Gene delivery with cationic lipids
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Interference of poly(ethylene glycol)-lipid analogues with cationic-lipid-mediated delivery of oligonucleotides; role of lipid exchangeability and non-lamellar transitions

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Abstract

Cationic liposomes are applied to transfer oligonucleotides (ODNs) into cells to regulate gene expression for gene therapeutic or cell biological purposes. In vivo, poly(ethylene glycol) (PEG)-lipid derivatives are employed to stabilize and prolong the circulation lifetime of nucleic acid-containing particles, and to improve targeting strategies. In this study, we have studied the effect of PEG-lipid analogues, i.e. PEG coupled to either phosphatidylethanolamine (PE) or ceramide, on cationic lipid/DNA complex (‘lipoplex’) assembly and the mechanism of cationic lipid-mediated delivery of ODNs in vitro. Inclusion of 10 mol % PEG-PE in ODN lipoplexes inhibited their internalization in Chinese hamster ovary cells by more than 70 %. The intracellular fraction remained entrapped in the endosomal/lysosomal pathway and no release of ODNs was apparent. Similar observations were made for complexes prepared from liposomes that contained PEG-ceramides. Interestingly, delivery resumed when lipoplexes had been externally coated with PEG-ceramides. In this case, the kinetics of delivery was dependent on the length of the ceramide acyl chain, consistent with a requirement for the PEG-lipid to dissociate from the complex. Moreover, although the chemical nature of the PEG-ceramides distinctly affected the net internalization of the complexes, impediment of delivery was largely related to an inhibitory effect of the PEG-lipid on the release of ODNs from the endosomal compartment. Cryo-electron microscopy and small angle X-ray scattering revealed that the PEG-lipids stabilize the lamellar phase of the lipoplexes, while their acyl-chain-length-dependent transfer from the complex enables adaptation of the hexagonal phase. Within the endosomal compartment, this transition appears to be instrumental in causing the dissociation and cytosolic release of the ODNs for their nuclear homing.

Keywords: antisense, gene therapy, lipoplex, hexagonal phase
Introduction

Short single-stranded DNA fragments like oligonucleotides (ODNs) provide a means for modulating gene expression, following their appropriate targeting to and hybridization with a given gene sequence. Both gene therapeutic and cell biological approaches will benefit from such applications. With the development of suitable delivery systems, the delivery efficiency of ODNs, which spontaneously translocate across the plasma membrane barrier very poorly if at all, has been greatly improved [1,2]. Although readily applicable *in vitro*, a programmable or controllable mode of delivery is particularly crucial for *in vivo* application, which demands stringent requirements for particle stability during circulation, and specific delivery to selected target tissue and/or cells.

Cationic lipid-DNA complexes (‘lipoplexes’) have been successfully used for gene delivery, and also cellular delivery of ODNs can be greatly improved in this manner, although the overall mechanism of delivery is still poorly defined. To overcome such drawbacks as relatively short circulation time *in vivo*, unspecific binding of serum components or an undesired interaction with non-target cells [3,4], lipoplexes are often coated with polymers, such as poly(ethylene glycol) (PEG) [5-7]. However, little insight is available as to how ‘PEGylation’ affects the overall cationic lipid-mediated delivery of a gene or ODNs into cells. In this regard, highly relevant issues concern the effect of the polymer on the physical properties of the lipid-DNA or lipid-ODN complex, and how such properties as well as the presence and membrane-anchorage of PEG as such, affects the (intra-)cellular interactions and processing, relevant to the eventual delivery of plasmid or ODN. Recent work has emphasized the close relationship between structure and function (i.e., transfectability) of lipid-DNA complexes. Thus the evidence indicates that the lipid-DNA complexes are highly ordered structures, and that an inverted hexagonal phase of the complexes strongly promotes transfection efficiency [8,9]. By contrast, a lamellar phase of the complexes correlates with stable particles, displaying substantially lower transfection potency. However, it is not unlikely that the additional inclusion of distinct lipids in the lipoplex, including the incorporation of PEGylated lipid may perturb the delicate balance of the transfection-supporting phase [10]. In addition, the bulky presence of PEG at the interface of interacting membranes will also pose as a steric barrier, which could, among others, frustrate the lamellar to hexagonal phase transition by precluding tight interaction of opposed membranes, necessary for such a transition to occur [11]. Accordingly, the PEG-lipid derivatives should eventually depart from the lipoplexes, when the complex has
reached the desired site of delivery. Such a dissociation could be accomplished, for example, when the polymer is ‘attached’ to membranes either via cleavable bonds, such as an S-S bond, or via lipid anchors, which in a time-dependent manner can exchange out of the lipoplexes. The latter class of lipids include ceramide-PEG, which in studies largely based on the use of liposomes, displayed exchange properties, the kinetics of which were governed by the length of the acyl chain [12].

