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Characterization and transfection properties of lipoplexes stabilized with novel exchangeable polyethylene glycol–lipid conjugates

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Abstract

The positive charge of cationic-lipid/DNA complexes (lipoplexes) renders them highly susceptible to interactions with the biological milieu, leading to aggregation and destabilization, and rapid clearance from the blood circulation. In this study we synthesized and characterized a set of novel amphiphiles, based on *N*-methyl-4-alkylpyridinium chlorides (SAINTs), to which a PEG moiety is coupled. Plasmids were fully protected in lipoplexes prepared from cationic SAINT-2 lipid and stabilized with SAINT-PEGs. Our results demonstrate that SAINT-PEG stabilization is transient, and permits DNA to be released from these lipoplexes. The rate of SAINT-PEG transfer from lipoplexes to acceptor liposomes was determined by the nature of the lipid anchor. Increased hydrophobicity, by lengthening the alkyl chain, resulted in a decrease of the rate of DNA release from the lipoplexes. Chain unsaturation had the opposite effect. Similarly, the in vitro transfection potency of lipoplexes containing PEG-SAINT derivatives was sensitive to the length and (un)saturation of the alkyl chain. However, the internalization of SAINT-PEG stabilized lipoplexes is determined by their charge, rather than by the concentration of the polymer conjugate. Lipoplexes targeted to cell-surface epithelial glycoprotein 2, by means of a covalently coupled monoclonal antibody, were specifically internalized by cells expressing this antigen.

Keywords: Cationic lipid; Polyethylene glycol; Steric stabilization; Gene delivery; Epithelial glycoprotein 2

1. Introduction

In recent years it has become apparent that synthetic amphiphiles provide a promising novel tool in the development of gene carrier systems. Introduction of nucleic acids into cells using cationic lipids results in efficient cellular transfection that is usually far superior over that accomplished via other non-viral systems. Moreover, synthetic amphiphiles are readily susceptible to chemical modifications, conveying specific properties to these molecules as such or providing possibilities for coupling with specific molecules.

However, the use of lipid-based carrier systems in vivo calls for some additional requirements to be fulfilled. For example, positively charged particles are especially prone to undesired interactions with plasma proteins, which can lead to destabilization and rapid clearance of the lipoplexes by macrophages before they reach the diseased tissue. Numerous approaches based on surface modifications have been proposed to decrease particle interactions with biological components resulting in increased blood circulation times. Currently the synthetic polymer polyethylene glycol (PEG), which can be easily synthesized in large amounts at high purity is the preferred substance to achieve this effect. The most beneficial characteristics of PEG are: chemical inertness, nonionic character, high water solubility and low cost [1,2].

The PEG moiety can be conjugated to phosphatidylethanolamines such as DSPE, serving as a bilayer anchor, via a carbamate linkage. However, it has been speculated that the net negative charge on the phosphate moiety may have undesirable effects on particle pharmacokinetics [3]. Furthermore, recent studies, in which the DSPE-PEG conjugate

Abbreviations: Cer, ceramides; DODAC, dioleyldimethylammonium chloride; DOPE, dioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DOPS, dioleoylphosphatidylserine; EGP-2, epithelial glycoprotein 2; GFP, green fluorescent protein; HBSS, Hank's balanced salt solution; PEG, polyethylene glycol; SAINT, *N*-methyl-4-alkylpyridium chloride; SATA, (*N*-succinimidyl-*S*-acetylthioacetate)

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was employed to stabilize lipid/DNA complexes, showed that the use of DSPE as a firm anchor brings along other drawbacks. It has been demonstrated that such a stable steric barrier strongly inhibits nucleic acid release from the endosomal compartment, precluding its delivery to the nucleus. Interestingly, the cell association and subsequent endocytosis of these lipoplexes were not altered by PEG [4–6]. Exchangeable PEG-derivatives, stabilizing a particle in a time-dependent manner, may circumvent these problems, as illustrated by the use of PEG-ceramides. It has been shown that the rate of PEG-ceramide release from the particle depends on the size of the PEG moiety as well as on the acyl chain length [7,8]. In biological membranes, ceramide-based lipids readily engage in H-bonding-driven interactions, an instrumental property in formation of sphingolipid-enriched domains, also known as 'rafts'. Whether such self-aggregating properties also affect their lateral behavior in cationic lipid complexes remains unknown, but such features may affect the controlled dissociation from lipoplexes. Taking also into account economical considerations for in vivo application, we therefore set out to develop novel exchangeable PEG-derivatives, which should closely resemble the lipophilic structure of the amphiphile in the lipoplex and whose synthesis should be relatively simple. To this end, we synthesized cationic lipids with a conjugated PEG moiety, relying on our previous studies in which we employed *N*-methyl-4-alkylpyridinium chlorides, a class of cationic lipids abbreviated as SAINT [9–11]. Their properties were characterized in terms of their exchangeability and potential for regulated and controlled gene delivery.

2. Materials and methods

2.1. Materials

The cationic lipid SAINT-2 was synthesized as described elsewhere [12]. DSPE-PEG2000, DOPE, DOPS, and DOPC were obtained from Avanti Polar Lipids (Alabaster, AL, USA). PEG-ceramides were from Northern Lipids (Vancouver, Canada). Polyethylene glycol monomethyl ether with molecular weight of 2000 was purchased from Fluka, and dried in vacuum above P_2O_5 before use. 2,4,4,6-Tetrabromo-2,5-cycloheadien-1-one (95%) was from Acros. All other chemicals were from Sigma, unless indicated otherwise.

2.2. Cell culture

Murine melanoma B16.F10 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 100 μg/ml penicillin/streptomycin (Gibco BRL, Life Technology, Merelbeke, Begium) at 37 °C (5% CO_2). EGP-2 expressing B16 melanoma cells (B16.C215) were cultured under the same conditions, but in the presence of 500 μg/ml G 418.

2.3. Transfection

B16 cells were plated 1 day prior to transfection in 6- or 12-well plates. The cells were incubated with the lipoplexes in FCS-supplemented cell culture medium (10%) for 4 h at 37 °C (50 μl of lipoplexes + 450 μl of medium—6-well plates; 100 μl of lipoplexes + 900 μl of medium—12-well plates). Then the cells were washed and treated with serum-containing medium, which was refreshed after 24 h of culture. Three days after transfection (unless indicated otherwise), the cells were washed with HBSS and examined by fluorescence-activated cell sorting (FACS). Note that the transfection capacity of the cationic lipid formulations employed in the present study was not significantly affected by the presence of serum, as previously reported [5,10].

2.4. Synthesis of SAINT-PEG derivatives

The overall synthetic pathway is illustrated in Fig. 1. Individual compounds were synthesized, purified and characterized as described in detail in the following subsections. ^{1}H-NMR and ^{13}C-NMR spectra were run on a Varian Gemini 200 or Varian VXR 300 spectrometer.

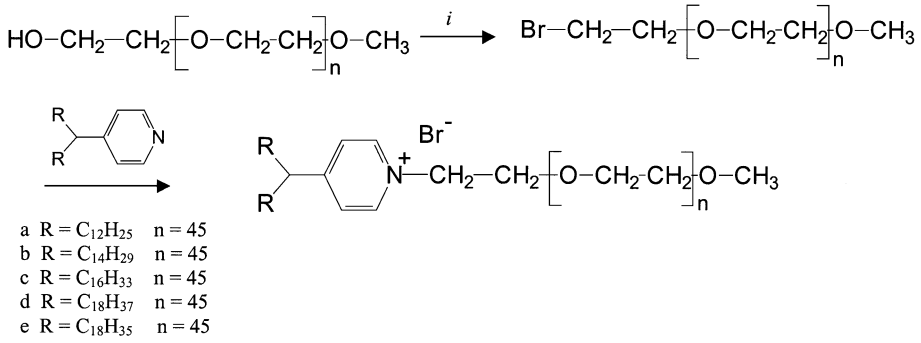

Fig. 1. Schematic representation of the synthesis of polyethylene glycol-coupled SAINT derivatives. The PEG-SAINTS consisted of SAINTs with dialkyl chains as indicated in a–e, to which PEG (Mw 2000 kDa (n = 45)) was coupled. For details see Section 2.

