Chapter 7

Epilogue

7.1 Achievements and outlook

The biological membrane is a complex system of phospholipids, steroids and proteins. It protects the inside of the cell form the outside world in a very specific way. Other important functions include transport of substances, signal transduction and recognition processes. Many diseases are due to mutations in membrane proteins. An altered membrane phospholipid composition is possibly a fundamental component of the abnormal signal transduction and neurodegradation in Alzheimer's disease. For a better understanding of the function of membranes often simplified systems are studied.

For this purpose vesicles are frequently examined, which are formed from synthetic amphiphiles. Synthetic amphiphiles often have simple chemical structures and vesicles can be prepared from one or a few amphiphilic compound. Since the discovery that vesicles could also be prepared from synthetic amphiphiles, they have been extensively used as model systems for biological membranes.

Protein-lipid interactions are important for an understanding of the different functions of the biological membrane. Cantor developed the lateral pressure profile concept for a better comprehension of protein-lipid interactions. Changes in the lateral pressure profile can lead to alterations in structure and function of membrane proteins. The major goal of the present thesis was to alter the lateral pressure in the membrane with the use of light-sensitive surfactants. Another target was the modulation of the activity of channel proteins by the use of light-sensitive surfactants.

Azobenzene was chosen as the light-sensitive functionality, because the trans-cis (E-Z) isomerisation of azobenzenes was known to be a clean process and easy to accomplish. Therefore novel surfactants were designed and their successful synthesis is described in Chapter 2. Various surfactants, single- and double-tailed, were prepared containing azobenzene moieties.

Studies of vesicles containing azobenzene-substituted amphiphiles are known in the literature. For instance, H-aggregation of azobenzene-substituted surfactants in membranes has been a recognised phenomenon for a long time. The results presented in Chapter 3 give more insights regarding the types of H-aggregates that can occur in
membranes. It became clear that UV-vis spectroscopy is an important tool for obtaining (indirect) information about the type of H-aggregates and the occurrence of domain formation.

Also trans-cis isomerisation experiments of azobenzene-substituted surfactants in membranes have already been reported in the literature. However, little research was performed to investigate the effect of trans-cis isomerisation on the membrane properties. In this thesis, results from different types of experiments indicate that disordered of the membrane is induced by trans-cis isomerisation of the azobenzene-substituted phosphates added to (phospholipid) vesicles. This effect was elucidated by DSC experiments, 2H NMR experiments, kinetic measurements of the thermal cis-trans back isomerisation and MD simulations.

Clearly, the modulation of the opening and closing of the channel protein MscL by isomerisation of the azobenzene-substituted phosphates is one of the highlights of this thesis (Figure 7.1). This result supports the hypothesis that the open probability of MscL depends on the order (or lateral pressure) in the membrane. It was already published in the literature that an increase in the order of the membrane was accompanied by a decrease in open probability. Despite new experiments which possibly strengthen this relation, it will probably always remain a rule of thumb. An approximation of the exact relation between the lateral pressure profile and the open probability of a channel protein can be obtained via (MD) simulations, chain order measurements by 2H NMR spectroscopy and the determination of the structures of the channel proteins. This combination looks the most promising, because determination of the lateral pressure profile by measurements has still not yet been accomplished.

Figure 7.1 Simplified representation of the modulation of the channel protein MscL.

Moreover, it is remarkable that the protein functions in a membrane which in part consists of synthetic amphiphiles that do not possess the basic structure of a phospholipid. Systems composed of a combination of synthetic amphiphiles and biological macromolecules [DNA, (membrane) proteins] are yet only poorly investigated, except for synthetic amphiphiles, which have been used for trafficking of DNA into cells. For
example, promising results have been achieved for non-toxic synthetic pyridinium-based amphiphiles\textsuperscript{9} and sugar-based gemini amphiphiles.\textsuperscript{10}

Future research on light-sensitive membranes can be focused on several issues. It is highly interesting to see if also other membrane proteins respond to trans-cis isomerisations of azobenzene-substituted amphiphiles, especially the channel proteins discussed in Chapter 1. The effect of the position of the azobenzene in the tail could be the goal of another interesting study. In this thesis, azobenzene-substituted phosphates were synthesised with the azobenzene at different positions in the tail. It was found that isomerisation of the azobenzene had different effects on the membrane. The expectation is that the effect of trans-cis isomerisation of the azobenzene on the function of membrane proteins depends on the position of the azobenzene in the tail. One could also envisage the incorporation of larger switches, which lead to even bigger disturbing effects on the vesicular bilayer.

