Lipitoids – novel cationic lipids for cellular delivery of plasmid DNA *in vitro*

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Background: Although synthetic nonviral vectors hold promise for the delivery of plasmid DNA, their gene-transfer efficiencies are far from matching those of viruses. To systematically investigate the structure–activity relationship of cationic lipids, a small library of cationic lipid–peptoid conjugates (lipitoids) was synthesized. The compounds were evaluated for their ability to form complexes with plasmid DNA and to mediate DNA transfer *in vitro*.

Results: Lipid–peptoid conjugates were conveniently prepared in high yield using solid-phase synthesis. Several lipitoids condensed plasmid DNA into 100 nm spherical particles and protected the DNA from DNase digestion. A subset of lipitoids with a repeated (aminoethyl, neutral, neutral) sidechain trimer motif conjugated with dimyristoyl phosphatidyl-ethanolamine (DMPE) mediated DNA transfer with high efficiency.

Conclusions: Automated solid-phase synthesis of cationic lipids allowed the rapid synthesis of a diverse set of transfection reagents. The most active compound DMPE–(*N*ae–*N*mpe–*N*mpe)$_3$ (*N*ae, *N*-aminoethyl glycine; *N*mpe, *N*-p-methoxyphenethyl-glycine) is more efficient than lipofectin or DMRIE-C (two commercial cationic lipid transfection reagents) and is active in the presence and absence of serum. The activity in the presence of serum suggests potential for applications *in vivo*.

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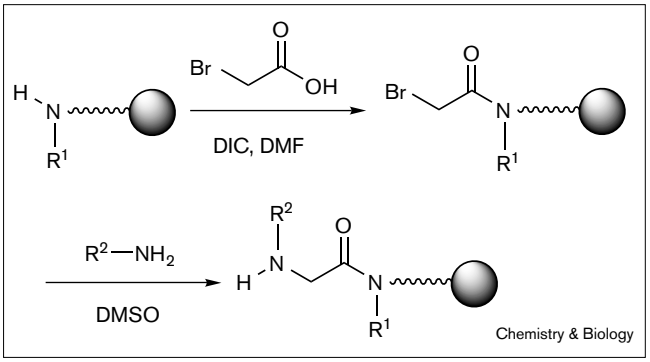
Introduction

Gene therapy has been receiving much attention due to its promise to prevent and treat many acquired and inherited diseases [1–4]. Viruses are the most effective DNA delivery vehicle for gene therapy, but they suffer from a number of undesirable properties for therapeutic applications, such as uncertainties about safety, immunogenicity, limited packaging capacity for genetic material and manufacturing difficulties. Nonviral DNA delivery vehicles have, therefore, also been under development in an effort to overcome these barriers [5–8]. There are mainly two classes of compounds used for the nonviral delivery of DNA: firstly, cationic polymers such as polylysines [9,10], cationic peptides [11,12], proteins [13,14], glucaramide-based polymers [15], polyetheleyneimines [16,17], or cationic dendrimers [18,19], and secondly, monocationic or polycationic lipids [20–23] such as lipofectin, lipofect-amine, lipofectace, and DMRIE-C. Both of these classes of compounds spontaneously form complexes with DNA (commonly called polyplex and lipoplex) through electrostatic interactions, condensing the DNA into a compact form, and protecting it from enzymatic degradation. So far, however, the existing nonviral DNA-delivery vehicles are far from matching the efficiency of viruses for DNA transfer, and often exhibit high cellular toxicity. Because a variety of cationic structures are efficient at transfecting cells, we decided to systematically investigate a diverse family of cationic lipids to find molecules with high DNA- transfer efficiency and low cytotoxicity. In order to rapidly synthesize a wide variety of cationic lipids, we used the solid-phase peptoid-synthesis methods that were developed for combinatorial drug discovery [24–26].

In this report, the term peptoid refers to poly *N*-substituted glycines. Because peptoids contain a secondary amide linkage rather than the primary amide bond found in peptides and proteins, peptoids are resistant to proteolytic enzymes [27], and can be employed for applications *in vivo* where peptides fail as a result of enzymatic degradation. Peptoids are prepared by solid-phase synthesis using a submonomer method that utilizes inexpensive starting materials (primary amines and bromoacetic acid) as building blocks, rather than costly monomers such as protected amino acids for peptide synthesis. In addition, a much wider choice of functional groups can be readily introduced as sidechains, generating peptoid oligomers with diverse physical, chemical and biological properties.

Figure 1



Scheme for synthesis of peptoids (poly *N*-substituted glycines) on the solid phase using the submonomer method. Each peptoid monomer is constructed on polymer beads by coupling bromoacetic acid to a secondary amine on the resin, followed by S_N2 displacement of the bromide by a desired amine. DIC, diisopropylcarbodiimide; DMF, dimthylformamide; DMSO, dimethylsulfoxide.

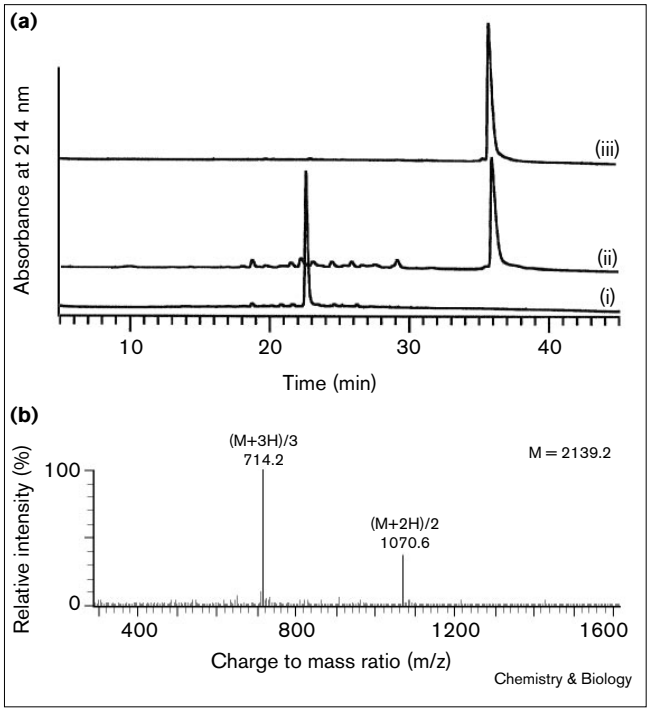
Automated synthesis can be used to generate libraries with little difficulty. To obtain new cationic-lipid compounds, phospholipids have been incorporated as sidechains into the peptoid structure using the submonomer synthetic method on solid phase, affording a new class of lipid compounds called lipopeptoids or lipitoids. These lipitoids have been evaluated for their ability to mediate DNA transfer to cells.