2.4.1. Bromo-PEG$_{2000}$-monomethylether [13,14]

Triphenylphosphine (1.180 g; 4.5 mmol) was dissolved in 13 ml of dry dichloromethane. 2,4,4,6-Tetrabromo-2,5-cyclohexadien-1-one (1.843 g; 4.5 mmol) was added. The mixture was stirred for 2 h. After salt formation was completed (checked with ^{31}P-NMR, solvent C_6D_6), 2.87-g polyethylene glycol monomethyl ether (2000) was added. Stirring was continued for 5 days. The solvent was removed by evaporation in vacuum and the residue was treated with 25-ml distilled water. The aqueous solution was filtered and centrifuged for 1 h. The clear solution was decanted and freeze-dried; yield: 2.576 g of a white powder (90%). If necessary, traces of triphenylphosphorousoxide can be removed by dispersing a dichloromethane solution of the compound in an excess of ether followed by filtration by suction and drying. ^{13}C-NMR (75.4 MHz, $CDCl_3$): δ=30.15, 58.83, 70.33, 70.99, 71.71 [15].

2.5. SAINT-PEG synthesis

To a solution of 0.2 g (0.1 mmol) Br-PEG$_{2000}$-OCH_3 in absolute ethanol was added the corresponding pyridine in a 10-fold excess. The solution was refluxed for 7–14 days. The reaction was followed by ^{1}H-NMR. The solvent was removed and the residue was purified by chromatography on Al_2O_3 (neutral, activity III), gradient elution with a mixture of dichloromethane/2–10% methanol, and crystallization from acetone/ether (1:1) gave the products in 60–80% yield.

2.5.1. 4-(Didodecylmethyl)-1-(PEG$_{2000}$OCH$_3$)-pyridinium bromide (a)

Crystallization from acetone/ether gave white crystals, mp 42–45 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ=0.85 (t, 6 H), 1.22 (m, 40 H), 1.54 (m, 2 H), 1.71 (m, 2 H), 2.74 (m, 1 H), 3.35 (s, 3 H), 3.62 (m, PEG$_{chain}$), 4.05 (m, 2 H), 5.17 (m, 2 H), 7.66 (d, J=6.6 2 H), 9.50 (d, J=6.6 2 H). ^{13}C-NMR (50.3 MHz, $CDCl_3$): δ=13.89, 22.44, 27.21, 29.10, 29.16, 29.38, 31.66, 35.45, 46.34, 58.78, 60.09, 69.39, 69.92, 70.11, 70.28, 71.66, 126.34, 145.06, 166.89.

2.5.2. 4-(Ditetradecylmethyl)-1-(PEG$_{2000}$OCH$_3$)-pyridinium bromide (b)

White crystals mp 45–46 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ=0.87 (t, 6 H), 1.24 (m, 48 H), 1.57 (m, 2 H), 1.73 (m, 2 H), 2.76 (m, 1 H), 3.37 s, 3 H), 3.64 (m, PEG$_{chain}$), 4.08 (m, 2 H), 7.68 (d, J=6.6 2 H), 9.51 (d, J=6.6 2 H). ^{13}C-NMR (50.3 MHz, $CDCl_3$): δ=14.00, 22.55, 27.30, 29.27, 29.51, 31.77, 35.57, 46.46, 60.09, 70.39, 71.77, 126.40, 145.14, 167.07.

2.5.3. 4-(Dihexadecylmethyl)-1-(PEG$_{2000}$OCH$_3$)-pyridinium bromide (c)

White crystals mp 46–47 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ=0.81 (t, 6 H), 1.18 (m, 56 H), 1.50 (m, 2 H), 1.66 (m, 2 H), 2.70 (m, 1 H), 3.32 (s, 3 H), 3.58 (m, PEG$_{chain}$), 4.01 (m, 2 H), 5.13 (m, 2 H) 7.64 (d, J=6.2 2 H) 9.46 (d, J=6.2 2 H). ^{13}C-NMR (50.3 MHz, $CDCl_3$), δ=13.92, 22.46, 27.21, 29.13, 29.18, 29.30, 29.46, 31.68, 35.48, 46.35, 58.80, 60.03, 69.47, 69.94, 70.32, 71.69, 126.30, 145.10, 166.89.

2.5.4. 4-(Dioctadecylmethyl)-1-(PEG$_{2000}$OCH$_3$)-pyridinium bromide (d)

White crystals mp 47–48 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ=0.84 (t, 6 H), 1.21 (m, 64 H), 1.54)m, 2 H), 1.68 (m, 2 H), 2.73 (m, 1 H), 3.35 (s, 3 H), 3.61 (m, PEGchain), 4.06 (m, 2 H), 5.16 (m, 2 H) 7.66 (d, J=6.2 2 H), 9.49 (d, J=6.2 2 H). ^{13}C-NMR (50.3 MHz, $CDCl_3$): δ=13.99, 22.55, 27.31, 29.22, 29.27, 29.39, 29.56, 31.77, 35.57, 46.47, 58.90, 60.04, 69.56, 70.02, 70.38, 71.77, 126.38, 145.38, 167.05.

2.5.5. 4-(Dioleylmethyl)-1-(PEG$_{2000}$OCH$_3$)-pyridinium bromide (e)

White crystals mp 44–45 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ=0.85 (t, 6 H), 1.23 (m, 52 H), 1.55 (m, 2 H), 1.71 (m, 2 H), 1.96 (m, 8 H), 2.74 (m, 1 H), 3.35 (s, 3 H), 3.61 (m, PEG$_{chain}$), 4.06 (m, 2 H), 5.16 (m, 2 H), 5.31 (m, 4 H), 7.66 (d, J=5.9 2 H), 9.47 (d, J=5.9, 2 H). ^{13}C-NMR (50.3 MHz, $CDCl_3$): δ=13.90, 22.44, 26.95, 27.21, 28.99, 29.06, 29.13, 29.26, 29.50, 31.65, 35.46, 46.36, 58.79, 60.02, 69.43, 69.94, 70.30, 71.68, 126.28, 129.48, 129.72, 145.05, 166.88.

2.6. Liposome and lipoplex preparation

Liposomes were formed by mixing lipids in a chloroform/methanol solution and drying under a stream of nitrogen gas. The remnants of solvents were removed by additional drying in vacuum for 2 h. The lipid film was subsequently re-suspended in deionized water by intense vortexing, resulting in the formation of multilamellar vesicles (MLVs). This suspension was sonicated in a bath-type sonicator until clarity. Vesicles were composed of SAINT-2/DOPE (50:50, mole ratio); SAINT-2/DOPE/PEG-lipid (45:45:10), SAINT-2/DOPE/RhPE (49.5:49.5:1); SAINT-2/DOPE/PEG-lipid/RhPE (45:45:10:1), unless indicated otherwise.

To visualize and quantify internalized lipoplexes, 1% of N-Rh-PE was added to the lipids prior to drying. To distinguish between cell-bound and internalized lipoplexes, cells were treated with a quencher (trypan blue) and analyzed by FACS or confocal microscopy.

