LIPOPLEXES: A NON-VIRAL GENE DELIVERY VEHICLE

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ABSTRACT
A lipoplex (also known as Genosome) is a lipid and DNA complex that is used to deliver genes. It can be a form of non-viral gene therapy as the complex does not require any components of a virus in order to transport genetic material. As a promising strategy for the treatment of many inherited and acquired diseases, gene therapy which is defined as the genetic modification of cells for therapeutic benefit. The aim for gene therapy is to deliver healthy exogenous drugs such as plasmid DNA and single-strand oligonucleotides to replace a missing gene that otherwise have a normal makeup so as to cure genetic diseases, for instance, cystic fibrosis, malignant melanoma, and gaucher's disease. The formation of lipoplexes was a result of the electrostatic interaction between cationic charge from lipids and anionic charges from DNA. It include both large scale production and small scale production. Mechanism of action of lipoplexes include Endocytosis of lipoplexes and then release of lipoplex from endosomes. Various characterization techniques are used for evaluation of lipoplexes like light scattering, electrophoretic mobility, microscopy, diffraction calorimetry, FT-IR, NMR, ESR, FRET and separate characterization parameters for lipoplexes and physicochemical properties and toxicity studies also done. Results shows that good bioavailability, biocompatibility, inhence permeability and targeting achieved. Future of this type of drug deliver system will explore the utilization of nanogenobiotics.

Key Words: gaucher's disease, nanogenobiotics, FT-IR, NMR, ESR, FRET.

INTRODUCTION
The use of cationic-lipids complex (lipoplexes) for gene transfer was introduced by Felgner et al. and Wu and Wu, respectively. Their use has moved rapidly from transfection of cell cultures to clinical gene therapy applications. Among about 60 synthetic transfection agents that are available commercially, more than half are based on the use of cationic reagents.
Cationic lipid/DNA complexes (lipoplexes) used in gene therapy are based on the hypothesis that the complexes adsorb more effectively to the anionic plasma membrane of mammalian cells via electrostatic interactions.

Compared with other non-viral delivery systems, including anionic liposomes that encapsulate nucleic acids, lipoplexes tend to mediate a higher level of transfection in numerous cell lines.

The aim for gene therapy is to deliver healthy exogenous drugs such as plasmid DNA and single-strand oligonucleotides to replace a missing gene that otherwise have a normal makeup so as to cure genetic diseases, for instance, cystic fibrosis, malignant melanoma, and gaucher's disease.

Based on these factors, reliable and efficient vectors delivering exogenous genes into target cells are urgently awaited. Non-viral vectors are widely used for their advantages over viral vectors in recent years, because they are safe and cheap, easy to manufacture in lager scale, and they can also deliver large pieces of DNA.

During the past years, designing new non-viral system for DNA transfer has become an interesting field attracting more and more researchers.

**Formation of cationic lipoplexes**

The formation of lipoplexes was a result of the electrostatic interaction between cationic charge from lipids and anionic charges from DNA. Lipoplex morphologies were determined by the competitive interactions between electrostatic forces and elasticity forces driven by the lipid hydrophobic moiety.
A single plasmid is surrounded by sufficient cationic lipids to completely neutralize the negative charges of DNA and provide a complex with a net positive charge that can associate with the negatively charged surface of cells, which may be correlated with effective transfection.

Cationic liposomes and DNA are driven by electrostatic interactions to form lipid–DNA complexes or lipoplexes.

Two processes occur during the formation of cationic lipoplexes, namely, DNA-induced membrane fusion and liposomes induced cooperative DNA collapse.

During the complex formation, both lipid and DNA undergo a complete topological transformation into compact quasispherical complex particles with ~0.2 mm diameter, and they are easy to form string-like colloidal aggregates, inside of which the complexes have an ordered multilamellar structure (LCα), when the complexes are neutrally charged.

**Lipoplex structures**

Two fundamentally different types of models have been employed in order to interpret the cationic lipoplex structures, an "external" model, in which DNA is adsorbed onto the surface of cationic liposomes, and an "internal" model, in which the DNA is surrounded or "coated" by a lipid envelope.