In the present work we have examined the structural and functional consequences of incorporating PEG-lipids in cationic lipid/ODN complexes, and the ensuing effect on cellular interaction and processing of such complexes, including ODN delivery. As demonstrated by small angle X-ray scattering and cryo electron microscopy, the presence of PEG-lipids interferes with the phase properties of the lipoplexes by stabilizing a lamellar-like morphology. This phase displays an inherent particle stability that strongly impedes intracellular ODN release, leading to particle entrapment in the endo/lysosomal pathway without cellular transfection.

Materials and methods

Materials
Poly(ethylene glycol) (PEG), Mw 2000 D, covalently attached to distearoyl-phosphatidylethanolamine (DSPE) [DSPE-PEG 2000], was obtained from Avanti Polar Lipids (Alabaster, AL, USA), and dioleoyl-phosphatidylethanolamine (DOPE) and N-(lissamine rhodamine sulphonyl)phosphatidylethanolamine (N-Rh-PE) were purchased from the same source. Ceramide-C8-PEG, ceramide-C14-PEG and ceramide-C20-PEG (Mw PEG is 2000 D) were obtained from Northern lipids (Vancouver, Canada). The cationic lipid SAINT-2 [N-methyl-4(dioleyl)methylpyridinium chloride] was synthesized as described in detail elsewhere [13]. Antisense ODN complementary to the mRNA of the corticotrophin-releasing factor receptor (CRF-R; GeneBank accession no. L25 438) targeted to bp 473-490 (sequence 5’GGA TGA AAG CCG AGA TG 3’), and a 17-mer randomized-sequence of ODN (sequence 5’-ACT ACG ACC TAC GTG AC-3’) were designed and manufactured by Biognostik (Göttingen, Germany). The randomized ODN sequence, labeled at the 5’-end with FITC, was used for cellular binding and uptake studies. All ODNs were thioated and purified by high performance liquid chromatography, cross-flow dialysis and ultrafiltration. All chemicals were from Sigma (Missouri, USA), unless stated otherwise.
**Cell culture**

Chinese hamster ovary (CHO) cells, stably expressing the CRF receptor under the control of CMV promoter were kindly provided by Solvay Pharmaceuticals (Weesp, The Netherlands). The cells were grown in CHO-S-SFM medium (Gibco) supplemented with 10 % heat inactivated fetal calf serum, 2 mM L-glutamine and penicillin (50 U/ml) /streptomycin (50 µg/ml) under the selection of 0.5 mg/ml geneticin in 5 % CO₂/95 % air at 37°C.

**Preparation of lipid vesicles and ODN-containing lipoplexes**

The lipids were dissolved in chloroform/methanol (1:1, volume ratio). SAINT-2 and DOPE (1:1, molar ratio) with or without various lipid-PEG analogues (at concentrations of 1, 5 or 10 mol %, relative to the total lipids) were mixed, and the solvent was removed by evaporation under a stream of nitrogen, followed by placing the vial under vacuum for at least 1 h. The lipids were then resuspended in MilliQ water at stock concentrations of 1 or 0.5 mM, and sonicated to clarity in a bath sonicator in a closed vial. When required for monitoring the (intra)cellular fate of the lipoplexes by fluorescence microscopy, 0.5 mol % N-Rh-PE was included in the lipid mixture. Insertion of the PEG-lipids into the lipoplexes was accomplished by two procedures. In procedure I, lipoplexes were prepared with PEG-lipid-containing SAINT-2/DOPE liposomes and ODNs as follows: 20 nmol of PEG-lipid-containing liposomes, suspended in 200 µl CHO-SFM medium (Life Technology, Breda, the Netherlands), were mixed with 0.1 nmol ODNs, diluted in 200 µl of the same medium. After 20 min at room temperature, the mixture was diluted with 600 µl pre-warmed medium, and added to the cells. Alternatively in procedure II, preformed lipoplexes of SAINT-2/DOPE and ODNs were coated with PEG (lipid-PEG coating). In this case 20 nmol SAINT-2/DOPE liposomes and 0.1 nmol ODNs were mixed in 100 µl CHO-SFM medium and incubated for 20 min at room temperature. Then 2 nmol lipid-PEG was added and the mixture was incubated at 60°C for 1 h, cooled to 37°C, and diluted with 900 µl medium prior to addition to the cells. Following lipoplex assembly, the packing efficiency of the ODNs was examined by determining ODN accessibility towards Oligreen, using the Oligreen ssDNA Quantitation kit (Molecular probe, OR, USA). The assay was performed according to instructions provided by the manufacturer.