Results

Synthesis of lipitoids

Peptoids are readily synthesized on the solid phase using the submonomer method [24–26]. Each *N*-substituted glycine monomer is constructed on polymer beads by coupling bromoacetic acid to secondary amine on the resin, followed by the S_N2 displacement of the bromide by a desired amine (Figure 1). These two steps are repeated using a fully automated robotic synthesizer until the desired sequence is completed. Lipitoids were synthesized by first elaborating the peptoid oligomer and coupling the lipid via its amino group as the last (amino-terminal) submonomer. Phosphatidylethanolamine in 15% (v/v) methanol in chlorobenzene was treated with 0.95 molar equivalents of KOH to liberate the free amine moiety prior to lipid addition. Due to the poor solubility of the phosphatidylethanolamine molecules, this material was added at 0.2 M concentration in 15% (v/v) methanol in chlorobenzene, and, accordingly, a longer (16 h) reaction time was used. The bromoacetylation steps and the S_N2 displacement steps proceeded smoothly, generally with > 98% yield at each step. The final displacement step by the phospholipid resulted in a somewhat lower yield of approximately 85%. This is probably due to the lower concentration of the nucleophile (phospholipid) and a different solvent system. Treatment of the resin with 95% (v/v) trifluoroacetic acid (TFA) in water at 25°C for 20 min resulted in cleavage of the desired peptoid from the resin and removal of the *tert*-butoxy carbonyl (BOC) group on the *N*-(2-aminoethyl)glycines (*N*ae) and *N*-(3-aminopropyl)glycines as expected. The crude products were purified using reverse-phase preparative high-performance liquid chromatography (HPLC). Analytical reverse-phase HPLC revealed > 95% homogeneity for the purified lipitoids. In order to make stock solutions, lipitoids were dissolved in water at a concentration of 5 mM and sonicated for 1 min, resulting in a clear solution.

Figure 2

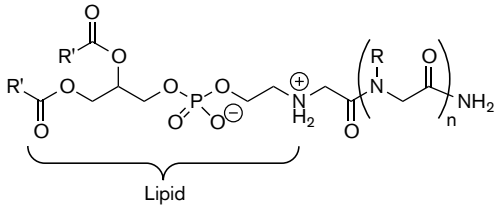


Analysis of lipitoid using reverse-phase–HPLC and electrospray ionization mass spectroscopy. **(a)** HPLC trace of (i) crude (*N*ae–*N*mpe–*N*mpe)$_3$, (ii) crude DMPE–(*N*ae–*N*mpe–*N*mpe)$_3$, and (iii) purified DMPE–(*N*ae–*N*mpe–*N*mpe)$_3$ (**14**). A linear gradient of 5–95% (v/v) solvent B in solvent A over 40 min was used at a flow rate of 100 µl/min (solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile) and a column temperature of 60°C. **(b)** Electrospray ionization mass spectrum of lipitoid **14**. The analysis was performed at a cone voltage of 30 V.

Figure 2a shows the HPLC traces of crude (*N*ae–*N*mpe–*N*mpe)$_3$, crude DMPE–(*N*ae–*N*mpe–*N*mpe)$_3$, and purified DMPE–(*N*ae–*N*mpe–*N*mpe)$_3$ (**14**; *N*mpe, *N*-*p*-methoxyphenethyl-glycine; DMPE, dimirystoyl phosphotidylethanolamine). The electrospray ionization mass spectrum displays the multiply charged species derived from the lipitoid **14**, which were used to determine the molecular weight of **14** (Figure 2b). The observed molecular weights were in good agreement with the calculated values for all the compounds that we prepared (Table 1).

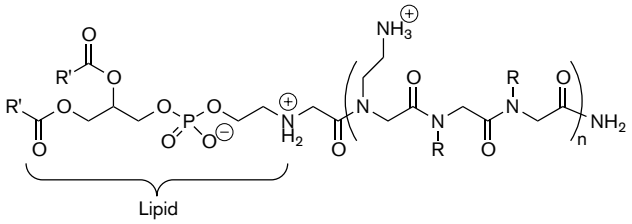
Table 1

(a) Synthetic cationic lipitoids for gene transfer: repeating monomer motif.



	Lipid*	R	n†	Designator‡	Molecular weight Calculated	Molecular weight found§
1	DMPE	2-Aminoethyl	2	DMPE-(*N*ae)$_2$	893.2	ND
2	DMPE	2-Aminoethyl	4	DMPE-(*N*ae)$_4$	1093.4	1092.9
3	DMPE	2-Aminoethyl	10	DMPE-(*N*ae)$_{10}$	1694.1	1693.4
4	DMPE	2-Aminoethyl	4	DPPE-(*N*ae)$_4$	1149.5	1148.9
5	DPPE	2-Aminoethyl	10	DPPE-(*N*ae)$_{10}$	1750.2	1749.4
6	DPPE	3-Aminopropyl	4	DMPE-(*N*ap)$_4$	1149.2	1148.8
7	DMPE	3-Aminopropyl	10	DMPE-(*N*ap)$_{10}$	1834.5	1833.7
8	DPPE	3-Aminopropyl	2	DPPE-(*N*ap)$_2$	977.4	ND
9	DPPE	3-Aminopropyl	4	DPPE-(*N*ap)$_4$	1205.6	1205.9
10	DPPE	3-Aminopropyl	10	DPPE-(*N*ap)$_{10}$	1890.6	1889.8

(b) Synthetic cationic lipitoids for gene transfer: repeating trimer motif.