In one model, lipopelx structures, the DNA is bound electrostatically to the outside of the cationic lipid vesicles, i.e., it is adsorbed onto the vesicle, gaining a beads on a string structure.

Lipoplexes were proposed to form either large aggregates surrounded by thin fibers, or condensed DNA coated by a lipid bilayer. The simplest case is one where the liposomes adhere to the DNA strand like “beads on a string,” similar to the structure of micelle complexes with oppositely charged poly electrolytes.

The appearance of the lipoplexes is often a highly ordered tubular structure when they are endocytosed by cells and assume perinuclear localization in these endosomes.

The observed ordered cationic lipoplexes mainly have multi lamellar structure LCα and hexagonal structures containing inverted hexagonal HCII and hexagonal HI.
Most complexes assume lamellar phase (LCα) structures with DNA sandwiched between the cationic lipids. A transient spaghetti-like structure existed between LCα and HCII, and it possibly served as a precursor to the phase HCII.

Mechanism of lipoplex-mediated transfection

Endocytosis of lipoplexes

Lipoplexes enter the cell primarily through endocytosis and most of the DNA of lamellar lipoplexes remains trapped in the endosome with their vector.

Lipoplex-mediated transfection and endocytosis.

Cationic lipids forming micellar structures called liposomes are complexed with DNA to create lipoplexes. The structures fuse with the cell membrane, at least sometimes after interactions with surface proteoglycans. The complexes are internalised by endocytosis, resulting in the formation of a double-layer inverted micellar vesicle. During the maturation of the endosome into a lysosome, the endosomal wall might rupture, releasing the contained DNA into the cytoplasm and potentially towards the nucleus. DNA imported into the nucleus might result in gene expression. Alternatively, DNA might be degraded within the lysosome.

Release of lipoplex from endosomes

Once in the endocytic pathway the plasmid may become degraded when reaching the lysosomes. Accordingly for productive transfection the plasmid needs to acquire cytosolic access at an earlier stage presumably by escape from endosomal Compartments.
In this process the hexagonal structure of the lipoplex is thought to play a crucial role and in this regard a parallel can be drawn with the mechanism by which adenovirus particles transfect cells. This virus also a popular tool in gene delivery enters cells via clathrin mediated endocytosis and macropinocytosis and the actual escape into the cytosol involves lysis of the endosomal membrane structure. The lipoplex lacks a protein machinery to destabilize the endosomal membrane.

At these conditions, the highest DNA dissociation was obtained as opposed to a lower dissociation upon interaction with PS-containing bilayers, which showed a transient formation of the micellar cubic phase.

**Method of Preparation of Lipoplexes**
No adequate method has been reported for the production of large-scale batches of lipoplexes with sufficient shelf-life. A continuous mixing method for the manufacturing of lipid/DNA-complexes on a large scale followed by lyophilisation for the development of stable lipoplex formulation with reproducible characteristics and increased shelf-life.

**Small-scale preparation of lipoplexes**
Enhanced green fluorescent plasmid pAH7-EGFP was mixed with DAC-30w in lipid/DNA ratios (w/w) of 4:1 and 5:1.

↓
Lyophilised DAC-30w was re dispersed in TM1 at a concentration of 1 mg/ml and incubated for 30 min at room temperature.

↓
Plasmid DNA and the respective amount of DAC-30w-dispersion were diluted separately in equal volumes of transfection medium (TM1 or TM2) to obtain the desired lipoplex amount in 1 ml of lipoplex preparation.

↓
Dilutions were combined discontinuously by pipetting the liposomes into the plasmid solution, gently mixing and incubating for at least 30 min at room temperature to allow the formation of lipoplexes.

**Large-scale preparation of lipoplexes**
Sterile lyophilised DAC-30w was dispersed with sterile TM1 or TM2 at a concentration of 1 mg/ml and incubated for 30 min.

↓
For the preparation of extruded liposomes the dispersion was pumped once through an 800 nm polycarbonate membrane using a peristaltic pump.