**ODN release assay**

Lipoplexes were prepared as follow: 20 nmol of liposomes (with or without PEG-lipid, see above) were mixed with 0.1 nmol of ODNs in 120 µl 150 mM NaCl /10 mM Heps, pH 7.4,
and incubated for 20 min at room temperature. The lipoplexes were then diluted in 880 µl of an Oligreen solution 1X (Molecular Probe, OR, USA). Fluorescence was subsequently monitored at excitation and emission wavelengths of 485 and 520 nm, respectively. After 100 s, 100 nmol of vesicles consisting of DOPE/dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylserine(DOPS), molar ratio 2:1:1, were added to trigger ODN release. After 400 s Triton X-100 was added at a final concentration of 0.2 %, reflecting the level of fluorescence obtained after complete dissociation. Data of release were calculated from the fluorescence level obtained at 400 s, corrected for the background value obtained prior to addition of anionic vesicles, relative to the fluorescence obtained upon total release (Triton value).

**Fluorescence microscopy studies with FITC-dextran and fluorescently labeled lipid-ODN complexes**

The (intra)cellular fate of ODNs and lipoplexes was determined by monitoring the fate of FITC-labeled ODNs and N-Rh-PE labeled lipoplexes by epifluorescence or confocal laser scanning microscopy. CHO cells were grown on coverslips in 6-well plates and treated with various lipoplexes as described, and incubated during a time interval as indicated. The cells were rinsed twice with HBSS, and analyzed directly or fixed for 10 min in 2.5 % paraformaldehyde in PBS, washed and mounted on microscope slides for examination.

The endosomal/lysosomal pathway in living CHO cells was labeled with FITC-dextran (MW 71,600, Sigma) by a 12 h incubation with the probe at 2 mg/ml. Subsequently, the cells were washed and incubated with N-Rh-PE-labeled lipid-ODN complexes for 5 h at 37ºC. Fluorescence microscopic examination of the samples was carried out using a TCS Leica SP2 confocal laser scanning microscope (Leica, Wetzlar, Germany).

**Antisense assay**

The antisense effect of ODNs delivered by SAINT-2/DOPE with or without lipid-PEGs was examined by Western immunoblot. Approx. 10⁶ CRF-R-expressing and control CHO cells were seeded in 10 cm dishes and grown for 24 h. The cells were then treated with the various complexes (5 nmol ODNs/100 nmol lipid) for 5 h, after which period the complexes were removed and fresh medium was given. The cells were harvested 72 h after ODN treatment, lysed, and the membranes were isolated as described [14]. Samples (25 µg of protein) were then analyzed on 12.5 % SDS-PAGE (Bio-Rad, Hercules, CA), blotted on pure nitrocellulose membrane (Trans-Blot Transfer medium, Bio-Rad, Hercules, CA) and probed
with goat anti-rat CRF-R (1:500, Santa Cruz), followed by horseradish peroxidase conjugate rabbit anti-goat antibody (Sigma, Steinheim, Germany). The blot was processed with ECL™ (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

**Turbidity measurements**

An aliquot of 87.5 nmol of SAINT-2/DOPE (1:1 molar ratio) or SAINT-2/DOPE/5 mol %DSPE-PEG was mixed with HBS (10 mM HEPES, 150 mM NaCl, pH 7.4) and 0.875 nmol ODNs in a final volume of 875 µl. The turbidity was measured at room temperature at a wavelength of 350 nm in a UVikon 930 spectrophotometer.

**Cryo-EM of lipoplexes**

The morphology of lipid-ODN complexes was determined by transmission cryo-EM. Portions of 2 µl of the various samples, indicated in the legends, were applied on glow discharged holey carbon-coated grids, and the excess of liquid was blotted away by Waterman paper. The specimen was frozen in liquid ethane and then mounted in a GatAn (mol 626) CRYO-STAGE and examined in a Philips CM 120 cryo-electron microscope, operating at 120 kV.

**Small angle X-ray scattering (SAXS) analyses**

To determine the lipoplex structure, SAXS measurements of PEG-devoid lipoplexes and PEG-containing lipoplexes were performed at 25°C using a NanoStar device (Brucker AXS and Anton Paar) with a ceramic fine-focus X-ray tube, operating in a point focus mode. The tube was powered with a Kristalloflex K760 generator at 35 kV and 40 mA. The primary beam was collimated using cross-coupled Göbel mirrors and a 0.1-mm pinhole providing a CuKα radiation beam (λ = 0.154 nm) with a full-width at half-maximum of about 0.2 mm in diameter at the sample position. The sample-detector distance was 0.65 m. The use of a Hi-Star position-sensitive area detector (Siemens AXS) allowed recording the scattering intensity in the q-range of 0.5 to 3.5 nm⁻¹. The scattering vector q is defined as \( q = \frac{4\pi}{\lambda} \cdot \sin(\theta/2) \), where \( \theta \) is the scattering angle. The measurements of the samples were performed using a sample cell of 2 mm thickness covered by two thin kapton films. For sample preparation, 1000 nmol SAINT-2/DOPE were gently mixed with 5 nmol ODNs in 60 µl of 150 mM NaCl/10 mM HEPES. After 20 min at room temperature, the samples were analyzed by SAXS.
**Results**

