Improved DNA/Emulsion Complex Stabilized by poly(ethylene glycol) Conjugated Phospholipid

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INTRODUCTION

Recently, finding correlations between the structure of lipid-DNA complexes and their biological activity is gaining more interest (1–3). One major drawback associated with *in vivo* lipid mediated gene delivery is relatively low transfection efficiency due to poor stability of the complex upon contact with serum (4,5). Given that the transfection efficiency of lipid-DNA complexes highly depends on its structural and physico-chemical properties, a detailed description of these particles is necessary.

Strong electrostatic interactions between positively charged lipid-DNA complexes and negatively charged proteins in the blood are responsible for the rapid aggregation of lipid-DNA complexes upon contact with serum. One way to overcome the problem of serum instability and to prolong their circulation time in the blood is to protect their surface by adding PE-PEG (6). Hong *et al.* (1) reported reduced aggregation of cationic liposome/DNA complex by incorporating a small amount of PE-PEG into the formulation, but the size of their particles is still very large. Recently, Blessing *et al.* (7) obtained small and negatively charged plasmid-detergent particles, which because of the absence of targeting ligand, failed to show any biological activity. More recently, Monck *et al.* (8) reported extended circulation time of small, stabilized plasmid-lipid particles following intravenous administration.

In vitro transfection studies performed by Liu *et al.* (9) showed that the transfection activity of tween 80-containing emulsions was not affected by the presence of serum. More recently, Yi *et al.* (10) developed a physically stable and serum-resistant, cationic oil-in-water emulsion containing DNA for *in vitro* delivery. Previously, we reported that intraportal injection of cationic DNA/emulsion resulted in high level of gene expression in the liver but poor transfection efficiency following intravenous administration (11). Based on this study, we report the first physico-chemical study on the development of a new DNA/emulsion formulation stabilized by PE-PEG for *in vivo* administration.

MATERIALS AND METHODS

Materials

DOTAP, egg PC and distearoylphosphatidylethanolamine polyethylene-glycol 2000 were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Olive oil, cholesterol and cholesteryl oleate were purchased from Sigma (St Louis, MO, USA). $Na^{125}I$ was obtained from DuPont NEN (Boston, MA, USA). All other chemicals were of reagent grade. Plasmid pCMVL, which contains the cDNA of firefly luciferase driven by a human cytomegalovirus immediate-early promoter, was amplified, purified and radioiodinated with ^{125}I as previously described (5). The specific radioactivity of the labeled DNA was 5 μCi/μg.

Preparation and Purification of Emulsions

The emulsions were prepared as previously described (11). Three different emulsion formulations containing DNA/cationic lipid (DNA/DOTAP, charge (-/+) ratio = 1/1.5) complexes were studied: olive oil, egg PC, cholesteryl oleate, cholesterol, PE-PEG (68.8:23.6:5.9:1.7:0 (a), 62.6:21.5:5.4:1.5:9.0 (b), 62.0:21.3:5.3:1.4:10.0 (c), w/w). Trace amount of ^{125}I-labeled DNA was included into the emulsion for quantification. Emulsions were purified by sucrose density gradient centrifugation as previously described (5).

Particle Size and Zeta Potential Analyses of the Emulsion

Emulsion purified by sucrose gradient centrifugation was analyzed for particle size distribution by a N4 PLUS apparatus (Coulter Corporation, Miami, FL, USA). The emulsion was diluted in 3 mL distilled water to obtain an intensity ranged between 10^4 and 10^6. Each measurement lasted for 2 min and repeated 3 times with 1 min of equilibrium period in between. The results are shown as the mean intensity-weighted particle size in nm. Purified emulsion was analyzed for Zeta potential by using a Zetasizer 4 apparatus (Malvern Instruments Ltd, Worcs, England). The emulsion was diluted in 2 mL sodium phosphate buffer (10 mM, pH 7.4) and 5 measurements were taken for each sample. The results are shown as the mean potential in mV. Significance was determined by Student *t*-test.

Freeze-Fracture EM

Purified emulsion was placed between gold stubs and shock frozen by immersion in liquid nitrogen-cooled propane.