↓

In order to obtain a lipid/ DNA ratio (w/w) of 4:1 or 5:1, plasmid and lipid-dispersion (not extruded or extruded) were diluted separately in sterile TM1 or TM2 to a concentration of 50 mg/ml DNA and 200 or 250 mg/ml DAC-30w, respectively.

↓

To achieve simultaneous mixing of lipid and DNA at a constant flow rate both preparations were mixed via a Y-connector using a Hundt PM pump.

↓

Lipoplex dispersion was collected in an appropriate reservoir.

↓

Lipoplex formation was characterised 30 min after mixing by size measurement.

↓

Lipoplex dispersions (1.5 ml) were transferred into 2 ml lyophilisation vials.

↓

Vials were placed on the shelf of an alpha 2–4 Christ lyophiliser.

↓

The freeze-drying process occurred with the following cycle: freezing of the samples at -50 °C for 3 hr at 1000 mbar chamber pressure. Main drying took place at -20 °C for 42 hr and -30 °C for 6 hr chamber pressure was reduced to 0.05 mbar.

↓

Cooling of samples at 5 °C, which was also the final storing temperature until rehydration, finished the cycle. Before removal, vials were capped under vacuum.
Schematic representation of a large-scale manufacturing process. Lipid and plasmid, respectively, are diluted separately in transfection medium. Lipid is extruded once through a membrane with defined pore size (here 800 nm) and continuously mixed with equal volumes of plasmid in a second pump system via a Y-connector. The resulting lipoplexes are bottled and then lyophilised.

Lipoplexes prepared on a large scale showed no change in size within the first 48 hr after preparation. Size stability of the lyophilized product was also maintained during 18 months of storage (4–8 °C). An important aspect is the maintenance of biological activity of the lyophilised product.

The lyophilisate has been stored for 18 months at 4–8 °C and the transfection efficiency of the stored lyophilisates is comparable to the initial value obtained after preparation. The difference in lipoplex preparation technique (small scale versus large scale) did not influence the morphology of the lipoplexes. Both preparations showed the same pattern.

**Characterization of Lipoplexes**

**Techniques used in biophysical and physicochemical characterization of Lipoplexes**

Parameters such as size, surface charge, and state of dehydration upon liophilization are commonly monitored using DLS, electrophoretic mobility and recently FT-IR techniques, respectively, to assess lipoplex physical stability upon storage.
Characterization of lipoplexes by fluorescence spectroscopy method

Fluorescence spectroscopy has received increasing attention as a tool for lipoplex characterization, due to the use of more sensitive probes and possibility to operate under physiological conditions.

The kinetics of lipoplex formation as a function of charge ratio and vesicle composition was followed by stopped-flow fluorescence studies, using ethidium bromide (EtBr).

Fluorescence correlation spectroscopy is a highly versatile technique that can easily be adapted to solve specific biological questions, unraveling molecular interactions in vitro and in vivo. It gives information on molecular mobility and photo physical and photochemical reactions, by recording and correlating the fluorescence fluctuations of single labeled molecules through the exciting laser beam. Moreover, using dual-color fluorescence cross-correlation, specific binding studies can be carried out.

Characterization of lipoplexes by Forster Resonance Energy Transfer (FRET)

Madeira et al. used FRET to characterize the structure of DNA/ DOTAP complexes (Lipoplexes) for varying DNA plasmid size, buffer ionic strength, and both in the presence and absence of helper lipid (DOPE) and serum.
Forster Resonance Energy Transfer (FRET) is an important process that occurs in the excited state whenever the emission spectrum of a fluorophore, called the donor, overlaps with the absorption spectrum of another molecule, called the acceptor.

The FRET rate from a donor to an acceptor depends on (i) the distance between the donor (D) and acceptor (A) molecules, (ii) the overlapping extension of acceptor absorption spectra and donor emission spectra,( iii) the donor quantum yield and (iv) the relative orientation of the acceptor and donor transition dipoles.

