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Cationic Amphiphiles as Delivery System for Genes into Eukaryotic Cells

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Abstract. Cationic liposomes, consisting of synthetic amphiphiles and a so-called helper lipid, rapidly form complexes with DNA, known as lipoplexes. When incubated with cells in culture, the DNA can be delivered into the cell and becomes expressed. Because of these properties, lipoplexes are considered a useful alternative for viral vectors for *in vivo* gene therapy. Yet, many hurdles have still to be taken. These are illustrated in the present overview, which briefly describes the critical steps involved in overall gene delivery *in vitro*, ranging from lipoplex formation to nuclear penetration and delivery of the desired gene.

1. Introduction: Cationic Amphiphiles for Transfection

When analyzed at the molecular level, numerous disorders appear to be due to genetic impairment. Therefore, treatment of the cause of such diseases will require manipulation at the level of the genetic machinery of the cell, including the possibility to substitute malfunctioning genes. This therapy may include replacement of one particular gene, but may also involve the introduction of a multigene complex, e.g. artificial chromosomes [1]. The validity of applying transfection technology is not restricted to medical purposes only. Also in biotechnology, transfection has become a versatile tool in order to achieve expression of particular proteins in cells (e.g. for quality improvement; production of proteins for therapeutic purposes) or in cell biology, to study the function of particular proteins or intracellular processes, mediated by a specific protein.

To introduce a gene or transfect a cell, several methods are available that give rise to more or less efficient transfection. Common methods for transfection are the co-precipitation of DNA using calcium phosphate [2], and membrane permeabilization with DEAE dextran. Both methods rely on osmotic pressure, exerted onto the cell membrane, and are neither selective nor applicable *in vivo*. Also, the efficiency of delivery is usually very low, and at best a few percent of the cells show expression of the desired protein. Physical methods, like electroporation and the use of the so-called "gene gun" [3] often suffer from the same shortcomings. Viruses, such as retroviruses and adeno- or adeno-associated viruses, are particles that require a transfection strategy for their own reproduction, for which purpose they exploit eukaryotic target cells. It is therefore that viruses can be seen as highly efficient vectors for gene-delivery. Unfortunately, in spite of their efficiency,

a number of safety limitations of these particles are apparent, including an immunological response, which precludes frequent administration (if required), and potential transformation risks of the host cell. Therefore, alternative carrier systems are investigated, including the application of synthetic amphiphiles or 'cationic' lipids.

2. Structure and Behaviour of Amphiphiles

The first report concerning a potential for amphiphiles to efficiently transfect eukaryotic cells was published in 1987 by Felgner and colleagues [4]. Amphiphiles are amphipathic compounds that consist of a hydrophilic head group and a pair of hydrophobic carbon chains, either saturated or unsaturated, that may vary in length (usually between 14-18 carbons). Because of this amphipathic structure, amphiphiles are able to form a bridge between organic and aqueous environments. The most common natural amphiphilic molecules are lipids. Under physiological conditions (25°C, pH~7.4) amphiphiles self-assemble into membrane structures, which can be lamellar, bilayer structures or non-bilayer structures, like inverted hexagonal phases. The molecular shape of the molecule dictates this phase preference, which implies that molecules with an overall cylindrical shape prefer a lamellar organization, whereas cone shaped molecules prefer inverted phases, such as the hexagonal phase. Amphiphiles with a relatively small head group compared to the surface area occupied by the hydrophobic region of the molecule prefer an inverted phase and as such can destabilize a bilayer structure [5, 6].

