Azobenzene-substituted phosphate amphiphiles
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Chapter 6

Photochemically-induced disturbance of the alkyl chain packing in vesicular membranes

6.1 Introduction

The topic of this chapter is a detailed study of the effects of trans-cis isomerisation of the azobenzene-substituted phosphates on the properties of a vesicular bilayer. Differential scanning microcalorimetry (DSC) and NMR are important techniques that have been used to study the structure and dynamics of bilayers. With the use of DSC, information concerning the packing of the tails in the bilayer can be obtained. $^2$H NMR can give information about the order of the chains in the membrane. Order parameters, which are measures of the chain packing in the membrane, can be derived from the $^2$H NMR data. The classical definition of the order parameter ($S_n$) is that it is a quantity which is unity in a perfectly ordered phase and zero in a completely disordered phase. Order parameters depend on $\theta_n$, the angular deviation of bond n from its orientation in the ordered, all-trans chain conformation. In the following equation the dependence of the order parameter on $\theta_n$ is given:

$$\langle S_n \rangle = \frac{1}{2} \left\langle 3 \cos^2 \theta_n - 1 \right\rangle$$  \hspace{1cm} (1)

Order parameters have also been estimated by computer simulations. Other experimental techniques which provide information on order parameters include infrared spectroscopy and fluorescence polarisation measurements.

Another measure for the packing efficiency is the fluidity. Fluidity is a measure of the viscosity, the ease of diffusion of the amphiphiles in the plane of the bilayer. It is assumed that all these quantities used for describing the packing of the amphiphiles in the membrane are related in a certain way with each other. A decreased packing of the amphiphiles corresponds with a decreased order, a lower viscosity and a higher fluidity.
6.1.1 Differential scanning microcalorimetry

As discussed in Chapter 1, the alkyl tails can reside in the membrane in two different main phases: the highly ordered gel state and the less ordered liquid crystalline state. The temperature at which the system, largely cooperatively, changes from the gel to the liquid crystalline phase is called the main phase transition temperature ($T_m$). The $T_m$ is characteristic for each bilayer-forming surfactant and can be determined using various techniques.\textsuperscript{4,5} DSC is one of the preferred methods because it is a highly sensitive, non-invasive technique and it is easy to perform.\textsuperscript{6,7} During the measurement a sample cell and a reference cell are heated at a constant rate, typically 1 degree per minute. Heat exchange with the environment is prevented by an adiabatic shield. The apparatus measures the excess heat that has to be added to the sample cell in comparison to the reference cell, in case of an endothermic transition. When an exothermic process takes place, less heat is added to the sample cell. So, the difference in heat capacity of the sample and the reference cell is recorded. In practice, the temperature at which the curve shows a maximum is taken as the $T_m$. In theory, it is more correct to take the temperature at which the melting starts as the $T_m$.\textsuperscript{8} When the concentration of the surfactant is known, the enthalpic change of the transition can be calculated via integration of the surface under the DSC curve.

The width of the transition is a measure for the cooperativity of the transition. It is assumed that a group of surfactant molecules, a patch, undergoes the transition at the same time. A narrow transition indicates that the patches are large. In contrast, a broad transition points to small patches and a less cooperative transition. As a measure of cooperativity, the patch number was introduced.\textsuperscript{9}

Pretransitions are often observed in DSC curves and they can be attributed to 1) inhomogeneous mixing of different constituents, 2) impurities, 3) inhomogeneous size distribution of the vesicles (vide supra), 4) a transition to an intermediate phase (e.g. rippled phase, vide supra).

The effect of the preparation method of the vesicles on the phase transition has been studied with DSC by several groups.\textsuperscript{5,9-12} Already in 1981, it has been reported\textsuperscript{10} that the $T_m$ depends on the size of the vesicles. The various available preparation methods produce vesicle solutions with different size distributions, therefore distinct DSC curves can be obtained with different preparation methods. Okahata \textit{et al.}\textsuperscript{11} found that sonication led to broader phase transitions at a lower temperature. This effect was only found for anionic and cationic surfactants, not for nonionic and zwitterionic surfactants. Biltonen and Lichtenberg\textsuperscript{12} stated that small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs) in coexistence give two separate maxima in
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the DSC scan, with the maxima of the SUVs at a lower temperature. A likely explanation is that due to the high curvature in the SUVs, the packing of the lipids is less efficient in comparison with LUVs. It was also found that incubation of the sample for sufficiently long periods of time below T_m led to fusion of the SUVs into LUVs. This conclusion was drawn from the fact that the transition of the SUVs became less intense upon longer incubation times. It has to be noted that the size-dependence was observed for DPPC, a zwitterionic lipid.