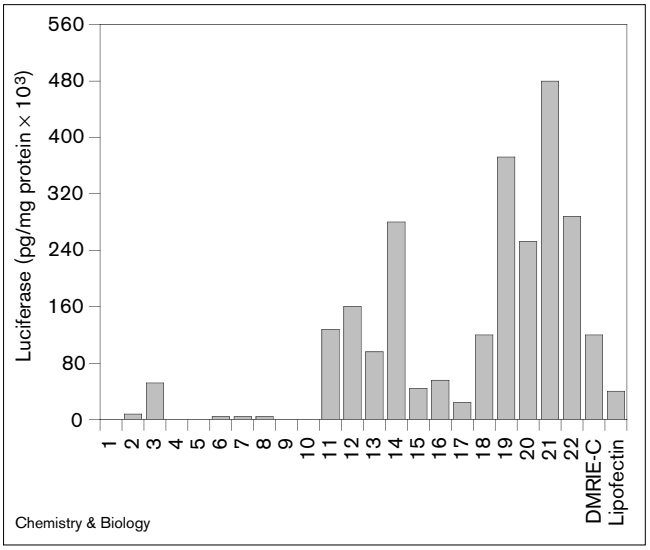
	Lipid*	R	n†	Designator‡	Molecular weight Calculated	Molecular weight found§
11	DMPE	2-Phenethyl	3	DMPE-(*N*ae*N*pe*N*pe)$_3$	1960.5	1959 .5
12	DMPE	(*S*)-1-phenylethyl	3	DMPE-(*N*ae*N*spe*N*spe)$_3$	1960.5	1959.6
13	DMPE	*p*-MeO-phenethyl	2	DMPE-(*N*ae*N*mpe*N*mpe)$_2$	1658.1	1658.0
14	DMPE	*p*-MeO-phenethyl	3	DMPE-(*N*ae*N*mpe*N*mpe)$_3$	2140.7	2139.7
15	DMPE	*p*-MeO-phenethyl	4	DMPE-(*N*ae*N*mpe*N*mpe)$_4$	2623.2	2622.0
16	DMPE	*p*-MeO-phenethyl	8	DMPE-(*N*ae*N*mpe*N*mpe)$_8$	4553.5	4551.3
17	DMPE	*p*-MeO-phenethyl	12	DMPE-(*N*ae*N*mpe*N*mpe)$_{12}$	6483.8	6480.8
18	DOPE	*p*-MeO-phenethyl	3	DOPE-(*N*ae*N*mpe*N*mpe)$_3$	2248.8	2248.0
19	DMPE	Pentyl	3	DMPE-(*N*ae*N*pn*N*pn)$_3$	1756.4	1755.4
20	DOPE	2-Methylbutyl	3	DOPE-(*N*ae*N*mb*N*mb)$_3$	1864.5	1864.4
21	DMPE	3-Methylbutyl	3	DMPE-(*N*ae*N*ia*N*ia)$_3$	1756.4	1755.5
22	DOPE	3-Methylbutyl	3	DOPE-(*N*ae*N*ia*N*ia)$_3$	1864.5	1864.3

*DMPE, dimyristoyl phosphatidylethanolamine; DPPE, dipalmitoyl phosphatidylethanolamine; DOPE, dioleoyl phosphatidylethanolamine. †Note that these values also represent the number of formal positive charges on each lipitoid. ‡*N*ae, *N*-(2-aminoethyl)glycine; *N*ap, *N*-(3-amino-propyl)glycine; *N*pe, *N*-(2-phenylethyl)glycine; *N*spe, *N*-[(*S*)-1-phenylethyl]glycine; *N*mpe, *N*-[2-(4'-methoxyl-phenyl)ethyl]glycine; *N*pn, *N*-pentylglycine; *N*mb, *N*-(2-methylbutyl)-glycine; *N*ia, *N*-(3-methylbutyl)glycine; §by electrospray mass spectrometry. ND, not determined.

Transfection efficiency

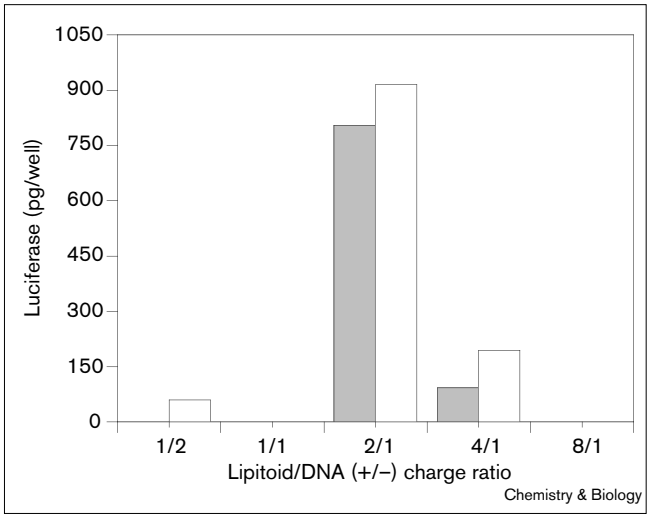
To evaluate the ability of the lipitoids to mediate DNA transfer *in vitro*, transfection experiments were performed using a luciferase reporter plasmid and the HT1080 cell line in the presence of 10% fetal calf serum in the media. DNA–lipitoid complexes were formed by mixing lipitoid with the DNA at a stoichiometry expressed as a charge ratio (+/–) between the lipitoid positive charge and the DNA negative charge. Comparison of the transfection activity of the different lipitoids was carried out at a 2/1 charge ratio. Complexes were prepared by mixing equal volumes of the DNA and lipitoid solutions, which generally affording clear solutions without any detectable precipitates. The transfection results are shown in Figure 3.

Figure 3



Comparison of DNA transfection mediated by synthetic cationic lipids and lipitoids. Transfections were mediated by lipitoids, DMRIE-C, and lipofectin in HT1080 cells in the presence of 10% fetal calf serum. A +/– charge ratio of 2/1 was used for formation of all complexes. Luciferase was quantified as described in the Materials and methods section.

Figure 4



Effect of altering the +/– charge ratio of lipitoid **14**–DNA on transfection efficiency on HT1080 cells. Shaded bars, with 10% serum in DMEM; open bars, without serum in OptiMEM. The amount of luciferase in 20 µl cell lysate was quantified as described in the Materials and methods section.

Lipitoids with only positively charged sidechains (Table 1a, **1**–**10**) did not mediate significant DNA transfer, regardless of the sidechain length (2-aminoethyl versus 3-aminopropyl) or the lipid moiety (DMPE versus dipalmitoyl phosphotidylethanolamine [DPPE]). In contrast, the lipitoids with a (2-aminoethyl, neutral, neutral) trimer motif (Table 1b, **11**–**22**) mediated DNA transfer to varying degrees in the presence of serum, and their efficiencies were generally higher than that of lipofectin. In fact, lipitoid **21** showed 10 to 20 times higher efficiency than lipofectin and threefold higher efficiency than DMRIE-C in HT1080 cells. These lipitoids also worked in the absence of serum (data not shown). Comparison of the lipitoids containing DMPE conjugated with the nine-mer amphiphilic peptoids (**11**, **12**, **14**, **19**, **21**) indicates that either aromatic or aliphatic sidechains (*o*-methoxyphenylethyl and branched or straight pentyl group) in the peptoid moiety were both effective, although the gel-mobility shift assay and the DNase I digestion assay demonstrated that the lipitoids with aromatic sidechains formed stronger complexes with DNA (see below). With respect to effect of the length of the peptoid moiety among the lipitoids DMPE–(*N*ae–*N*mpe–*N*mpe)$_n$ (n = 2, 3, 4, 8, 12 for **13**, **14**, **15**, **16** and **17**, respectively), the nine-mer (**14**, n = 3) was found to be the most active. Finally, DMPE was consistently more effective than dioleoyl phosphotidylethanolamine (DOPE) as the lipid moiety in these lipitoids.

