Recombinant DNA techniques together with the mapping of the human genome have made it feasible to treat genetic disorders at their cause instead of treating the symptoms. Viruses are, exploiting their natural ability to enter cells and deliver their genes into the nucleus, used to deliver genetic material into mammalian cells to correct for the genetic disorders. However, the human body is likely to elicit an immune response against these viruses, which will limit the efficacy of the treatment and, moreover, may seriously endanger the patient’s health. Therefore, non-viral gene carrier systems are under development that ultimately should be as efficient as viral vectors in delivering their genetic cargo into cells, while being less toxic. Cationic lipids have proofed to be safe delivery systems, although their efficiency of delivery is still lower compared to viral vectors. To be able to enhance the transfection efficiency of cationic lipid-DNA complexes (lipoplexes), a thorough understanding of the mechanism of transfection is essential. The transfection process can be divided into three steps, each of which poses a barrier to the efficiency of gene transduction, i.e., (i) binding of lipoplexes to cells, followed by their internalization, (ii) release of DNA from the lipoplex and into the cytoplasm and (iii) nuclear translocation of the DNA, followed by transcription. The ultimate challenge will be to design lipoplexes that meet such requirements, that each step proceeds with the highest efficiency possible.

To begin with, to achieve cellular binding of lipoplexes a positive charge ratio of the cationic lipid to the DNA may be optimal. This will result in binding of the lipoplexes to the negatively charged cell membrane by electrostatic interaction. Alternatively, ligands can be incorporated onto the lipoplex surface that may specifically interact with cellular receptors. Subsequently, the lipoplexes have to be internalized. Internalization of lipoplexes by mammalian cells most likely occurs through an endocytic process. Clathrin-mediated endocytosis was shown to represent the main entry pathway for lipoplexes. Clathrin coated vesicles (CCVs) that mediate receptor uptake can reach sizes up to 200 nm. This implies that lipoplexes, in order to be internalized via CCVs, should not exceed a size of 200 nm. The positive charge ratio of cationic lipid to DNA results in the formation of relatively small, condensed particles compared to particles that are neutral. Thus, next to mediating cell binding, the positive charge ratio of the lipoplex may be important in controlling the size of the lipoplexes. However, it has been reported that opsonized bacteria (Escherichia coli) can be internalized via the pathway of clathrin-mediated endocytosis, suggesting that clathrin-mediated endocytosis of particles exceeding diameters of 200 nm is not unprecedented.
The cellular uptake of lipoplexes via an endocytic process implies that the lipoplex and/or the DNA have to escape from the endosomal compartment prior to reaching the lysosomal compartment, where degradation may occur. It was shown that lipoplex size and/or surface properties influence the trafficking of lipoplexes towards lysosomes. Small lipoplexes (200-250 nm) bearing DNA protrusions being transported more rapidly into lysosomes than bigger (200-400 nm - 1 μm) and smooth lipoplexes, which could diminish their transfection efficiency. In addition, the relatively big lipoplexes expose a greater surface for interaction with the endosomal membrane than small lipoplexes. This would enhance the chances of escape from the endosomal compartment, assuming that lipid mixing between the lipoplex and the endosomal membrane is instrumental in the endosomal release of the lipoplex. Moreover, DNA protrusions may hamper the intimate contact between the lipoplex and the endosomal membrane, which is required for lipid mixing between these two structures. Alternatively, it could be envisaged that lipoplexes that cause endosomal membrane perturbations affect (arrest) the endosomal trafficking. Thus the absence of (large) lipoplexes in lysosomes presumably reflects the consequence of an efficient endosomal escape.