The effect on the phase transition of vesicles upon addition of alcohols,\textsuperscript{6,8,13-18} (oppositely charged) surfactants,\textsuperscript{19-25} equally charged surfactants,\textsuperscript{23,25-27} sugar surfactants,\textsuperscript{20} amino acids,\textsuperscript{26} small organic molecules,\textsuperscript{29,30} cholesterol,\textsuperscript{31,32} carboxylic acids\textsuperscript{14,33} and ureas\textsuperscript{34} has been studied. The effects on the phase transition of the various additives is often complex and difficult to explain, but some trends are generally observed. Addition of short-chain n-alcohols (n<8) decreases the T_m, an increase is observed for long-chain n-alcohols (n>12).\textsuperscript{29} Often, high concentrations of long-chain n-alcohols also induce a decrease in the T_m.\textsuperscript{8} Carboxylic acids show the same trend as the n-alcohols at low concentrations.\textsuperscript{14,33} Usually the T_m increases upon mixing with opposite charged surfactants.\textsuperscript{24,25} The decreased head group repulsions lead to a decrease in the intermolecular distance between the alkyl chains and therefore to more ordering of the tails.

6.1.2 Static solid state $^2$H NMR

A measure of the chain order in the bilayer can be obtained using static solid state $^2$H NMR.\textsuperscript{35,36,37} For this purpose vesicles are prepared from lipids with (partially) deuterated tails. The movements of the tails in the bilayer are restricted and therefore are the static electric-quadrupolar and magnetic-dipole interactions not completely averaged out and an anisotropic signal is observed.\textsuperscript{38} In contrast, in ordinary fluids, the motions are not significantly restricted, the interactions are averaged out, and one isotropic signal is observed. In case of an anisotropic system, the quadrupolar splitting is easily measurable. The quadrupolar splitting provides information about the average orientation and the fluctuations of the C-D bond vector according to the equation:

$$\Delta V_q = \frac{3}{4} \left( \frac{e^2 q Q}{h} \right) S_{CD}$$

in which $\Delta V_q$ is the quadrupolar splitting, $(e^2 q Q/h)$ is the static quadrupolar coupling constant (170 kHz) and $S_{CD}$ is the C-D bond order parameter.\textsuperscript{39} $S_{CD}$ depends on the angle between the carbon-to-deuterium vector and the director, which is the normal to the bilayer surface.\textsuperscript{36,40}
The C-D bond-order parameter for a distinct nucleus depends on two contributions. Firstly, the chain order parameter, which depends on the position of the nucleus in the chain, originates from trans-gauche isomerisations of the chain parts together with the librational motions within the various conformational states. Secondly, contributions due to additional slower motions independent of the position in the chain occur and these originate from molecular motions and/or collective disturbances of the bilayer.

The proton nucleus does not possess a quadrupolar moment and is not suitable for studies based on the anisotropy of the dipolar interactions. The variations of the dipolar interactions along the chain are of the same order as the splitting due to the nearest-neighbour dipolar interactions and this usually leads to a broad peak. This renders it difficult to extract detailed information about the protons at different positions in the chain. The deuterium nucleus is particularly suitable because the quadrupolar splitting is, in most cases, much larger than the dipolar interactions between deuterons. An extra advantage of deuterium is that in systems with lipids with several deuteriums often the individual lines can be resolved. A disadvantage is the availability/preparation of the (partially) deuterated lipids.

Recently, the effect of several additives on the order parameters were studied. Addition of small alcohols to $^2\text{H}_4\text{-DOPC}$ led to a decrease of the quadrupolar splitting. Unsaturations of the tails also promoted disordering. Cholesterol and DOPE had the opposite effect. These recent results are all in line with the conclusions of Gawrisch and Holte.