Optimization of transfection conditions

In an effort to optimize the DNA transfection efficiency, lipitoid **14** and DNA were complexed at different +/– charge ratios ranging from 8/1 to 0.5/1, and the transfection ability evaluated in the presence and absence of serum. A +/– ratio of 2/1 was found to be the most efficient at transfecting cells. Serum did not have a significant influence on efficiency at the ratios tested (Figure 4).

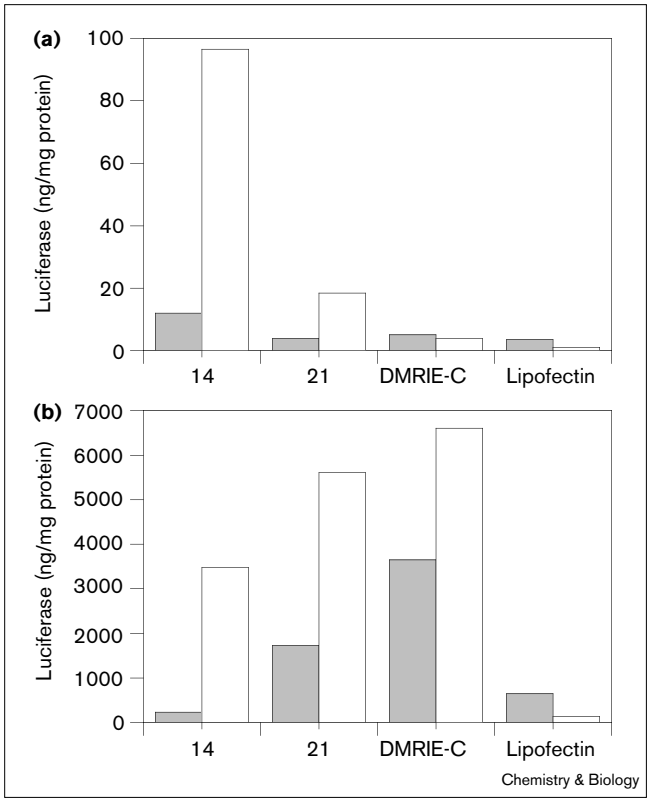
Cell dependence of transfection and toxicity

Efficiency and cell toxicity of transfection mediation compounds have been found to depend on the cell lines used. For example, we studied the transfection efficiency of lipitoids **14** and **21** on COS6M, NIH3T3 and HT1080 cell lines using the optimal lipitoid/DNA ratio of 2/1. Lipitoid **21** was very efficient (Figure 3) and also barely toxic on HT1080 cells, but not very efficient on NIH3T3 cells. In contrast, lipitoid **14** showed very high activity and was essentially nontoxic on NIH3T3 cells. This was especially true in the absence of serum when compared with lipitoid **21**, DMRIE-C, or lipofectin (Figure 5a). When COS6M cells were transfected, lipitoids **14** and **21** were comparable or slightly less effective than transfection with DMRIE-C, although both lipitoids worked much better than lipofectin (Figure 5b). When green fluorescent protein (GFP) was used to evaluate the transfection efficiency for COS6M cells, lipitoid **21** consistently resulted in a higher percentage of fluorescent cells (approximately 30%) than lipofectin, but produced approximately as many fluorescent cells as DMRIE-C (Figure 6).

Electron microscopy of lipitoid and lipitoid–DNA complexes

Because size measurement of a clear solution of lipitoid **14** by dynamic light scattering failed, we used negative-stain

Figure 5



Comparison of transfection efficiency in different cell lines. Transfection was mediated by lipitoids **14**, **21**, DMRIE-C, or lipofectin on **(a)** NIH3T3 cells and on **(b)** COS6M cells. Shaded bars, with 10% serum in DMEM; open bars, without serum in OptiMEM. A +/– charge ratio of 2/1 was used for formation of all complexes. Luciferase and total protein in cell lysate were quantified as described in the Materials and methods section, and data were expressed in ng of luciferase per mg of total protein.

electron microscopy to characterize the lipitoids. Analysis of lipitoid **14** revealed aggregates of mostly cylindrical micelles with some spherical micelles, whose diameters were 10–15 nm (Figure 7a).

When complexed with plasmid DNA, lipitoid **14** underwent a dramatic morphological change. Electron microscopy showed the formation of relatively homogeneous, spherical particles with diameters ranging from 50–150 nm for a 2/1 (+/–) ratio of **14**/DNA complex (Figure 7b).

Particle size and zeta potential

Particle size and zeta potential of complexes of plasmid DNA with different amount of lipitoid **14** were measured using dynamic light scattering and Doppler electrophoretic light scattering analysis (Figure 8). As expected, the particle size became smaller as more lipitoid **14** became associated with DNA, decreasing to the smallest diameter of approximately 120 nm at a +/– charge ratio of 2/1. When the charge ratio increased further, the particle size increased slightly. The zeta potential (a measurement of particle surface charge) of the complex increased steadily as the lipitoid/DNA ratio increased, and charge neutralization was realized at a lipitoid/DNA charge ratio between 0.5 and 1 (Figure 8). A similar trend was observed for complex formation between lipitoid **21** and DNA; the particle size reached a minimum diameter of 120 nm when the +/– charge ratio was around 2/1, and slightly increased at the higher charge ratio.

Gel-mobility-shift assay

When negative charges in the phosphate backbone of DNA are neutralized by positively charged lipitoid upon complex formation, the resulting electroneutral or positively charged complexes do not migrate towards the cathode during agarose gel electrophoresis. Gel mobility shift assays demonstrated that the plasmid DNA was completely

Figure 6

Expression of green fluorescent protein (GFP) in COS6M cells. Transfection of COS6M cells using a plasmid coding for the GFP gene mediated by **(a)** lipitoid **21**, **(b)** DMRIE-C, or **(c)** lipofectin. A +/– charge ratio of 2/1 was used for formation of all complexes.

