Synthetic amphiphiles as gene delivery agents
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The objective of the research described in this thesis was to develop novel synthetic amphiphiles, to examine the possible application of the corresponding vesicles as drug carrier systems and to investigate their mechanism of action. Because it is anticipated that gene therapy will play an increasingly important role in future treatment it is necessary to develop carriers that can transport DNA and oligonucleotides into cells. The advantage of using synthetic amphiphiles as such carrier system is tremendous. They have been shown to protect nucleic acids from degradation. Because the vesicles made from synthetic amphiphiles have the ability of drug entrapment or drug association of a variety of drugs due to the variation in the structure of the amphiphiles, they can concentrate drugs in order to lower the dose. This structural variation, i.e. the ability to be modified with each group of interest at each available place in the molecule, can also lead to a possible targeting device. Moreover, amphiphiles are thought to be non-immunogenic and are relatively easy to synthesize (and thus applicable for large scale production). In this thesis, plasmid DNA was used as a model drug because the synthetic amphiphiles are highly efficient DNA carriers. Chapter 2 describes different parameters which can influence the transfection efficiency of the transfection complex using DOTMA/DOPE as the delivery system. The size of the vesicles does not influence the transfection efficiency, in contrast to the length of the DNA, even when taking into account the number of copy DNA available. The shortest DNA (4.7 kB) gave the best transfection results. Further correlation between the length of the DNA and the transfection efficiency could not be observed. Chapter 2 also describes the ability of DOTMA/DOPE vesicles to create pores in the cellular membrane. For these studies erythrocytes were used. Upon membrane rupture or pore formation, hemoglobin leaks out of the cell, which can be measured spectroscopically. The amount of released hemoglobin is then a measure of the degree of membrane rupture. In this way a good model was created to determine whether pore formation could be involved in the mechanism of transfection. DOTMA/DOPE vesicles showed a
correlation between the concentration of the vesicles and the percentage of leakage. An increase in the concentration leads to an increase in hemoglobin release. It was observed that upon addition of DNA to the vesicles, at low amphiphile concentration, the cell membrane was protected from permeabilization by the amphiphiles, while at higher amphiphile concentrations pore formation was more pronounced, emphasizing the role of the amphiphiles and their accessibility in this process.

In 1944 it was found that pyridinium-derived amphiphiles showed antiseptic and antibiotic properties. In 1982 it was shown that these types of amphiphiles could form vesicles when they were suspended in an aqueous medium. Chapter 3 describes the transfection efficiencies of a novel series of pyridinium-derived amphiphiles. These amphiphiles proved to be very effective in bringing about transfection. The transfection efficiency is increased up to 12 times in comparison with DOTMA/DOPE-induced transfection. The transfection ability of these vesicles turned out to be applicable to a broad range of cell lines. Chapter 3 describes also in more detail the relationship between the structure of an amphiphile and its transfection ability, as examined by systematically modifying the alkyl chain and the head group of the amphiphile. The length of the alkyl chains is an important parameter. The alkyl chains should be long enough to form vesicles and to create hydrophobic domains in order to stabilize the complex. When the alkyl chains are too long, however, the transfection efficiency decreases, probably due to an excess of hydrophobicity or to steric hindrance. The amphiphiles need a certain degree of hydrophobicity in order to form stable vesicles and stable complexes. Upon increasing the hydrophobicity by increasing the alkyl chain length, the formed complex will not be stable, because the increased hydrophobicity will cause a repulsion of the DNA. The length of the alkyl chain seems to reach an optimum around 16 carbon atoms. An unsaturation in the alkyl chain of the amphiphile leads to an increase in transfection efficiency, especially when this unsaturation is in an isomerically pure conformation, i.e. 100% cis-orientation or 100% trans-orientation. Using different methods it was shown that these amphiphiles were non-toxic when used in vitro. This chapter further describes the pore-forming capacity of these amphiphiles, using the erythrocyte membrane. However, these amphiphiles create pores to a lesser extent than the DOTMA/DOPE amphiphiles. This could indicate that the decreased toxicity of the new amphiphiles is probably due to a decreased membrane permeabilization. Because the new amphiphiles are far more effective in transfecting cells, compared
Summarizing discussion

to DOTMA/DOPE vesicles, it is likely that a larger amount of cells interacts with the complex, although the interaction per cell is lower. In total this will lead to a higher transfection and to a lower toxicity.