Lipoplexes were prepared at a SAINT to DNA molar charge ratio 2.5:1, unless indicated differently: 0.5 ml of medium containing 1 μg/ml of pDNA (pEGFP-N1, Clonetech) was added to 0.5 ml of medium containing a solution of cationic liposomes. The average size of the lipoplexes employed in this work ranged from 200 to 250 nm as determined by dynamic light scattering (not shown).

2.7. Plasmid preparation

The plasmid (pEGFP-N1; Clontech, Palo Alto, USA) was isolated form *Escherichia coli* with the Maxi-Prep Kit from Qiagen (Hilden, Germany). Isolated DNA was stored in TE buffer (100mM NaCl, 10 mM Tris–HCl) at a concentration of 1 mg/ml after verifying its purity by determining the ratio of absorbance at 260/280 nm and by gel electrophoresis.

2.8. Agarose gel electrophoresis of lipoplexes

Lipoplexes were prepared with plasmid DNA and SAINT-2/DOPE or SAINT-2/DOPE/PEG-lipid vesicles, as described above. The lipoplexes were incubated with 100 units of DNase I for 30 min at 37 °C in the presence of $MgCl_2$. 1% Zwittergent (Calbiochem, La Jolla, CA, USA) was used to release DNA from the lipoplexes. Samples in glycerol were loaded on a 0.8% agarose gel containing 1.25 μM EtBr immersed in TBE buffer.

2.9. Monitoring of DNA accessibility by a picogreen assay

Lipoplexes were added to a picogreen solution (Molecular Probes, Eugene, OR, USA) prepared according to the manufacturer's protocol. The amount of DNA accessible for labeling was measured with a fluorometer (Perkin Elmer LS 55, λ_{ex} 502 nm, λ_{em} 520 nm). Triton X-100 was added to determine the amount of total DNA present in each sample.

2.10. Anionic vesicle-induced release of DNA from lipoplexes

Lipoplexes prepared in HBSS were added to a picogreen solution, prepared according to the manufacturer's instructions, followed by addition of PS/PE/PC (1:2:1) liposomes in a fivefold molar excess. The change in fluorescence, indicating an increase in the amount of accessible DNA, was subsequently measured online with a fluorometer (Perkin Elmer LS55, λ_{ex} 502 nm, λ_{em} 520 nm). The value of total DNA present was determined by the addition of Triton X-100 solution at the end of each measurement.

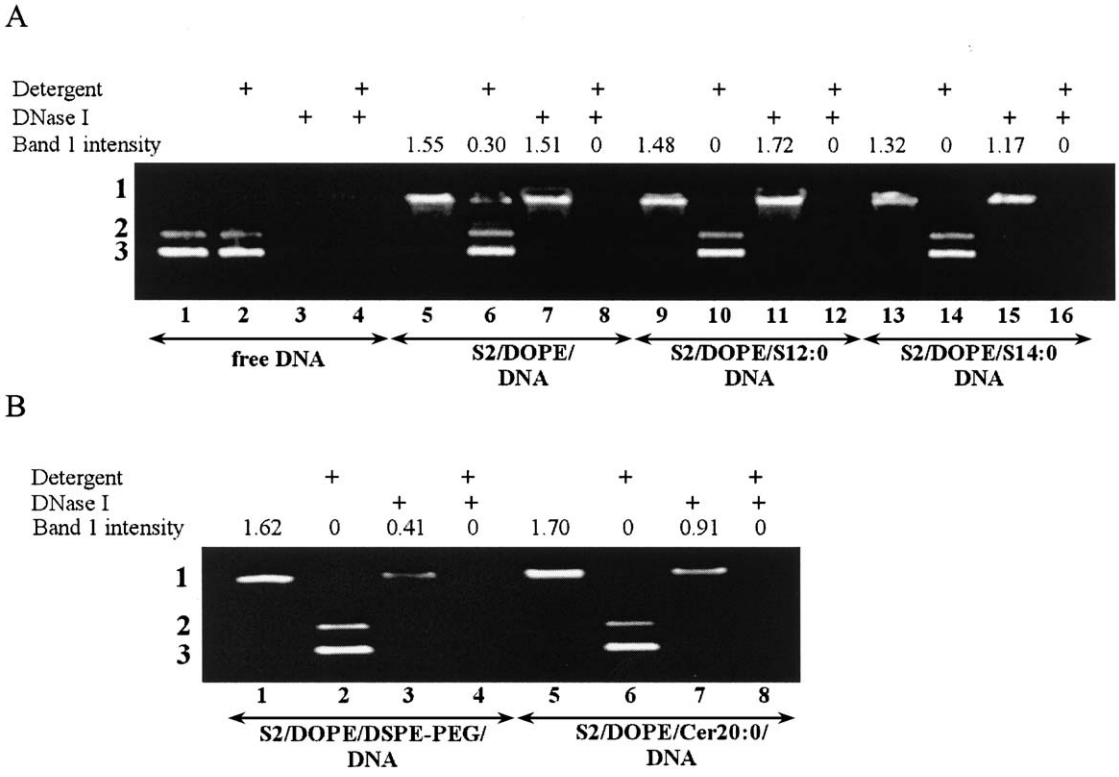

Fig. 2. Effect of PEGylated lipid stabilizers on plasmid accessibility in lipoplexes. (A) C14:0 and C12:0 SAINT-PEG (10 mol%) containing lipoplexes were prepared and the plasmids accessibility was determined by an incubation in the presence of DNase, as described in Section 2. The extent of DNA degradation was then analyzed by agarose chromatography, and quantified (indicated as Band 1 intensity) by using ScionImage computer software provided by Scion Corporation. Treatments of each sample were as indicated at the top of each lane, as follows: Lanes 1, 5, 9, 13—samples without any treatment; Lanes 2, 6, 10, 14—samples were treated with detergent alone (1% Zwittergent); Lanes 3, 7, 11, 15—samples exposed to DNase I (100 units, 30 min, 37 °C); Lanes 4, 8, 12, 16—samples treated with a detergent followed by DNase I digestion. (B) Lipoplexes, containing DSPE-PEG or PEG-Cer20 (10 mol%), were analyzed for plasmid accessibility by an incubation with DNase I, as in (A). Conditions were as follows: Lanes 1, 5—samples without any treatment; Lanes 2, 6—samples were treated with detergent alone (1% Zwittergent); Lanes 3, 7—samples were incubated with DNase I (100 units, 30 min, 37 °C); Lanes 4, 8—samples were treated with 1% Zwittergent, followed by DNase I.

2.11. FACS analysis

FACS analysis was performed on a Coulter Elite flow cytometer. The cells were screened for the reporter gene 3 days after transfection, unless indicated differently. Cells were washed three times with HBSS, trypsinized, centrifuged and resuspended in HBSS. 5000–10 000 cells were measured in each sample (λ_{ex} 488, λ_{em} 530).

2.12. Coupling of antibody

The MOC-31 antibody was coupled to MPB-containing lipoplexes by a sulfhydrylmaleimide coupling method described previously [16]. Briefly, SATA (*N*-succinimidyl l-*S*-acetylthioacetate) was used to introduce free sulfhydryl groups in the antibody. Free SATA was separated by gel permeation chromatography using Sephadex G-50. MPB-containing lipoplexes were incubated with antibody containing reactive sulfhydryl groups for 4 h at room temperature (0.3 mg antibody/μmol of lipid). Unreacted sulfhydryl groups were capped by *N*-ethylmaleimide (8 mM). Unconjugated antibody was separated from lipoplexes by flotation on a metrizamide gradient as described elsewhere [17]. Subsequently, lipoplexes were dialyzed against HN-buffer, pH 7.4. The average amount of antibody coupled to lipoplexes was 30 μg/μmol lipid.