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ABBREVIATIONS: Phosphatidylethanolamine conjugated poly(ethylene glycol), PE-PEG; 1,2-dioleoyl-3-trimethylammonium-propane, DOTAP; egg phosphatidylcholine, egg PC; Dioleoylphosphatidylcholine, DOPC; Dioleoylphosphatidylethanolamine, DOPE; Electron microscopy, EM.

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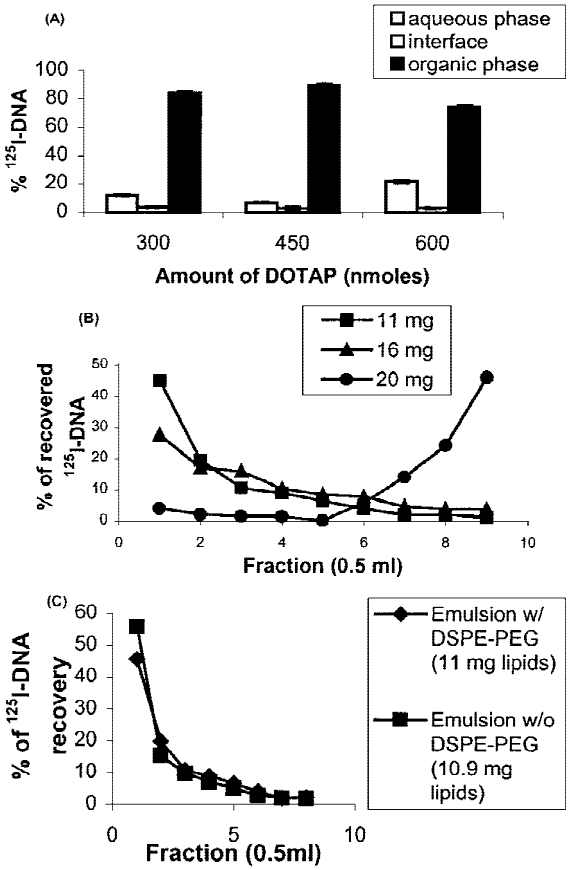

Fig. 1. (A) Effect of DOTAP on the partition of DNA into organic phase. ^{125}I-DNA (100 μg) was incubated with indicated amounts of DOTAP in the Bligh and Dyer monophase at room temperature for 30 min. The monophase was separated into two phases by the addition of $CHCl_3$ and water. Partition of DNA into the aqueous phase, the interface and the organic phase were determined by measuring the radioactivity. (B) Sucrose density gradient sedimentation profile of DNA incorporated into emulsion containing PE-PEG with increasing amount of total emulsion lipid. (C) Sucrose density gradient sedimentation profiles of DNA incorporated into emulsion with and without PE-PEG.

Frozen samples were fractured using a hinged double replica device in a Cressington CFE50 (Cranberry, PA, USA) freeze fracture unit, at −172°C and 6×10^{-6} Torr, shadowed at 45° with platinum-carbon, then at 90° with carbon. Replicas were immediately cleaned in deionized water, collected on 200 mesh copper grids and examined in a JEOL (Peabody, MA, USA) JEM 1210 transmission electron microscope operated at 80 kV.

Small-Angle X-Ray Diffraction

Purified emulsion was loaded on the top of a centricon 100 centrifugation tube (Millipore Corporation, Bedford, MA, USA). The tube was centrifuged at 2000 rpm for 20 min in a Beckman centrifuge (Beckman Coulter, Inc., Somerset, NJ, USA). After centrifugation, the concentrated emulsion was loaded inside a capillary tube and centrifuged again at 5000 rpm for 30 min. The X-ray source was the synchrotron of the LURE center (University of Paris-South). The wavelength of the radiation was 0.145 nm. Small-angle diffraction analysis was performed on all samples with a sample-film distance of 272 mm. A two-dimensional detection system with image plates was used (Molecular Dynamics Scanner). Intensity profiles I(s) were extracted ($s = 2\sin\Theta/l$ where 2Θ is the scattering angle and l is the sample-film distance) and the s value can be converted into distance d by the relationship $d = 1/s$.