The transfer efficiency (E) is the fraction of photons absorbed by the donor that are transferred to the acceptor and is given by:

\[ E = \frac{R_0^6}{R_0^6 + r^6} = 1 - \left( \frac{I_{DA}}{I_D} \right). \]

Schematic representation of the lipoplexes multilamellar structure with the fluorescent probes within DNA and lipid. A) Acceptor (a) on the DNA and donor (d) on the lipid. B) Acceptor (a) on the lipid and donor (d) on the DNA.

The microscopic structure of stable lipoplexes has been mainly studied by small-angle X-ray scattering (SAXS). With this technique, it was possible to determine for the first time the interlamellar distances in different lipoplex formulations in water or saline buffers.
Fluorescence spectroscopy, through the use of FRET methodology, has also provided valuable information about lipoplex microscopic structure by using a dye on the liposomes and another on the DNA.

Application of FRET to retrieve meaningful structural information in lipoplexes is not a trivial matter. Indeed, in the particular case of lipoplexes, and as previously described for membranes instead of an isolated donor/acceptor pair at a single defined distance.

**Physico-chemical properties of lipoplexes**

**Particle size distribution and zeta potential analysis**

The physico-chemical properties of the different liposomes and lipoplexes by measuring the particle size range by Dynamic Light Scattering (DLS) and the surface charge by zeta potential. Particle size and zeta potential were measured using a Malvern Zetasizer Nano ZS apparatus.

Zeta potential measurements were carried out using the M3-PALS technology.

Width at half peak height is indicative of the homogeneity of size and charge distribution. To assess whether the surface charge of lipoplexes could influence the choice of cell internalization pathway we used two different formulations of liposomes to prepare lipoplexes: Dynamic Light Scattering (DLS) that allows to obtain cationic lipoplexes with a low net charge ratio and Neutraplex (Nx) to prepare positively or negatively charged lipoplexes.

No particles larger than 600 nm were detected in any formulation. The global net charge ratios of positive charges in DOGS to negative charges in cardiolipin (CL) and Oligonucleotides (ON) were calculated.

Global net charge ratios of 1.8 for Nx+20 and 1.0 for Nx240 were obtained, suggesting that Nx240 is the lipoplex preparation closest to neutrality. In comparison, a ratio of 7.3 with the DLS formulation.

ON are fully protected in DLS or Nx lipoplexes when incubated for 4 hours in serum with medium as assayed by electrophoresis (PAGE).
PAGE and cryo-electron microscopy analysis revealed that no excess material was detected in Nx lipoplexes containing 10 to 40 mg Oligonucleotides (ON).

Physico-chemical characterization of the liposomes and lipoplexes used in this study.

<table>
<thead>
<tr>
<th>TYPE OF FORMULATION</th>
<th>ON</th>
<th>SIZE, nm</th>
<th>ζ POTENTIAL (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIPOSOMES</td>
<td>DLS</td>
<td>137 (149)</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>Nx</td>
<td>170 (22)</td>
<td>N.D.</td>
</tr>
<tr>
<td>LIPOPLEX</td>
<td>DLS/ON</td>
<td>179 (25)</td>
<td>+44</td>
</tr>
<tr>
<td></td>
<td>Nx+20/ON</td>
<td>278 (245)</td>
<td>+22</td>
</tr>
<tr>
<td></td>
<td>Nx+40/ON</td>
<td>203 (200)</td>
<td>−37</td>
</tr>
</tbody>
</table>

Particles mean diameter was measured by dynamic light scattering and surface charge by zeta potential. Particle size data are expressed as hydrodynamic diameter vs. intensity. Width, half peak height. Nx+, cationic Nx lipoplexes. Nx2, anionic Nx lipoplexes. Nx20 or 40, 20 or 40 mg ON mixed with 156 mg total lipids, respectively.

**Lipoplex molar mass, geometric size and density**

Time-resolved multi angle laser light scattering (TR-MALLS) is used to monitor the temporal variation of DNA/ cationic liposome lipoplex molar masses and geometric sizes throughout the complexation process.

Charge ratios near unity lead to a growing kinetic regime in which initially formed primary lipoplexes undergo further aggregation eventually forming large molar mass lipoplexes of high density, while charge ratios very far from unity yield low molar mass lipoplexes of lower density.