The charge of the head group of an amphiphile can be either negative (e.g. phosphate) or positive (nitrogen). However, for the purpose of delivery of DNA it is obvious that the latter is preferred given the charge of the nucleic acid, which per base pair contains a double negative charge. Hence, electrostatic interactions represent a determining factor in the interaction between the amphiphiles and the DNA molecules. As will be discussed below, for delivery *per se*, other factors are involved as well. One of these parameters must involve the structure of the amphiphile. Dozens of different compounds have been synthesized, which show differences in head group as well as hydrophobic chains, attached to the head group via ester, ether, C-N or C-C bonds. The nature of these bonds may be related to toxicity, a feature prominently associated with several amphiphile formulations. Toxicity has been reported to be more prominent for amphiphiles with stable ether linkages than for those containing less stable ester linkages [7]. Interestingly, a recently synthesized and highly efficient amphiphile called 'SAINT', which contains the chemically highly stable C-C bonds (fig.1), shows little if any toxicity [8], implying that the dogma that a high transfection is correlated with a high level of cytotoxicity, is not necessarily correct.

Each compound often gives rise to differences in transfection activity, the efficiency of which is frequently dependent of the cell type, and the presence of exogenous factors such as serum.

The hydrophilic head groups are based in all cases on quaternized nitrogen-atoms including different amine and pyridinium groups (SAINT) [8]. The length and state of saturation of the alkyl chains also varies, although data presented thus far suggest that mono-unsaturated alkyl C₁₈ chains are often most effective. We have investigated molecular parameters that determine transfection efficiency of a series of novel pyrimidium surfactants, in which we systematically modified either the head group structure, including the distribution of charges within this region, as well as the length and state of saturation of the alkyl chains. The highest transfection efficiency was obtained when the pyridinium ring containing the single charged nitrogen was left unaltered, while the double alkyl chains consisted of *cis* C_{18:1} (figure 1). However, the presence of such aliphatic tails

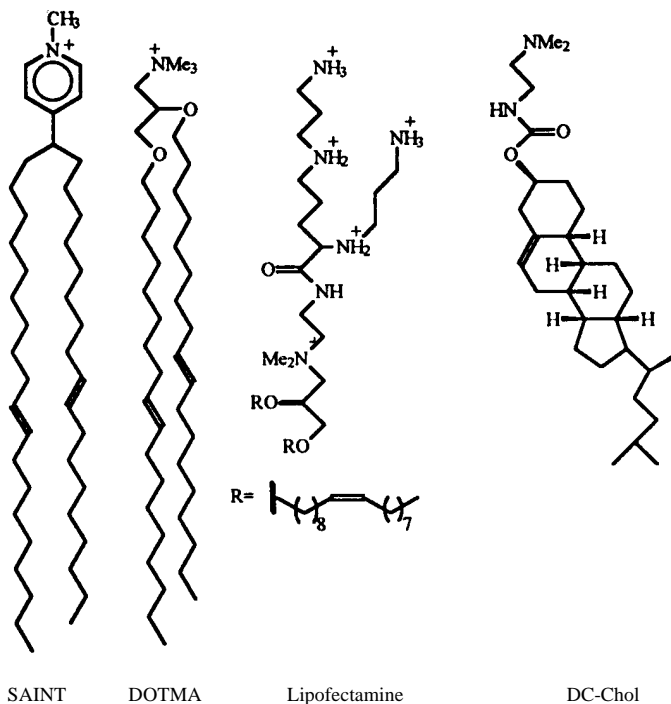


Figure 1. Structures of some representative cationic amphiphiles

is not a prerequisite for constructing an effective delivery vehicle. Also other hydrophobic anchors have been employed, as in derivatives based upon cholesterol [9] and cholic acid anchors [10].

