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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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 effects 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 were 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.

Key words: antisense, gene therapy, hexagonal phase, lipoplex.

INTRODUCTION

Short single-stranded DNA fragments such as 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 tissues and/or cells.

Cationic-lipid–DNA complexes ('lipoplexes') have been successfully used for gene delivery, and cellular delivery of ODNs can also be greatly improved in this manner, although the overall mechanism of delivery is still poorly defined. To overcome such drawbacks as a 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 ODN into cells. In this regard, highly relevant issues concern the effects 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, affect the (intra-)cellular interactions and processing relevant to the eventual delivery of plasmid or ODN. Previous work has emphasized the close relationship between structure and function (i.e. transfection efficiency) of lipid–DNA complexes. Thus the evidence indicates that 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 a steric barrier, which could, among other effects, 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

Abbreviations used: Cer-C₈ (etc.), ceramide with a C₈ fatty acyl chain length (etc.); CHO, Chinese hamster ovary; CRF-R, corticotropin-releasing factor receptor; DOPE, dioleoyl-phosphatidylethanolamine; DSPE, distearoyl-phosphatidylethanolamine; EM, electron microscopy; lipoplex, cationic-lipid–DNA complex; N-Rh-PE, N-(lissamine rhodamine sulphonyl)phosphatidylethanolamine; ODN, oligonucleotide; PE, phosphatidylethanolamine; PEG, poly(ethylene glycol); SAINT-2, N-methyl-4-(dioleoyl)methylpyridinium chloride; SAXS, small-angle X-ray scattering.

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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 includes 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 into cationic-lipid-ODN complexes, and the ensuing effects on the cellular interaction and processing of such complexes, including ODN delivery. As demonstrated by small-angle X-ray scattering (SAXS) and cryo-electron microscopy (cryo-EM), 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

PEG (M_r 2000), covalently attached to distearoyl-phosphatidylethanolamine (DSPE) (DSPE-PEG 2000), was obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.), and dioleoyl-phosphatidylethanolamine (DOPE) and *N*-(lissamine rhodamine sulphonyl)phosphatidylethanolamine (N-Rh-PE) were purchased from the same source. Ceramide- C_8 -PEG (Cer- C_8 -PEG, where C_8 denotes the fatty acyl chain length), Cer- C_{14} -PEG and Cer- C_{20} -PEG (PEG M_r 2000) were obtained from Northern Lipids (Vancouver, Canada). The cationic lipid SAINT-2 [*N*-methyl-4-(dioleoyl)methylpyridinium chloride] was synthesized as described in detail elsewhere [13]. Antisense ODN complementary to the mRNA of the corticotropin-releasing factor receptor (CRF-R; GenBank accession no. L25 438), targeted to bp 474–490 (sequence 5' GGATGAAAGCCGAGATG 3'), and a 17-mer randomized-sequence ODN (sequence 5' ACTACGACC-TACGTGAC 3') were designed and manufactured by Biognostik (Göttingen, Germany). The randomized ODN sequence, labelled at the 5'-end with FITC, was used for cellular binding and uptake studies. All ODNs were thioated and purified by HPLC, cross-flow dialysis and ultrafiltration. All chemicals were from Sigma, unless stated otherwise.

Cell culture

Chinese hamster ovary (CHO) cells, stably expressing the CRF-R under the control of the cytomegalovirus promoter, were kindly provided by Solvey 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 units/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, v/v). 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 Millipore 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 of CHO-SFM medium (Life Technology, Breda, The Netherlands), was mixed with 0.1 nmol of ODNs, diluted in 200 µl of the same medium. After 20 min at room temperature, the mixture was diluted with 600 µl of 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 of SAINT-2/DOPE liposomes and 0.1 nmol of ODNs were mixed in 100 µl of CHO-SFM medium and incubated for 20 min at room temperature. Then 2 nmol of lipid-PEG was added and the mixture was incubated at 60 °C for 1 h, cooled to 37 °C, and diluted with 900 µl of 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 Probes, Eugene, OR, U.S.A.). The assay was performed according to instructions provided by the manufacturer.