To better illustrate this observation, the final values of the apparent molar mass and geometric size were determined for each DNA/ cationic liposome pair considered here and are plotted as a function of the DNA/ cationic lipid charge ratio.
The spread in the data reflects the fact that the DNA concentration was varied from 1.08 to 10.8 mg/ml (five concentrations were considered for each formulation) as fact that the cationic liposomes considered here were all the same mean size.

![Graph showing temporal behavior of apparent molar mass](image)

The temporal behavior of the apparent molar mass for complexing with cholesterol cationic liposomes is illustrated here. The DNA/cationic lipid charge ratios are as indicated in the plot. The total DNA concentration is kept fixed at 1.08 mg/ml. The DNA plasmid size is 8.1 kb. Similar trends are observed for the temporal behavior of the apparent geometric size.

**Thermodynamics of lipoplexes**

The thermodynamics of lipoplex formation is not well established, although it has been conclusively demonstrated that in most cases cationic lipid-DNA binding is endothermic.

The complete thermodynamic description of the process was only available through theoretical approaches and had not been obtained in a direct experiment.

The free energy of lipoplex formation by analyzing the dissociation of the complex at elevated ionic strength.

By using relatively short oligonucleotides, to characterize the full relationship between degree of association and ionic strength, and hence determine the binding energy per nucleotide.

**Entropy-driven lipoplex formation**

The lipoplex formation is endothermic, i.e., the enthalpy of complex formation, $\Delta H$,
is positive.

Since, for any process to be thermodynamically favored, the free energy change must be negative,

$$\Delta G = \Delta H - T\Delta S < 0,$$

It is clear that lipoplex formation must be driven by an increase of entropy. The association of cationic lipid and DNA at physiological ionic strength is very tight and therefore one can expect that under those conditions, the entropic term will be large and determining.

The enthalpy change per mole of lipid in lipoplex formed at typical temperatures is ~1 RT (600 cal/mole).

The overall free energy of binding is of about the same magnitude, thus leading to the conclusion that the entropic term in the above equation should be ~2 RT per mole of complex, the direct determination of the binding entropy.

**Toxicity of lipoplexes**

**Inflammatory toxicity of lipoplex**

Inflammatory toxicity represents a typical toxicity associated with systemic administration of cationic liposome/DNA complex (lipoplex).

That the lipoplex gene delivery system mediates an uptake of plasmid DNA by the liver, mainly by Kupffer cells, in which a large amount of cytokine is produced.

Opsonization effect in the stimulation of Kupffer cell uptake is proposed as an explanation for the differences in the pharmacokinetic properties of plasmid DNA after lipoplex injection and sequential injection.

**Inflammatory toxicity of lipoplex via systemic administration**

**General observations on the lipoplex-induced toxicity**

Little toxicity was observed, when lipoplex was administered in moderate doses by either local or systemic injection. Mortality occurs in animals after systemic administration of several cationic lipid-based gene delivery systems at high dose.

The systemic toxicity of lipoplex has been comprehensively studied, in which various cationic lipids were explored, such as GL-67, GL-62, DMRIE, DOTMA, DOTAP. The
toxicities observed in these studies include (1) inflammatory toxicity, (2) hepatotoxicity, as evidenced by elevated serum levels of the transaminases, such as alanine amino transferase (ALT) and aspartate amino transferase (AST) and (3) hematologic and serologic toxicity, as typified by leukopenia and thrombocytopenia. The complexes formed between cationic liposomes and DNA is likely to be responsible for these toxicities.

The mechanism underlying lipoplex induced inflammatory toxicity was extensively studied. However, the mechanisms of hepatotoxicity, leukopenia and thrombocytopenia are still not completely revealed.

**Inflammatory toxicity and its relation to gene expression**

Systemic administration of lipoplex induces a rapid activation of the innate immune system. The inflammatory response was observed in patients as a flu-like symptom: myalgia, headache and high temperature 6 hr after nebulization of lipoplex into the lung in a clinical trial of cystic fibrosis.