### 6.2 DSC measurements on the double-tailed azobenzene-substituted phosphates

To study the effect of trans-cis isomerisation on the $T_m$ of a vesicular bilayer, the azobenzene-substituted phosphates have to be mixed with a carrier surfactant with a $T_m$ above 10°C. A first choice for a mixing surfactant is DSP (distearylphosphate), the unfunctionalised homolog of the double-tailed azobenzene-substituted phosphates. DSP has a $T_m$ of 78°C (see Chapter 3), which is too high for a study of the effects of trans-cis isomerisation. The trans isomer of azobenzene is thermally more stable than the cis isomer. It is expected that the thermal cis-trans isomerisation rate will increase significantly at elevated temperatures. So, due to the high $T_m$ of DSP, other vesicle-forming amphiphiles were preferably chosen with lower $T_m$’s. Another drawback of DSP is that it can only be mixed with azobenzene-substituted phosphates up to a concentration of 5 mol%.
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Useful amphiphiles are SAINT-1 and SAINT-5 with $T_m$ values of 29.8 °C and 43.5 °C, respectively (structure: Figure 6.1).\cite{46,47}

**Figure 6.1** Basic structure of the SAINT amphiphiles. $R=C_{16}H_{33}$: SAINT-1; $R=C_{18}H_{37}$: SAINT-5.

In Figure 6.2 and Figure 6.3 the DSC graphs for SAINT-1 and SAINT-5, using different preparation methods, are given (the "hot-water method" is defined in Table 3.1). It is clear that the preparation method has a significant influence on the phase transition. It was already shown\cite{31} that a decreasing size of the vesicles leads to broadening of the transition. A likely reason for the observation of the pretransition involves formation of SUVs and LUVs (see also section 6.1.1).

**Figure 6.2** DSC graphs of SAINT-1. Preparation methods: (a) hot water method; (b) bath sonication; (c) tip sonication.

Lipid films were prepared which consist of 25 mol\% of DT Azo-xP and 75 mol\% of SAINT-1 or SAINT-5. To avoid the occurrence of domain formation of H-aggregates, all the DSC samples with the double-tailed azobenzene-substituted phosphates were tip sonicated. It was impossible to prepare stable vesicle solutions for combinations of DT Azo-9P with SAINT-1 or SAINT-5. In Figure 6.4 and Figure 6.5 the DSC results for mixtures of DT Azo-3P and DT Azo-5P are presented.
Figure 6.3 DSC graphs of SAINT-5. Preparation methods: (a) hot water method; (b) bath sonication; (c) tip sonication.

Figure 6.4 DSC graphs of (a) pure SAINT-1; (b) 25% mol% trans of DT Azo-5P and 75 mol% of SAINT-1; (c) the same as (b) but now with cis DT Azo-5P. All samples were tip sonicated.
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Figure 6.5 DSC graphs of (a) pure SAINT-5; (b) 25% mol% of trans DT Azo-3P and 75 mol% of SAINT-5; (c) the same as (b) but now with cis DT Azo-5P. All samples were tip sonicated.

The DSC graphs of 25 mol% of DT Azo-3P in SAINT-1 are similar to the results of DT Azo-5P in SAINT-1 presented in Figure 6.4. Similar graphs, presented in Figure 6.5, were found for 25 mol% of DT Azo-5P in SAINT-5. In Table 6.1 and Table 6.2 the results are summarised. Between the individual scans of the mixed vesicles (DT Azo-xP and SAINT) there was more variation than between the individual scans of the pure SAINTs. The temperatures for the pure SAINTs are therefore more exact. The isomerisation to the cis isomer was accomplished by irradiation with light of 365 nm.

Table 6.1 Summary of the phase transitions of pure SAINT-1 and mixtures of SAINT-1 and trans or cis DT Azo-xP. All samples were tip sonicated. m: major peak.

