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Gene delivery with cationic lipids

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Summary

The replacement of a deficient gene by its normal copy has been the basis of the treatment envisioned for gene therapy of inherited disorders. With improving and rapidly advancing insight, showing that the products of genes and their regulation also play a crucial role in many other diseases, including acquired diseases, gene therapy now holds promises and expectations for the treatment of numerous disorders that range from cancer to infectious diseases like AIDS. The principle of gene therapy has therefore been reconsidered and refocused as bearing relevance not only to replacing a potentially deficient gene but also, and more importantly, to influencing the physiology and signal transduction in the cell by over-expressing or down-regulating one or several genes, for example those expressing membrane receptors. Down-regulation of a gene can be achieved by the transfer of short antisense oligonucleotides (ODNs) into cells or by intracellular delivery of the more recently developed small interfering RNA (siRNA). In addition, a gene can be expressed by translocating plasmid DNA into cells containing the sequence for a gene of interest under the influence of a promoter.

The methods for delivering nucleic acids (ODNs, siRNA or plasmid DNA) to the cells are divided into two groups, i.e., viral and non-viral methods. Viral vectors were developed first and take advantage of the natural ability of a virus to penetrate into cells and use their machinery for the production of viral proteins. Although very efficient *in vitro* and *in vivo*, viral vectors may pose problems in terms of safety for the use as therapeutic drugs because of their immunogenicity and in some cases the potential risk of insertion of the gene into the host genome that can lead to the development of cancer as some recent clinical studies have shown [1]. Non-viral vectors that comprise cationic lipids and cationic polymers are thought to be safer *in vivo* because they are immunologically inert. They can also accommodate a greater variety of cargo and are not limited to coding sequences of nucleic acid as viral vectors but can deliver directly antisense ODNs and siRNA. These vectors are also easier to produce and can be readily chemically modified for the purpose of improving therapeutic applications. The efficiency of these vectors, however, is lower than that of viral vectors and has until now precluded their extensive use for *in vivo* therapeutic applications. Therefore a better understanding of the parameters that govern transfection efficiency of non-viral vectors will be crucial for the development of *in vivo* applications.

The aim of this thesis was to characterize the mechanisms of transfection mediated by lipoplexes prepared from different cationic lipids and to study potential applications of these

vectors for gene therapy. Two cationic lipid systems are used in this thesis: the SAINT-2 cationic lipid system used in combination with dioleoylphosphatidylethanolamine (DOPE) for the delivery of antisense ODNs (Chapters 3 and 4) and a cationic lipid system that consists of pH sensitive sugar-based gemini surfactants (Chapters 5 and 6). **Chapter 2** presents a review on the use of cationic lipids for gene delivery. The biophysical parameters important for the formation of the complexes, interaction with the cell membrane and further intracellular delivery of nucleic acids in the cells are discussed in this chapter. It emphasizes the importance of structural properties of the lipoplexes in each step of the transfection process and discusses the potential role played by a helper lipid, like DOPE. Furthermore endocytosis of lipoplexes, mediating cellular entry, and the mechanism of endosomal disruption are considered in this chapter. Finally, the important role of non-bilayer phases of lipoplexes (inverted hexagonal, hexagonal and cubic micellar phases) in endosomal disruption is discussed.

Poly(ethylene glycol) (PEG)-lipid analogues are used in gene delivery to stabilize lipoplexes, thereby preventing interaction with serum components and non-target cells and thus promoting a longer blood circulation time of such (PEG-) coated lipoplexes. In **chapter 3**, the effect of different PEG-lipid analogues on lipoplex formation with ODNs, the structural phases of PEGylated lipoplexes and the efficiency of intracellular delivery of ODNs is investigated. The non-exchangeable DSPE-PEG is compared to ceramide-PEG analogues of variable chain lengths with correlated variable exchangeable properties. It is shown that DSPE-PEG prevents the nuclear translocation of ODNs by preventing their release from the endosomal compartment. Because of its higher exchangeability, the short ceramide-C8-PEG analogue did not preclude as extensively as DSPE-PEG the release and translocation of ODNs to the nucleus. Finally, cryo-EM and SAXS measurements showed that DSPE-PEG stabilizes the lamellar phase of lipoplexes.