Further fundamental research can give insights into the effect of trans-cis isomerisation on the properties of the membrane. For instance, a more thorough study of the thermal half-life time of the cis isomer of the azobenzene-substituted surfactants in the membrane will lead to more detailed information on the properties of the bilayer upon trans-cis isomerisation. Variables that can be examined include temperature, composition of the bilayer and size of the vesicles.

In Chapter 3 it was shown that upon trans-cis isomerisation at higher concentrations of the azobenzene-substituted dialkyl phosphate, release of calcein occurred via a rupture-like process of the vesicle. Therefore, light-sensitive vesicles can, in principle, be used as a controlled-release drug delivery system. The encapsulated drug can be released at a desired place with the use of local irradiation. The use of this system in medicine is still science fiction. Azobenzene is toxic and is suspected to be carcinogenic. Furthermore, release would only be convenient close to the skin, although specific methods are available for deeper irradiation.\textsuperscript{11}

Trans-cis isomerisation of the single-tailed azobenzene-substituted surfactants had a larger effect on the phase transition temperature in comparison with the double-tailed analogues. This is in contrast with the results of the calcein leakage experiments. Rupture of the vesicles was possible upon trans-cis isomerisation of the double-tailed azobenzene-substituted phosphates. In case of the single-tailed azobenzene-substituted phosphates, leakage of calcein could not be induced. Further experiments, for example, using \textsuperscript{2}H NMR spectroscopy, may clarify these observations.

In this thesis, the properties of light-sensitive membranes were investigated. These types of membranes are interesting systems for a study of non-specific lipid-protein interactions. For further research it is
important to know if trans-cis isomerisation induces a significant change in the membrane thickness. MD simulations predicted no significant change, additional $^2$H NMR experiments can give more support for this observation.

The results in this thesis are stimulating for further research. As research on membranes continues, it becomes more and more clear that their functioning is more complex than initially envisaged. With the present research a contribution has been made to a better understanding of membranes and membrane proteins, and we hope that the thesis may be an initiator for further research with light-sensitive membranes.

7.2 References

4 Cantor, R.S. *Biochemistry* **1997**, *36*, 2339.
Summary

Living organisms are built up from at least one cell. A human being is build up from ca. 10^{13} cells with ca. 220 different cell types. All cells are surrounded by a cell membrane. The basic compounds of the cell membrane are phospholipids, cholesterol and proteins. The lipid molecules contain a hydrophilic and a hydrophobic part. Molecules with this combination of properties are also called surfactants or amphiphiles. These molecules have a strong tendency for self-assembly and form aggregates in water. The hydrophobic parts of the different molecules attract each other and form the hydrophobic part of the assembly. The hydrophilic parts of each molecule (head group) point to the water phase. The driving force behind this process is largely provided by the water molecules. The water molecules prefer to have interactions with themselves rather than with hydrophobic parts of the surfactants and they, consequently, exclude the hydrophobic parts, this is called hydrophobic interactions. Different types of aggregates can be formed in water by the surfactants. One type of aggregate is the vesicle with inside an aqueous compartment. In principal, the biological cell is also a vesicle. Therefore vesicles are often used as model systems for the study of biological cell membranes.

The cell membrane has several functions like: protection of the content of the cell against influences from outside, passage of nutrients and waste products, recognition processes and signal transduction. Only small molecules (O_2, CO_2, H_2O) can diffuse directly through the membrane, larger molecules are usually transported via membrane proteins. Also transport against the gradient is managed by membrane proteins.