<table>
<thead>
<tr>
<th></th>
<th>T (°C) Trans isomer</th>
<th>T (°C) Cis isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure SAINT-1</td>
<td>19.6</td>
<td>26.3 (m)</td>
</tr>
<tr>
<td>25 mol% DT Azo-3P</td>
<td>19</td>
<td>24* 28 (m)</td>
</tr>
<tr>
<td>25 mol% DT Azo-5P</td>
<td>18</td>
<td>24* 28 (m)</td>
</tr>
</tbody>
</table>

* Appears as a shoulder in the DSC graph. † Appears as a broad peak from 12-17°C with the maximum value at 13°C.

In all experiments there was a tendency that the major phase transition temperature increased upon addition of 25 mol% of trans DT Azo-xP. This observation was already made previously for a similar system.²⁰
The increase was attributed to favourable electrostatic interactions between the head groups. In contrast, the pretransition peaks shifted a little to lower temperatures. In case of SAINT-1, a shoulder appeared at 24°C. Additional peaks can be attributed to other phase transitions (e.g. rippled-phase) or microdomains.

**Table 6.2** Summary of the phase transitions of pure SAINT-5 and mixtures of SAINT-5 and trans or cis DT Azo-xP. All samples were tip sonicated. m= major peak.

<table>
<thead>
<tr>
<th></th>
<th>T (°C) Trans isomer</th>
<th>T (°C) Cis isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure SAINT-5</td>
<td>37.6 44.2 (m)</td>
<td></td>
</tr>
<tr>
<td>25 mol% DT Azo-3P</td>
<td>36 45 (m)</td>
<td>29 40 (m)</td>
</tr>
<tr>
<td>25 mol% DT Azo-5P</td>
<td>36 45 (m)</td>
<td>34 43 (m)</td>
</tr>
</tbody>
</table>

Upon trans-cis isomerisation of azobenzene-substituted phosphates, all phase transitions shifted to lower temperatures. In case of SAINT-1, only one (broad) peak is left. In the DSC graphs of SAINT-5, two peaks are still observed. It is concluded that isomerisation of the azobenzene-substituted phosphates to the cis isomer leads to disturbance in the bilayer (less order). Remarkable is that isomerisation of DT Azo-3P apparently leads to a higher disorder.

**6.3 DSC measurements on the single-tailed azobenzene-substituted phosphates**

The same type of experiments as described in section 6.2 were performed with the single-tailed azobenzene-substituted phosphates (Chapter 2). Tip sonication of the vesicles was not necessary, because the single-tailed azobenzene-substituted phosphates have less tendency to form H-aggregates (Chapter 5). All samples were extruded through 200 nm filters before use and no H-aggregates were observed by UV-vis spectroscopy. First, vesicles were prepared consisting of 25 mol% of ST Azo-xP and 75 mol% of SAINT-1. In Figure 6.6, Figure 6.7 and Figure 6.8 the DSC results for ST Azo-3P, ST Azo-5P and ST Azo-9P in SAINT-1 vesicles are presented.

The first observation is the broadening of the transition upon addition of ST Azo-xP indicating that the transition becomes less cooperative. Secondly, after isomerisation to the cis isomer of single-tailed azobenzene-substituted phosphate, a slight increase in the cooperativity of the transition is observed for all three samples. It is particularly noteworthy that upon isomerisation the transition becomes more cooperative but the transition temperature decreases.
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Figure 6.6 DSC graphs of (a) pure SAINT-1; (b) 25% mol% trans ST Azo-3P and 75 mol% SAINT-1; (c) the same as (b) but now with cis ST Azo-3P. All samples were extruded through 200 nm filters.

Figure 6.7 DSC graphs of (a) pure SAINT-1; (b) 25% mol% trans ST Azo-5P and 75 mol% SAINT-1; (c) the same as (b) but now with cis ST Azo-5P. All samples were extruded through 200 nm filters.
**Figure 6.8** DSC graphs of (a) pure SAINT-1; (b) 25% mol% trans ST Azo-9P and 75 mol% SAINT-1; (c) the same as (b) but now with cis ST Azo-9P. All samples were extruded through 200 nm filters.