3. Self Assembly of Amphiphile/DNA Complexes

The complexes consisting of DNA and amphiphile are relatively easy to prepare. First, liposomes are made of equimolar amounts of the amphiphile of choice and a ‘helper’ lipid, dioleoylphosphatidylethanolamine (DOPE). These vesicles are usually prepared by sonication. Then DNA is added and the charge interaction leads to a total reassembly of lipids around the DNA, often interpreted as resulting from DNA-induced fusion of the amphiphilic vesicles (see below). The structure of the resulting DNA-amphiphile-complexes (lipoplexes) seems to be dependent on both the type of amphiphile and the DNA. Especially the physical properties of the cationic amphiphile plays an important role in complexation. During the complex formation, the cationic amphiphiles should be in the fluid state as this state greatly facilitates the interaction with the DNA. Amphiphiles, whose bilayers are in a solid state, usually give rise to the formation of lipoplexes, which are loosely packed. In case of the SAINT amphiphiles with saturated chains, the resulting complex shows up like ‘beads on a (DNA) string’, suggesting a poor packing of the DNA by the amphiphile [our unpublished observations; c.f. 11]. We suppose that the lower transfection efficiency of these lipoplexes is caused by that property, i.e., the inability to properly

**Uncovered plasmid-DNA
(visible on the surface of complexes)**

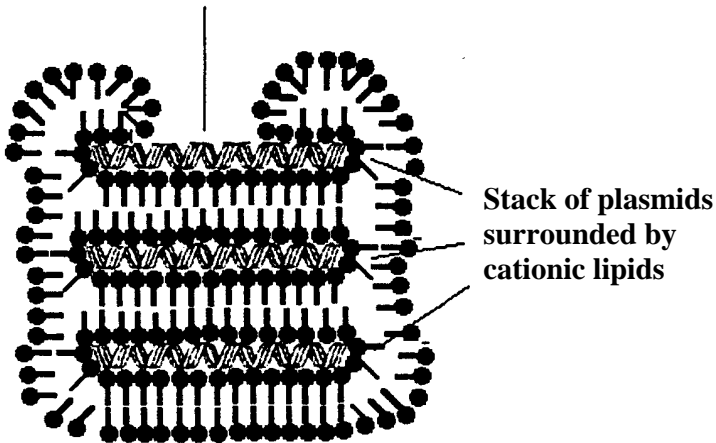


Figure 2. Crossection of a multilayered Lipoplex

wrap a supercoiled plasmid, and to maintain its DNA in a condensed state. With amphiphiles in the fluid state, the lipoplex formation follows very fast kinetics, which can be monitored when using lipid mixing assays [12]. Thus a total reorganization of the initial vesicular structures takes place, and, interestingly, we observed that the eventual transfection efficiency of the complex was independent of the initial size of the amphiphilic vesicles, i.e., prior to their addition of DNA [13].

The exact role that membrane fusion plays, or whether membrane fusion plays a role at all in the assembly of the complex, is not clear. This is related to the fact that lipid mixing is monitored as measure of fusion. Fusion implies however that also contents mixing occurs of two volumes that were initially separated. Amphiphilic vesicles, however, are often highly leaky, and hence, preclude verification of fusion by a contents mixing assay [6]. Moreover, the final structure that is obtained upon lipoplex formation has little resemblance of a well-defined bilayer that has grown in surface area as a result of fusion. Therefore, more detailed studies will be needed to define separate steps that eventually lead to the assembly of the lipoplex. Without additional evidence, lipid mixing as such does not suffice to prove the involvement of membrane fusion. As an alternative mechanism for vesicle-vesicle fusion, it is possible that the assembly involves molecular recruitment, the donor membranes providing a direct source for the DNA to recruit the 'amphiphilic coat'. Whatever the precise mechanism, for some of these complexes it has been demonstrated that their basic structure encompasses multilayers of organized plasmids surrounded by cationic lipids, as revealed by x-ray crystallography [14]. In the final preparations, often differently sized lipoplexes are observed. This may result from imperfect coating of the DNA (see Fig.2; ref.[15]). Thus adjacent complexes may become aggregated by exposed DNA, which may lead to larger complexes. From the point of view of achieving efficient transfection, it is important to note that in mixtures that have been allowed to equilibrate, the size of the complexes usually increases, particularly after long storage. At the same time, the transfection efficiency decreases.