Studies on animals have shown that lipoplex is immunologically active when administered locally or systemically. The immune response is characterized as induction of large quantities of proinflammatory cytokines, such as tumor necrosis factor a (TNF-a), interferon-g (IFN-g), interleukin 6 (IL-6) and interleukin 12 (IL-12). TNF-a is the earliest produced cytokine with peak value of 2000–3000 pg/ml in serum after 2 h of injection; while IL-12 and IFN-g peak at 6 h with a value of 2000 pg/ml and 50000 pg/ml, respectively.

**Strategies to reduce the inflammation induced by lipoplex**

Many strategies have been developed for overcoming the inflammatory toxicity of lipoplex. These strategies can be summarized as three different categories:

1. Eliminating immune stimulatory CpG motifs in the plasmid DNA;
2. Decreasing the interactions of lipoplex with immune cells;
3. Suppressing the immune response to lipoplex by using immunosuppressant agents.

Since CpG motif of plasmid DNA is the major source of immune stimulation, direct modification or reduction of the number of unmethylated CpG motifs should be an effective approach.
Mechanism related to the reduced immune response

The reduced liver uptake of DNA following sequential injection may explain the effect of sequential injection on decreasing the cytokine induction.

The introduction of CpG motifs of plasmid in lipoplex into the Kupffer cells via the opsonization of lipoplexes by some opsonin proteins in the serum is most likely to contribute to the mechanism of inflammatory response associated with lipoplex administration.

Therefore, serum proteins seem to play an important role in mediating the liver uptake of the DNA particle in the presence of cationic liposomes. The involvement of serum proteins in sequential injection and lipoplex injection, liposome–serum complex, which formed with the initial interaction of liposomes and serum, were able to further complex with DNA, and the resulting ternary complex led to similar low inflammatory response to the sequential injection.

Based on the observations from the pharmacokinetics and biophysical study of the formation of the particles in vitro, a model is given to emphasize the protein binding to the DNA delivery system and its relationship to the uptake by RES, including liver and spleen, in cases of sequential and lipoplex injections (Fig. 8).

In case of lipoplex, DNA is condensed by cationic liposomes to form lipoplex, in which most of DNA is in the interior of the lipoplex.

When the lipoplex is injected into the circulation, the lipoplex will be recognized and labeled by the blood opsonins, and the labeled complex will be guided to RES.

After sequential injection, complex of cationic liposomes/serum proteins/DNA is formed in such way that DNA will be exposed on the surface of the complex. Further binding of DNA to the liposome–serum complex might mask the action of opsonization.

The resulting ternary complex could also readily release DNA during the first-pass through the lung, and bring less DNA into the liver. As a result, DNA mainly distributes in the lung with the sequential injection, comparing to the major deposition of DNA in the liver with lipoplex injection.
Schematic illustration of interactions of serum proteins with liposomes and DNA, as well as the resulting DNA disposition involved in lipoplex injection and sequential injection.

Pharmacokinetic studies can provide useful information about the mechanisms underlying inflammatory toxicity associated with the systemic administration of lipoplex. Opsonization of lipoplex by certain serum proteins may assist the immunostimulatory plasmid DNA in being selectively guided to Kupffer cells; therein resulting in high level of cytokine induction. As a successful example of minimizing the inflammatory toxicity, sequential injection of liposomes and DNA leads to a significant reduction of liver uptake of plasmid DNA and much reduced inflammatory toxicity.

Emphasis is placed on the discussion of the correlation between the pharmacokinetic behavior of DNA and the inflammatory toxicity by comparing the differences in the pharmacokinetics of DNA following lipoplex injection and sequential injection. Sequential injection is one of the strategies used to ablate diminish the toxicity associated with the application of cationic liposomes for gene transfection in vivo.

**Cytotoxicity of lipoplexes**

Nguyen et al clearly indicated that there was a synergism between cationic lipid and pDNA in causing cytotoxicity. They found that cationic lipid (Lipofectamine 2000) alone induced only a slight cellular toxicity, irrespective of the absence or presence of serum, and free pDNA did not show any cytotoxicity.