The ability of the lipoplex to adopt a hexagonal conformation has been correlated with efficient transfection. Such a hexagonal phase may induce perturbations in the endosomal membrane, which will allow the release of DNA into the cytoplasm. The presence of the helper lipid dioleoylphosphatidylethanolamine (DOPE), which forms an inverted hexagonal phase in isolation, in the lipoplex facilitates transfection. Next to playing a role in the hexagonal organization of the lipoplex, DOPE facilitates the release of DNA from the lipoplex by weakening the interaction between the cationic lipid and the DNA. More accurately, the PE-headgroup of DOPE is responsible for the efficient release of DNA from lipoplexes. Recently, it was shown that cationic lipids can adopt a hexagonal phase when mixed with anionic lipids, while both populations of lipids form lamellar phases in isolation. Thus the hexagonal phase of the lipoplex may well be promoted by the presence of acidic lipids from the endosomal membrane. The flip-flop of phosphatidylserine (PS) from the endosomal membrane into the lipoplex, as suggested by Xu and Szoka, thus may serve a dual purpose. First, PS neutralizes the electrostatic interaction between the cationic lipid and the DNA, which results in lipoplex disassembly. Second, the combination of PS with cationic lipids may promote the formation of a hexagonal structure, which allows the transfer of DNA over the endosomal membrane into the cytoplasm of the cell. The dual function of PS implies that the release of DNA from the
lipoplex and into the cytoplasm occur concomitantly. It should be noted that the DNA has to be present in a condensed state to efficiently translocate over the endosomal membrane. The mechanism by which lipoplexes, organized in a hexagonal phase, perturb the endosomal membrane remains unclear. Does fusion take place or is the hexagonal phase relevant to translocation of cationic lipids into the target membrane, thereby causing a destabilization? The relevance of lamellar to hexagonal phase transitions for the mechanism of lipoplex-mediated transfection sets requirements to the shape, and hence the structure of the cationic lipid, implying that the synthesis of novel compounds may take such features into account. Currently, hexagonal phase formation is determined by small angle X-ray- and $^{31}$P NMR-measurements, which are techniques that are often not routinely available. A simple screening assay for the presence of hexagonal phases, e.g. based on fluorimetry, would be of great help to those who try to design transfection-active cationic lipids.

Following its endosomal escape the DNA has to be transported into the nucleus for expression to occur. Similarly, antisense oligonucleotides have to reach the nucleus in order to downregulate gene expression. Alternatively, antisense constructs may exert their function in the cytoplasm of the cells. The half-time of DNA present in the cytoplasm is approximately 90 minutes. In order to reach the nucleus intact it should travel relatively fast through the cytoplasm of the cell into the nucleus. Whereas oligonucleotides can freely diffuse into the nucleus, the nuclear transfer of DNA most likely requires active transport through the nuclear pore complex (NPC). As macromolecules with a diameter of up to 36 nm were shown to pass the NPC, the average width of a supercoiled DNA molecule of 20-80 nm would allow NPC-mediated nuclear transfer of (exogenous) DNA. However, efficient transfection of synchronized cell cultures has been shown to coincide with the onset of mitosis. During mitosis the nuclear envelope disassembles. The absence of the nuclear membrane during mitosis would greatly facilitate the uptake of DNA into the nucleus, and most likely is responsible for the majority of DNA transfer into the nucleus during transfection. While the condensed conformation of DNA is important for its translocation over cellular membranes, condensed (supercoiled) DNA is also known to be transcriptionally more active than decondensed (linear) DNA.

In summary, in order to achieve efficient transfection the lipoplex should harbor the following properties. First, the DNA in the lipoplexes should be in a condensed state, in order to allow efficient translocation over the endosomal (and nuclear) membrane. A positive charge ratio of cationic lipid to DNA may aid to form such condensed particles and in addition mediate
binding of the lipoplexes to the negatively charged cell surface. To allow release of the lipoplexes from the endosomes intimate contact between these two structures should be promoted. Herein a hexagonal conformation of the lipoplex could be helpful. Subsequently, the transfer of acidic lipids into the lipoplex may promote the formation of a hexagonal phase which may result in membrane perturbations, that allow DNA translocation into the cell’s cytoplasm. Little is known about the subsequent transfer of exogenous DNA into the nucleus. Piggybacking on proteins, e.g. histones, containing a nuclear localization signal (NLS) should be considered as a possibility. Alternatively, during the absence of the nuclear membrane at the time of mitosis DNA may be taken up into the nucleus.

Next to optimizing non-viral vectors for gene delivery, attention should be paid to the development of the gene constructs. If sustained expression of the transgene is required, integration of the gene into the host genome is necessary. However, aspecific integration of a gene into the host genome could lead to the disturbance of normal gene functioning. Transfection with artificial chromosomes as well as episomally stable plasmids are promising alternatives.

While in general adherent cells can be efficiently transfected with cationic lipids, cells that grow in suspension seem to be more resistant to lipoplex-mediated transfection. With the current knowledge of the requirements that have to be met for cellular uptake and endosomal release of lipoplexes, an attempt can be made to elucidate the resistance to transfection of suspension cells. *(Ex vivo)* transfection of suspension cells would be of great interest for the treatment of, for example, blood-borne diseases such as leukemia. Moreover, transfection of stem cells would provide a tremendous (gene)therapeutic potential for the treatment of several disorders, such as Parkinson’s disease, graft rejection, and cancer.