**DSPE-PEG inhibits cellular delivery and prevents intracellular dissociation of ODNs from lipoplexes**

As demonstrated previously [1], after internalization of SAINT-2/DOPE lipoplexes, the associated ODNs readily dissociate and accumulate in the nuclei. Thus in the absence of PEG-lipid, effective delivery of the FITC-labeled ODNs is observed, following an incubation for either 5 (Fig. 1A) or 24 h (not shown). Note that at these conditions ODNs and the lipids, marked by N-Rh-PE-labeling (red), become separated (insert Fig. 1A). A virtually identical image was obtained (not shown; cf Fig. 1A) when 10 % DSPE-PEG was included in the medium. By contrast, when 10 % DSPE-PEG had been incorporated into the lipoplexes by exogenous addition (procedure II, coating), the ODNs were mostly seen associated with the plasma membrane, often showing a clustered appearance, whereas nuclear staining was virtually negligible (Fig. 1B). When lipoplexes were used prepared from liposomes that contained 10 % DSPE-PEG (procedure I), the uptake of ODNs was similarly strongly inhibited (Fig. 1C). However, rather than a localization at the cell surface, in this case a fine punctate distribution was seen, largely limited to intracellular compartments that are clearly distinguishable from the nucleus. Interestingly, the uptake increased when the DSPE-PEG concentration was reduced to 5 %, but no shift in intracellular ODN distribution was observed (Fig. 1D). In fact, even at a density as low as 1 mol % DSPE-PEG nuclear accumulation of ODNs was still effectively prevented (not shown). Accordingly, the presence of the PEGylated lipids had two effects. First, in a PEG-lipid concentration dependent manner, the net uptake of lipoplexes was inhibited. Secondly, once intracellular, the presence of the PEG-lipids effectively inhibited complex dissociation. To support the latter notion, PEGylated lipoplexes were prepared that contained both fluorescently-tagged ODN (FITC) and N-Rh-PE (0.5 mole %), as a marker of the lipoplex lipid phase. The merged pictures revealed (Fig. 1E) that dissociation of the complex does not take place (c.f. Fig. 1A), even at DSPE-PEG concentrations as low as 1 mole %. Thus escape of ODNs from lipoplexes was abolished when DSPE-PEG was present.
The distribution pattern of the internalized complexes, seen in Fig. 1C and 1D, suggests that the ODNs are trapped in the endo/lysosomal pathway. Indeed, when loading this pathway with FITC-Dextran (Fig. 1F), followed by Rh-PE-labeled complexes, a colocalization of both probes was observed, implying that the particles are processed towards lysosomes, which typically localize in perinuclear regions of the cells. Apparently,
the hydrolytic activity of the lysosomes did not suffice to release ODNs or fragments thereof after long incubation times, since as noted above, even after 24 h the distribution of the FITC-labeled ODNs was identical to that observed after 5 h (not shown; c.f. Figures 1D and F).

These data indicate that although DSPE-PEG may represent a useful tool for inhibiting ODN-lipoplex interaction with cells (thereby prolonging the circulation time of particles in vivo), the lipid analogue is not suitable for targeting (by coupling of tissue-specific antibodies) or controlled delivery of the therapeutic cargo. Thus, once reaching the cellular destination, the PEGylated lipid remains firmly associated with the lipoplex, which precludes ODN release and causes the complex to be processed into the degradation compartment. This notion emphasizes the need for exchangeable derivatives. In this regard, claims have been made [12] that, depending on the length of their fatty acyl chain, PEGylated ceramides may display the desired exchange properties, necessary for the process that eventually leads to a destabilization of the lipoplex structure that favors ODN release.

**Dissociation of PEGylated lipids is required for nuclear delivery of ODNs**

Lipids may display facilitated exchange when the length of one of their acyl chains is shortened [15]. This will diminish hydrophobic interactions within the lipidic core of a membrane and cause an increase in their relative water solubility.