Apart from a connection between the size of lipoplexes and their transfection efficiency, the net charge of the complex is also an important factor, codetermining transfection efficiency.

Experiments with cationic amphiphiles and anionic DNA, mixed at different charge ratios revealed that a more positive net charge leads to a higher transfection efficiency. An excess of positive charge is likely relevant for the initial interaction of the complex with the cell surface, presumably involving negatively charged sulfated proteoglycans, which have been suggested to promote cationic lipid-mediated transfection [16]. On the other hand, with an excess positive charge, it is also possible that lipidic particles, not containing DNA, co-exist in the mixture, that could facilitate transfection, as has been demonstrated *in vivo* [17]. The effect of noncomplexed amphiphiles and/or DOPE upon transfection in cell cultures is, however, unknown.

Another feature that should be taken into account when correlating amphiphile properties and transfection efficiency is their ability to condense open circular and linear DNA molecules [18]. As already noted, compacted particles, containing condensed DNA, should likely lead to higher transfection efficiency. This is particularly related to the ability of complexes to be processed along the endocytic pathway, thought to represent a major entry pathway for productive transfection, while taking into account the dimensions of the transport vesicles involved. Given an upper limit of approx. 100-150 nm for coated or uncoated endocytic vesicles, it is difficult to reconcile an endocytic processing of complexes exceeding sizes of 300 nm. However, the influence of DNA condensation on the size of the resulting lipoplexes appears marginal. Our own studies indicate that the major fraction of the plasmid DNA is already in a (supercoiled) condensed state, and does not significantly condense further upon complexation with cationic amphiphiles [unpublished observations].

Moreover, by employing different cationic amphiphiles that displayed different transfection capacities, we were unable to correlate those differences to differences in compaction ability, which was essentially the same for all amphiphiles tested. Hence further compaction of routinely used supercoiled E.coli-derived plasmids, that contain the desired reporter genes, does not seem to be a dominant parameter in governing transfection efficiency.

4. Role of the Helper Lipid in Complex Formation and Transfection Efficiency

As noted above, synthetic amphiphiles are often mixed with the zwitterionic phospholipid DOPE, which has been shown to greatly facilitate transfection relative to that obtained for the amphiphile *per se* [e.g. 4]. At neutral pH, DOPE in isolation does not form a bilayer structure, and hence requires the presence of a bilayer-stabilizing environment, as provided by the bilayer-forming amphiphiles. However, in isolation, or when becoming enriched in domains upon phase separation in mixed bilayers, DOPE will adopt an inverted hexagonal phase, dictated by the shape concept, as explained above. The presence of DOPE is also often related to an enhancement of bilayer destabilization in general and membrane fusion in particular [19, 20]. At present, it is largely unknown how and via which property DOPE is involved in the delivery of genes into cells. Evidence is essentially lacking which would support a role in fusion of the complex with a cellular membrane. In fact, direct measurements of lipid dilution, as a measure of membrane fusion, are not consistent with a high fusion potential of the DNA/lipid complexes [18, 21]. Rather, the membrane destabilizing properties of DOPE, resulting in pore-like structures [8] may be instrumental in the ultimate delivery of genes into the cell's cytoplasm, a crucial step in the eventual delivery to the nucleus. In this regard, it is also relevant to note that phosphatidylcholine, a lamellar phase promoting lipid, inhibits transfection efficiency. This further suggests that the bilayer destabilizing features of DOPE are of importance in obtaining productive DNA delivery. In this regard, further work will also be needed to examine the miscibility of the helper lipid with the cationic amphiphiles, and how this miscibility may influence the stability of the DNA-amphiphile interaction, as upon ultimate nuclear delivery, the presence of residual amphiphile

inhibits transcription efficiency (see below). It is thus possible that DOPE may also be involved in weakening the intensity of the charge interaction between DNA and the cationic amphiphile, thereby eventually promoting an effective dissociation of plasmid from the complex.