Lipoplexes induced a significant cytotoxicity toward HeLa cells, B16BL6 cells and RGC-6 cells compared to cationic lipid alone, and the cytotoxicity increased as the cationic lipid content in the lipoplex increased.
Cationic liposomes formulated from DOPE and cationic lipids (such as DOTAP, DMTAP, DPTAP, DSTA), whether or not they were complexed with DNA, were highly toxic in vitro toward macrophages, but not toward non-phagocytic T cells.

The incorporation of DNA marginally reduced cationic liposome toxicity toward macrophage. Other factors affecting cytotoxicity of lipoplexes include zeta potential, incubation time, cell type and cell density.

Toxicity of the lipoplex may depend upon the nature of the aggregates formed. For example, the same lipoplex has been shown to exhibit a significantly reduced toxicity when present in a vesicular as opposed to a micellar solution.

Applications of Lipoplexes

Selective gene delivery for cancer therapy using cationic lipoplexes

The cationic lipoplexes have been shown to be selective for tumour vascular endothelial cells (VECs).

For cancer, for generic drug delivery, limited targeting has been achieved via selective delivery using upstream intra arterial administration use of immune liposomes exploitation of ligand–receptor interaction hypoxic induction of gene expression in solid tumours and use of tumour cell-specific promoters.

In general, only those ideas that are realised by the merging of different concepts seem to proceed and deliver results, albeit hardly impressively.

There is a real need for better ways to deliver therapeutics to tumours in vivo. This looks at these issues and ways to improve on the current situation for cationic lipoplex, an entity that has various attributes which place it among one of the most promising delivery agent for gene therapy for cancer.

Post-pegylated lipoplexes are promising vehicles for gene delivery in (Retinal Pigment Epithelium) RPE cells

Cationic lipoplexes strongly aggregate when exposed to the vitreous. Such aggregates of lipoplexes are completely immobilized in the vitreous gel and, consequently, the lipoplexes are not able to reach the neural retina or the RPE. We have shown that the aggregation of
cationic lipoplexes in vitreous can be avoided by coating them with the hydrophilic (neutral) polymer polyethyleneglycol (PEG).

The incorporation of PEG in gene therapy vectors is a well recognized strategy to avoid aggregation of lipoplexes in extracellular matrices, like vitreous and mucus, and to reduce the vector toxicity.

While “pegylation” of lipoplexes improves their “journey” through the extracellular matrices, it has, however, been reported that pegylation lowers the transfection efficiency of lipoplexes in different cell types.

Schematic representation of the preparation of pre-pegylated lipoplexes (Panel A) and post pegylated lipoplexes (Panel B). Pre-pegylated lipoplexes result from the complexation between pegylated liposomes and pDNA. Post-pegylated lipoplexes are made by the mixing of PEG-containing lipids (i.e. PEG-ceramides) and preformed non-pegylated lipoplexes.

The aim of the first part of the present study was to evaluate whether pegylated lipoplexes succeed or fail in the transfection of RPE cells. Indeed, it turned out that lipoplexes pegylated in a ‘conventional manner (termed ‘pre-pegylated’ lipoplexes) fail to do so. As a result, the second and major part of study was to design pegylated lipoplexes that cause an efficient transfection of RPE cells.
A novel siRNA-lipoplex technology for RNA interference in the mouse vascular endothelium

The siRNA-lipoplexes comprising of positively charged liposomes in combination with different target-specific siRNA molecules. The effect of different amounts of PEGylation on siRNA activity in vitro. Short interfering RNA mediated gene silencing was completely abolished in the presence of 5 mol% of DSPE-PEG-2000 but was maintained in the presence of 1–2 mol% in the formulation.

In contrast, at 5 mol% of PEGylation, when no siRNA was observed, cellular uptake appeared to be blocked, because lipoplexed siRNA-Cy3 mainly became attached to the cell’s surface.

This unspecific inhibition of a non target protein level is probably due to a more pronounced toxic effect of the non-PEGylated lipoplex in vitro. To analyze whether the PEGylation of the siRNA-lipoplexes can also reduce the toxicity in vivo.