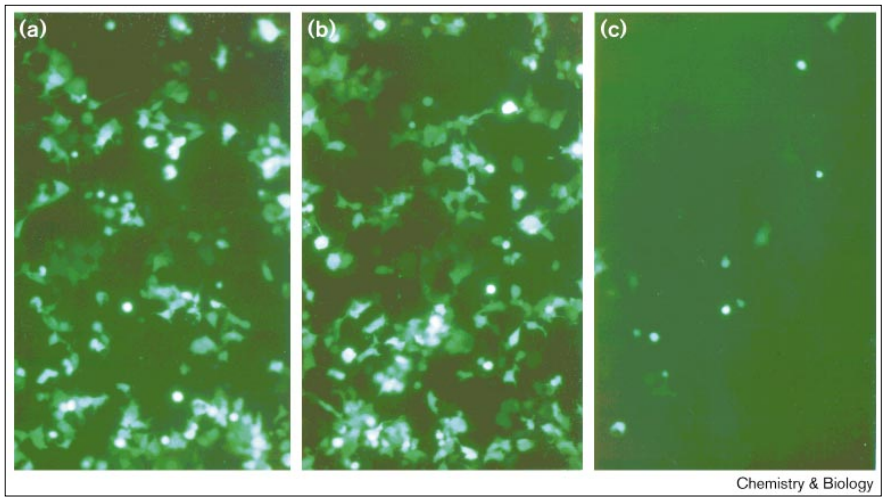
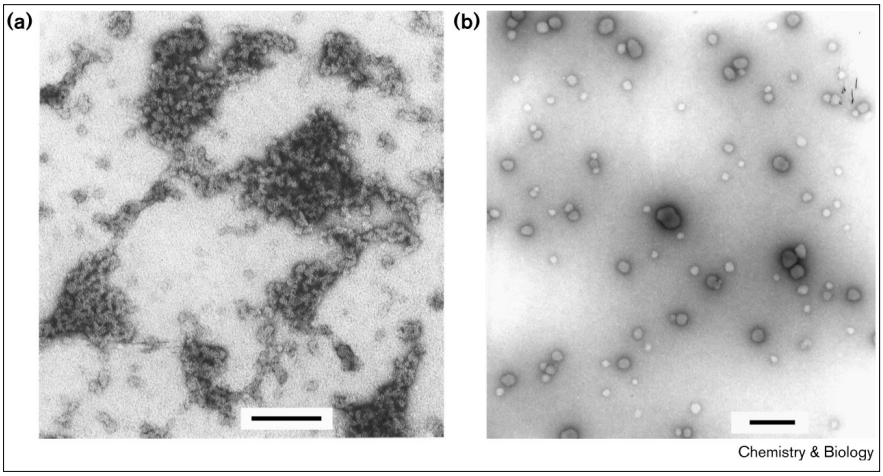


Figure 7



Electron microscopy of lipitoid **14** and lipitoid **14**–DNA complex. Transmission electron microscopy **(a)** of lipitoid **14** and **(b)** of lipitoid **14**–DNA complex at a +/– charge ratio of 2/1. Scale bars, 200 nm.

retained in the loading well when mixed with lipitoid **14** at a +/– charge ratio greater than or equal to 1/1 (Figure 9a). In contrast, lipitoid **21** did not form a strong and/or stable complex with the plasmid DNA (Figure 9a), and a +/– charge ratio of 8/1 was required to achieve complete retardation of the plasmid DNA, although electron microscopy revealed spherical lipitoid–DNA complex formation at +/– charge ratios of both 2/1 and 4/1 (data not shown).

Complex sensitivity to DNase I digestion

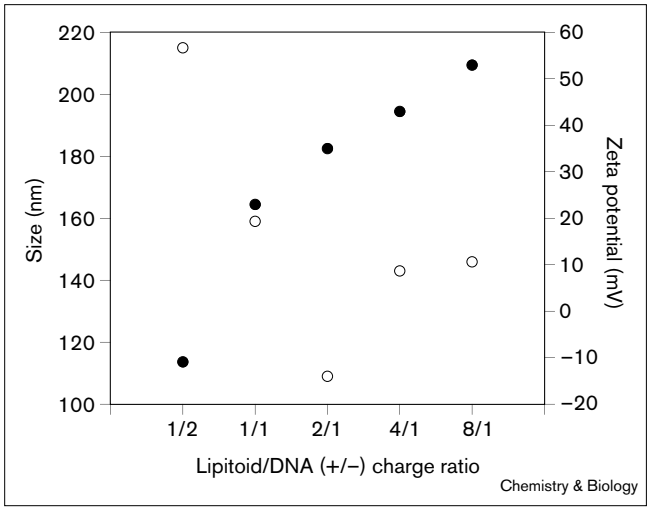
Lipitoid–DNA complexes with a +/– charge ratio of 2/1 were treated with DNase I, followed by 1% sodium dodecyl sulfate (SDS) to inactivate the nuclease and to liberate the DNA from the lipitoids. The results were analyzed using agarose gel electrophoresis. Among all the lipitoids in Table 1b, lipitoids containing the *N*ae–*N*mpe–*N*mpe motif (**13**, **14**, **15**, **16**, **17** and **18**) protected DNA to a higher extent than lipitoids containing other structural motifs regardless of the length of the lipitoid or the structure of phospholipid moiety (data not shown). In fact, when the effect of charge ratio was examined for lipitoid **14**, no significant degradation was observed even for the complex with a +/– charge ratio as low as 1/2, although the supercoiled conformation was maintained somewhat better at higher charge ratios (Figure 9b). In contrast, lipitoid **21** poorly protected DNA from DNase I digestion (Figure 9b), which is consistent with the fact that lipitoid **21** did not form a stable complex with the plasmid DNA (according to the gel-shift assay).