5. Association of Complexes with the Cell Membrane; Mechanisms of Gene Entry

There is still a fragmentary understanding of the biological processes involved in the transfer of the transgene into the cell and its subsequent expression. In principle, cell surface-attached complexes could either directly translocate their plasmid into the cells at the level of the plasma membrane or prior to that, might be internalized, followed by delivery via the endosomal compartment. This scenario bears similarity to the mechanisms exploited by viruses to deliver their nucleic acids into cells for viral replication [22]. Thus pH-independent viruses, such as paramyxoviruses, after attachment to distinct surface receptors, directly fuse with the plasma membrane, mediated by specific viral spike proteins, thereby delivering their genetic material into the cytoplasm. However, there are numerous virus families (orthomyxovirus, retroviruses) that require a mild acidic pH for productive infection, as provided by the environment of the endosomal compartment, which is reached after internalization of the viral particle by endocytosis. The requirement of a low pH is dictated by special requirements that are imposed on the viral fusion protein, present on the viral surface, before it can display fusion properties that trigger the merging of the viral and the endosomal membranes, thereby ejecting the nucleic acid into the cytoplasm. At present it is still not entirely clear at which cellular site, lipoplexes carry their DNA into the cytoplasm. There seems to be no obvious reason why a mild acid pH would be required, as there is no measurable physical effect of mild acid pH on the amphiphile/DNA complex. Yet, many investigators appear to favor a mechanism that involves endocytic internalization, followed by perturbation of the endosomal compartment [23, 24], the release of the nucleic acid occurring via competitive interaction with negative charges. The latter can be induced, for example, by the acidic phospholipid phosphatidylserine, which has been shown of being capable to displace the nucleic acid from the amphiphile [25]. It is unclear whether such interactions, which do not seem to be dictated by acid pH, can also take place at the cell surface of eukaryotic cells. It is apparent though, that at neutral pH, the complexes are capable of perturbing plasma membrane integrity. Thus DNA/amphiphile complexes, consisting of SAINT or lipofectin have been shown to cause hemolysis, emphasizing that these complexes do not remain attached as inert particles on surfaces of cells lacking endocytic capacity [8, 26]. Since significant lipid mixing is not observed at these conditions, the question arises as to whether complexes can engage in transfer of amphiphile and DOPE, either as monomers or during contact, which upon insertion into the target membrane can weaken its integrity. In light of revealing mechanisms of gene translocation, it would be of interest to determine the molecular transfer capacity of amphiphiles and the potential role of DOPE in facilitating this process.

It should be noted that most of the evidence favoring endocytosis is still largely circumstantial. The demonstration of endocytosis *per se* is insufficient for proving that endocytosis is required for transfection to occur, while it has often been difficult to exclude that the inhibition of transfection by endocytic inhibitors was related to an inhibition of endocytosis rather than to replication. Furthermore, it has not always been possible to clearly define the lipoplex-labeled intracellular compartments and to unambiguously visualize lipoplex-containing endosomes. Experiments with fluorescent lipoplexes have shown an accumulation of fluorescence around the nucleus [23, 27]. It is possible that this fluorescence corresponds to lipoplexes, which are located in late endosomal/lysosomal structures. If so, it is not clear how effective the DNA can still escape

from these compartments, as the nucleic acid is readily degraded in the lysosomal compartment [25].

