{14}\text{H}_{29}\text{OPO}_3\text{Na}_2 \) upon lowering the concentration. An interpretation could involve the formation of unneutralised monoalkyl phosphate, which is poorly soluble in water. This phenomenon may also be due to the presence of small amounts of unneutralised alkyl phosphate present as an impurity. Later, Arakawa and Pethica\(^3\) found that the formation of quarter salts induced the higher Krafft temperatures. Quarter salts have the composition \( \text{RPO}_4\text{H}_2\cdot\text{RPO}_4\text{HNa} \) and are stable in the solid phase.

Walde et al.\(^4\) investigated the aggregation behaviour of \( \text{C}_{12}\text{H}_{29}\text{OPO}_3\text{H}_2 \) and \( \text{C}_{16}\text{H}_{29}\text{OPO}_3\text{H}_2 \) as a function of the pH. Spontaneous vesicle formation was observed at pH-values lower than 3 for \( \text{C}_{12}\text{H}_{29}\text{OPO}_3\text{H}_2 \). Under these conditions half of the surfactants are completely protonated.
and half are monoionic. There is an indication that hydrogen bonding increases the stability of the vesicles.\textsuperscript{5} Between pH=3 and pH=7-9 crystallisation of the compound occurred. In the alkaline region the solution was clear and micelles were formed. It is evident that the charge of the head group strongly influences the aggregation behaviour. If the charge of the head group is increased, the packing parameter will decrease, and this will lead to micelle formation. Stable vesicles for C_{16}H_{29}OPO_{3}H_{2} were only observed at high temperatures and pH<4. Storage at room temperature led to partly crystallised structures.

In Table 5.1 the CMC values of sodium and disodium alkyl phosphates reported in the literature are shown. Upon increasing the tail length a decrease in CMC is observed for both salts. A linear relationship between log CMC and the number of carbons in the tail was found for the disodium alkyl phosphates at 45°C.

**Table 5.1 CMC values of mono-n-alkyl phosphates determined by electrical conductivity measurements.**

<table>
<thead>
<tr>
<th></th>
<th>CMC (mM)</th>
<th>CMC (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monosodium salt</td>
<td>Disodium salt</td>
</tr>
<tr>
<td>C8</td>
<td>141 (25°C)\textsuperscript{5}</td>
<td>142 (30°C)\textsuperscript{7}</td>
</tr>
<tr>
<td></td>
<td>142 (30°C)\textsuperscript{7}</td>
<td></td>
</tr>
<tr>
<td>C10</td>
<td>35 (25°C)\textsuperscript{6}</td>
<td>36.4 (60°C)\textsuperscript{3}</td>
</tr>
<tr>
<td></td>
<td>36.4 (60°C)\textsuperscript{3}</td>
<td>40 (30°C)\textsuperscript{7}</td>
</tr>
<tr>
<td>C12</td>
<td>3.5 (40°C)\textsuperscript{2}</td>
<td>40 (20°C)\textsuperscript{2}</td>
</tr>
<tr>
<td></td>
<td>40 (20°C)\textsuperscript{2}</td>
<td>57 (25°C)\textsuperscript{3}</td>
</tr>
<tr>
<td></td>
<td>57 (25°C)\textsuperscript{3}</td>
<td>44.3 (35°C)\textsuperscript{8}</td>
</tr>
<tr>
<td></td>
<td>44.3 (35°C)\textsuperscript{8}</td>
<td>49.2 (45°C)\textsuperscript{8}</td>
</tr>
<tr>
<td>C14</td>
<td>15.9 (35°C)\textsuperscript{8}</td>
<td>18.4 (45°C)\textsuperscript{8}</td>
</tr>
<tr>
<td>C16</td>
<td>7.00 (45°C)\textsuperscript{8}</td>
<td></td>
</tr>
</tbody>
</table>

Brackman and Engberts\textsuperscript{6} measured the CMC of alkyl phosphates by monitoring the pH as a function of surfactant concentration. Below the CMC the pH decreases upon addition of surfactant and above the CMC the pH increases. The pK\textsubscript{a} of the phosphate head group is higher in the micelle than for the surfactant monomer. To reduce the head group
repulsions counterion binding or protonation is favoured. The increase in pH was most pronounced for the sodium alkyl phosphates but also clear for disodium alkyl phosphates. It can be concluded that the pH varies with the concentration of the alkyl phosphate.