Schematic drawing of the highly positive charged liposomes and the siRNA-lipoplexes. The negatively charged siRNAs are complexed by electrostatic interaction with the positive charges of the cationic lipid.

Applied identical doses of PEGylated (1mol% DSPE-PEG-2000) and non-PEGylated siRNA-lipoplexes by tailvein injection into the mice. Consecutive daily treatments (days 1–5) of non-PEGylated siRNA-lipoplexes by systemic administration (i.v.) caused loss in body weight over time, whereas mice treated with the same daily doses of PEGylated variants (1 mol% DSPE-PEG-2000) appeared unaffected. To elucidate the differences in body weight loss after
treatment with PEGylated and non-PEGylated siRNA-lipoplexes, a possible immune reaction upon lipoplex treatment was analyzed.

For this reason, interleukin-12 level (IL-12) was assayed in immune-competent mice after single i.v. bolus of non complexed mPoly(I:C) (positive control) or PEGylated and non-PEGylated siRNA-lipoplexes (siRNAPTEN, siRNALuc). The enzyme-linked immune sorbent assay (ELISA) analysis revealed that no increase of IL-12 occurred upon siRNA-lipoplex treatment regardless of PEGylation.

**Non-viral gene therapy by lipoplex for spinal cord regeneration**

Pathology of Spinal cord injury (SCI) and cell targeting for gene therapy

Microglia are among the first such cells to migrate into the lesion from the surrounding tissue. Neutrophils arrive between three and 24 hours and monocytes are the next to arrive two to three days post-injury. These cells damage the neural tissue by releasing pro-inflammatory cytokines and proteases.

However, they also help to remove injured tissue debris and they release protective cytokines that promote neuronal regeneration and tissue repair.

In response to demyelination, oligodendrocyte pro-genitor cells that produce the proteoglycan neuroglycan 2 (NG2) and express platelet-derived growth factor-a receptor on the cell membrane are recruited from the gray and white matter for remyelination of regenerated spinal cord axons at the site of injury.

Damage to the spinal cord meningeal surface also initiates invasion of fibroblastic meningeal cells that restore the continuous layer of cells around neural tissue. Multipotential progenitor cells line the central canal of the adult rodent spinal cord.

In response to SCI, these multipotential progenitor cells (also known as ependymal cells) proliferate dramatically and differentiate into astrocytes and oligodendrocytes. Astrocytes become the main cellular component of the glial cell because they proliferate and migrate into the injured area to eventually fill the vacant space.

The strategy of non-viral gene therapy for regeneration of injured spinal cord is to transfec the endogenous cells with gene vectors that lead to the expression of functional molecules,
such as growth factors, in a sustained fashion and thereby generate long-lasting biological effects.

Plasmid delivery using nanocarriers. Polyplexes, lipoplexes or PEI-complexed plasmids can bind to specific receptors on the cell surface and then be internalized through endocytosis. The unbound DNA enters the nucleus through NPC, undergoes transcription in the nucleus, and eventual RNA translation and gene expression in the plasma. Abbreviations: NLS: nuclear localization signals; NPC: nuclear pore complex; PEG: polyethylene glycol; PEI: polyethylenimine.

Three days after implantation, the gene was expressed at the injury site. This study demonstrated successful transfection of reactive and proliferative cells with non-viral vectors and that such delivery of a therapeutic gene may regulate cell and tissue organization and promote axonal growth.

CONCLUSION
As stated above, to achieve such a goal, attempts have been made to confer viral attributes to lipoplexes, namely through the association of certain proteins or peptides. Whether these improvements result in a system that, while inhibiting satisfactory ability to mediate in vivo transfection, would lead to such a complexity that could endanger its versatility and large scale production or could limit extended repeated in vivo use due to immunogenicity, are important questions that remain to be addressed.
Taking together the variables affecting the formation and structure of lipoplexes, their biological stability, and thus their bio distribution and pharmacokinetics, and those affecting their mode of interaction with cells, it will be rather laborious and difficult to design a non-viral vector capable of fulfilling the conflicting requirements imposed by each of the different stages involved in the gene delivery process.

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