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

The (intra)cellular fate of ODNs and lipoplexes was determined by monitoring the fate of FITC-labelled ODNs and N-Rh-PE-labelled 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 then incubated for a time interval as indicated. The cells were rinsed twice with Hanks balanced salt solution, and then analysed 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 labelled with FITC-dextran (M_r 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-labelled lipid-ODN complexes for 5 h at 37 °C. 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 or 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 of ODNs/100 nmol of lipid) for 5 h, after which period the complexes were removed and fresh medium was supplied. 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 analysed on SDS/12.5%-PAGE (Bio-Rad, Hercules, CA, U.S.A.), blotted on a pure nitrocellulose membrane (Trans-Blot® Transfer Medium; Bio-Rad) and probed with goat anti-(rat CRF-R) (1:500; Santa Cruz), followed by horseradish peroxidase-conjugated rabbit anti-goat antibody (Sigma, Steinheim, Germany). The blot was processed using 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 of ODNs in a final volume of 875 μ l. 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 μ l of the various samples, as indicated in the Figure 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.

SAXS analyses

To determine 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 CuK α radiation beam ($\lambda = 0.154$ nm) with a full-width at half-maximum of approx. 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 of the scattering intensity in a q -range of 0.5–3.5 nm⁻¹. The scattering vector q is defined as $q = (4\pi/\lambda) \cdot \sin(\theta/2)$, where θ 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 of SAINT-2/DOPE was gently mixed with 5 nmol of ODNs in 60 μ l of 150 mM NaCl/10 mM Hepes. After 20 min at room temperature, the samples were analysed 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-labelled ODNs was observed, following incubation for either 5 h (Figure 1A) or 24 h (results not shown). Note that, under these conditions, the ODNs and lipids, marked by N-Rh-PE labelling (red), become separated (Figure 1A, inset). A virtually identical image was obtained (results not shown; cf. Figure 1A) when 10 mol % DSPE-PEG was included in the medium. By contrast, when 10 mol % 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 (Figure 1B). When lipoplexes were used prepared from liposomes that contained 10 mol % DSPE-PEG (procedure I), the uptake of ODNs was similarly strongly inhibited (Figure 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 mol %, but no shift in intracellular ODN distribution was observed (Figure 1D). In fact, even at a density as low as 1 mol % DSPE-PEG, nuclear accumulation of ODNs was still effectively prevented (results 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 mol %) as a marker of the lipoplex lipid phase. The merged pictures revealed (Figure 1E) that dissociation of the complex did not take place (cf. Figure 1A), even at DSPE-PEG concentrations as low as 1 mol %. Thus escape of ODNs from lipoplexes was abolished when DSPE-PEG was present.

The distribution pattern of the internalized complexes, seen in Figures 1(C) and 1(D), suggests that the ODNs are trapped in the endo/lysosomal pathway. Indeed, when loading this pathway with FITC-dextran (Figure 1F) followed by Rh-PE-labelled complexes, co-localization of the two 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, the distribution of the FITC-labelled ODNs after 24 h was identical with that observed after 5 h (results not shown; cf. Figures 1D and 1F).

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 it reaches 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 emphasizes the need for exchangeable derivatives. In this regard, claims have been made [12] that, depending on the length of their fatty acyl chains, PEGylated ceramides may display the desired exchange properties necessary for the process that eventually leads to destabilization of the lipoplex structure and favouring 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 relative water solubility.