5.1.2 Single-tailed azobenzene-substituted surfactants

Different types of single-tailed azobenzene-substituted zwitterionic surfactants have been studied in the past years (cationic,\textsuperscript{9-15} zwitterionic,\textsuperscript{16} anionic,\textsuperscript{17-19} non-ionic\textsuperscript{20}). The aggregation behaviour of cationic surfactants with the structure given in Figure 5.1 (RazoC\textsubscript{n}N\textsuperscript{+}) has been studied as a function of the substituent R and the spacer length (n).\textsuperscript{9-14}

![Figure 5.1 Structure of RazoC\textsubscript{n}N\textsuperscript{+}.\textsuperscript{9-14}](image)

Bilayer structures were observed when R was C\textsubscript{12}H\textsubscript{25}O and n=2, 4 and 10. The samples were irradiated to induce trans-cis isomerisation. At a ratio of 55\% trans and 45\% cis short rods were observed. Upon decreasing the length of R to C\textsubscript{4}H\textsubscript{9} and C\textsubscript{2}H\textsubscript{5}, micelles were found (n=2)\textsuperscript{11-14}. For R= C\textsubscript{2}H\textsubscript{5} and n=2 a CMC of 10 mM was observed. Increasing the concentration of the cis isomer by irradiation led to an increase of the CMC. At a ratio of 58\% cis and 42\% trans the CMC was 22 mM. Also, the slope of the concentration-conductivity plot above the CMC became steeper as the irradiation time increased. Extrapolation of the counterion binding versus the concentration of the cis isomer to 100\% cis isomer indicates that the micelles of the pure cis isomer may be nearly without counterion binding, which could imply small micelles.\textsuperscript{11,12} Recently, a group of structurally closely related surfactants (AZMS) was studied.\textsuperscript{15}

![Figure 5.2 Structure of AZMS.\textsuperscript{15}](image)

A linear relationship was found between the logarithm of the CMC and the number of carbon atoms in the alkyl group. Also for this series an
increase in CMC was observed upon isomerisation. The ratio of CMC\textsubscript{cis} to CMC\textsubscript{trans} was found to be 1.87-2.85. The area per molecule was calculated for the trans and cis isomers from the surface tension data and was found to be 0.60 and 0.74 nm\textsuperscript{2}, respectively.

Hatton \textit{et al.}\textsuperscript{20} studied the nonionic surfactants shown in Figure 5.3.

\textbf{Figure 5.3} Structure of C\textsubscript{4}AzoOC\textsubscript{n}E\textsubscript{2}. (n = 2,4,6,8)

For n = 2, 4 and 6 micelles were observed but for n = 8 precipitation occurred at high concentrations. From the surface tension data, a large molecular area was calculated for n = 8 in comparison with that for the shorter spacers. The authors attribute this deviation to the adsorption of the azobenzene group at the interface whereby the alkyl spacer forms a loop.

Kunitake \textit{et al.}\textsuperscript{19} prepared and studied azobenzene-substituted amphiphiles with a malonate head group Figure 5.4.

\textbf{Figure 5.4} Structure of C\textsubscript{n}AzoC\textsubscript{m}(COO\textsuperscript{−})\textsubscript{2}.

Rod-like aggregates were observed for C\textsubscript{11}AzoC\textsubscript{5}(COO\textsuperscript{−})\textsubscript{2} and fibers for C\textsubscript{7}AzoC\textsubscript{10}(COO\textsuperscript{−})\textsubscript{2} and C\textsubscript{12}AzoC\textsubscript{10}(COO\textsuperscript{−})\textsubscript{2}. The dispersions were not stable and tend to precipitate after a few hours at room temperature. For all three surfactants H-aggregation (see Chapter 3) was observed by UV-vis spectroscopy.