3. Results

3.1. Plasmid DNA in lipoplexes coated with SAINT-PEGs is effectively protected

To verify the relative extent to which SAINT-PEGs potentially perturbed the lipoplex structure and hence caused undesirable plasmid exposure, lipoplexes containing either SAINT-PEGs, ceramide-PEG and DSPE-PEG were examined for DNase I sensitivity.

Samples containing 1 μg DNA (pEGFP-N1) complexed with SAINT-2/DOPE or SAINT2/DOPE/lipid-PEG were exposed to 100 units of DNase I for 30 min at 37 °C. As shown in Fig. 2A, plasmid complexed with SAINT-2/DOPE (50:50) is insensitive to DNase I activity (compare lane 6 vs. lane 7). Essentially identical results were obtained for complexes containing SAINT-PEGs. Examples are presented in lanes 9–16 for the C12:0 and C14:0 derivatives. These data were confirmed when plasmid DNA accessibility was assayed for labeling with picogreen. Both in control and SAINT-PEG-containing samples, the degree of labeling amounted approximately 20% (not shown). By contrast, as shown in Fig. 2B and reflected by the picogreen assay, inclusion of C20:0 ceramide-PEG or PEG-DSPE almost doubled the plasmids accessibility, showing an enhanced degradation (compare band I intensities in Fig. 2B) and degree of picogreen labeling (40–50%; not shown). Thus, relative to DSPE-PEG and Cer-PEG, the SAINT-PEG

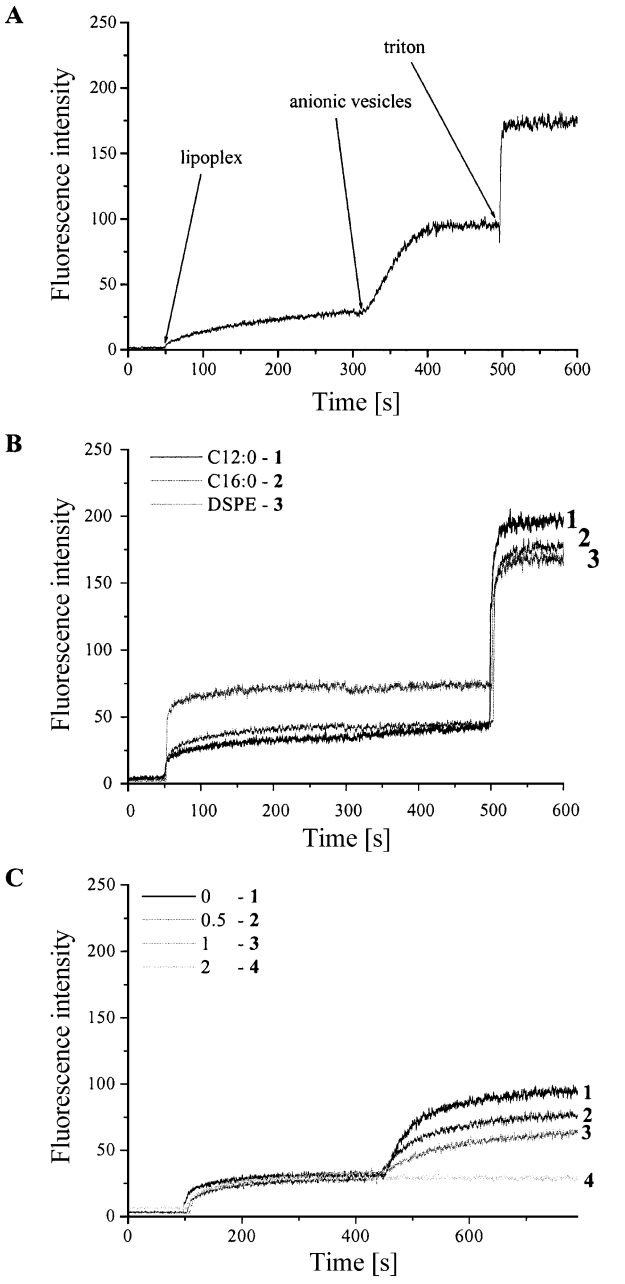

Fig. 3. Accessibility of DNA towards picogreen in non-PEGylated and PEGylated lipoplexes. Lipoplexes were added to a picogreen solution, followed by the addition of PS/PE/PC (1:2:1) liposomes in fivefold molar excess. The changes in fluorescence, indicating an increase in the amount of accessible DNA, were measured with a fluorometer. The value of total DNA accessibility was determined by the addition of a Triton X-100 solution (1%, final concentration) at the end of each measurement. (A) Fluorescence development obtained for lipoplexes consisting of SAINT-2/DOPE (50:50). (B) Lipoplexes were prepared with various PEGylated derivatives (10 mol%) as follows: SAINT-2/DOPE/SAINT$_{12:0}$-PEG (1), SAINT-2/DOPE/SAINT$_{16:0}$-PEG (2) and SAINT-2/DOPE/DSPE-PEG (3). Anionic lipid vesicles were added after 300 s. (C) SAINT-2/DOPE/SAINT$_{12:0}$-PEG; PEG content: 0 (1), 0.5 (2), 1 (3) or 2 (4) mol%. Anionic vesicles were added after 450 s. Note that the presence of PEG inhibits DNA release from lipoplexes induced by the addition of anionic vesicles (A vs. B), and that release of DNA requires reduction in PEG content to 1 mol% or less (C).

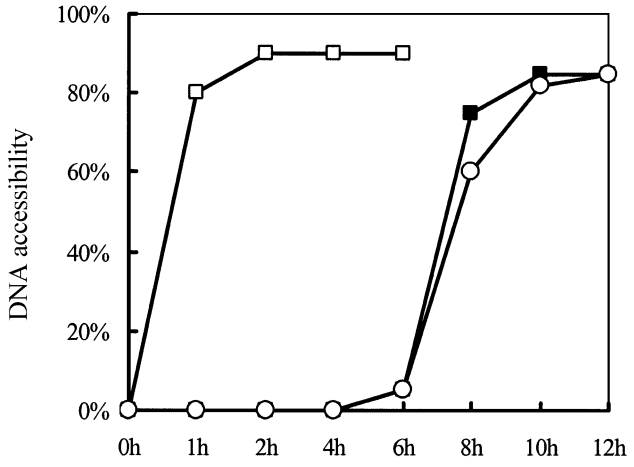

Fig. 4. Time-dependent transfer properties of SAINT-PEGs. Lipoplexes were incubated with POPC liposomes (25-fold excess) for different periods of time. Subsequently, they were transferred into a picogreen solution followed by addition of PS/PE/PC (1:2:1) liposomes in a fivefold molar excess. The changes in DNA accessibility for labeling with picogreen were measured with a fluorometer. The 100% value reflects the fluorescence intensity obtained after addition of Triton X-100 (1%, final concentration) to non-PEGylated lipoplexes. Lipoplexes employed were as follows: SAINT-2/DOPE/SAINT$_{12:0}$-PEG (□); SAINT-2/DOPE/SAINT$_{14:0}$-PEG (■); SAINT-2/DOPE/SAINT$_{18:1}$-PEG (○). The PEG–lipid concentration was 5 mol%.

derivatives considerably improved plasmid protection in the SAINT lipoplexes.