In Table 6.3 a summary of the $T_m$ values is given. In contrast with the results of the double-tailed azobenzene-substituted phosphates, a decrease in the $T_m$ values is observed upon addition of the trans single-tailed azobenzene-substituted phosphates.

Addition of the single-tailed phosphates apparently leads to more disturbance in the bilayer. It is also clear that ST Azo-9P has only a minor effect in comparison with ST Azo-5P and ST Azo-3P. The effect on the broadening is the least outspoken and also the decrease in $T_m$ is the smallest.

**Table 6.3** Summary of the $T_m$ values of pure SAINT-1 and mixtures of SAINT-1 and trans or cis ST Azo-xP. All samples were extruded through 200 nm filters.

<table>
<thead>
<tr>
<th></th>
<th>$T_m$ (°C) Trans isomer</th>
<th>$T_m$ (°C) Cis isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure SAINT-1</td>
<td>27.3</td>
<td></td>
</tr>
<tr>
<td>25 mol% ST Azo-3P</td>
<td>19.0</td>
<td>14.5</td>
</tr>
<tr>
<td>25 mol% ST Azo-5P</td>
<td>19.5</td>
<td>16.3/17.1</td>
</tr>
<tr>
<td>25 mol% ST Azo-9P</td>
<td>23.5</td>
<td>23.6</td>
</tr>
</tbody>
</table>
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It is rather amazing to see that the effect of isomerisation of the azobenzene on $T_m$ increases as the azobenzene group in the alkyl chain is closer to the head group. In case of ST Azo-9P only a narrowing of the transition is observed, there was no change in $T_m$. Table 6.3 summarizes the results of the DSC experiments for the single-tailed azobenzene-substituted phosphates.

To see whether the effect of trans-cis isomerisation is reversible, a cis-isomerised sample was irradiated with light of 436 nm. Indeed, the DSC graph became equal to the original DSC graph of the trans isomer.

In the DSC experiments discussed so far, lipid films were prepared with SAINT-1 and ST Azo-xP. To make a comparison, another preparation method was used. Vesicles were first prepared from pure SAINT-1 and then the appropriate amount of trans or cis ST Azo-xP solubilised in water was added. Due to the low solubility of ST Azo-9P only ST Azo-3P and ST Azo-5P were used. DSC experiments were performed, which showed that addition of trans or cis ST Azo-3P or ST Azo-5P had a similar effect on the phase transition as found in case of the first preparation method.

In all the DSC experiments with combinations of SAINT-1 and ST Azo-xP, scans were taken up to 40°C. Separate DSC experiments were performed with scans up to 100°C. Surprisingly, a large exothermic peak was observed during the first scan between ca. 50 and ca. 80°C (Figure 6.9).

![Figure 6.9](image)

**Figure 6.9** DSC scans of 25% mol% trans ST Azo-3P and 75 mol% SAINT-1. (a) first scan; (b) second scan.
In the second scan a higher $T_m$ was observed ($21.9^\circ$C). UV-vis spectroscopy was used to check for the presence of domains of $H$-aggregates, but a blue shift was not observed. Cryo-TEM was used to study the sample before and after heating to $95^\circ$C. In Figure 6.10 some representative cryo-TEM pictures are given.

![Cryo-TEM images of 25% mol% trans ST Azo-3P and 75 mol% SAINT-1. (a) and (b) without heating; (c) and (d) after heating to 95°C. Bar represents 100 nm.](image)

**Figure 6.10** Cryo-TEM images of 25% mol% trans ST Azo-3P and 75 mol% SAINT-1. (a) and (b) without heating; (c) and (d) after heating to 95°C. Bar represents 100 nm.

In the unheated sample, closed vesicles are observed with a double layer which looks like the surface of an orange. Orange-like double layers were observed before\(^{48}\) and were attributed to the rippled phase ($P_{\beta}'$).\(^{49,50}\) Between the gel state and the liquid-crystalline state often an intermediate phase is observed, the ripple phased. Although the structure of the rippled phase is still under dispute,\(^{51,52}\) it is clear that the chains are predominantly in the all-trans conformation, thus a gel-like conformation, and that they are tilted with respect to the bilayer normal.\(^{53}\) The vitrifications of the samples for the cryo-TEM (Figure 6.10a,b) were initiated at room temperature, that is close to the phase
transition and, therefore, the existence of the rippled phase is not unexpected.