Overall it can be concluded that single-tailed azobenzene-substituted surfactants with longer tails aggregate to form rod-like structures or bilayers. Surfactants with shorter tails preferentially form micelles. When micelles are formed normally no H-aggregation was observed. Upon isomerisation an increase in CMC was reported in all cases.
5.2 Aggregation behaviour of the single-tailed azobenzene-substituted phosphates

ST Azo-3P and ST Azo-5P (Figure 5.5) are soluble in water. Clear solutions were obtained but after a while crystallisation occurred. A 1 mM solution is stable for at least 1 day but a 8 mM solution shows crystal formation already after half an hour. Addition of NaOH to the solutions, in which crystal formation occurred, led to an increase in solubility but no clear solutions were observed. To obtain more information about these crystals, the melting point of these crystals was compared with that of ST Azo-3P. To this end, the crystals formed in solutions of ST Azo-3P were separated from the liquid and were dried. The crystals started to melt at 195°C and the final melting temperature was above 300°C. ST Azo-3P has no melting point but decomposes at ± 340°C. It is clear that the crystals formed in the solutions have a different composition than ST Azo-3P. A likely explanation could be the formation of quarter salts as reported by Arakawa and Pethica. It is expected that these salts (RPO₄H₂·RPO₄HNa) have a lower melting point than the disodium salts.

![Figure 5.5 Structure of the single-tailed azobenzene-substituted phosphates.](image)

ST Azo-9P is only partly soluble in water. A mixture of water and ST Azo-9P was visually observed at 60°C and the solution was not clear. However, more ST Azo-9P was solubilised than at 20°C. Above 80°C the solution became clear. Two peaks were observed by DSC at 42.2°C and 90.6°C. The solubility of ST Azo-9P was studied as a function of the pH (Figure 5.6). NaOH was added in small portions. First, the turbidity decreased upon increasing the pH, but crystals were also observed by eye at pH ca. 12. Above pH=12.3 a sudden increase in turbidity was detected corresponding to formation of more crystals.
All three single-tailed surfactants were subjected to dynamic light scattering (DLS) experiments. Particles with sizes between 50-800 nm were observed for all three surfactants at low concentrations (0.5-1.0 mM). The average size was roughly 200 nm. Also after extrusion through filters of 200 nm, larger particles were still observed. There was almost no change in the particle size distribution.

Aggregates formed in 4 mM aqueous solutions of ST Azo-3P and ST azo-5P were characterised with cryo-TEM for both the trans and the cis conformation (Figure 5.7). Sheets/crystals with sizes larger than 200 nm were observed for all samples. The cryo-TEM data appear to suggest that the trans samples form cylindrical micelles and the cis samples spherical micelles.

Figure 5.8 and Figure 5.9 show the surface tension plot of ST Azo-3P and ST Azo-5P, respectively. The CMC corresponds to the break in the plot and was found to be 1.4 mM for ST Azo-3P and 1.8 mM for ST Azo-5P. Hayashita et al. found the same effect with RazoC\textsubscript{N}\textsuperscript{+} (Figure 5.1). For R=4 and n=2 a CMC of 2.4 mM and for R=2 and n=4 a CMC of 4.6 mM was obtained. The closer the azobenzene in the alkyl chain is to the head group, the smaller the CMC, and the more stable the micelle.

The saturation adsorption values, $\Gamma_{max}$, at the air/water interface and the minimum area per molecule, $A_{min}$, were estimated using the Gibbs isotherm,

$$
\Gamma_{max} = \frac{-1}{2.303nRT} \left( \frac{\partial \gamma}{\partial \log C} \right)_T
$$

(1)
where $R = 8.31 \text{ J mol} \text{ K}^{-1}$ and $N_A = \text{Avogadro's number}$. The value of $n$ represents the number of species at the interface whose concentrations change with the surfactant concentration.\textsuperscript{22} For dilute solutions of a nonionic surfactant or for an ionic surfactant (ratio surfactant: counterion, 1:1) in the presence of a swamping amount of electrolyte, the value of $n$ is 1.