As referred to above, and described in detail elsewhere [13], another possibility to explain the mechanism of lipoplex uptake and DNA delivery relies on the permeabilization of the target membrane. Such permeabilization could arise from lipid phase separations, induced upon the transfer of amphiphile/DOPE molecules into the plasmamembrane, or from insertion of the latter as (unstable) domains. However, in spite of lack of knowledge concerning the mechanism, perturbation of the erythrocyte membrane occurs upon their interaction with lipoplexes, causing the release of hemoglobin (65 kDa). Whether the size of such defects is large enough to allow passage of a (partly released) plasmid, is unknown. As will be discussed below, the size exclusion range of the nuclear membrane is around 70 kDa, which translates into pores of approx. 10 nm. Lipoplexes permeabilize erythrocytes, causing the release of molecules of a similar size. It is in this regard of interest to mention that there is a clear limit in the size of the plasmid that can be translocated via a lipoplex-mediated mechanism [8]. This size dependence, showing optimal transfection for plasmid DNA of 4.7 kb and little if any for plasmids of 18 kb (and intermediate values for sizes in between) would be consistent with the formation of a 'pore' of a distinct size, allowing cytoplasmic entry of the plasmid. However, at the same time, such a distinct size dependence would not be consistent with entry via a rupture mechanism, as we do not anticipate that rupture would be discriminatory to the access of plasmids in the size range of 4-18 kbp [8]. Hence, although the exact mechanism remains to be determined, the mechanism of delivery likely involves a membrane destabilization and 'pore' formation step, either taking place at the level of the plasma membrane or at the endosomal membrane or both. At the level of the endosome, one would thus anticipate a more subtle mechanism of delivery than one that would involve delivery via endosomal rupture.

In the process of cytoplasmic delivery, the issue remains as to whether free plasmid, i.e. devoid of any associated amphiphile, is entering the cytoplasm or whether plasmids are still partly covered by the cationic lipid. The latter might be necessary for providing protection against rapid degradation by cytoplasmic nucleases. Remaining amphiphile might also be needed for enabling the eventual entry of the complex into the nucleus, i.e., the amphiphile playing an active role in nuclear penetration. It is evident though, that for transcription to occur, complete dissociation has to take place. In a cell-free system, we observed little if any transcription of plasmid DNA in the presence of SAINT amphiphiles [unpublished observations]. Also, microinjection of plasmids, but not complexes, into the nucleus leads to gene expression, implying that the lipoplex coat is inhibitory [23]. It would appear therefore, that complete dissociation of the plasmid from the lipoplex, possibly mediated by negative counter charges (phosphatidylserine, see above), is crucial for obtaining the most efficient transfection. The completeness of this dissociation will depend on the stability of the lipoplex in terms of the stability of the interaction between DNA and amphiphile/DOPE mixture, as determined by electrostatic and structural interactions. These parameters, in turn, may be effected by the interaction of (extra)cellular components with the complex (including serum). Taken together, in this manner the amphiphile *per se* may very much determine the stability of the complex, and hence the relative ease of its dissociation. It is thus possible that these parameters are crucial in explaining differences in transfection capacities of various synthetic amphiphiles.

6. Trafficking of the Complex in the Cytoplasm

To become expressed, the DNA has to reach the nucleus. How the lipoplexes or the plasmid, free or partly covered by amphiphile traffics through the cytoplasm to the nucleus is still unknown.

Interestingly however, when microinjected into the cytoplasm, naked DNA will not significantly become expressed, and it seems that, in the absence of a nuclear localization signal, it poorly if at all reaches the nucleus [23, 28, 29]. By extrapolation, this would imply that the plasmid, in order to accomplish efficient transcription, should preferably still have attached at least part of its amphiphilic coat. In passing, it is then tempting to suggest that complete dissociation in one of the previous steps described above would be highly disadvantageous to the efficiency of a particular amphiphile: if uncoating is complete before reaching the nucleus, the plasmid might never become expressed. The latter will also be the case, as inferred from expression in cell free systems or nuclear injection, when an efficient level of coating is still present. Hence, it could be suggested that there is a delicate balance between the level of coating and uncoating of the plasmid/amphiphile complex, which eventually governs transfection efficiency. A priori, for nuclear entry, the plasmid may well require part of the membrane stability perturbing amphiphilic coat. This suggestion stems from the notion that *signal-mediated* nuclear import allows passage of particles up to 25 nm [30]. With atomic force microscopy, we noted that GFP- and CAT-containing plasmids, in the absence of amphiphile/DOPE, display a supercoiled structure with sizes of 50x200 nm. Hence, unless the the supercoiled structure unwinds into a free hydrated DNA double helix, which is about 3 nm in width, it is not very likely that such particles, irrespective of the presence of the nuclear signal, readily traverse the nuclear membrane. These considerations would therefore provide a rationale as to why at least some amphiphilic remnants would be beneficial for plasmids in order to accomplish transfection as efficiently as possible. On the other hand, it cannot be excluded that nuclear integration and transcription will mainly occur after cells have divided. This notion would be in line with observations that it is virtually impossible to transfect non-dividing cells.