Therefore, we next examined the effect on delivery when the ceramide-PEGs were inserted exogenously. Thus, 10 mole % ceramide-C8-PEG, ceramide-C14-PEG or ceramide-C20-PEG was incorporated exogenously into preformed lipoplexes, as described in the Materials and methods section (procedure II), and the PEGylated lipoplexes were incubated with cells for 2 h, 5 h and overnight. The presence of ceramide-PEGs affected the net cell-association of the lipoplexes, similarly as observed for DSPE-PEG. The highest uptake was seen for short-chain ceramide-containing lipoplexes, suggesting that uptake efficiency might be related to the relative differences in the kinetics of ceramide-PEG exchange, i.e. with more rapid exchange (occurring during the incubation of the lipoplexes with the cells) leading to a higher uptake. In this case nuclear accumulation of ODNs was seen after 2 h for control lipoplexes and for lipoplexes coated with ceramide-C8-PEG. With the ceramide-C14 and C20 derivatives, complexes could only be detected in close association with the plasma membrane (data not shown). As demonstrated in Fig. 2, when extended to a 5 h incubation, the nuclear labelling of cells treated with control (Fig. 2A) and ceramide-C8 lipoplexes (Fig. 2B) was prominently apparent, it gradually appeared in cells treated with ceramide-C14-
PEG complexes (Fig. 2C), whereas for ceramide-C20 complexes, the ODNs were still largely associated with internalized and cell surface-bound complexes (Fig. 2D). After an overnight incubation (Fig. 2E-H), effective nuclear delivery of ODNs was seen for both the ceramide-C8 and ceramide-C14 derivatives, whereas some delivery was apparent in case of complexes that contained the ceramide-C20 derivative. The time and ceramide-PEG species dependence of nuclear delivery implied a chain-length dependent dissociation of the ceramide-PEG from the lipoplexes, thereby activating the capacity of lipoplex-mediated delivery of ODNs.

![Figure 2](image)

**Figure 2.** Effect of exogenous coating of lipoplexes with ceramide-PEG on ODN Delivery. CHO cells were incubated with SAIN T-2/DOPE-FITC-ODN complexes (A, E) which had been coated (procedure II) with 10 mol % ceramide-C8-PEG (B, F), ceramide-C14-PEG (C, G) or ceramide-C20-PEG (D, H). After 5h (A-D) or an overnight (O/N) incubation (E-G), the localization of ODNs was visualized by fluorescence microscopy (right panel). The left panel shows the corresponding phase contrast images.

This possibility was next examined by simulating such a release by incubating PEGylated ODN lipoplexes with anionic lipid vesicles [16].
Chapter 3

PEG-lipids modulate the release of ODN from lipoplexes

As shown in table 1 the presence of PEG-lipids prevents the release of the ODNs from the lipoplexes, induced by the interaction of anionic vesicles with the lipoplexes. In a density-dependent manner, the presence of DSPE-PEG effectively prevents the release of ODNs upon addition of anionic vesicles, irrespective of whether the PEGylated lipid was incorporated in vesicles prior to lipoplex assembly or when pre-assembled lipoplexes had been coated subsequently. Note that addition of free DSPE-PEG did not affect the release. Also coating (procedure II) or prior incorporation of PEG-ceramide derivatives (procedure I) effectively prevented ODN release. Interestingly, in the presence of non-incorporated ceramide analogues, effective inhibition of release was also observed when either ceramide-C8 or C14 was included in the mixture, but not in case of the ceramide-C20 analogue. These differences very likely reflect differences in the transfer properties as free monomers of the short chain derivatives (C8 and C14) versus the essentially non-exchangeable properties of the C20 and DSPE-PEG derivative [12], which leads to rapid integration into the lipoplex of the former, but not of the latter.

PEGylation interferes with functional delivery of ODNs into eukaryotic cells

The data thus far demonstrate that PEGylated lipids inhibit or delay lipoplex interaction with cell surfaces and/or intracellular delivery of ODNs. To correlate the intracellular localization of ODNs with their potential antisense effect, the efficiency of down-regulation of CRF overexpression in CHO cells (see the Materials and methods section) was examined by

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<td>5 % CER20-PEG</td>
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Table 1: Effect of PEGylated lipids on the release of ODNs from lipoplexes in the presence of anionic vesicles. PEG-coated lipoplexes were incubated with anionic vesicles (DOPE/DOPC/DOPS, 2/1/1). The release of ODNs (expressed as percentage) was monitored by measuring fluorescence arising when Oligreen binds to the liberated ODNs, as described in the Materials and methods section. For calibration, the 100 % value was set to the fluorescence obtained upon total disruption of the complexes with Triton X-100. The percentage of release is expressed as the ratio of fluorescence, measured after 5 min of incubation with anionic vesicles, relative to total release. With control, PEG-lipid devoid complexes, a release of 95% was obtained.
Western immunoblotting. As shown in Fig. 3, down regulation of CRF-R expression was seen when antisense ODNs were delivered with SAINT-2/DOPE, compared to the levels of expression seen in untreated cells, cells treated with complexes containing mismatch ODNs or in cells treated with antisense ODNs alone. When the same amounts of antisense ODNs were delivered with 5 mol % DSPE-PEG containing complexes, the antisense effect on CRF-R was completely abolished, consistent with the observation that no ODNs were released from lipoplexes in this case.