3.2. SAINT-PEG lipids stabilize lipoplexes by precluding membrane–membrane interactions

According to the model proposed by Xu and Szoka [18] and further extended by Hafez et al. [19] as well as based upon the experimental data obtained by other groups [4–6], it became evident that a close contact between endosomal membrane and lipoplex is required for nucleic acid release and subsequent transfection. Thus, steric stabilization of lipoplexes has to be transient for efficient transfection to occur, implying that SAINT-PEG derivatives should dissociate from lipoplexes in a time-dependent manner. To mimic the endosomal escape of DNA, the interaction of PEGylated lipoplexes with anionic vesicles was investigated by recording changes in the amount of nucleic acid accessible for the fluorescent dye picogreen. As shown in Fig. 3A, the addition of anionic vesicles to SAINT-2/DOPE lipoplexes results in an abrupt enhancement of the pool of plasmid DNA, which becomes accessible to the dye. This is due to both release of the DNA from the complex as a result of electrostatic competition of the anionic lipid and DNA for association with the cationic amphiphile, and destabilization of the complex following anionic lipid-induced non-lamellar transitions upon mixing with the cationic lipids [5,20]. The presence of the PEG coating effectively inhibited this process, as demonstrated for SAINT-PEGs (Fig. 3B) and DSPE-PEG (Fig. 3B). Interestingly, the $C_{5:0}$ SAINT-PEG derivative did not show any inhibitory effect (data not shown), and the kinetics of picogreen fluorescence development was identical to that shown in Fig. 3A.

These data emphasize the need for dissociation of the PEGylated lipid derivative in order to destabilize the lipoplex structure, necessary for plasmid release, which requires a direct interaction between lipoplex and target membrane. As shown in Fig. 3C, concentrations as low as 2 mol% SAINT-PEG effectively shield the lipoplexes from interaction with anionic lipid vesicles, as reflected by the poor picogreen fluorescence development at such conditions.

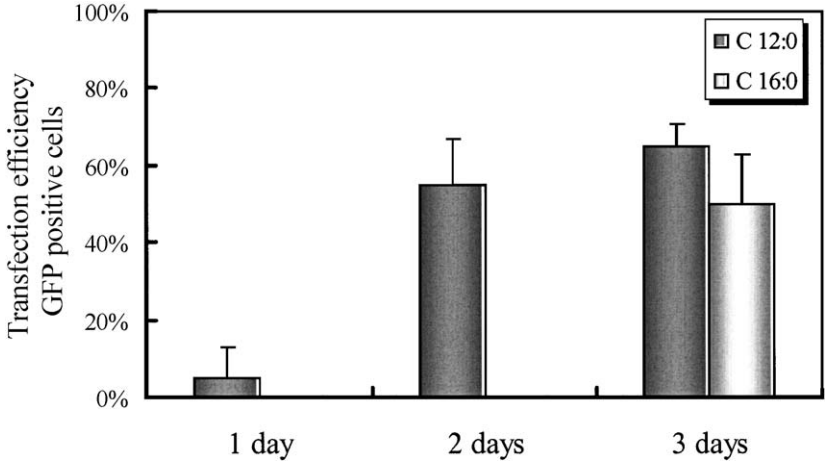

Fig. 5. Time-dependent transfection of B16 cells mediated by SAINT-2/DOPE lipoplexes, stabilized with SAINT-PEG derivatives. B16 cells were plated 1 day prior to transfection. The cells were incubated with the lipoplexes for 4 h at 37 °C. After this period, the cells were washed and treated with serum-containing medium, which was refreshed after 24 h of culture. The cells were washed with HBSS and examined by FACS, 1, 2 or 3 days after transfection. Lipoplexes used were: SAINT-2/DOPE/SAINT$_{12:0}$-PEG (SAINT-2/DOPE (1:1); PEG 10 mol%); SAINT-2/DOPE/SAINT$_{16:0}$-PEG (SAINT-2/DOPE (1:1); PEG 10 mol%).

3.3. Alkyl chain length and (un)saturation govern dissociation of SAINT-PEG conjugates from lipoplexes

The foregoing results imply that the PEG shield requires an almost complete disappearance in order to allow a direct interaction between lipoplex and target membrane. Therefore, we assumed that the time needed for DNA to become accessible to picogreen labeling, following addition of anionic lipid vesicles and in the presence of an appropriate acceptor for the PEGylated lipids, could be taken as an indirect measure of the PEG–lipid exchange rate. POPC liposomes, which were included in the incubation mixture, were utilized as acceptor vesicles for SAINT-PEGs. In this set of experiments, the initial molar ratio of PEG–lipids was reduced to 5 mol%. A 25-fold excess of POPC liposomes would theoretically result in a maximal reduction of the PEG concentration to 0.2 mol%. We estimated that such a reduction would be sufficient for DNA release (Fig. 3C). Since the acyl chain length of lipids is known to exhibit the most prominent effect on monomeric lipid transfer, we compared the results for $SAINT_{12:0}$-PEG and $SAINT_{14:0}$-PEG. As seen in Fig. 4, the time required to the onset of DNA release was different for the two derivatives. Lengthening the alkyl chain by two carbons resulted in a prolongation of the lag period before the lipoplexes were able to release their DNA, which occurred over a period of several hours. In the case of $SAINT_{12:0}$-PEG (open squares) the release was completed after 1–2 h, while for a longer lipid anchor this was accomplished only after 8–10 h (filled squares). The amount of DNA released relative to the release from PEG-devoid complexes was the same for both compounds, i.e. 85% of the plasmid DNA was released from the PEGylated complexes.

Unsaturation in the acyl chain is another factor affecting the rate of PEG–lipid migration between membranes. As shown in Fig. 4, introduction of a double bond shortens the lag period, preceding DNA release. With the $SAINT_{18:0}$-PEG derivative, plasmid accessibility towards picogreen labeling could not be detected up to incubation periods of at least 18 h, suggesting its relative immobile behavior, once inserted into the SAINT-lipoplexes. However, for the unsaturated derivative (18:1), the process of PEG migration was essentially completed within 10 h (Fig. 4, open circles).

3.4. Transient stabilization of lipoplexes with SAINT-PEG enables efficient cellular transfection

After having established the alkyl-chain length and saturation-dependent release of SAINT-PEG from lipo-