In previous work, it was demonstrated that SAINT-2/DOPE-ODN lipoplexes are efficient carriers in the delivery of antisense ODNs to neuronal cell lines, leading to an effective and functional down-regulation of neuronal membrane receptors [2]. Accordingly, we considered the option to exploit the principle of this approach in designing a non-invasive antisense therapy to the brain, and aimed at the construction of means and devices for lipoplexes to cross the blood-brain barrier (BBB). The purpose of the studies described in **chapter 4** of this thesis was to set up an experimental in vitro model for the BBB in our laboratory, to study the interaction of SAINT-2/DOPE-ODN lipoplexes with this BBB model, and to investigate lipoplex transport across the cell monolayer. The first part of the

chapter describes the successful introduction of this BBB model, including its morphological and structural characterization.. Adsorption-mediated transcytosis is a well characterized mechanism for cationic entities to cross cell monolayers, but despite the cationic nature of the lipoplexes, transcytosis and subsequent secretion at the opposite side of the cells was not observed. Furthermore we show in this chapter that coupling of a model protein BSA precluded the capacity of SAINT-2/DOPE cationic liposomes to form complexes with ODNs. Therefore, coupling strategies for the purpose of triggering receptor-mediated transcytosis, a mechanism that has been characterized for transferrin and its receptor, will require improvement in order to eventually accomplish successful translocation of lipoplexes across endothelial cell layers.

In **chapter 5** mechanistic features and the transfection efficiency of different pH sensitive sugar-based gemini surfactants were investigated, both in vitro and in vivo. These sugar-based gemini surfactants contain two protonatable amino moieties in the head group, the charge of which depends on the pH. Thus, the extent to which the two amino moieties are charged will influence the relative size of the head group of the cationic lipid, which is reflected by a pH-dependent phase behavior [3,4]. Out of the five compounds examined, two surfactants with an ethylene oxide spacer, GS1 and GS2, were found to effectively transfect cells in vitro with a relatively low level of toxicity. Both compounds were shown to adopt at physiological pH a lamellar phase and to display a good colloidal stability in salt and serum. In vivo transfection experiments showed that because of this relative stability at physiological pH, both compounds were capable of avoiding ‘preliminary capture’ as large clustered complexes in the lung capillaries, as inferred from the absence of transfection in the lungs, evidenced by bioluminescence imaging.

Finally, in **chapter 6** a study is presented on the potential mechanism of transfection, mediated by the two pH sensitive gemini surfactants, GS1 and GS2. Of particular interest, these studies revealed that the gemini lipoplexes undergo a lamellar-to-non-inverted micellar (H_I) phase transition with decreasing pH as occurs in the endosomal compartment. This observation is novel, since it contrasts the common observation reported for numerous other lipoplexes, which display a lamellar-to-hexagonal H_{II} transition. A lipid mixing assay and plasmid DNA release studies demonstrated that these lipoplexes disrupt model membranes particularly at acidic rather than at neutral or basic pH. Furthermore, the presence of PS in the target membrane was necessary for disruption to occur. From these studies, we conclude that the gemini-based lipoplexes undergo at mild acidic pH a lamellar- to- normal (non-

inverted) hexagonal H_I phase, and that next to complexes that display an H_{II} phase, also the H_I phase can lead to membrane disruption, endosomal release and subsequent transfection.

Discussion and perspectives

We show in this thesis how PEG-lipid analogues, although capable of prolonging the circulation time of lipoplexes and preventing interaction with blood components and cells, can also negatively affect the effectiveness of intracellular delivery of lipoplex cargo. We also show that lipoplexes, formed from pH sensitive sugar-based gemini surfactants mimic some of the properties of the PEGylated complexes by similarly displaying a good stability at neutral pH, while they are able to disrupt (endosomal) membranes at acidic pH and transfect cells. Experiments to determine the circulation time of these lipoplexes in vivo and their biodistribution, as compared to PEGylated lipoplexes, would be of interest in order to evaluate whether these compounds would be an interesting alternative to the use of PEGylated lipids. In addition to maintaining a lamellar phase, PEG-lipids also confer stealth property by providing a hydrophilic coating to the lipoplexes and creating a steric barrier. With the gemini surfactants this hydrophilic coating is provided by the reduced sugar in the head group. For further development, the steric barrier properties of other gemini surfactants with longer sugar chains could be tested for their interaction with blood components.