As demonstrated above, the delivery of fluorescently tagged ODNs was timedependent, when they were entrapped in complexes coated with PEG-ceramides of different fatty acyl chain lengths. Thus, after a 5 h incubation, ODN delivery was largely restored when complexes had been coated (procedure II) with ceramide-C8-PEG, partly restored when coated with ceramide-C14-PEG, whereas nuclear delivery still did not occur after this time interval in case of ceramide-C20-PEG. Consistently, as shown in Fig. 3, the antisense effect of ODN in down-regulating CRF-R expression was inversely proportional to the fatty acyl chain length of the employed PEG-R expression in down-regulating CRF-R expression (Fig. 3B). Thus relative to untreated cells

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**Figure 3: Down-regulation of CRF-R expression by antisense ODN, delivered by lipoplexes, with or without lipid-PEGs.** CHO cells, stably expressing CRF-R (lane 5, ‘untreated cells’), were treated with antisense-containing lipoplexes, containing the various PEGylated lipid derivatives. The antisense effect was evaluated by Western immunoblotting. The cells were incubated with lipoplexes or free antisense ODNs for 5 h. Then fresh serum-containing medium was added and after 72 h CRF-R expression was examined. The cell membranes were isolated, and proteins were separated on PAGE and probed with goat anti-CRF-R and then with horseradish peroxidase-conjugated rabbit anti-goat antibodies. The blot was processed by ECL. In (A), reduced CRF-R expression is seen following lipoplex-mediated antisense delivery (lane 1; AS + S/D). The antisense effect was abolished when non-exchangeable DSPE-PEG was included in the lipoplex formulation (lane 2; AS + S/D/DSPE-PEG). MAS + S/D (lane 3) reflects treatment of the cells with mismatch antisense, while AS (lane 4) indicates treatment with free antisense ODN. In (B), the effects of PEG-ceramides on lipoplex-mediated antisense delivery was examined. Antisense ODNs were associated with cationic lipids (lane 1; AS + S/D) or ceramide-PEG-coated complexes [lane 2 – 4; (AS + S/D)-Cer-C8-PEG, (AS + S/D)-Cer-C14-PEG, (AS + S/D)-Cer-C20-PEG, respectively]. Note that the degree of down-regulation is correlated with the length of the ceramide acyl chain length.
(lane 5) and ODN treatment by delivery in PEG-devoid complexes (lane 1), an effective reduction of CFR-R expression was seen with ceramide-C8-PEG-coated complexes. The efficiency was diminished when employing ceramide-C14-PEG-coated complexes, whereas no significant effect was seen with ceramide-C20-PEG coated complexes. These functional data are therefore fully consistent with the observations on the effectiveness of ODN delivery when employing fluorescently tagged markers to monitor the effect of PEGylated lipids on the (intra)cellular processing of ODN-containing lipoplexes (see Fig. 2). An identical trend as with transfection efficiency was observed when ODNs were replaced by a reporter gene, pGFP (plasmid encoding enhanced green fluorescent protein; Clontech) (results not shown).

In spite of the presence of PEGylated lipids such as DSPE-PEG or ceramide-PEG, lipoplexes are (at least partly) internalized by cells, implying that the presence of PEG does not necessarily prevent lipoplex-cell membrane (receptor) interaction, necessary for endocytic internalization, the pathway along which SAINT-2-containing lipoplexes deliver plasmids [17]. However, once internalized, this interaction apparently does not suffice for ODNs to be released and requires the dissociation of the PEGylated lipid from the complex, implying that additional factors must be involved, like the intimacy of complex-endosomal membrane interaction (as noted above), and in addition lipid structural requirements (e.g. the lamellar to hexagonal phase changes) that may codetermine overall nucleic acid release efficiency [8,9]. The effect of PEGylated lipids on lipoplex structure was therefore examined next.

**Figure 4: Effect of DSPE-PEG on vesicle aggregation in water and physiological salt solutions.** SAINT-2/DOPE (S/D) and SAINT-2/DOPE/5 mol % DSPE-PEG (S/D/DSPE-PEG) vesicles, prepared in water, were suspended in salt solutions (HBS), and then mixed with ODNs. Turbidity was monitored as a function of time at a wavelength of 350 nm. The maximal turbidity levels obtained were plotted, and the time required to reach this plateau value is indicated on top of the bars.
The presence of PEGylated lipids stabilized the lamellar phase of ODN complexes