Figure 5.7 Cryo-electron micrographs of aqueous solutions of trans ST Azo-3P (top left), cis ST Azo-3P (top right), trans ST Azo-5P (bottom left) and cis ST Azo-5P (bottom right). The concentration for all samples was 4 mM. The bars represent 100 nm. The cis samples were prepared by irradiation with light of 365 nm for 7 min.
**Figure 5.8** Surface tension plot of ST Azo-3. \( T = 25°C. \)

**Figure 5.9** Surface tension plot of ST Azo-5. \( T = 25°C. \)
For solutions of monoionic surfactant and a monovalent counterion (1:1) in the absence of any other solutes the value of $n$ is 2.\textsuperscript{21} For other systems, the value is ambiguous since it is not known to what extent the different ions contribute. For ST Azo-3P and ST Azo-5P, the estimated values of $A_{\text{min}}$ are 57 Å$^2$ and 49 Å$^2$ for $n=2$, and 86 Å$^2$ and 73 Å$^2$ for $n=3$.

The location of the azobenzene can also have an influence on the pKa of the surfactants as part of a micelle. Therefore comparison of the results can be complicated because the value of $n$ can be different for the two surfactants.

To confirm that ST Azo-3P and ST Azo-5P form micelles, an experiment with the fluorescent probe Nile Red (Figure 5.10) was performed.

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{nile_red_structure.png}
\caption{Structure of Nile Red.}
\end{figure}

The maximum emission wavelength of Nile Red depends strongly on the polarity of the medium. In water Nile Red has an emission maximum at 660 nm. When micelles are present, Nile Red interacts with the micelles and a change to a lower emission wavelength is observed combined with an increase of the fluorescence. For both ST azo-3P and ST azo-5P (3 mM), a shift to 650 nm and a 4-fold increase of the fluorescence intensity was observed in accordance with presence of micelles.

In sum, ST Azo-9P is only partly soluble in water. ST Azo-3P and ST Azo-5P are soluble in water but tend to precipitate at higher concentrations. The precipitates have a different composition from that of the starting material and could be Quarter salts (RPO$_4$H$_2$·RPO$_4$HNa). From the experiments it became clear that ST Azo-3P and ST Azo-5P form micelles.

### 5.3 Isomerisation of the single-tailed azobenzene-substituted phosphates

UV-vis spectra of the single-tailed surfactants were taken at a concentration of 25 μM. Presumably, the surfactants reside in solution at this concentration as free monomers. The adsorption maxima of the trans and cis isomer are given in Table 5.2.
Table 5.2 Absorption maxima of the trans and cis isomer of ST Azo-xP. [ST azo-xP]=25 µM. For an assignment of the maxima, see Figure 5.11.

<table>
<thead>
<tr>
<th></th>
<th>ST Azo-3P</th>
<th>ST Azo-5P</th>
<th>ST Azo-9P</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_1$ / nm (trans)</td>
<td>241</td>
<td>241</td>
<td>240</td>
</tr>
<tr>
<td>$\lambda_2$ / nm (trans)</td>
<td>353</td>
<td>354</td>
<td>347</td>
</tr>
<tr>
<td>$\lambda_3$ / nm (cis)</td>
<td>244</td>
<td>243</td>
<td>243</td>
</tr>
<tr>
<td>$\lambda_4$ / nm (cis)</td>
<td>316</td>
<td>318</td>
<td>318</td>
</tr>
<tr>
<td>$\lambda_5$ / nm (cis)</td>
<td>440</td>
<td>433</td>
<td>434</td>
</tr>
</tbody>
</table>

As expected, the surfactants show absorption maxima ($\lambda_1, \lambda_2$) for the trans isomer that belong to free monomers in aqueous solution. ST Azo-9P has a slightly lower $\lambda_2$ than the other two, this effect was not observed for the azobenzene-substituted, double-tailed phosphates (Chapter 3). The trans isomer of ST Azo-3P could be readily isomerised into the cis isomer with the use of light with a wavelength of 365 nm (Figure 5.11). Isosbestic points were observed. No complete isomerisation of the single amphiphile back to the trans isomer was possible, even after 10 min. of irradiation with light of 436 nm. The complete cis-trans isomerisation of the DT Azo-xP in vesicular membranes possibly reflects the additional stability of the trans isomer in a membrane. A second round of irradiation gave similar spectra. No further lowering of the trans absorption band was observed. The same features were found for ST Azo-5P and ST Azo-9P.