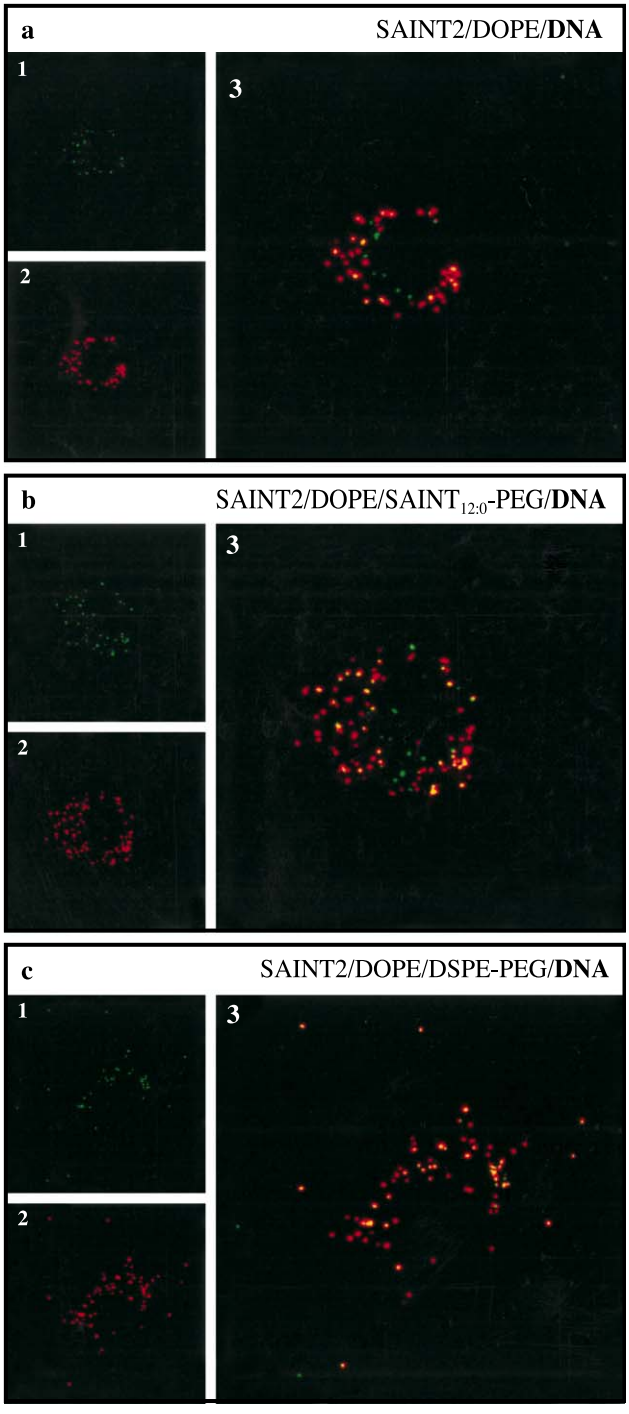

Fig. 6. Intracellular dissociation of SAINT-PEG stabilized lipoplexes. SAINT-2/DOPE and SAINT-2/DOPE/PEG-lipid liposomes were labeled with RhPE (red) and complexed with pDNA (green), labeled with Fasttag nucleic acid labeling kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Subsequently, the lipoplexes were added to B16 cells and incubated at 37 °C for 3 h. The samples were analyzed by confocal microscopy (Leica TCS SP2, Germany). Lipoplexes were composed of SAINT-2/DOPE/RhPE (49.5:49.5:1); SAINT-2/DOPE/PEG-lipid/RhPE (45:45:10:1). To distinguish between cell-bound and internalized lipoplexes, cells were treated with a quencher (trypan blue) before analysis by confocal microscopy. (a) SAINT-2/DOPE-DNA; 1—DNA (green), 2—RhPE (red), 3—merged (yellow); (b) SAINT-2/DOPE/$SAINT_{12:0}$-PEG-DNA; 1—DNA (green), 2—RhPE (red), 3—merged (yellow); (c) SAINT-2/DOPE/DSPE-PEG-DNA; 1—DNA (green), 2—RhPE (red), 3—merged (yellow). Cells transfected with lipoplexes, coated with exchangeable $SAINT_{12:0}$-PEG, show a (time-dependent) separation of lipids and DNA (b). This was not observed for lipoplexes, stabilized with DSPE-PEG (c).

plexes in a synthetic environment, i.e. with liposomes as acceptors, we next investigated how these properties affected cellular transfection. To this end, B16 cells were transfected with lipoplexes containing different SAINT-PEG species and DSPE-PEG, and screened for the reporter gene (GFP) expression 3 days later. For control (i.e. PEG-devoid) SAINT-2/DOPE lipoplexes, the transfection efficiency amounted 80% in terms of GFP-positive cells in the culture. The transfection efficiency for SAINT-PEG derivatives with saturated alkyl chains, which varied from 12 to 16 carbons, was around 60%. No transfection was observed for the $SAINT_{18:0}$-PEG derivative and for lipoplexes stabilized with DSPE-PEG. Introduction of a double bond ($SAINT_{18:1}$-PEG) caused the number of transfected cells to increase to 70%. Accordingly, these data indicate that also in association with cellular membranes, SAINT-PEG derivatives can dissociate from the lipoplexes in a chain-length- and chain-saturation-dependent manner. To reveal the time dependence of this process, GFP reporter gene expression was determined after 1, 2 or 3 days, following transfection with lipoplexes coated with either $SAINT_{12:0}$-PEG or $SAINT_{16:0}$-PEG. As shown above, in the presence of anionic liposomes, DNA was released from the former within 2 h, while in the case of $SAINT_{16:0}$-PEG, an incubation of more than 12 h was required for complete release. As shown in Fig. 5, consistent with a chain-length-dependent kinetics of SAINT-PEG release, transfection was readily seen for $SAINT_{12:0}$-PEG lipoplexes, even after 24 h, whereas with lipoplexes containing SAINT16:0-PEG, positive cells (60%) were detected only when the duration of the experiment had been extended to 3 days.

Finally, a lipid-structure-dependent dissociation of PEGylated stabilizers from lipoplexes, a prerequisite for intracellular destabilization of the lipoplexes, causing DNA release at the endosomal level, could also be visualized by confocal microscopy. As shown in Fig. 6, compared to cells transfected with control lipoplexes (A), cells transfected with complexes coated with exchangeable $SAINT_{12:0}$-PEG reveal DNA release from the complex (B), whereas no dissociation occurs for complexes containing non-exchangeable DSPE-PEG (C).

3.5. Charge determines internalization of SAINT-PEG stabilized lipoplexes by cells

Previously, we [5] and others [6,21] reported that the presence of a DSPE- or Ceramide-based PEG stabilizer (up to 10%) in lipoplexes does not prevent their association with cells and subsequent internalization. We observed that also our standard SAINT-PEG-coated complexes (SAINT-2/DOPE/SAINT-PEG, +/− charge ratio 2.5) were efficiently internalized by the cells, even when further increasing the SAINT-PEG content. Compared to non-PEGylated complexes, the net uptake of lipoplexes stabilized with SAINT-PEG, up to 20 mol%, was only reduced by 20–25%. However, a steric stabilization of SAINT-PEG, in terms of diminished internalization of lipoplexes, did become apparent when the charge ratio was reduced. Fig. 7 presents the data for DSPE-PEG and $SAINT_{16:0}$-PEG as constituents of SAINT-2/DOPE/DNA complexes in which the charge ratio varied from 2.5 to 1.5 (+/−). At a charge ratio of 2.5, the uptake of PEG-stabilized complexes was reduced only slightly (~10%) compared to control, i.e., PEG-devoid lipoplexes. By contrast, when the net positive charge ratio of the lipoplexes was decreased to 1.5, their uptake was reduced by almost 80% and 60% for DSPE- and SAINT-PEG-containing lipoplexes, respectively. These conditions were then exploited to investigate whether internalization could be restored via specific targeting.

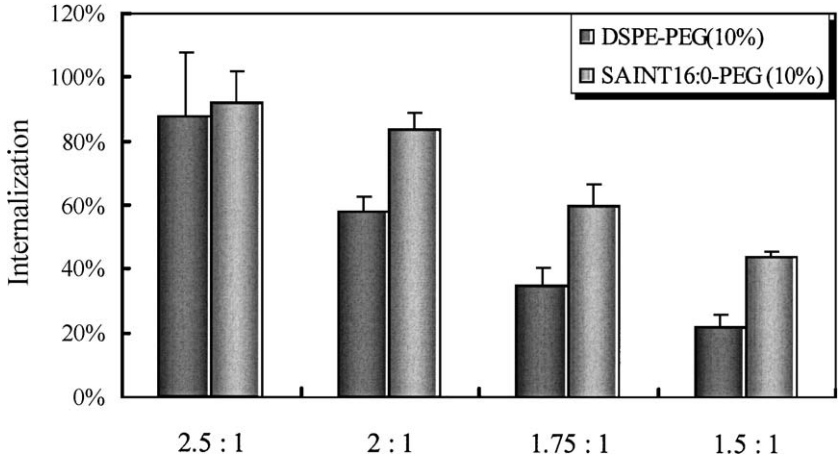

Fig. 7. The influence of the charge ratio (+/−) on the internalization of lipoplexes, stabilized with PEG. RhPE labeled vesicles were mixed with pDNA at four different charge ratios and their internalization was determined by FACS analysis. Lipoplexes were incubated with B16 cells for 3 h. Trypan blue (0.04%) was employed as an extracellular fluorescence-quencher. The 100% value represents the fluorescence intensity obtained for non-stabilized SAINT-2/DOPE-DNA complexes. Vesicles were composed of SAINT-2/DOPE/RhPE (49.5:49.5:1); SAINT-2/DOPE/PEG-lipid/RhPE (45:45:10:1). Note that the internalization of stabilized cationic lipids/DNA complexes depends on the net positive charge of particles.