After heating to 95°C, large aggregates are observed without definite size and shape. Apparently the heating leads to aggregation of the vesicles.

In sum, addition of the trans double-tailed azobenzene-substituted phosphates leads to small increases in $T_m$ and of the trans single-tailed azobenzene-substituted phosphates to significant decreases in $T_m$. In both mixtures, isomerisation of the azobenzene-substituted phosphates leads to a lowering of the $T_m$, indicating a disturbance of the bilayer.

6.4 Static solid state $^2$H NMR measurements

For the first measurements vesicles composed of 20 mol% of DT Azo-5P and 80 mol% of $^2$H$_2$-POPC (structure: see Figure 6.11, $T_m=-2^\circ C$) were used. Part of the vesicle solution was irradiated with light of 365 nm. In Figure 6.12 the $^2$H-NMR spectra of the unirradiated and irradiated sample are presented. The isotropic signal at 0 kHz is due to the presence of small vesicles and/or a small amounts of HDO present in the sample. A quadrupolar splitting is observed, which is due to the existence of large multilamellar vesicles (LMLVs).

![Figure 6.11 Structure of $^2$H$_2$-POPC.](image)

A quadrupolar splitting of 8.8 kHz was measured for LMLVs of the pure $^2$H$_2$ POPC, this value is slightly lower than the value measured for the mixture with 20 mol% of trans DT Azo-5P (9.0 Hz). So, introduction of 20 mol% of trans DT Azo-5P in POPC vesicles has a small effect on the order in the membrane.

It is evident that upon irradiation, leading to trans-cis isomerisation of the azobenzene-substituted phosphates, the quadrupolar splitting decreases from 9.0 kHz to 4.8 kHz. From these results it is concluded that the chain order in the membrane decreases upon trans-cis isomerisation of the azobenzene-substituted phosphate DT Azo-5P. The thermal back isomerisation from cis to trans was also followed by $^2$H-NMR. In Figure 6.13 the quadrupolar splitting is plotted as a function of time. As anticipated, the quadrupolar splitting slowly returns to its
original value corresponding with a thermal isomerisation from cis to trans.

**Figure 6.12** $^2$H-NMR spectra of vesicles composed of 80 mol% of $^2$H$_2$-POPC and 20 mol% of DT Azo-5P; (a) unirradiated; (b) irradiated with light of 365 nm.

Similar experiments with DT Azo-5P and POPC were performed in the group of Professor Glaubitz$^{55}$ and also a decrease in the chain order was observed upon trans-cis isomerisation of our azobenzene-substituted phosphate DT Azo-5P.

**Figure 6.13** The quadrupolar splitting as a function of the time of vesicles composed of 80 mol% of $^2$H$_2$-POPC and 20 mol% of DT Azo-5P. In time, thermal cis-trans isomerisation of DT Azo-5P occurs. The line is drawn as a guide to the eye. $T= 25$°C.
Is the decrease in order upon trans-cis isomerisation comparable with the effect of a substitution of a trans with a cis carbon-carbon double bond, at position 9? There is a controversy in the literature whether the chain order changes upon introducing cis double bonds in the alkyl chains. Róg et al.\textsuperscript{56} conclude, after combining experimental results\textsuperscript{57,58} and MD simulations,\textsuperscript{56} that at temperatures above the \( T_m \), the configuration of the carbon-carbon double bond has little effect on the order in the membrane. This is in contradiction with results of Gawrisch,\textsuperscript{42,45,59} in which he observed a lowering of the chain order upon introduction of a cis unsaturation. Róg\textsuperscript{56} notes that the packing in a POPC bilayer is looser in comparison with that in a DMPC (C\textsubscript{14:0}, C\textsubscript{14:0}) or PEPC (C\textsubscript{16:0}, C\textsubscript{18:1}) bilayer and that the lateral self-diffusion of the POPC is slower than that of DMPC and PEPC. Overall, there is little effect on the chain order. The work of both authors seems reliable and so it can be concluded that a cis configuration leads to looser packing but the magnitude of the decrease in order is still unclear.