Recently, single-tailed azobenzene-substituted surfactants were studied. In this study also an incomplete back isomerisation to the trans isomer was observed. In previous work also a partial recovery of the trans absorption band was found, even after a long (more than 30 min.) vis-light irradiation time ($\lambda=410-510$ nm). This is an indication that the photo-stationary state of the cis isomer is remained to some extent.

In contrast, when vesicles were prepared from 20 mol% of ST Azo-3P and 80 mol% of DOPC complete recovery of the trans band was observed. (Figure 5.12) The same holds for ST Azo-5P. Apparently, is it more favourable for the azobenzene-substituted surfactants in a membrane to have an almost complete recovery of the trans isomer.
Figure 5.11 UV-vis spectra of ST Azo-3P. (A) Before irradiation, (B) after irradiation with 365 nm light (7 min.), (C) after irradiation with 436 nm light (2 min.). [ST Azo-3P]=25 µM.

Figure 5.12 UV-vis spectra of 20 mol% of ST Azo-3P and 80 mol% of DOPC. (A) Before irradiation, (B) after irradiation with 365 nm light (7 min.). After subsequent irradiation with 436 nm light, an absorbance curve identical to the one in curve A was obtained. [ST Azo-3P]=25 µM.
CHAPTER 5

The values for $\lambda_2$ and $\lambda_4$ found for 20 mol% of ST Azo-xP in 80 mol% of DOPC are given in Table 5.3. The values of $\lambda_1$, $\lambda_3$ and $\lambda_5$ were similar to those of the free monomer in solution (Table 5.2). Trans ST Azo-9P shows an absorption around 321 nm, indicating H-aggregation and domain formation. Isomerisation was possible and after one round of irradiation a $\lambda_2$ of 335 nm was detected, but H-aggregation was still observed.

Table 5.3 Absorption maxima of 20 mol% of ST Azo-xP in 80 mol% of DOPC for the trans and cis isomer. [ST azo-xP]=25 $\mu$M.

<table>
<thead>
<tr>
<th></th>
<th>ST Azo-3P</th>
<th>ST Azo-5P</th>
<th>ST Azo-9P</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_2$ / nm (trans)</td>
<td>351</td>
<td>352</td>
<td>321, 345*</td>
</tr>
<tr>
<td>$\lambda_4$ / nm (cis)</td>
<td>313</td>
<td>313</td>
<td>311</td>
</tr>
</tbody>
</table>

* After extrusion at 40°C.

Gentle heating of the sample with ST Azo-9P to 40°C during the extrusion shifted $\lambda_2$ to 345 nm. It seems that heating breaks up the H-aggregates.

The half-life time for thermal isomerisation of the cis isomer of ST Azo-xP was determined for three different situations (Table 5.4). The vesicles were irradiated with 365 nm light for 25 min., so that a maximal isomerisation to cis was obtained. The vesicles were quickly transferred into the cuvettes and the absorbance at 350 nm was monitored for several days. A typical plot is shown in Figure 5.13. The isomerisation reaction followed first-order kinetics and the data were fitted (Figure 5.13) with the software program Origin. As the half-life time increases the accuracy decreases. Thus, half-life times larger than 35 h. are not indicated in the table with their absolute value. For samples with a half-life time shorter than 35 h. a standard deviation of 0.5 h., or less was found.