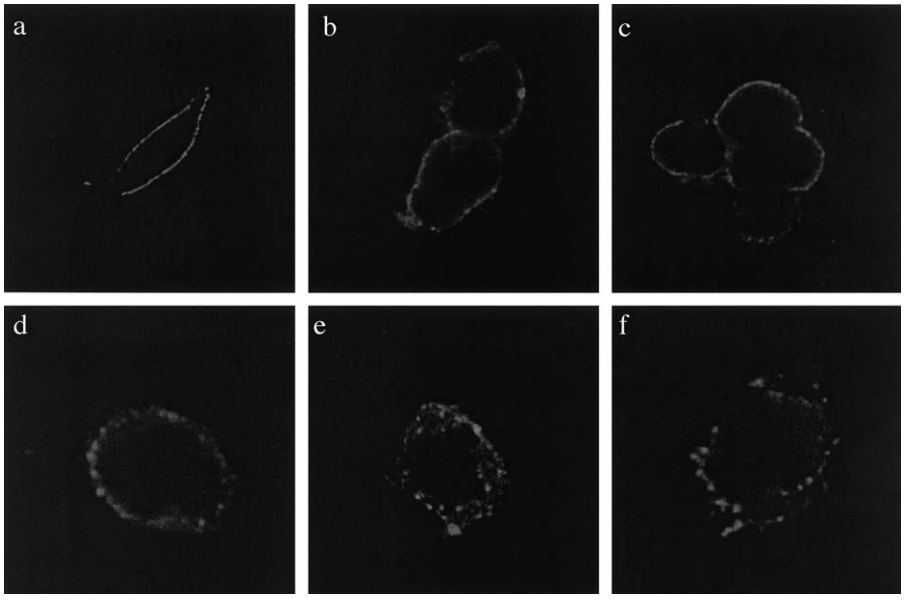

Fig. 8. Epithelial glycoprotein 2 targeted delivery mediated by SAINT-2/DOPE lipoplexes stabilized with the SAINT14:0-PEG, analyzed by confocal microscopy. The EGP-2 expressing B16 melanoma cells (B16.C215) were incubated with antibody (MOC-31)-coupled lipoplexes (SAINT-2/DOPE/$SAINT_{14:0}$-PEG-DNA) for 1 h in serum-free medium. Subsequently, the cells were intensely washed with HBSS and further incubated at 37 °C for 0.5 (a), 1 (b), 2 (c), 4 (d) or 12 h (e). To distinguish between cell-bound and internalized lipoplexes, cells presented in panel f were treated with a quencher (trypan blue). Lipoplexes: SAINT-2/DOPE ratio 1:1; $SAINT_{14:0}$-PEG 10 mol%; +/− ratio 1.5:1. The lipoplexes remained at the plasma membrane for up to 4 h (a–d), without being internalized. Images acquired after 12 h (e) of incubation revealed intracellular localization of lipoplexes, as verified by quenching of the extracellular fluorescence (f).

3.6. Targeted delivery mediated by SAINT-PEG-stabilized lipoplexes

To investigate potential targeting of SAINT-PEG stabilized complexes in an in vitro assay, we utilized a cell line expressing the human epithelial glycoprotein 2 (EGP-2), one of the best characterized tumor-associated antigens present in lung carcinoma. Anti-EGP-2 antibody (MOC31) was coupled to MPB-PE-containing lipoplexes by a sulfhydryl-maleimide coupling technique as described in Section 2. $SAINT_{14:0}$-PEG was used as a stabilizer. Cationic lipids and DNA were mixed at a charge ratio of 1.5. The cells were first incubated with antibody-coupled lipoplexes for 1 h. Subsequently, they were extensively washed and further cultured for different periods of time (0.5, 1, 2, 4 or 12 h). The fate of the RhPE labelled lipoplexes was monitored as a function of time by confocal microscopy and quantified by FACS analysis. As shown in Fig. 8, the lipoplexes remained at the plasma membrane for up to 4 h, without being internalized. The initially even distribution of the dye-marked complexes gradually transformed into a clustered appearance of complexes. This suggested that the lipoplexes, after initial random attachment, subsequently migrated and clustered into specific regions on the plasma membrane. Microscopic images acquired after 12 h of incubation revealed intracellular localization of lipoplexes, which was confirmed by applying an extracellular fluorescence quencher, trypan blue (Fig. 8f). As quantified by FACS analysis, extending the incubation time up to 12 h resulted in substantial internalization, which was further increased after 24 h (Table 1).

Table 1
Epithelial glycoprotein 2 targeted delivery mediated by SAINT-2/DOPE lipoplexes stabilized with the SAINT14:0-PEG

	24 h		12 h		4 h		1 h	
	Ab^+	Ab^-	Ab^+	Ab^-	Ab^+	Ab^-	Ab^+	Ab^-
Internalization (fluorescence intensity)	185 ± 10	11 ± 5	93 ± 5	10 ± 2	9 ± 5	4 ± 5	5 ± 5	5 ± 2
Transfection efficiency (GFP positive cells; %)	47 ± 10	0						

The EGP-2 expressing B16 melanoma cells (B16.C215) were incubated for 1 h in serum-free medium with antibody (MOC31)-coupled lipoplexes (SAINT-2/DOPE/$SAINT_{14:0}$-PEG-DNA). Subsequently, the cells were intensely washed with HBSS and further incubated at 37 °C for 1, 4, 12 or 24 h. To distinguish between cell-bound and internalized lipoplexes, cells were treated with a quencher (trypan blue) and analyzed by FACS. SAINT-2/DOPE ratio 1:1; $SAINT_{14:0}$-PEG—10 mol%, +/− ratio 1.5:1. Ab^+-antibody-coupled lipoplexes; Ab^--lipoplexes without coupled antibody. Transfection efficiency was determined for the cells incubated with lipoplexes for 24 h.
For each experiment mean values were obtained from two independent experiments, performed in triplicate.

Moreover, the lack of uptake of non-targeted but SAINT-PEG-stabilized lipoplexes, regardless of the time of incubation, indicates that the internalization was antibody-dependent. Additionally, when prior to the incubation with the targeted lipoplexes, the cells were incubated with free antibody, lipoplex uptake was strongly reduced. Furthermore, the uptake of antibody-coupled and antibody-free lipoplexes by the B16 cell line, which does not express EGP-2, was negligible.

Antibody-coupled lipoplexes were competent to transfect cells, with an efficiency of around 50% (Table 1).

4. Discussion

In this study a novel set of PEGylated lipid analogues has been synthesized. The PEG-SAINT derivatives display alkyl-chain length and saturation-dependent exchange properties. This property provides the possibility for transient stabilization of lipoplexes, which, moreover, can be specifically targeted to appropriate cellular receptors.