### 6.5 MD simulations

MD simulations were performed to obtain insights into the conformations of the trans and cis DT Azo-5P molecules incorporated into a DOPC membrane. In addition, highly useful quantitative data can be calculated like area per lipid, thickness of the bilayer and electron density profiles.

MD simulations were performed on a small patch of a membrane, containing 32 DOPC, 6 DT Azo-5P (16 mol\%) and 1482 water molecules and 12 Na\textsuperscript{+}-ions.\textsuperscript{60} It was found that the trans isomer of DT Azo-5P is more elongated than the cis isomer and this can lead to a smaller cross-sectional surface area of the headgroup and a larger bilayer thickness. The overall electron distribution across the bilayer is similar for bilayers containing the trans or cis isomer of DT Azo-5P. From these results, it is concluded that the thickness of the bilayer is not significantly different for the two isomers of DT Azo-5P. The electron distributions of the separate isomers of DT Azo-5P are different, corresponding to a difference in shape. In Figure 6.14 snapshots of the simulation are presented. It is observed that, in time, the two chains of trans DT Azo-5P are on average more in each others proximity than the chains of cis DT Azo-5P.
Figure 6.14 Snapshots of the simulation of DT Azo-5P (16 mol%) in a matrix of DOPC. DT Azo-5P is highlighted as space filling. The water molecules are coloured green, the carbon atoms grey, the nitrogen atoms blue, oxygen atoms red and the phosphate atoms orange. Upper two pictures: trans DT Azo-5P; lower two pictures: cis DT Azo-5P.

6.6 Discussion

The main conclusion is that trans-cis isomerisation of the azobenzene-substituted phosphates leads to a decrease in the order of the membrane. In Chapter 3, a four times higher open probability for MscL upon trans-cis isomerisation was observed. Is there a relation between the results of Chapter 3 and those in this chapter, and is there a match with results from the literature? To answer these questions the model of Cantor\textsuperscript{61,62} (lateral pressure profile concept) and the conclusions of Gawrisch\textsuperscript{63} are used in our discussion.
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In Figure 6.15a,b a simplified presentation of their most important proposals are shown. Roughly, the membrane can be characterised in two different ways, i.e. by an increase or decrease in chain order or in terms of the lateral pressure, namely an increase or decrease of the lateral pressure in the chain region. Up to now, a relation is assumed to exist between the two parameters.\(^\text{63}\)

\[\text{a} \quad \begin{array}{c}
\text{3 DOPC} \\
\text{2 DOPC + 1 DOPE}
\end{array} \]

- release of headgroup repulsion.
- reduction of the area per lipid
- increase in chain order
- increase in chain repulsion

\[\text{b} \quad \begin{array}{c}
\text{3 DOPC} \\
\text{2 DOPC + 1 DOPE}
\end{array} \]

- increase of headgroup repulsion
- increase of the area per lipid
- decrease in chain order
- decrease in chain repulsion

\[\text{c} \quad \begin{array}{c}
\text{2 DOPC + 1 trans DT 4-Azo-5P} \\
\text{2 DOPC + 1 cis DT 4-Azo-5P}
\end{array} \]

- increase of the area per lipid
- decrease chain order

\textbf{Figure 6.15} Simplified proposals for understanding changes in the packing of the membrane upon introduction of an additive.

Introduction of DOPE (Figure 6.15a) leads to release of headgroup repulsions, a reduction of the area per lipid and therefore an increase in the chain order. In contrast, introduction of short-chain alcohols (Figure 6.15b) leads to an increase in repulsion in the headgroup region, an increase of the area per lipid and therefore a decrease in chain order. Upon trans-cis isomerisation of the azobenzene-substituted dialkyl phosphates (Figure 6.15c) a decrease in chain order was observed (this Chapter).