Surprisingly, little research has been done on thermal isomerisation rates of azobenzenes as part of an aggregate (vesicle, micelle etc.). In contrast, the rates of thermal cis-trans isomerisation of azobenzenes doped in or covalently attached to polymers have been investigated in more detail and are known to depend on polymer morphology.\textsuperscript{24-28} The thermal cis-trans isomerisation rates for donor-acceptor-substituted azobenzenes were determined in the presence of micelles and vesicles.\textsuperscript{29,30,31} These azobenzenes contain a nitro- and an amino-substituent and therefore the thermal isomerisation rate depends strongly on the polarity, viscosity and hydrogen-binding capacity of the solvent.
The thermal isomerisation rates of cis azobenzene-substituted surfactants incorporated into membranes have only been studied previously by Moss and Jiang.\textsuperscript{32,33} They studied the thermal isomerisation rate of an azobenzene-substituted surfactant (Figure 5.14) which was part of a membrane.\textsuperscript{33}

\begin{figure}[h]
\centering
\includegraphics[width=0.7\textwidth]{figure5_13.png}
\caption{Typical plot of absorbance at 350 nm vs time for ST Azo-3P (25 mol\%) in DOPC (75 mol\%) at 25°C. The fitted graph is plotted over the data points.}
\end{figure}

The membrane consisted of 10 mol\% of surfactant 1 and 90 mol\% of surfactant 2. The phase transition temperature of the mixed membrane was 68°C. So, at the measured temperatures (21-60°C) the membrane was in the gel phase. The kinetics were studied at different temperatures and compared with the rates in acetonitrile. Excellent first-order kinetics were obtained. The isomerisation of cis-1 in the mixed vesicles occurred 2-3 times faster than in acetonitrile at each of the investigated temperatures. A more favourable entropy of activation for the isomerisation in the membrane was found. Possibly, the spatially more demanding cis-1 present in the membrane hinders the vibrations and rotations of the neighbouring surfactants. Previous studies have shown that solvent effects on the thermal cis-trans isomerisation of these types of azobenzenes are minor.\textsuperscript{34}
In Table 5.4 the calculated half-life times for ST Azo-xP in different situations are given. The vesicles with SAINT-1 (structure shown in Chapter 6) are just above their phase transition temperature and it can be assumed that the vesicles with DOPC are far above their phase transition temperature.

**Table 5.4 Half-life times of the cis isomers of ST Azo-xP in different situations. T= 25.0°C.**

<table>
<thead>
<tr>
<th>t(\frac{1}{2}) (h.)</th>
<th>ST Azo-3P</th>
<th>ST Azo-5P</th>
<th>ST Azo-9P</th>
</tr>
</thead>
<tbody>
<tr>
<td>monomer in water</td>
<td>27.0</td>
<td>26.9</td>
<td>&gt;35</td>
</tr>
<tr>
<td>75 mol% SAINT-1</td>
<td>10.1</td>
<td>12.1</td>
<td>17.8</td>
</tr>
<tr>
<td>80 mol% DOPC</td>
<td>19.7</td>
<td>25.7</td>
<td>&gt;35*</td>
</tr>
</tbody>
</table>

* The vesicle were extruded at 40°C.

The longest half-life times were recorded for cis ST Azo-9P, presumable is cis ST Azo-9P less unfavourable than ST Azo-3P and ST Azo-5P in both a membrane and free in solution. It is likely that a cis azobenzene at the end of the alkyl chain gives less disturbance than a azobenzene located in the middle of the alkyl chain. Cis ST Azo-3P has the shortest half-life times in the membrane and it seems that the membrane is the most disturbed by cis ST Azo-3P. These results are in accordance with the results presented in Chapter 3. For the double-tailed azobenzene-substituted phosphates also a decrease in half-life time was found upon positioning the azobenzene in the alkyl chain closer to the head group.

In sum, ST Azo-xP can be isomerised easily with light of 365 nm. It appears that the cis isomer in solution is less unfavourable than in the membrane because back isomerisation to the trans is only partially possible and the half-life times are longer than that of the azobenzene-substituted phosphates in the membrane (especially in the SAINT-1
membranes). H-aggregation was only observed for the combination of 80 mol% of DOPC and 20 mol% of ST Azo-9P.