7. Summary

In this brief overview, we have exclusively focussed on the use and fate of synthetic amphiphiles as gene delivery systems *in vitro*. An effort was undertaken to discuss the pitfalls in the various steps involved in transfection, ranging from complex assembly to nuclear delivery. By being able to carefully define the requirements of each of these steps, it might become possible to design amphiphiles that might compete with viral counterparts in terms of efficiency. It is evident though, that although progress made thus far is promising, there are a considerable number of hurdles that have yet to be taken before effective gene delivery can be rationalized and realized *in vivo*, as discussed in [28], employing synthetic amphiphiles as delivery vehicle. Yet, although a therapeutic application may not be imminent, for cell biological purposes, transfection based upon the application of synthetic amphiphiles has been quite successful, allowing the (over)expression of a host of proteins, also in cells that were difficult to transfect by classical means [see e.g. 31].

A variety of amphiphiles is currently available. This makes it possible to screen and compare their efficiency, which may be of help in understanding the mechanism of their action, a crucial issue in further advancing the application *in vivo*. From a practical point of view, such an application would be advantageous as most amphiphiles used thus far display essentially no immunological response, are readily produced in large quantities, show a high chemical stability, and are easy and economical in their use, compared to other transfection procedures.

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References

- [1] J.J.Harrington *et al.*, Formation of de novo centromeres and construction of first generation human artificial microchromosomes, *Nat. Genet.* 15 (1997) 345-355.
- [2] W. Song and D.K. Lahiri, Efficient transfection of DNA by mixing cells in suspension with calcium phosphate, *Nucleic Acids Res.* 23 (1995) 3609-3611
- [3] E.L. Rech *et al.*, Biolistic-mediated gene expression in guinea pigs and cattle tissues *in vivo*, *Braz J Med Biol Res* 29 (1996) 1265-1267.
- [4] P.L. Felgner *et al.*, Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure, *Proc.Natl.Acad.Sci.U.S.A.* 84 (1987) 7413-7417.
- [5] J.N.Israelachvili *et al.*, Physical principles of membrane organization, *Q. Rev. Biophys.* 13 (1980) 121-200.
- [6] J.B.F.N. Engberts and D.Hoekstra, Vesicle-forming synthetic amphiphiles, *Biochim. Biophys. Acta* 1241 (1995) 323-340.
- [7] P.L. Felgner *et al.*, Improved cationic lipid formulations for *in vivo* gene therapy, *Ann.NY.Acad.Sci.* 772 (1995) 126-139
- [8] I. van der Woude *et al.*, Novel pyridinium surfactants for efficient, nontoxic *in vitro* gene delivery, *Proc Natl Acad Sci U. S. A.* 94 (1997) 1160-1165.
- [9] C. Kawaura *et al.*, Atomic force microscopy for studying gene transfection mediated by cationic liposomes with a cationic cholesterol derivative, *FEBS Lett* 421 (1998) 69-72.
- [10] R.K. DeLong *et al.*, Novel cationic amphiphiles as delivery agents for antisense oligonucleotides, *Nucleic Acids Res* 27 (1999) 3334-3341.
- [11] B.Sternberg *et al.*, New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy, *FEBS Lett.* 356 (1994) 361-366.