MscL is an abundant membrane protein which is important for regulation of the turgor in the cell. An osmotic downshock leads to swelling of the cell. To prevent lysis, the channel opens, and both ions and small molecules can leave the cell. In this manner the cell survives a osmotic downshock. The opening of MscL is induced by changes in the membrane, as a consequence of the swelling of the cell. Therefore these channels are called “mechano-sensitive” (MscL: mechano-sensitive channel of large conductance).

Various scientists published (simplified) models to represent the changes in the membrane. An example is the lateral pressure profile model. This model is based on the assumption that at the various depths in the membrane different lateral pressures are present. The variation of the pressure as a function of the position in the membrane is called the lateral pressure profile. At the moment, lateral pressure profiles can only be obtained via computer simulations. Experimental techniques are not available yet.
The idea, that the function of some membrane proteins is regulated by changes in the packing of the chains in the membrane, led to the interest in membranes in which these changes can be induced by simple chemical manipulation. The goal of this thesis was the preparation of membranes of which the packing properties could be changed by e.g. irradiation with light. A second aim was to explore the possibilities to regulate to opening and closure of MscL using these light-sensitive membranes.

For the construction of light-sensitive membranes, surfactants are necessary containing a light-sensitive group. The azobenzene group was chosen because it is known that this functionality can easily be switched from the trans (E) to the cis (Z) isomer and vice versa, using light of the appropriate wavelength (Figure 1). During the isomerisation (in both directions), normally no side reactions occur. The trans isomer of azobenzene is thermally the most stable form.

![Figure 1](image)

**Figure 1** The trans and cis isomer of azobenzene.

In Chapter 2 the synthesis of these light-sensitive surfactants is described. Phosphate based surfactants were synthesised with one or two azobenzene-substituted alkyl tails. The position of the azobenzene group in the tail was varied. In Figure 2 an example is given.

![Figure 2](image)

**Figure 2** An azobenzene-substituted surfactant, DT Azo-5P.

Unfortunately, the pure double-tailed azobenzene-substituted surfactants did not form vesicles. However, vesicle formation was possible when the light-sensitive double-tailed surfactants were mixed with vesicle-forming surfactants, like DOPC (a phospholipid).

The properties of bilayers containing azobenzene surfactants were studied in detail, special attention was, of course, paid to the effect of trans-cis isomerisation of the azobenzenes. It was already known from the literature that H-aggregation can occur in membranes containing trans azobenzene surfactants. H-aggregation is characterised by a
parallel alignment of the azobenzene moieties, which induces dipole-dipole interactions accompanied with a change in the UV-vis absorption spectrum. It became clear (Chapter 3) that two types of H-aggregation can occur, with and without domain formation. H-aggregation without domain formation can only occur at higher concentrations of the azobenzenes. The occurrence of H-aggregation with domain formation depends on the method of preparation of the vesicles and the ionic strength of the solution. The formation of H-aggregates with domain formation was undesirable for later experiments. Moreover, trans-cis isomerisation of these types of aggregates was sometimes not possible. A relationship was proposed between the wavelength of absorption and the extent of H-aggregation and possible domain formation.

Trans-cis and cis-trans isomerisation induced by light of the appropriate wavelength was easily accomplished for vesicles containing azobenzene-substituted surfactants without domain formation (Chapter 3). The isomerisation was found to be clean and was maximal within minutes. The thermal half-life times of the cis isomer were also determined. It became clear that the half-life times depend on the position of the azobenzene group in the tail. When the azobenzene group is situated closer to the head group, shorter half-life times were measured. An increase in the amount of double-tailed azobenzene surfactant in the bilayer led to a faster thermal cis-trans isomerisation.

The effect of trans-cis isomerisation of the azobenzene-substituted surfactants on the permeability of the membranes was investigated using fluorescence spectroscopy. A high concentration of a fluorescent probe was encapsulated in vesicles composed of DOPC and DT Azo-5P. The non-encapsulated fluorescent probe molecules were removed by filtration. The leakage of the probe from the vesicles can then be followed by fluorescence spectroscopy because at high concentrations of the probe in the vesicle the fluorescence is quenched. When leakage of the probe occurs, the fluorescence will increase as a result of the dilution of the probe. Without trans-cis isomerisation no release of the probe was observed during at least one hour.