Previous results\(^\text{64,65,66}\) showed that DOPE shifts the open probability curve for MscL to higher pressures and that cone-shaped surfactants lower the open probability curve. As discussed in Chapter 4, the effect of addition of the cone-shaped surfactants on the membrane is unclear. DOPE increases the chain order and trans-cis isomerisation of DT Azo-5P reduces the chain order, so the four times higher open probability of MscL upon trans-cis isomerisation of DT Azo-5P (Chapter 4) fits into the
analysis. It seems that there is a relation between the chain order and the open probability of MscL. However, more experiments are necessary to prove this relationship.

In Figure 6.15b, the increase of headgroup repulsions leads to a decrease in chain order. In our system (Figure 6.15c) a decrease in headgroup repulsions is more plausible. Overall, the description of the effect of trans-cis isomerisation as a change in the lateral pressure profile is complicated.

Normally, a decrease in the order is accompanied by thinning of the membrane. Therefore, possible lipid-protein interactions can be due to membrane thinning and/or changes in lateral pressure. From the MD simulations is was concluded that the thickness of the bilayer was not affected significantly upon trans-cis isomerisation of the azobenzene-substituted dialkyl phosphates. It looks more reasonable that the increase in open probability of MscL is due to changes in the order or lateral pressure in the chain region. More experiments (e.g. $^2$H NMR) are necessary to obtain unambiguous data about the magnitude of thinning of the membrane upon trans-cis isomerisation of the azobenzene-substituted surfactants.

Looking at the results, it seems that trans-cis isomerisation of the azobenzene-substituted double-tailed surfactants has a larger effect on the permeability of the membranes than found for the azobenzene-substituted single-tailed surfactants (Chapter 3,5). In contrast, a large effect of trans-cis isomerisation on the $T_m$ was observed for both the single- and double-tailed surfactants.

The change in shape of MscL upon opening is still under debate, correlations with changes in the membrane that might induce opening are therefore difficult to establish.

6.7 Conclusions

Trans-cis isomerisation of the azobenzene-substituted phosphates leads to a lowering of the $T_m$ of vesicles formed from SAINT and DT Azo-xP or ST Azo-xP. A larger decrease is observed when the azobenzene in the chain is positioned closer to the head group. The decrease in the chain order, as measured by $^2$H NMR, strengthens the DSC results that the packing of the bilayer is significantly disturbed upon trans-cis isomerisation.

6.8 Acknowledgments

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6.9 Experimental section

General techniques. UV-vis spectra were recorded as described in Chapter 3. $^2$H$_2$-POPC has been previously synthesised.^{69}

Vesicle preparation. The vesicles were prepared as described in Chapter 3. After vortexing the hydrated sample, tip sonication or extrusion was applied. The samples were extruded 11 times through 200 nm filters at room temperature. Tip sonication was applied for 2 min. as described in Chapter 3.

Differential scanning calorimetry (DSC). DSC experiments were performed following the procedure outlined in Chapter 3. For the trans-cis isomerisation experiments the samples were irradiated with light of 365 nm for 15 – 30 min to obtain a maximum isomerisation. For the cis-trans isomerisation light of 436 nm was used to irradiate the sample for 15 min.

Cryo-TEM. Cryo-TEM experiments were performed according to the procedure described in Chapter 3.

Static solid state $^2$H NMR. A thin lipid film of 20 mol% of DT Azo-5P and 80 mol% of $^2$H$_2$-POPC (or d31-POPC) was prepared as described elsewhere (Chapter 3). The film was hydrated with 2 ml of deuterium-depleted water and was incubated for 20 min. without stirring or vortexing. The final total lipid concentration was 10 mM. The MLVs were vortexed 5 times for 1 s. and half of the sample was irradiated with light of 365 nm for 60 min. Spectra were recorded on a Bruker Avance 500 WB (Bruker BioSpin Corp., Billerica, MA) NMR spectrometer (operating frequency for $^2$H is 76.8 MHz), using a quadrupolar echo technique.^{70} The recycling delay was 200 ms, echo delay was 30 $\mu$s, the 90° pulse was 6 $\mu$s, and 20,000-50,000 scans were collected. Typically, before Fourier transformation, an exponential multiplication with a line-broadening factor of 100 Hz was used.

MD simulations. Dr. Alex de Vries performed the MD simulations and the detailed experimental details have been published.^{60}

6.10 References

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