5.4 Binding and permeability

The binding of ST Azo-5P to DOPC vesicles and the flip-flop rates were studied by fluorescence spectroscopy using a method published by de la Maza et al.\textsuperscript{35} A fluorescent probe, TNS (Figure 5.15), was used for which the fluorescence in water is very low. When the probe binds to the bilayer surface of vesicles, an increase in fluorescence is observed.

\[ \text{Figure 5.15 Structure of TNS.} \]

The fluorescence is quenched when negative charges (i.e. sodium dodecyl sulfate, SDS) are added. When flip-flop of the anionic surfactant occurs the fluorescence increases. No detectable flip-flop of the probe itself was measured for at least 6 h. and it is be assumed that the probe resides at the interface of the outer leaflet.\textsuperscript{35} De la Maza et al.\textsuperscript{35} studied the rate of flip-flop of SDS in a phosphatidylcholine bilayer. The incorporation of the SDS monomers was a very rapid process (within seconds). It has to be noted that these experiments were performed below the CMC of SDS. The rate of flip-flop of SDS was very low, after approximately 45 min. a quarter of the SDS monomers was in the inner leaflet. The results correspond well with those for different surfactants used in other studies.\textsuperscript{36,37}

In our experiments we have used DOPC vesicles (1 mM) to which ST Azo-5P was added. A drawback of this experiment is that ST Azo-5P absorbs light at the excitation wavelength of the probe TNS (325 nm). Since the concentration of ST Azo-5P (8 or 16 \( \mu \)M) was in the same region as that of the probe (10 \( \mu \)M). It can be assumed that ST Azo-5P will not adsorb all the light and its concentration stays constant. A change in emission is then due to a change in the quenching of the probe TNS. As a control, disodium stearylphosphate (ST SP, C\textsubscript{18}H\textsubscript{37}OPO\textsubscript{3}Na\textsubscript{2}) was used.

After addition of TNS to the DOPC vesicles, fluorescence emission was observed and binding occurred of the TNS to the DOPC vesicles. After an incubation time of 30 min., a stable fluorescence signal was recorded. ST SP or ST Azo-5P was added and a decrease in the fluorescence was directly observed. The amount of quenching of the fluorescence depended on the concentration of the additives. The decrease in
fluorescence was in the same order for ST SP and ST Azo-5P and the fluorescence was followed in time. No changes in the fluorescence signal were observed for a period of 150 min. for both the ST SP and the ST Azo-5P sample at the two different concentrations used (8 or 16 µM). From these results it may be concluded that ST Azo-5P binds fast to the DOPC vesicles (within a second) and that the flip-flop rate is very slow.

The permeability of DOPC vesicles containing ST Azo-5P was tested with the use of calcein efflux experiments. ST Azo-5P was added to DOPC vesicles with encapsulated calcein. Firstly, trans ST Azo-5P was added up to 80 mol% (in ratio with DOPC), no leakage of calcein was observed. Also the addition of cis ST Azo-5P up to 95 mol% did not lead to leakage of calcein. The concentration of trans ST Azo-5P was further increased to 0.8 mM and still no leakage of calcein was observed. Despite the fast binding, trans ST Azo-5P could induce leakage of calcein. It was not possible to monitor the effect of addition of cis ST Azo-5P at higher concentrations because then cis Azo-5P absorbs part of the excitation light (490 nm).

5.5 Calcein efflux experiments with MscL

During the reconstitution of MscL, Biobeads are added to remove the detergent. From some simple UV-vis spectroscopic mixing experiments of ST Azo-5P and Biobeads it became clear that ST Azo-5P binds to the Biobeads. Therefore, it is better to add ST Azo-5P after the reconstitution process. In the calcein efflux experiments with MscL, ST Azo-5P was added to the proteoliposomes.

The experiments were performed with liposomes which contained WT MscL and a control sample without the channel. Firstly, the concentration of ST Azo-5P was varied and it was observed that at concentrations above 0.1 mM, the fluorescence signal was influenced by cis ST Azo-5P and also precipitation occurred for both the trans and cis isomer of ST Azo-5P. Therefore the experiments were performed at 0.1 mM of ST Azo-5P. In this setup leakage was neither observed upon addition of trans or cis ST Azo-5P nor after trans-cis isomerisation of the sample containing trans ST Azo-5P.