- [12] D.K. Struck *et al.*, Use of resonance energy transfer to monitor membrane fusion. *Biochemistry* 20 (1981) 4093-4099
- [13] I. van der Woude *et al.*, Parameters influencing the introduction of plasmid DNA into cells by the use of synthetic amphiphiles as a carrier system, *Biochim. Biophys. Acta* 1240 (1995) 34-40.
- [14] J.O. Radler *et al.*, Structure of DNA-cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes, *Science* 275 (1997) 810-814.
- [15] S. Huebner *et al.*, Lipid-DNA complex formation: reorganization and rupture of lipid vesicles in the presence of DNA as observed by cryoelectron microscopy, *Biophys.J.* 76 (1999) 3158-3166
- [16] K.A. Mislick and J.D. Baldeschwieler, Evidence for the role of proteoglycans in cation-mediated gene transfer, *Proc.Natl.Acad.Sci.U.S.A.* 93 (1996) 12349-12354.
- [17] Y.K. Song and D. Liu, Free liposomes enhance the transfection activity of DNA/lipid complexes *in vivo* by intravenous administration, *Biochim. Biophys. Acta* 1372 (1998) 141-150.
- [18] H.G. Hansma *et al.*, DNA condensation for gene therapy as monitored by atomic force microscopy, *Nucleic Acids Res* 26 (1998) 2481-2487.
- [19] M Vidal and D. Hoekstra, *in vitro* fusion of reticulocyte endocytic vesicles with liposomes, *J. Biol. Chem.* 270 (1995) 17823-17829.
- [20] M.Otter-Nilsson *et al.*, Cytosolic ATPases, p97 and NSF, are sufficient to mediate rapid membrane fusion, *EMBO J.* 18 (1999) 2074-2083.
- [21] T. Stegmann and J.Y. Legendre, Gene transfer mediated by cationic lipids: lack of a correlation between lipid mixing and transfection, *Biochim. Biophys. Acta* 1325 (1997) 71-79.
- [22] D. Hoekstra, Membrane fusion of enveloped viruses: especially a matter of proteins, *J.Bioenerg. Biomembr.* 22 (1990) 121-155.
- [23] J. Zabner *et al.*, Cellular and molecular barriers to gene transfer by a cationic lipid, *J Biol Chem* 270 (1995) 18997-19007.
- [24] A. El Ouahabi *et al.*, Intracellular visualization of BrdU-labeled plasmid DNA/Cationic liposome complexes, *J Histochem Cytochem* 47 (1999) 1159-1166.
- [25] Y. Xu and F.C. Szoka, Jr., Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection, *Biochemistry* 35 (1996) 5616-5623.
- [26] H. Gershon *et al.*, Mode of formation and structural features of DNA-cationic liposome complexes used for transfection, *Biochemistry* 32 (1999) 7143-7151.
- [27] X. Gao and L. Huang, Cytoplasmic expression of a reporter gene by co-delivery of T7 RNA polymerase and T7 promoter sequence with cationic liposomes, *Nucleic Acids Res* 21 (1993) 2867-2872.
- [28] S. Simoes *et al.*, Cationic liposomes as gene transfer vectors: Barriers to successful application in gene therapy, *Curr. Opin. in Mol.Therap.* 1 (1999) 147-157.

- [29] H.Pollard *et al.*, Polyethylenimine but not cationic lipids promote transgene delivery to the nucleus in mammalian cells, *J.Biol.Chem.* 273 (1998) 7507-7511.
- [30] F. Melchior and L. Gerace, Two-way trafficking with Ran, *Trends Cell Biol.* 8 (1998) 175-179.
- [31] S.C.D. van IJzendoorn and D.Hoekstra, (Glyco)sphingolipids are sorted in sub-apical compartments in HepG2 cells: A role for non-Golgi-related intracellular sites in the polarized distribution of (glyco)sphingolipids, *J. Cell Biol.* 142 (1998) 683-696.