In Vivo Biodistribution

^{125}I-DNA was incorporated into the emulsion. Purified emulsion was administered into mice at a dose of 50 μg DNA per mouse via the tail vein. After 30 min following injection, mice were bled from the retroorbital sinus under anesthesia and killed by cervical dislocation. Lungs, liver, heart, kidney, and spleen were collected and assayed for radioactivity and the results were expressed as the percentage of injected dose in a given organ. Significance was determined by Student *t*-test. The protocol was in accord with the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

Table I. Particle Size, Zeta Potential and *In Vivo* Biodistribution (30 min) of the Emulsion Formulations

Formulation	Naked DNA	Emulsion a	Emulsion b	Emulsion c
Percent PE-PEG		0% PE-PEG	9% PE-PEG	10% PE-PEG
Size(nm ± S.D.)	—	303.8 ± 83.8	225.2 ± 65.7[b]	231.0 ± 79.0[b]
Zeta potential (mV ± S.D.)	—	30.1 ± 0.6	14.0 ± 0.5[c]	9.2 ± 0.6[c,d]
In vivo biodistribution (% ± S.D.*)				
Blood	9.3 ± 0.8	6.7 ± 1.6	21 ± 3.5[a,c]	23.9 ± 1.3[a,c]
Liver	5.8 ± 0.8	42.1 ± 5.2[a]	22 ± 2.4[a,c]	14.1 ± 2[a,c]
Lung	0.7 ± 0.15	4.5 ± 0.6	1.1 ± 0.1	1.2 ± 0.2
Heart	0.5 ± 0.1	0.4 ± 0.2	0.6 ± 0.1	0.8 ± 0.2
Spleen	0.8 ± 0.2	3.2 ± 0.7	2.3 ± 0.5	2.6 ± 0.5
kidney	2.0 ± 0.2	1.7 ± 0.4	2.6 ± 0.4	2.4 ± 0.2

* results expressed as the percentage of injected dose in a given organ (n = 3 mice per group).
[a] $P < 0.05$; statistical significance compared to the naked DNA group.
[b] $P < 0.1$; statistical significance compared to the emulsion (a) group.
[c] $P < 0.05$; statistical significance compared to the emulsion (a) group.
[d] $P < 0.05$; statistical significance compared to the emulsion (b) group.