In recent years it has become apparent that the lipid phase of lipoplexes may play an important role in bringing about lipoplex-mediated transfection. Specifically, lamellar-phase complexes bind stably to membranes while hexagonal-phase complexes are unstable and such features are thought to be instrumental in productive transfection [8,9]. Accordingly, this could imply that lipoplexes prepared from PEG-liposomes and ODNs display a lipid phase that lacks the ability to disturb the endosomal membrane and/or to release ODNs. As shown previously [9], in water SAINT-2/DOPC liposomes appear as unilamellar vesicles with a diameter of 100-200 nm. However, when suspended in salt, a transition from the lamellar to the hexagonal phase takes place. As shown in Fig. 4, the turbidity of a suspension of SAINT-2/DOPC vesicles in water increased more than 20-fold upon addition of salt, reflecting vesicle clustering upon charge neutralization and a concomitant transition to the hexagonal phase upon membrane-membrane interaction [9]. The presence of ODNs did not interfere with this transition. However, when 5 mol % DSPE-PEG was present in the SAINT-2/DOPC vesicles under otherwise the same conditions, the aggregation of the lipid vesicle was evidently prevented. Accordingly, since the hexagonal phase transition requires close interactions between opposed membranes, the data suggest that the PEGylated lipid stabilizes the lamellar phase by preventing such interactions.

To further clarify and support these observations, the morphology of cationic lipid-ODN complexes was investigated by cryo-EM and SAXS. Control SAINT-2/DOPC-ODN complexes appeared as fingerprint-like structures initially showing particles with a diameter of 100-200 nm, which cluster into larger complexes as a function of time (> 15 min; Figures 5A and B). The fingerprint structure is typical of the hexagonal phase. To support this conclusion, we analyzed the samples by SAXS. As shown in Fig. 6A, three diffraction maxima were apparent at $q = 0.105, 0.181$ and $0.201 \text{Å}^{-1}$, implying that the location of the peaks is in the ratio of $1: \sqrt{3}: \sqrt{4}$, i.e., fully consistent with a hexagonal morphology. The periodicity of the phase is about 7 nm (Fig. 6A). By contrast, the morphology of DSPE-PEG-containing complexes is different. Cryo-EM revealed the presence of small homogenous complexes with a diameter of 30-50 nm, which did not display a tendency to aggregate (Figures 5C and D). The structures appeared less densely packed than PEG-devoid complexes, as reflected by a less electron-dense appearance of the images, while often only a few membrane layers were seen, surrounding an aqueous volume, i.e., an internal space lacking significant internal structure. These structures more closely resemble a
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lamellar than a hexagonal organization. Indeed, as shown in Fig. 6B, the SAXS diffraction pattern differed considerably from that obtained for the non-PEGylated complexes (Fig. 6A). Only one peak at $q = 0.111$ (Å$^{-1}$) was obtained, corresponding to a distance of 5.6 nm. This distance is compatible with a lamellar phase of similar periodicity, observed for lamellar complexes obtained for plasmid-containing lipoplexes [8,9]. A similar periodicity was obtained when ODNs were substituted for plasmid or when the concentration of DSPE-PEG was reduced from 5 to 1 mole %.

When 10% DSPE-PEGs was incorporated into preformed SAINT-2/DOPE-ODN complexes, their presence evidently controlled the size of the complexes by preventing clustering, observed to occur for complexes devoid of PEG-lipid. The PEG-lipid being restricted to the outer periphery of the complex, the typical fingerprint structure was largely maintained in this case (Figures 5E and F). However, as shown above (Fig. 1), in line with the impeding effect of externally exposed non-exchangable PEG-lipid, such complexes give rise to poor release of ODNs.
To further define the effect of the PEGylated lipid on hexagonal phase formation, we examined the lipid phase of SAINT-2/DOPE/5 mol % DSPE-PEG vesicles in water, which consist of a mixture of particles of various sizes and shapes, as shown in Fig. 5G. Following their incubation in a physiological salt environment, the transition into a hexagonal texture was not seen (Fig. 5H). Rather, vesicles in salt appeared as well defined unilamellar liposomes and particles with invaginations, indicating that DSPE-PEG apparently prevents the SAINT-2/DOPE from adopting a hexagonal morphology by interfering with the close approach of opposed membranes.

**Discussion**

The present work demonstrates that as a function of their structure, PEGylated lipid analogues can strongly interfere with the functional properties of lipoplexes, in terms of antisense or gene delivery. This interference is only partly due to an inhibition of complex internalization by cells, which has prompted their development and application in the first place. Rather, our data reveal that PEG-lipid analogues strongly interfere with structural features of the complex, involving a stabilization of the lamellar phase and precluding an intimate interaction with the endosomal membrane, thereby impeding cytosolic release of ODN or genes. The latter was apparent from the marked abrogation of down-regulation of a