With the introduction of cationic amphiphiles as a transfection reagent in 1987 it was believed that fusion was the mechanism by which these vesicles interact with the cell membrane. Chapter 4 therefore describes the fusion behavior of the pyridinium-derived amphiphiles. Using different techniques it was shown that these vesicles can fuse rapidly and to a high extent with themselves. Asymmetric fusion, either with phospholipid liposomes or with erythrocyte ghosts was also observed. Fusion appears to be dependent on the structure of the amphiphile. Amphiphiles with saturated alkyl chains fuse to a lesser extent and with slower kinetics than those with unsaturated alkyl chains. Although these vesicles showed to be quite fusogenic, it is not likely that fusion is the main event involved in the interaction of the amphiphiles with the cell membrane. When the complex of vesicles and DNA is made, as is done prior to the transfection experiments, no fusion ability could be observed anymore, indicating that the transfection complex as such is not fusogenic. Chapter 5 describes in more detail the interactions of the vesicles and of the complex with the cellular membrane. To this end, the synthetic amphiphiles were labeled with $^{14}$C and the DNA was labeled with $^{32}$P. Then the cell-associated material was determined as a function of time at $4^\circ\text{C}$ (to determine the binding) and at $37^\circ\text{C}$ (to determine the binding and the uptake). It was shown that the vesicles as well as the DNA (i.e. the total complex) were only taken up by the cells after an initial incubation of at least two hours. Moreover, it was shown that vesicles with inferior transfection abilities also associated poorly with the cells. In this chapter it was also shown that the charge ratio of amphiphile over DNA as exits in the final complex, is an important factor for optimal transfection. An unsaturation in the alkyl chain leads to a decrease of the charge ratio. The unsaturation in the alkyl chain also influences the size of the complex, which becomes larger and more heterogeneous. It was found that the more heterogeneous the complex, the lower the transfection. This chapter furthermore describes the involvement of specific membrane surface molecules. Addition of pronase abolished the transfection completely, calcium inhibited the transfection by 85% and an incubation with an antibody against an adhesion molecule reduced the transfection to 30%. These experiments may well indicate that calcium-mediated proteoglycans might be involved in the uptake of the complex and act as a receptor,
or that they trigger a specific reaction or a specific compound needed for the uptake of the complex.

In the final chapter, the amphiphiles described in chapter 3 were used to deliver oligonucleotides to ovarian carcinoma cells in order to inhibit the expression of the P-glycoprotein, which is responsible for the multidrug-resistant phenotype. This chapter first describes the over-expression of a functional P-glycoprotein on resistant tumor cells and the absence of it on sensitive tumor cells. Then the functionality of the P-glycoprotein was determined, using a fluorescent dye, showing that the P-glycoprotein which is present on the resistant cells is active. It has not been possible yet to inhibit the amount of this protein or to inhibit its function, but further research is in progress.

Taken together, the present studies show that synthetic amphiphiles are very efficient carriers for introducing DNA into a variety of cells and are therefore suitable carriers for the delivery of genetic material to cells. Particularly, the pyridinium amphiphiles described in chapter 3, showed to be very potent and to be non-toxic. In general this thesis supplies evidence that a surface-protein dependent pore formation mechanism might be involved in the transfection.

A challenging step will be to unravel the complete mechanism. This might be achieved by screening proteins and lipids at the cell surface, which are involved in the binding and/or uptake of the vesicles, the DNA or of the complex as a whole. It is also interesting to examine cells which are difficult to transfect or cannot be transfected at all, whether they show a decreased level of specific proteins or lipids.

The work described in this thesis implies that the transfection increases when an unsaturation is introduced in the alkyl chains of the amphiphiles. The role of this unsaturation should be investigated in more detail. The next goal would be to study the fate of the amphiphiles and that of the DNA in the cell. Therefore, the vesicles and the DNA (or any drug of interest) could be labeled with a non-exchangeable label, for instance a radiolabel, and given to the cells. At different time points and at different temperatures cell organelles should be isolated and screened for radioactivity. Co transport and co localization with other compounds can then be determined as well. It will also be relevant to investigate the fate, stability and toxicity of these amphiphiles in vivo. Up till now several studies have been performed in which plasmid DNA is complexed to cationic vesicles and injected into mice. Most of the activity is located in liver and lung and some activity in spleen, heart and kidney.
The final goal (targeting the complex to specific cells or specific tissues) can then be accomplished by investigating whether a targeting device, like an antibody or specific sugar residues, can be incorporated in the vesicles without affecting their properties. One must bear in mind however, that the correlation between in vitro and in vivo studies is very poor. The correlation between in vivo studies in different species (for example mice versus human) is also very poor. The experiments in human cell culture should therefore be explored, including co-cultures and simultaneous influences of various compounds.