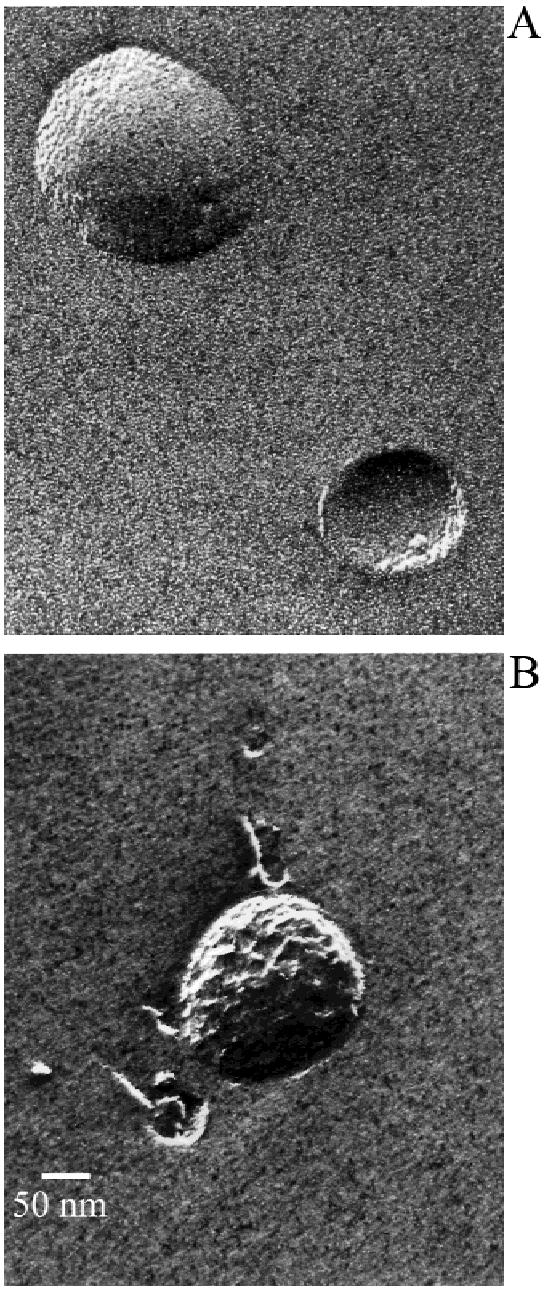

Fig. 2. Transmission electron micrographs of DNA/emulsion containing 10% PE-PEG. Panel A shows the control emulsion containing no DNA. Panel B shows DNA/emulsion depicting some fractured-lipid covered DNA inside the emulsion droplet.

RESULTS AND DISCUSSION

Physico-Chemical Characterization of the Emulsion Formulation

At a DNA/DOTAP charge ratio 1/1.5 (-/+), 80% of DNA can be extracted into the $CHCl_3$ phase (Fig. 1A), indicating that DNA has formed a hydrophobic complex with cationic lipid. Such complex can be readily incorporated into an oil-in-water emulsion. After centrifugation, the profiles of DNA/emulsions with and without PE-PEG were similar with about 70% of the ^{125}I-DNA associated with the emulsion at the top of the sucrose density gradient (Fig. 1C). Furthermore, incorporation of DNA/DOTAP complexes was dependent on the total amount of lipid in the emulsion, with maximal DNA incorporation achieved by using 11 mg of total lipid. Higher amounts of lipid resulted in lower levels of DNA incorporation (Fig. 1B). At 20 mg lipid, no DNA was associated with the emulsion, probably because the excess PE-PEG acted as a strong surfactant and destabilized the emulsion.

The size of the emulsion containing no PE-PEG (approximately 300 nm) was larger than those containing PE-PEG (225–230 nm) (Table I). Emulsions containing 9 or 10% PE-PEG showed no difference in size (225–230 nm in diameter). However, the zeta potential of the emulsion containing 10% PE-PEG was significantly reduced compared to the emulsion containing 9% PE-PEG (14 vs 9.2 mV, $P < 0.05$). The emulsion containing no PE-PEG exhibited the highest zeta potential (about 30 mV), indicating the charge shielding activity of poly(ethylene glycol) coating the emulsion.

Emulsions containing 10% PE-PEG were further characterized by freeze fracture EM. The rough surface found inside the emulsion containing DNA corresponds to the DNA/DOTAP complex fractured during the freeze-fracture process (Fig 2). The same emulsion without DNA (Fig. 2) showed a smooth structure typical of an oil droplet as previously described by Wheeler *et al.* (12).

X-Ray Diffraction Analysis of the Emulsion Formulation

For the emulsion formulated without PE-PEG, the X-ray diffraction pattern displayed a lamellar arrangement between the DNA and the lipid (Fig. 3A). Two sharp peaks appeared at 69.7 Å and 34.8 Å corresponding, respectively, to the first and second order of a lamellar periodic structure with DNA intercalated between cationic lipid bilayers. The half width at half maximum of the first order diffraction peak was equal to 0.00131 $Å^{-1}$, which was significantly higher than the half width at half maximum of the instrumental resolution (0.00056 $Å^{-1}$). This indicates that the correlation length associated with the lamellar organization was about 940 Å, corresponding to a lamellar order containing approximately 14 bilayers. In a recent study, Koltover *et al.* (13) reported a detailed X-ray diffraction study of the lamellar arrangement between DNA and DOTAP/DOPC cationic liposome as a function of the lipid composition and lipid/DNA ratio. Their results showed that the lamellar periodicity of isoelectric cationic liposome/DNA complex increased from 50 Å for DOTAP cationic liposome without DOPC to 69 Å for DOTAP/DOPC liposome (3:7 mole:mole). From their results, we can assume that the lamellar periodicity of 69 Å we have observed is not entirely due to the DOTAP cationic lipid. Other neutral lipids including egg PC and olive oil may have been included with DOTAP in the lamellar arrangement incorporating DNA inside the emulsion.