During the experiments, the vesicles are diluted and this is normally accomplished by addition of buffer to maintain osmotic equivalence inside and outside the vesicle. In subsequent experiments the dilution was made with pure water to gain a small osmotic pressure across the membrane. Although a small osmotic pressure could lead to faster openings of the channels, no release of calcein was observed.

The leakage of calcein was monitored for a longer time. Long incubation times were sometimes necessary to observe channel activity as previously shown by Martinac and Perozo. After 105 min, a small
amount of calcein was released, including the control samples. A long incubation time did not induce opening of the MscL channel.

Finally, the moment of isomerisation of ST Azo-5P was changed. First, trans ST Azo-5P was added to the DOPC vesicles and then ST Azo-5P was irradiated to achieve trans-cis isomerisation. No leakage of calcein was observed with fluorescence spectroscopy.

5.6 Conclusions

The ST Azo-xP surfactants have a limited solubility in water. ST Azo-3P and ST Azo-5P form micelles but also larger aggregates with sizes larger than 200 nm. The surfactants could easily be isomerised into the cis isomer, both as free monomer in solution or as part of a bilayer. Longer half-life times, for the thermal cis-trans isomerisation, were found for the ST Azo-xP monomers free in solution.

Although fast binding of ST Azo-5P to DOPC vesicles was observed, the permeability was not affected by either trans or cis ST Azo-5P. Induction of opening of MscL could not be accomplished by isomerisation of the ST Azo-5P as monitored by calcein efflux.

Unfortunately because of time limitations, no patch clamp experiments with the single-tailed azobenzene-substituted phosphates have yet been performed.

5.7 Acknowledgment

Joost Folgering is acknowledged for preparing the proteoliposomes and Anno Wagenaar for providing disodium stearylphosphate (ST SP).

5.8 Experimental section

General remarks. Water was distilled twice in an all-quartz distillation unit.

Preparation of the vesicles. The vesicles were prepared following the procedure described in Chapter 4.

Cryo-TEM. Cryo-TEM experiments were performed according to the procedure described in Chapter 3. Extrusion was not applied.

Dynamic Light Scattering. Size distributions were determined at a fixed angle of 90°C with a Malvern Instruments Zeta Sizer 5000 using the Contin analysis mode. The concentration of the surfactant solutions was 0.5 or 1 mM. Measurements were performed at room temperature.
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Surface tension experiments. Critical micelle concentrations (CMCs) were determined by drop tensiometry using a Lauda TVT1 drop tensiometer equipped with a Lauda RM6 thermostat bath (25°C).

Half-life time measurements. The half-life time measurements of the cis ST Azo-xP were performed as described in the experimental section of chapter 3.

Fluorescence spectroscopy binding experiments. Vesicles dispersions (20 mL, 1 mM) of DOPC were prepared by tip sonication for 30 min. A bluish solution was obtained. Stock solutions of TNS and ST Azo-5P (both 1 mM) were prepared. During the measurements, the concentration of DOPC was 1 mM and that of TNS 10 µM. The concentration of ST Azo-5P and ST SP was 8 or 16 µM. ST SP was added via a stock solution, which was kept at a temperature above the Krafft temperature of ST SP (62.0°C). The solution was buffered with 5 mM Tris-Cl, pH=8.0. The fluorescence measurements were performed using an SLM Amico spectrofluorometer. The excitation wavelength was set to 325 nm and the emission wavelength to 450 nm. The excitation and emission band passes were 4 and 2 nm, respectively.

Calcein efflux experiments without MscL. Vesicles were prepared by adding calcein solution (40 mM calcein, 10 mM KPi, 1 mM EDTA, pH=8.0) to the lipid film of DOPC (final concentration 5 mM). After vortexing, the sample was tip sonicated for 2 min. The free calcein was removed by means of column chromatography (Sephadex G75) using an aqueous buffer solution containing 10 mM KPi, 1 mM EDTA and 58 mM NaCl of pH 8.0. The measurements were performed as described in chapter 3.

Calcein efflux experiments with MscL. The experiments were carried out as described in Chapter 4.

5.9 References

SINGLE-TAILED AZOBENZENE-SUBSTITUTED PHOSPHATES