Discussion

Although a variety of cationic lipids can mediate DNA transfer to cells, few systematic studies have been carried out to assess the structure–activity relationship of these lipids [28–30], which is partly because the synthesis of lipids is often time consuming and labor intensive. Solid-phase synthesis of lipid analogs offers an attractive alternative, as the reaction workup is greatly simplified [31]. In addition, solid-phase chemistries and combinatorial library approaches facilitate the rapid generation of a wide array of structures. The 'peptoid approach' can access an extremely diverse family of structures that are protease resistant. A separate study in our laboratory revealed that high molecular weight peptoids with certain repeating motifs are effective DNA-delivery vehicles [32]. To gain further insight into the requirements for efficient DNA transfer by cationic lipids and to expand the scope of peptoid chemistry, we have prepared a small library of peptoid–phospholipid conjugates (lipitoids). The first series has a peptoid moiety consisting only of positively charged sidechains (Table 1a, **1**–**10**). These are analogous to polylysine–lipid conjugates and certain polycationic lipids [33–35]. The second series has neutral sidechains as well as positively charged sidechains. Specifically, we have studied a repeating trimer motif with one aminoethyl sidechain and two neutral sidechains (*N*ae–*N*xx–*N*xx) that is effective in gene transfer (Table 1b, **11**–**22**). As a lipid moiety, phosphatidylethanolamine was chosen for two reasons. First, naturally occurring lipids can ameliorate the toxic effect generally associated with unnatural synthetic cationic lipids. Secondly, phosphatidylethanolamine has a primary amino group, and thus is compatible with the submonomer method for peptoid preparation. We note that many different classes of synthetic or natural lipids can also be incorporated by direct or indirect coupling methods, however, thanks to the flexibility of peptoid chemistry.

Synthesis of the lipitoids was performed without difficulties. Because we used solid-phase synthesis, no tedious handling of lipids or work-up of the reaction was necessary, and thus this methodology greatly facilitated the synthesis of lipid molecules. In addition, the resulting products were readily purified to homogeneity using reverse-phase HPLC, because the desired lipitoid product is much more lipophilic than the impurity (presumably unconjugated

Figure 8



Particle size and zeta potential of lipitoid–DNA complexes. Effect of altering the +/− charge ratio of **14**/DNA complex on the complex particle size (open circles) and zeta potential (filled circles).

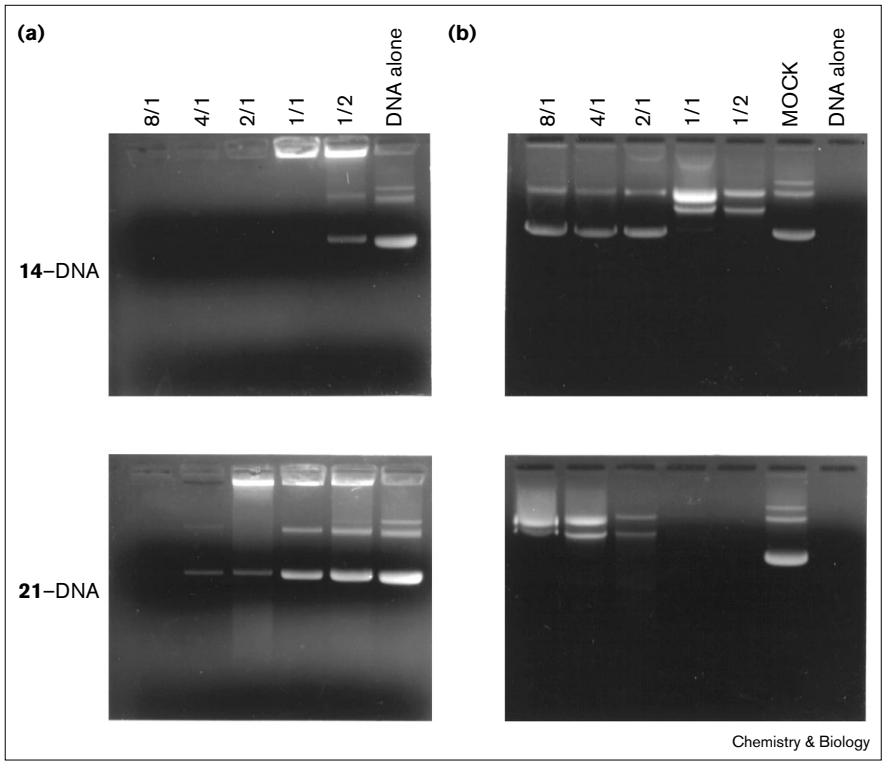
peptoid) that do not contain the lipid moiety. HPLC traces of crude (*N*ae–*N*mpe–*N*mpe)$_3$, crude DMPE–(*N*ae–*N*mpe–*N*mpe)$_3$, and purified DMPE–(*N*ae–*N*mpe–*N*mpe)$_3$ (**14**) indicate the high yield of nine-mer synthesis and the ease of isolation of the lipitoids (Figure 2a). The structures of the lipitoids were confirmed using electrospray mass spectrometry (Figure 2b, Table 1).

We have found that the transfection efficiency of the lipitoids is dependent both on the lipid moiety and on the sequence and length of the peptoid moiety. The lipid moiety is critical for the activity, as nine-mer peptoids such as (*N*ae–*N*mpe–*N*mpe)$_3$ have been shown to fail to condense DNA and to transfer it to cells. This is probably due to the lipid's ability to self-assemble through hydrophobic interactions to generate a positively charged extended surface. Furthermore, the lipid moiety could also play an active role in releasing the DNA into the cytoplasm through interactions with cell membranes, because the lipitoids with longer peptoids are less effective (compare **14** and **17**). DMPE is more effective than DOPE as a lipid moiety. This could relate to the shape of the alkyl chains, their hydrophobicity, and/or the phase-transition temperature of these lipids. Although DOPE and/or cholesterol are often added to the formulation of cationic lipids for DNA delivery, they did not enhance the transfection activity of the lipitoids (data not shown).

The cationic peptoid moiety does not only interact with DNA electrostatically — it must also actively mediate DNA transfer. The lipitoids with only positively charged sidechains did not mediate DNA transfer significantly, despite the fact that these lipitoids formed a complex with

Figure 9

Studies of DNA–lipitoid complex formation and DNA protection from DNase I by lipitoid. **(a)** Agarose gel-shift assay. The plasmid DNA (1 μg) and different amount of lipitoid **14** and **21** were mixed, followed by electrophoresis. Charge ratios (+/−) are indicated by the values above each lane. **(b)** DNase I protection assay. Lipitoid–DNA complexes were treated with DNase I and then 1% SDS, followed by electrophoresis.



the plasmid DNA and protected it from enzymatic degradation. In contrast, lipitoids with an amphiphilic trimer motif (Table 1b, **11**–**22**) did mediate DNA transfer to varying degrees, regardless of the neutral sidechains in the peptoid moiety. Consistent with this result, the high molecular weight peptoids containing an amphiphilic trimer motif were efficient DNA transfer agents whereas the peptoids containing only positively charged sidechains were not [32].