When DNA was incorporated into the emulsion containing 10% PE-PEG, the X-ray diffraction pattern no longer exhibited structural characteristics compatible with a lamellar periodic arrangement. Instead, the scattering pattern of DNA/DOTAP complexes was arranged in an inverted hexagonal phase with DNA located inside the water filled tubes surrounded by the lipids, forming inverted micelles. The fit of the scattering profile represented in Fig. 3B to a sum of three Lorentzian functions, giving rise to three peaks, can be indexed on a 2D hexagonal lattice because their positions are in

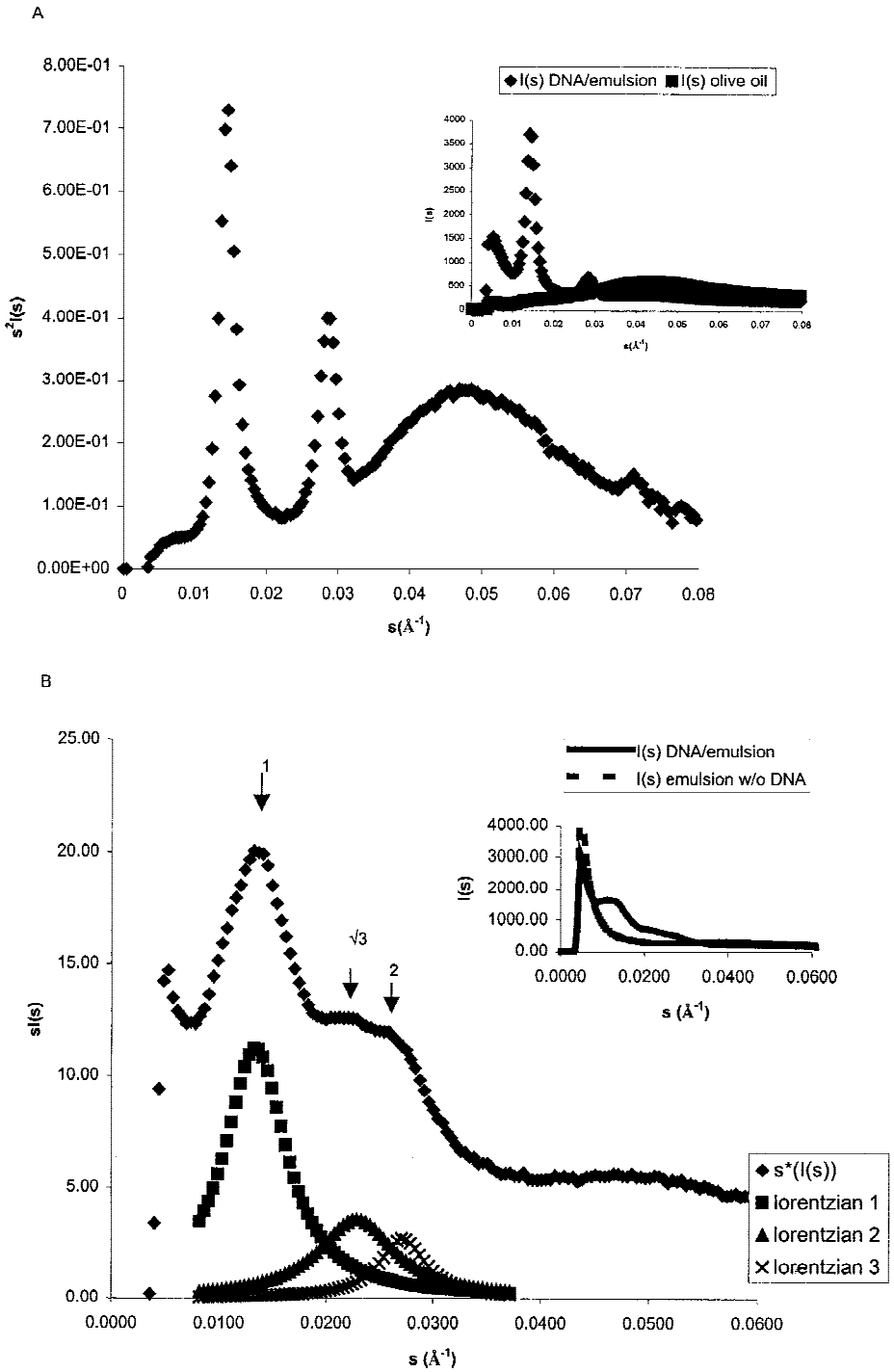

Fig. 3. (A) Small-angle X-ray diffraction study of DNA/emulsion without PE-PEG. Scattering profile $s^2I(s)$, where I(s) represents the measured scattered intensity. The s^2 normalization factor arises from the random orientation of the lipid bilayer systems. It is well established that the scattering function S(s) of one planar layer system is given by $I_{exp}(s) = S(s)/s^2$, where $I_{exp}(s)$ is the experimental intensity for an assembly of disoriented layer systems. Then, the relevant quantity is $s^2I(s)$. In the inset, scattering profiles I(s) which represents the measured scattered intensity. (B) Small-angle X-ray diffraction analysis of DNA/emulsion with 10% PE-PEG. Scattering profile sI(s): in the case of a 2D columnar hexagonal phase, the normalization factor arising from the disorientation is equal to s. In the inset, scattering profiles I(s), which represents the measured scattered intensity.

a typical ratio 1:√ 3: √4. The first Bragg peak is located at $s_1 = 0.0134$ Å^{-1} which corresponds to a unit cell spacing of $a_H = 2/\sqrt{3}/s_1 = 86$ Å. The very high value of the half width at half maximum of this peak ($\xi 1 = 0.0035$ Å^{-1}) indicates that the 2D hexagonal organization extends only in short distances, of the order of 250–300 Å. Because of the very high value of the half width at half maximum of the first peak, the data cannot exclude possible coexistence of a lamellar ar-