Above a concentration of 20 mol% of DT Azo-5P in DOPC vesicles, leakage of the probe occurred after light-induced trans-cis isomerisation. The leakage of calcein, as a result of the trans-cis isomerisation, increased with an increasing concentration of DT Azo-5P. Cryo-EM revealed that trans-cis isomerisation leads to rupture of the bilayer of the vesicle.

The effect of trans-cis isomerisation of DT Azo-5P on the opening and closure of MscL is described in Chapter 4. The purification of the channel protein is described and also the reconstitution of the protein in a membrane composed of 20 mol% of DT Azo-5P and 80 mol% DOPC. Two techniques were used for monitoring the opening and closure of the
channel: fluorescence spectroscopy (leakage of a probe) and patch clamp. After irradiation of the liposomes, leading to trans-cis isomerisation of DT Azo-5P, a release of 10% was monitored in comparison with the control-experiment.

A larger effect of trans-cis isomerisation of DT Azo-5P on the opening and closure of MscL was observed using patch clamp. During a patch clamp experiment, a small part of the membrane is sucked into a small glass pipette. The membrane-fragment forms a seal in the pipette. Exchange of ions between outside and inside of the pipette is only possible if the protein channel opens. The current, which is induced by opening of the channel, is recorded. It turned out that MscL, after trans-cis isomerisation of DT Azo-5P, was on average four times more often in the open conformation then before the irradiation. The open probability decreased upon switching back to the trans isomer. These results make clear that it is possible to regulate the opening and closure of the membrane protein MscL with the use of light-sensitive membranes.

The aggregation behaviour of the single-tailed azobenzene-substituted surfactants in water was studied in Chapter 5. The single-tailed azobenzene-substituted surfactants were more soluble in water than the double-tailed azobenzene surfactants. The surfactants form micelles as was observed by cryo TEM, surface tension measurements and fluorescence spectroscopy. With cryo TEM also larger particles were observed. Vesicles were prepared consisting of DOPC and the single-tailed azobenzene-substituted surfactants. In this system it was also possible to isomerise the light-sensitive surfactants from trans to cis and vice versa. Higher thermal half-life isomerisation rates where observed for the cis isomer when the azobenzene was positioned closer to the head group. The isomerisation was also faster in the membrane in comparison with the free monomer in solution. In other experiments the effect of addition of the single-tailed azobenzene-substituted surfactants to DOPC vesicles was studied. Binding of the single-tailed surfactants to DOPC vesicles was observed using fluorescence spectroscopy. Despite the large effect on the phase transition temperature, the permeability of the vesicles was not affected by trans-cis isomerisation as measured by calcein efflux experiments. The opening of MscL could not be induced by trans-cis isomerisation as monitored by fluorescence spectroscopy.

The effect of trans-cis isomerisation of the light-sensitive surfactants on the properties of the membrane was more extensively studied in Chapter 6. The effect of trans-cis isomerisation on the phase transition temperature was studied using DSC. For this purpose, vesicles were prepared which consisted of the light-sensitive surfactants and a matrix lipid with an phase transition temperature well above 0°C. For both the vesicles containing the single- and the double-tailed azobenzene-substituted phosphates a lowering of the phase transition temperature
was observed upon trans-cis isomerisation. The effect increased as the azobenzene moiety was closer situated to the head group. $^2$H NMR measurements revealed also that the chain order decreased upon trans-cis isomerisation. These findings strengthen the results described in Chapter 4. In the literature it is assumed that opening of MscL is more difficult if there is an increase in the order of the membrane. In the present research, a decrease in the order of the chain packing was found as a consequence of trans-cis isomerisation. This decrease led apparently to an increase in the open probability of MscL.

The results described in this thesis illustrate that the trans-cis isomerisation of the light-sensitive surfactants has a significant effect on the properties of membranes. It became clear that it is possible to regulate the opening and closing of the membrane protein MscL. In Chapter 7 the results are placed in a broader perspective and some suggestions for further work are presented.