For targeting purposes, such sugar-based gemini surfactants could be functionalized at the level of the head group, involving the coupling of a targeting ligand. The possibility of such coupling and the capacity to form efficient lipoplexes should be studied. Evidently, the potential spectrum for applications of such targeting strategies would be numerous, ranging from the targeting of tumors for cancer gene therapy to the exploitation of receptor-mediated transcytosis for strategies of blood-brain barrier crossing.

On a more fundamental basis, concerning the mechanism of transfection, studies of the intracellular fate of gemini lipoplexes could bring more insight into the intracellular trafficking of lipoplexes in general. Since the transfection properties of such sugar-based gemini surfactants, in contrast to other surfactant systems, is dependent on the pH, caveolae-mediated endocytosis of these lipoplexes might be induced, for example by further manipulating their size, which may serve as a trigger for such an event [5]. Importantly, since this mechanism of internalization likely avoids exposure of lipoplexes to a mild acidic pH environment, this pathway should not lead to transfection. Accordingly, such experiments would allow to sort out whether such a caveolae-mediated pathway is relevant to endocytosis of lipoplexes and whether this pathway could be instrumental in transcytosis

in endothelial cell crossing, given that this cell type is particularly enriched in caveolae on its surface.

To increase the potential of cationic lipid systems as vectors for the use in gene therapy, precise insight is needed into the different barriers involved in eventual expression of the delivered gene. In this context, some very recent observations indicate that nucleic acid release via endosomes to the cytosol occurs as efficiently with lipoplexes as with adenovirus particles [6]. This would suggest that intracellular delivery of nucleic acid is not the limiting step in transfection but that other parameters are involved. Studies on the effect of cationic lipids on the condensation of genes and their ensuing stability in the cytosol, in conjunction with an effect on transcription efficiency, may provide more insight into issues such as why viral vectors can be more efficient in transfection, in spite of delivering a substantially lower number of gene copies into the nucleus.

On a more general basis, gene therapy using cationic lipids represents 8.3 % of the gene therapy clinical trials worldwide (updated from January 2006 at www.wiley.co.uk/wileychi/genmed/clinical/) against 49 % for adenovirus and retrovirus systems together. This emphasizes that more insight is needed to address questions as to why cationic lipid-mediated transfection is less efficient than virus-mediated transfection, with approaches as described above and throughout this thesis. Moreover, in addition to developing new cationic lipid vectors, it would be necessary to broaden and diversify applications for existing vectors. Testing different cell types and different cargos, and exploiting different ways of administration will allow further development of these vectors for in vivo applications of gene therapy.

The resemblance of non-viral vectors with traditional drugs, in the way that they can be developed or produced, is their greatest advantage. A specialization of cationic lipids, to a disease or to an administration route, might bring more realism in the development of these vectors as effective drugs for gene therapy. For example, in case of the pH sensitive sugar-based gemini surfactants used in this thesis, a non-anticipated observation in the in vivo experiments might trigger a new range of applications for these compounds. Thus, in these studies, we observed in the mouth of the animal, injected with gemini lipoplexes, expression of the reporter gene, suggesting efficient transfection. This probably resulted from ingestion of the gemini lipoplexes by the mice, which also might have led to some transfection of the gastrointestinal tract. Further experiments with oral delivery of these lipoplexes to verify this hypothesis will be needed but could well lead to novel applications in oral gene therapy. For instance, oral gene therapy can be considered for the treatment of recurrent or refractory oral

cancers, like the oral squamous cell carcinoma [7]. Oral gene therapy could also be an alternative in the treatment of type 1 diabetes, such treatment would aim at delegating the production and secretion of insulin to the small intestine [8,9]. These approaches represent interesting potential therapeutic applications for sugar-based gemini surfactants.

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