Therefore we next examined the effects on delivery when ceramide-PEGs were inserted exogenously. Thus 10 mol % Cer-C₈-PEG, Cer-C₁₄-PEG or Cer-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 or overnight. The presence of ceramide-PEGs affected the net cell association of the lipoplexes, similar to that observed for DSPE-PEG. The highest uptake was seen for lipoplexes containing short-chain ceramides, suggesting that uptake efficiency might be related to 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 higher uptake. In this case

Figure 1 Intracellular localization of ODNs delivered by DSPE-PEG-containing lipoplexes

CHO cells were incubated with lipoplexes containing FITC-labelled ODNs, with or without DSPE-PEGs, for 5 h. ODN localization was visualized by confocal laser scanning microscopy. **(A)** Cells were incubated with FITC-ODN-containing SAINT-2/DOPE lipoplexes. Note that most ODNs were seen in the nuclei (bright green), while the carrier, marked by N-Rh-PE, remained localized in the perinuclear region (inset). **(B)** Cells were incubated with DSPE-PEG-coated SAINT-2/DOPE-ODN complexes (procedure II; using 10 mol% PEGylated lipid). Note the virtual absence of ODN nuclear localization in this case. **(C)** Cells were incubated with FITC-ODNs complexed with SAINT-2/DOPE/10 mol% DSPE-PEG liposomes (procedure I). **(D)** Cells were incubated with FITC-ODNs complexed with SAINT-2/DOPE/5 mol% DSPE-PEG. Note the absence of nuclear localization of ODNs. **(E)** Cells were incubated as in **(D)**. The lipid phase of the complex was visualized by including 0.5 mol% N-Rh-PE (red). Note that the ODNs and lipids co-localized in the cytosol (yellow dots), but dissociation of ODNs (green) and lipids (red) was not apparent. **(F)** Cells were preincubated with FITC-dextran (green) to mark the endosomal/lysosomal pathway. After washing, the cells were incubated with N-Rh-PE-labelled ODN-containing lipoplexes (red) that had been prepared from DSPE-PEG (5 mol%) containing SAINT-2/DOPE liposomes (procedure I). Note that the complexes have mostly co-localized with dextran at the perinuclear region.

Figure 2 Effect of exogenous coating of lipoplexes with ceramide-PEGs on ODN delivery

CHO cells were incubated with SAINT-2/DOPE-FITC-ODN complexes (**A**, **E**) that had been coated (procedure II) with 10 mol% Cer- C_8 -PEG (**B**, **F**), Cer- C_{14} -PEG (**C**, **G**) or Cer- C_{20} -PEG (**D**, **H**). After 5 h (**A–D**) or an overnight (O/N) incubation (**E–H**), the localization of ODNs was visualized by fluorescence microscopy (right panels). The left panels show the corresponding phase-contrast images.

nuclear accumulation of ODNs was seen after 2 h for control lipoplexes and for lipoplexes coated with Cer- C_8 -PEG. With the Cer- C_{14} and Cer- C_{20} derivatives, complexes could only be detected in close association with the plasma membrane (results not shown). As demonstrated in Figure 2, when extended to a 5 h incubation, nuclear labelling of cells treated with control (Figure 2A) or Cer- C_8 (Figure 2B) lipoplexes was prominently apparent; it appeared gradually in cells treated with Cer- C_{14} -PEG complexes (Figure 2C), whereas for Cer- C_{20} complexes the ODNs were still largely associated with internalized and cell-surface-bound complexes (Figure 2D). After an overnight incubation (Figures 2E–2H), effective nuclear delivery of ODNs was seen for both the Cer- C_8 and Cer- C_{14} derivatives, whereas some delivery was apparent in the case of complexes that contained the Cer- C_{20} 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 for lipoplex-mediated delivery of ODNs.