rangement of lipids intercalating DNA between planar bilayers. As control, the same emulsion without DNA only displayed a broad peak around 23 Å, corresponding to the intermolecular distance between two molecules of olive oil (Fig. 3A). This broad peak was not observed on the corresponding emulsion containing DNA, indicating that the sample contained no or very little DNA-free oil droplets. Koltover *et al.* (14) has shown previously that DNA intercalated between planar bilayers can be converted into inverted hexagonal complex by changing the membrane spontaneous curvature or the membrane flexibility. In our system, PE-PEG acts as a strong surfactant and reduces the rigidity of the lamellar arrangement formed by the lipid bilayers intercalating DNA.

In Vivo Biodistribution of the DNA/Emulsion Complex

Upon systemic administration, naked DNA was rapidly cleared from the circulation with only 10% of the injected dose remaining mainly in the liver (Table I). When emulsions without PE-PEG were administered systemically, they predominantly accumulated in the liver. As demonstrated by Sakaeda *et al.* (15), this can be explained by its large mean size resulting in a strong recognition of the emulsion by the mononuclear phagocytes in the liver (Kupfer cells). A second mechanism involving the adsorption of lipoproteins on the emulsion cannot be ruled out. Indeed, because of the large positive zeta potential associated with the emulsion, negatively charged lipoproteins present in the blood would easily adsorb on its surface and accelerate the clearance from the circulation (16). When 9 or 10% (w/w) PE-PEG was added to the lipidic formulation, the plasma clearance as well as the liver uptake were considerably decreased (Table I). The addition of PE-PEG helps to prolong the circulation of the emulsion in the blood by shielding the positive surface charge and by providing a more hydrophilic surface (17,18). However, these improvements are still not enough to obtain a DNA/emulsion delivery system that remains circulating for an extended period of time. In future experiments, this new phospholipid based emulsion containing internal DNA will be further developed to improve DNA retention, reduce size, and for the attachment of targeting ligands to allow specific gene delivery to target cells.

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