Lipitoid **14** showed the highest DNA-transfer efficiency at a +/– charge ratio of 2/1. The particle measurement experiment demonstrated that lipitoid **14** condenses DNA the most at this charge ratio. The low efficiency at the lower charge ratio does not result from incomplete charge neutralization, because the gel-shift assay, as well as the zeta potential measurement, confirmed the formation of a positively charged complex at a +/– charge ratio greater than or equal to 1/1. Also, the DNase I digestion assay demonstrated that lipitoid **14** can protect DNA effectively at a +/– charge ratio as low as 1/2. Thus, protection from serum degradation is not the only determinant of transfection efficiency. It is likely that the lipitoid also facilitates entry of DNA into the cytoplasm. Transfection efficiency and toxicity of lipitoids depend on the cell type, as is often the case with other cationic transfection agents. Lipitoids **14** and **21** showed considerably better activity than lipofectin for all three cell lines used in this study, however, and were also more efficient than DMRIE-C depending on the cell line tested.

To characterize the lipitoid–DNA complex particles, electron microscopy and light scattering studies were performed. Upon rehydration, lipitoid **14** resulted in a clear solution. In contrast to many cationic lipids, which form liposomes in aqueous media [36], lipitoid **14** formed aggregates of mostly cylindrical micelles with some spherical micelles. The diameters of these assemblies were 10–15 nm (Figure 6). Electron microscopy revealed that lipitoid **14** and **21** both formed relatively homogenous, individuated spherical particles whose diameters were around 50–150 nm, when complexed with DNA at a 2/1 (+/–) charge ratio. The homogeneity of the sample is an indication that there is little uncomplexed lipitoid present in the sample. These results are consistent with the data from the particle size measurement by dynamic light scattering. Although the fine structure of these complexes has yet to be determined, plasmid DNA is probably encapsulated inside the complex formed with lipitoid **14**, because the DNA was well protected in the complex from DNase I. These results are in contrast to the fact that formulation of DNA with common cationic transfection agents often results in a heterogeneous population of complexes with different shapes and different sizes including insoluble aggregates even under optimized conditions [37–39].

Significance

Gene therapy promises to prevent and treat many diseases. Although viruses are the most effective DNA-delivery vehicles for gene therapy, there are a number of drawbacks to their use in therapeutic applications, such as uncertainties about safety. Consequently, synthetic nonviral vectors are also being developed. In order to systematically investigate the structural requirement of cationic lipids for efficient nontoxic DNA transfer to cells, a small library of lipitoids (peptoid–phospholipid conjugates) has been generated. This is one of the first attempts to synthesize a wide variety of lipid structures on the solid phase. The submonomer synthesis methods of peptoid chemistry are well suited for this purpose. We have found that the transfection efficiency of the lipitoids is dependent both on the lipid moiety and on the sequence and length of the peptoid moiety. DMPE–(*N*ae–*N*xx–*N*xx)$_3$ (DMPE, dimirystoyl phosphotidylethanolamine; *N*ae, *N*-aminoethyl glycine; *N*xx, glycine with a neutral sidechain on the nitrogen) was the most effective structure studied. We expect that the amphiphilicity of the peptoid moiety plays an important role in transferring DNA into cells. These lipitoids mediate DNA transfection not only in the absence of the serum but also in its presence, and their transfection efficiencies are generally higher than those of lipofectin or DMRIE-C. The activity in the presence of serum offers the potential for its use in DNA-delivery applications *in vivo*. Furthermore, lipitoids are virtually nontoxic to cells under the optimized conditions. Structural studies of the lipitoid–DNA complexes revealed the formation of relatively homogeneous spherical particles, which protect the nucleic acid from DNase degradation. These particles are expected to serve as a core structure onto which additional components such as targeting ligands and/or colloidal stabilizers could be grafted in order to construct an artificial virus using a modular approach. Also, it should be possible to target these particles to specific organs by formulation with another lipid that is conjugated with appropriate targeting ligands. The design and synthesis of targeting ligands and the development of the conjugation chemistry for the lipitoids, as well as *in vivo* transfection assays are currently in progress.

Materials and methods

Materials

DMPE and DPPE were purchased from Genzyme (Cambridge, MA), DOPE from Avanti Polar Lipids Inc. (Alabaster, AK), and cholesterol from CalBiochem (San Diego, CA). Fmoc-Rink amide resin was purchased from NovaBiochem (San Diego, CA). Solvents (*N*,*N*′-dimethylformamide (DMF), dimethylsufoxide (DMSO), 1,2-dichloroethane (DCE), dicloromethane (DCM), methanol, 1,4-dioxane, acetic acid, acetonitrile) were purchased from Fisher Scientific (Pittsburgh, PA). Amines (phenethylamine, 2-(4′-methoxyphenyl)ethylamine, amylamine, isoamyl-amine, and ethylenediamine), other reagents (piperidine, *N*,*N*′-diisopropylcarbodiimide (DIC), bromoacetic acid, trifluoroacetic acid (TFA), and di-*tert*-butyl dicarbonate), and chlorobenzene were purchased from Aldrich (Milwaukee, WI). Plasmid DNA pCMV-km-LUC

was prepared as described by Murphy *et al.* [32]. Green fluorescent protein plasmid, pEGFP–N1, was purchased from Clontech (Palo Alto, CA). Tissue culture cells HT1080 (human fibrosarcoma), COS6M (African green monkey kidney), and NIH3T3 (murine fibroblasts) were obtained from ATCC. Dulbecco's modified Eagle's medium (DMEM), sodium pyruvate, L-glutamine, and Dulbecco's phosphate-buffered saline (DPBS) were purchased from JRH Bioscience (Lenexa, KS). Lysis buffer and the luciferase assay kit were purchased from Promega (Madison, WI). OptiMEM, Lipofectin (a 1:1 [w/w] mixture of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and dioleyl phosphatidylethanolamine (DOPE)) and DMRIE-C (a 1:1 molar ratio of 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide (DMRIE) and cholesterol) were purchased from Gibco/BRL (Gaithersburg, MD). Fetal calf serum (FCS) was purchased from Summit Biotechnologies (Fort Collins, CO). DNase I was purchased from Boehringer Mannheim (Indianapolis, IN). Ethylenediamine and 1,3-diaminopropane monoprotected with *tert*-butoxycarbonyl group (*t*-BOC) were prepared by the method of Krapcho and Kuell [40].