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-R overexpression in CHO cells (see the Materials and methods section) was examined by Western

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 a mismatch antisense ODN, while AS (lane 4) indicates treatment with free antisense ODN. In **(B)**, the effects of ceramide-PEGs on lipoplex-mediated antisense delivery were examined. Antisense ODNs were associated with cationic lipids (lane 1; AS + S/D) or with ceramide-PEG-coated complexes [lanes 2–4; (AS + S/D)-CerC₈-PEG, (AS + S/D)-CerC₁₄-PEG and (AS + S/D)-CerC₂₀-PEG respectively]. Note that the degree of down-regulation is correlated with the length of the ceramide acyl chain length.

immunoblot. As shown in Figure 3, down-regulation of CRF-R expression was seen when antisense ODNs were delivered with SAINT-2/DOPE, compared with the levels of expression seen in untreated cells, cells treated with complexes containing mismatch ODNs or cells treated with antisense ODNs alone. When the same amounts of antisense ODNs were delivered with complexes containing 5 mol% DSPE-PEG, 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 time-dependent 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 with complexes coated (procedure II) with CerC₈-PEG and partly restored with complexes coated with CerC₁₄-PEG, whereas nuclear delivery still did not occur after this time interval in the case of CerC₂₀-PEG. Consistently, the antisense effect of ODN in down-regulating CRF-R expression was inversely proportional to the fatty acyl chain length of the ceramide-PEG employed (Figure 3B). Thus, relative to untreated cells (lane 5) and ODN treatment by delivery in PEG-devoid complexes (lane 1), an effective decrease in CRF-R expression was seen with CerC₈-PEG-coated complexes. The efficiency was diminished when employing CerC₁₄-PEG-coated complexes, whereas no significant effect was seen with CerC₂₀-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 Figure 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 the lipoplex-cell-membrane (receptor) interaction that is necessary for endocytic internalization, the pathway along which SAINT-2-containing lipoplexes deliver plasmids [16]. However, following internalization, this interaction apparently does not suffice for ODNs to be released, which requires the dissociation of the PEGylated lipid from the complex. This implies that additional factors must be involved, like the intimacy of the complex-endosomal-membrane interaction (as noted above), and also lipid structural requirements (e.g. lamellar-to-hexagonal phase changes) that may co-determine overall nucleic acid release efficiency [8,9]. The effect of PEGylated lipids on lipoplex structure was therefore examined next.

The presence of PEGylated lipids stabilizes 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-containing 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/DOPE 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 Figure 4, the turbidity of a suspension of SAINT-2/DOPE 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/DOPE vesicles under otherwise identical conditions, the aggregation of the lipid vesicle was 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/DOPE-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 5B). The fingerprint structure is typical of the hexagonal phase. To support this conclusion, we analysed the samples by SAXS. As shown in Figure 6(A), three diffraction maxima were apparent at $q = 0.105, 0.181$ and 0.201 ($\text{\AA}^{-1}$), implying that the location of the peaks is in the ratio $1 : \sqrt{3} : \sqrt{4}$, i.e. fully consistent with a hexagonal morphology. The periodicity of the phase is approx. 7 nm (Figure 6A). By contrast, the morphology of DSPE-PEG-containing complexes is different. Cryo-EM revealed the presence of small homogeneous complexes with a diameter of 30–50 nm, which did not display a tendency to aggregate (Figures 5C and 5D). 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

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 (min) required to reach this plateau value is indicated on top of the bars.

resemble a lamellar than a hexagonal organization. Indeed, as shown in Figure 6(B), the SAXS diffraction pattern differed considerably from that obtained for the non-PEGylated complexes (Figure 6A). Only one peak at $q = 0.111 \text{ (Å)}^{-1}$ was obtained, corresponding to a distance of 5.6 nm. This distance is compatible with a lamellar phase of similar periodicity, as observed for lamellar complexes obtained with 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 mol%.