For lipoplexes to be effective in vivo, they require mutually exclusive properties. They need to be small, stable and display a prolonged circulation time for appropriate targeting. However, once internalized, they need to become destabilized in order to allow DNA delivery. PEG-ceramides may convey such properties to lipoplexes, but when employed for stabilization of DODAC/DOPE-DNA complexes, they were reported to strongly inhibit the transfection efficiency in HepG2 cells [6]. Together with the high costs of PEG-ceramides synthesis, these considerations prompted us to search for new PEG-derivatives, combining temporal stability with good retention of transfection efficiency.

Anchoring the PEG moiety to the cationic lipid SAINT-2 and its insertion into a relative homogenous environment, as provided by the SAINT-2/DOPE mixture, led to an improved protection of the plasmid (Fig. 2A). Thus, as revealed by a picogreen assay, in SAINT-2/DOPE/DNA complexes stabilized with DSPE-PEG and PEG-ceramides, DNA was (much) less protected (Fig. 2B; Audouy, S. and Hoekstra, D., unpublished observations). Thus, the structure of a properly, i.e. transiently, stabilized lipoplex is (co-) determined by the nature of the anchoring lipid.

Our experimental data show that, contrary to some other PEG conjugates (e.g. DSPE-PEG), SAINT-PEG stabilization allows DNA to be released from lipoplexes in a time-dependent manner. Increasing the hydrophobicity of the lipid anchor by lengthening the alkyl chain resulted in a decrease of the rate of DNA release (Fig. 4). As expected, introduction of a double bond had the opposite effect (Fig. 4). These dependencies were similar to those reported for spontaneous dissociation of phospholipids [22–24] and PEG–lipids [25,26] from vesicles.

The lag period between the addition of PEG acceptor liposomes and the onset of DNA release together with the observation that effective DNA release requires reduction of the PEG content to 1 mol% suggests that the PEG shield must be almost entirely removed before DNA release can occur. The PEG-devoid surface of lipoplexes will permit close contact between cationic and anionic lipids, thus allowing lipid transfer between the two systems, thereby promoting non-bilayer structure formation [27]. Indeed, the absence of PEG in the lipoplexes is likely to restore the formation of an inverted hexagonal phase (H_{II}), a feature which has been proposed to be instrumental in efficient gene delivery [5,11,28], as it destabilizes the lipoplex structure and, presumably, the endosomal membrane. Moreover, it has been reported that PEG–lipids are able to stabilize lamellar phases in mixtures with DOPE, a phospholipid preferring the inverted hexagonal phase (HII) [29,30].

Lipoplexes stabilized with DSPE-PEG or long-chain PEG-ceramides (C14 and C20) are internalized by cells to similar extents as unstabilized lipoplexes. However, such complexes display relatively low transfection efficiency because their stabilization refrains them from interacting with the endosomal membrane, thus preventing nucleic acid release and gene expression [5,6]. In the present study we observed that the number of GFP positive cells transfected with lipoplexes stabilized with SAINT-PEGs was higher than 50% for derivatives with alkyl chain lengths of C5:0, C12:0, C14:0, C16:0 and C18:1. No expression of the reporter gene occurred for the longest saturated derivative tested (18:0), presumably due its very slow release, and hence DNA, from the complex.

The transient SAINT-PEG stabilization was also revealed by the time-dependent expression of the GFP reporter gene (Fig. 5). Thus, when using the relatively short $SAINT_{12:0}$-PEG derivative, transfected cells were detected already after 24 h, transfection reaching its maximum on the second day. In case of $SAINT_{16:0}$-PEG, GFP-positive cells were detected only after 3 days. A SAINT-PEG chain-length- and, accordingly, a time-dependent destabilization of the complex, as reflected by a time-dependent (partial) separation of lipids and DNA, was also demonstrated by confocal microscopy (Fig. 6). Note that such redistribution patterns were not observed for lipoplexes containing DSPE-PEG, which confirmed earlier studies indicating that such a firm stabilization inhibits DNA release from lipoplexes, resulting in its entrapment in the endosomal compartment [5,6]. Therefore, we conclude that SAINT-PEG stabilization is transient, and allows in a time-dependent manner DNA to be released from lipoplexes and to escape from the endosomal compartment, which results eventually in efficient transfection. The correlations between alkyl chain length and unsaturation and exchange rate are very similar to those reported for the transfer of phospholipids [22,24] and ceramide-PEGylated derivatives [25,26] between vesicles.

Our data demonstrated that the transfection efficiency of lipoplexes stabilized with the SAINT-PEG derivatives (C12:0, C14:0, C16:0, C18:1) was diminished by 10–20% as compared to control, that is PEG-free SAINT2/DOPE lipoplexes. This could be explained by the observation that

in the presence of anionic vesicles, DNA was never entirely released from SAINT-PEG stabilized lipoplexes. As compared to non-stabilized lipoplexes, the maximal release of DNA from PEGylated complexes was around 85% (Fig. 4).

Evidently, SAINT-PEG does not entirely 'shield' the positive charge of lipoplexes at a charge ratio of 2.5 (+/−). As a consequence, the SAINT-PEGylated lipoplexes still interact with the net negatively charged cell membranes, resulting in subsequent complex internalization. Neither cell association nor subsequent endocytosis were altered by the polymer. In fact, even an enhanced association of PEGylated lipoplexes with cells has been reported, thus arguing against the inertness of distinct PEGylated lipid analogs [4–6,31,32].

The slight decrease (~ 15%) in the internalization of stabilized SAINT/DOPE lipoplexes (+/− 2.5) could be due to the fact that the presence of a 5-nm-thick coating might weaken the electrostatic interactions between complexes and the plasma membrane. The somewhat stronger effect observed for DSPE-PEG could be attributed to the presence of the negative charge on its phosphate moiety, which neutralizes positive charge of lipoplexes. Our data demonstrated the transfection efficiency of lipoplexes, the stronger the effect of PEG, in terms of decreased internalization (Fig. 7).

Since the uptake of SAINT-PEG stabilized lipoplexes could only be reduced by decreasing the +/− charge ratio (Fig. 7), we applied these conditions to investigate specific targeting of our complexes in an in vitro model. Thus, the internalization of EGP-2-targeted lipoplexes by EGP-2-transfected cells was antibody dependent, as revealed by competition experiments, whereas non-targeted lipoplexes were not taken up by the cells. Both quantitative (FACS) and qualitative (confocal microscopy) studies revealed a clear time-dependence of the uptake of the Ab-coupled lipoplexes. This can be attributed to the slow internalization of antibody bound to the EGP-2 as reported by Woo et al. [33]. However, when this antigen was utilized for targeting adenoviruses, increased luciferase expression was detected already after 24 h [34]. Our confocal studies also demonstrated that the initially random and homogeneously distributed attachment of targeted lipoplexes was subsequently replaced by a patch-wise appearance. This might imply that they cluster into certain regions on the plasma membrane involved in their internalization, as discussed previously in the context of clathrin-mediated endocytosis, which mediates lipoplex internalization [35]. Moreover, clustering prior to internalization also matches the mode of internalization described for polyplexes [36].

In conclusion, in the current work we characterized lipoplexes stabilized with a novel PEGylated lipid, SAINT-PEG, in terms of their utility for gene delivery. Our data demonstrate that stabilization provided by SAINT-PEG is transient and permits DNA release from the complex, resulting in expression of the marker gene (GFP). The structural features of the SAINT lipid acting as an anchor for the PEG moiety constitute the main determinant of the rate of PEG conjugate release. Therefore, a rational selection may result in the construction of stabilized lipoplexes with optimal structural features simultaneously meeting the requirements of retained stability in the vascular compartment and efficient release of the protective shield once inside the target cell.

Acknowledgements

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