Chemical synthesis of lipitoid

In order to construct *N*-(2-aminoethyl)-glycine and *N*-(3-aminopropyl)-glycine, mono-*t*-BOC ethylenediamine and 1,3-diaminopropane were used as submonomers, respectively, whose BOC protecting groups are ultimately removed at the cleavage step by 95% (v/v) TFA in water. Peptoids were synthesized on 100 mg (50 μmol) of Rink amide resin and elaborated under normal submonomer conditions until the second to the last position using an automated robotic synthesizer [24–26]. The lipid was then introduced at the amino terminus as follows. The amino-terminal amino group was acylated at 35°C for 45 min with 0.85 ml of 1.2 M bromoacetic acid in DMF and 0.2 ml of DIC. The resin was thoroughly washed with DMF (3 × 2 ml). DMPE (254 mg) was suspended in 2 ml of 15% (v/v) methanol in chlorobenzene, and treated with 0.95 equivalent (30 μl) of 12.8 N aqueous KOH. The resulting 0.2 M solution of DMPE was added to the bromoacetylated peptoid on resin, and the reaction was gently agitated for 14 h at 35°C. The resin was thoroughly washed with 15% (v/v) methanol (6 × 2 ml) in chlorobenzene to remove the unreacted DMPE, followed by DCM wash (2 × 2 ml). The lipitoid was cleaved from the resin and deprotected using 95% (v/v) TFA in water, and lyophilized to afford the crude product. The lyphophilized crude lipitoid was dissolved in 5 ml of 50% (v/v) acetonirile in water, and the solution was applied to a Delta-Pak™ C4 (15 μm, 300Å) equilibrated with 80% (v/v) solvent B (0.1% [v/v] TFA in acetonitrile) in solvent A (0.1% [v/v] TFA in water) on a Waters Prep LC3000 system (Waters, Milford, MA). Peaks were eluted with a linear gradient of 80% (v/v) solvent B in solvent A to 100% solvent B over 20 min followed by 100% solvent B for 15 min at a flow rate of 50 ml/min. Peaks were monitored at 220 nm. The fractions containing the desired lipitoid were combined and concentrated *in vacuo*. Products were characterized using analytical reverse-phase HPLC using a Vydac C4 column (5 μm, 300 Å. 1.0 × 150 mm) on a MAGIC 2002 liquid chromatography system (Michrom BioResource, Auburn, CA) and electrospray ionization mass spectrometry (Platform II, Micromass, Bevely, MA). DPPE and DOPE were introduced using a similar method.

Transfection assay

Lipitoids were dissolved in sterile water at a concentration of 5 mM and sonicated for 1 min, resulting in clear stock solutions. In order to obtain the concentration of negative charge on plasmid DNA, average formula weight of 330 was used per nucleotide, corresponding 3.03 nmol of phosphate (or negative charge) per 1 μg DNA. Lipitoids were formally considered to be fully ionized to calculate a +/− charge ratio with DNA. The plasmid DNA stock solution and the lipitoid stock solution were diluted with OptiMEM to the desired concentration before formulation, and complex was prepared by mixing equal volumes of the DNA and lipitoid solutions. All assays were performed in duplicate and the data were averaged. HT1080 and COS6M cells were maintained in DMEM supplemented with 10% (v/v) FCS, 0.5 mM sodium pyruvate, and 2 mM L-glutamine. NIH3T3 cells were maintained in DMEM supplemented with 10% (v/v) calf serum. Two ml each of cell suspension (1×10^5 cells/ml) was plated into a six-well plate 24 h prior to the transfection, and incubated in an atmosphere of 7% CO_2 at 37°C. Two hours prior to the addition of DNA to cells, the medium was completely removed from each well and replaced with 2 ml of DMEM containing 10% (v/v) FCS for serum positive experiments. For serum-free experiments, wells were rinsed with 2 ml of OptiMEM, and another 2 ml of OptiMEM was added to each well. Lipitoid–DNA complex was prepared to give a final DNA concentration of 10 μg/ml, and 100 μl of the solution was added to each well within 30 min after the preparation. The medium was completely removed from each well after incubation at 37°C for 3 h, and 2 ml of DMEM with 10% (v/v) FCS was added. After incubation at 37°C for 48 h, the medium was discarded, and cells were rinsed with DPBS (2 × 2 ml). The cells were shaken in 300 μl of lysis buffer at 25°C for 15 to 20 min, transferred to an eppendorf tube using a cell scraper, and frozen and thawed quickly in an ethanol–dry ice bath. The cell lysate was centrifuged at 14,000 rpm for 2 min. The firefly luciferase content of the supernatant was measured using a luciferase assay kit using a automated luminometer (Dynatech, ML2250, Chantilly, VA) according to the instructions provided by the manufacturer. The total protein content of the supernatant was determined using BCA assay (Pierce BCA protein assay reagent). GFP expression was performed using pEGFP–N1 instead of pCMV–km–LUC in a similar manner without cell lysis, and evaluated using a Zeiss Axiovert 135 microscope, light source and filter by following the instructions from Clontech. The toxicity of the transfection reagent was determined by visual examination of cells and by quantitating total protein recovered from cells 48 h post-transfection.

Characterization of DNA–lipitoid complex

Negative-stained transmission electron microscopy was performed using a Zeiss 10C transmission electron microscope. The sample solution was placed on a grid precoated with carbon and Formvar, and removed by blotting to a Kimwipe after 5 min. To stain the sample, 4% aqueous uranyl acetate was placed on the grid for 2–5 s, removed by blotting and allowed to dry. Particle size of the complexes was measured by dynamic light scattering using a N4 Plus instrument (Coulter Corp., Miami, FL), and the zeta potential was determined by Doppler electrophoretic light scattering analysis using a Delsa 400SX instrument (Coulter Corp.) according to the instructions provided by the manufacturer.

Agarose gel mobility shift assay

Complexes containing 1 μg DNA (pCMV–km–LUC) with different amount of lipitoid were loaded onto a 1% agarose gel containing ethidium bromide, and electrophoresed at 70V for 1 h to examine the retardation of the plasmid DNA.

DNase I protection assay

DNA (pCMV–km–LUC, 10 μg) alone and DNA–lipitoid complexes containing the same amount of DNA with different amount of lipitoids were treated at 37°C with 10 units of DNase I in a total volume of 50 μl of 10 mM aqueous $MgCl_2$ for 15 min. A 5 μl aliquot of reaction mixture was loaded with 1% SDS onto a 1% agarose gel containing ethidium bromide, and electrophoresed at 70V for 1 h to examine the integrity of the DNA.

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