When 10 mol% DSPE-PEGs were incorporated into preformed SAINT-2/DOPE-ODN complexes, their presence evidently controlled the size of the complexes by preventing the clustering that is observed to occur for complexes devoid of PEG-lipid. As the PEG-lipid is restricted to the outer periphery of the complex, the typical fingerprint structure was largely maintained in this case (Figures 5E and 5F). However, as shown above (Figure 1), in line with the impeding effect of externally exposed non-exchangeable 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 Figure 5(G). Following their incubation in a physiological salt environment, the transition into a hexagonal texture was not seen (Figure 5H). Rather, vesicles in salt appeared as well defined unilamellar liposomes and particles with invaginations, indicating that DSPE-PEG apparently prevents 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 prompted their development and application in the first place. Rather, our data reveal that PEG-lipid analogues strongly interfere with structural

Figure 5 Morphology of ODN lipoplexes as revealed by cryo-EM

The various ODN lipoplexes were investigated by cryo-EM, as described in the Materials and methods section. (A, B) Control complexes consisting of SAINT-2/DOPE-ODN. Note the striated structures which are typical of hexagonal morphology. These complexes tended to aggregate, and the average size was approx. 100–200 nm. (C, D) DSPE-PEG-containing complexes (procedure I). These appeared as small, round particles with an average size of 30–50 nm. Striated structures were far less frequent, and often particles were seen with a few lamellae. Such complexes did not tend to aggregate. (E, F) Preformed SAINT-2/DOPE-ODN complexes were coated with 10 mol% DSPE-PEG (procedure II). Most complexes showed the striated structures typically seen prior to coating, as in (A) and (B). (G) SAINT-2/DOPE/DSPE-PEG vesicles in water, revealing a mixture of particles with different sizes, with little if any electron-dense material inside. (H) When 150 mM NaCl/10 mM Hepes, pH 7.4, was added, the giant liposomes disappeared and the unilamellar liposome and particles with invaginations co-existed. Clearly there was no appearance of a hexagonal texture. Bars are 100 nm.

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 ODNs or genes. The latter was apparent from the marked abrogation of the

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 $0.210 \text{ (\AA)}^{-1}$, 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 one peak at $q = 0.111 \text{ (\AA)}^{-1}$ was obtained. This peak corresponds to a distance of 5.7 nm.

down-regulation of a 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 to be 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 [17,18]. Importantly, the capacity to release ODN or plasmid does not rely solely on the fact that the lipoplexes have adopted the hexagonal phase overall and have acquired access into the endosomal compartment. Thus our data suggest (Figures 5E and 5F) that direct membrane-membrane interactions between such complexes and the endosomal membrane are necessary, a step that 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 is triggered upon addition of DNA [19,20]. Alternatively, lipid-PEGs can be inserted into the preformed lipoplexes or liposomes [21–23]. Upon exogenous addition the PEGylated lipid will be 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 C_8 -Cer 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 rapid processing of relatively small particles, as obtained by this procedure, along the endocytic pathway [24].

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 ceramide-PEG exchange, and in parallel with that the ceramide-PEG lipid-dependent down-regulation of CRF-R expression (Figure 3). Thus Cer- C_8 -PEG displayed little if any inhibition of either parameter, while effective inhibition increased with increasing fatty acyl chain length, and became comparable with the effects of non-exchangeable DSPE-PEG in the case of Cer- C_{20} -PEG. The data suggest that, soon after the onset of the incubation, Cer- C_8 -PEG readily transfers from the complex to,

presumably, the cell surface, since substantial nuclear accumulation of ODNs was apparent after only 2 h.

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 [7,25]. Indeed, the primary and secondary energy minima for such interactions, taking place when membranes come into close proximity, are localized in a range of distances of 3–10 nm [26]. 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 probably should not exceed a distance of 1 nm; this event appears to be precluded by the steric interference of the PEGylated lipid. It is particularly interesting in this context to note that the release of ODNs from PEGylated lipoplexes is also inhibited when adding anionic liposomes (results not shown), a feature that might mimic the mechanism involved in nucleic acid release from lipoplexes in the endosomal compartment [27]. This observation could suggest that a simple flip-flop and electrostatic displacement of ODNs following their substitution by phosphatidylserine 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.

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