**Figure 6. Structure of cationic-lipid-ODN complexes, determined by SAXS.** The cationic-lipid-ODN complexes were prepared as described in the Materials and methods section, and the structures of the complexes were determined by SAXS. (A) Diffraction pattern of control SAINT-2/DOPE-ODN complexes, revealing three peaks at $q = 0.105, 0.180$ and $0.210$ (Å)$^{-1}$, which indicate a hexagonal morphology with a periodicity of 7.0 nm. (B) Diffraction pattern obtained with SAINT-2/DOPE/DSPE-PEG-ODN complexes. In this case only 1 peak at $q = 0.111$ (Å)$^{-1}$ was obtained. This peak corresponds to a distance of 5.7 nm.
target membrane receptor and a decrease in expression of a reporter gene. Clearly, the bilayer organization *per se* does not affect particle internalization, but non-bilayer features appear crucial in events that subsequently govern intracellular release of ODNs or reporter genes, events that appear to be triggered when the complexes are processed along the endosomal track [18,19]. Importantly, the capacity to release ODN or plasmid does not solely rely on the fact that the lipoplexes have overall adopted the hexagonal phase, and that they have acquired access into the endosomal compartment. Thus our data suggest (Figures 5E and F) that direct membrane-membrane interactions between such complexes and the endosomal membrane are necessary, a step which is prevented when PEGylated lipids are present. In that case, the complexes arrive in the lysosomal compartment, without the occurrence of cytosolic release of ODN or plasmid.

Currently, two procedures have been described for the incorporation of PEGylated lipids into gene delivery complexes. One involves prior incorporation of lipid-PEGs into liposomes, i.e. before lipoplex assembly triggered upon addition of DNA [20,21]. Alternatively, lipid-PEGs are inserted into the preformed lipoplexes or liposomes [22-24]. Upon exogenous addition the PEGylated lipid was limited to the outer periphery of the complex, and only such PEGylated complexes appear to display a fertile use in delivery, provided that the PEG-lipids are exchangeable. Thus even when exchangeable C8-ceramide is contained in the entire complex, i.e. when using PEGylated liposomes for lipoplex assembly, no delivery is observed and the lipoplexes reach the lysosomes, which correlates well with a rapid processing of relatively small particles, as obtained by this procedure, along the endocytic pathway [25].

When located in the periphery of the complex following exogenous insertion, ceramide-PEG derivatives may still inhibit cellular association of the complexes. Yet the efficiency of inhibition clearly showed a dependence on the kinetics of PEG-ceramide exchange, and in parallel to that the ceramide-PEG-lipid dependent down-regulation of CRF-R expression (Fig. 3). Thus C8-ceramide-PEG displayed little if any inhibition of both parameters while with increasing fatty acyl chain length the effective inhibition increased, and became compatible with effects of the non-exchangeable DSPE-PEG in case of ceramide-C20-PEG. The data suggest that, soon after the onset of the incubation, C8-ceramide-PEG readily transfers from the complex to, presumably, the cell surface, since substantial nuclear accumulation of ODNs was apparent after only two hours.

The present work indicates that electrostatic interactions between the complexes and the cell surface need not necessarily be prevented by the presence of moderate
concentrations of PEGylated lipids (up to 10 mol %), which is in line with similar observations reported by others [7,26]. Indeed, the primary and secondary energy minimum for such interactions, taking place when membranes come in close proximity, are localized in the range of distances of 3-10 nm [27]. For non-lamellar transitions to take place, possibly occurring when the lipid phase of the lipoplex interacts with the endosomal membrane, the inter-membrane distance should likely not exceed a distance of 1 nm; this event appears to be precluded by the steric interference of the PEGylated lipid. It is in this context particularly interesting to note that the release of ODNs from PEGylated lipoplexes is also inhibited when adding anionic liposomes (table 1), a feature that might mimic the mechanism involved in nucleic acid release from lipoplexes in the endosomal compartment [28]. This observation could suggest that a simple flip-flop and electrostatic displacement of ODNs following their substitution by PS in the cationic lipid complex does not occur or, alternatively, that such a translocation requires the complex to adopt the non-lamellar phase at the complex surface as well, a lamellar phase being stabilized when coated with PEGylated lipid (Figures 5 and 6).

Rather, the present work emphasizes an absolute requirement for dissociation of the PEG-lipid analogue to abrogate its effect on the ability of cationic lipids in conjunction with DOPE to adopt non-bilayer phases of the lipoplex that promote ODN or plasmid dissociation.

Abbreviations
ODNs, oligonucleotides; lipoplexes, cationic lipid-DNA complexes; CHO, Chinese hamster ovary; HII, inverted hexagonal phase; Lα, Lamellar phase; PEG, poly(ethylene glycol); SAINT-2, N-methyl-4-(dioleyl)methylpyridinium; DSPE, distearoyl-phosphatidylethanolamine; DOPE, dioleoyl-phosphatidylethanolamine; CRF-R, corticotropin releasing factor receptor; SAXS, small angle X-ray scattering.

References


