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Development of plasmid and oligonucleotide nanometric particles

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Nucleic acids delivery vectors have shown promising therapeutic potential in model systems. However, comparable clinical success is delayed essentially because of their poor biodistribution and of their ineffective intracellular trafficking. The size of condensed DNA particles is a key determinant for in vivo diffusion, as well as for gene delivery to the cell nucleus. Towards this goal, we have developed cationic thiol-detergents that individually compact plasmid DNA molecules into anionic particles. These particles are then 'stabilized' by air-induced dimerization of the detergent into a disulfide lipid on the template DNA. The particles all measure approximately 30 nm, which corresponds to the volume of a single molecule of plasmid DNA. The gel electrophoretic mobility of the anionic particles was found to be higher than that of the plasmid DNA itself. Similarly, particles formed with a 31-mer oligonucleotide measured 19 nm. Improved in vivo diffusion, as well as improved intracellular trafficking may be inferred from the faster migration of the complexes. Moreover, the size of the particles remains compatible with nuclear pore crossing. Finally, in an attempt to improve the biodistribution of these particles, we have coated the monomolecular particles with a poly(ethylene glycol) corona.
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Keywords: *gene delivery; cationic detergent; oligonucleotide; DNA condensation; poly(ethylene glycol)*

Introduction

Gene therapy relies on DNA carriers. Size and stability of DNA particles have been considered to be critical parameters for *in vivo* gene delivery because of known physiological constraints, such as extravasation through fenestrated endothelium and/or diffusion within tissues and the cell cytosol.[1,2] Synthetic vectors, such as cationic polymers and lipid mesophases, interact with DNA molecules through a highly cooperative process, leading to condensation of the anionic polyelectrolyte. The process is quasi-irreversible and leads to microprecipitates containing hundreds of DNA molecules per particle.[3] Large cationic multimolecular aggregates are efficient for delivery of DNA to cells in culture, but their *in vivo* diffusion is severely restricted due to their size.

Unlike the polycationic species mentioned above, cationic detergents interact with DNA reversibly. Equilibration ensures monodispersity and entropy tends to direct the system towards the largest number of condensed DNA particles. As a consequence, each particle should ultimately be made of a single nucleic acid molecule. This tendency has been observed experimentally for cationic detergents.[4,5] Detergents, however, have a much higher water solubility than lipids. Upon addition to cells, their fast exchange with the medium or the cell membrane leads to DNA unfolding, hence their poor transfection properties.[6,7]

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We have succeeded in combining the advantages of cationic detergents with those of lipids, through a two-step process. First, DNA molecules were individually condensed with a cationic cysteine-based detergent. The resulting particles were then stabilized by oxidative dimerization of the detergent into a gemini lipid on the template DNA.[8,9] Clean monomolecular DNA collapse occurred for detergent concentrations below the critical micelle concentration (cmc). Recently, we synthesized and investigated the properties of a series of novel dimerizable cationic detergents with various combinations of aliphatic chains and headgroups. The tetradecane-based detergent exhibited both monomolecular DNA condensation and efficient cell transfection properties.[10] Here, we present the most prominent features of these nanoparticles together with ongoing efforts to increase their bioavailability.

Results and discussion

Monomolecular plasmid DNA condensation

As schematically depicted in Figure 1, anionic DNA molecules were individually condensed with the cationic thiol-detergent C_{14}COrn. The resulting particles were then stabilized by oxidation of the thiol-detergent into a disulfide lipid on the template DNA. Figure 2 presents the electrophoresis migration pattern of plasmid DNA observed with increasing initial concentrations of detergent. It is well known that complexes formed with cationic lipids are too large to migrate into the gel, so they remain trapped in the well. Surprisingly, we found DNA particles to be mobile when formed by oxidation of the

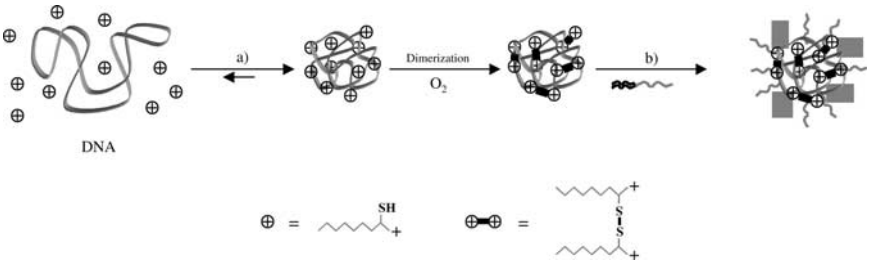

Figure 1 *(a) Cationic detergents with low binding cooperativity are in fast thermodynamic equilibrium and condense DNA into small particles. Then the particles are frozen by DNA template-assisted oxidation of thiols into disulfides. (b) Monomolecular DNA complexes are subsequently modified by inserting a lipid-PEG.*

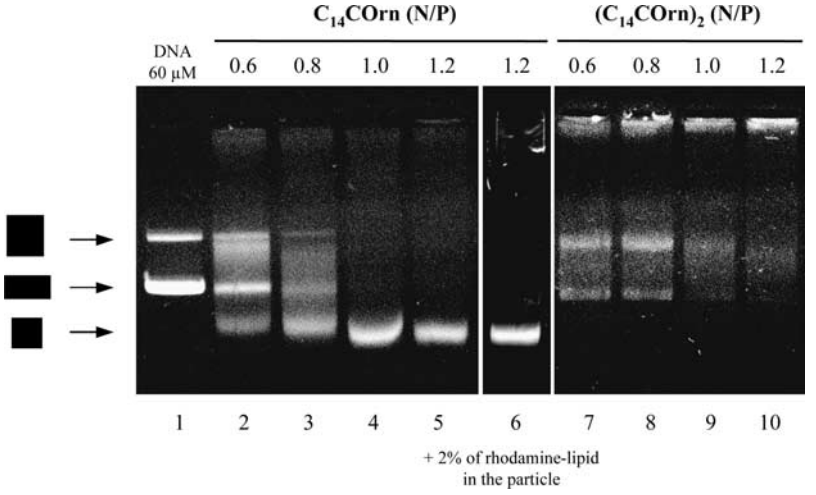

Figure 2 *Agarose gel electrophoresis of $(C_{14}COrn)_2$/DNA complexes. Free DNA (lane 1) and its complexes with $C_{14}COrn$ at various N/P ratios: 0.6, 0.8, 1.0 and 1.2 (lanes 2–5) prepared according to the procedure outlined in Figure 1. The gel was stained with ethidium bromide to visualize DNA. The lipidic component of the particles (N/P 1.2) was revealed by addition of 2% of rhodamine-labeled lipid to the initial mixture (lane 6). Rhodamine-labeled lipid was examined under fluorescence illumination (λ Ex/Em: 538/580 nm). When DNA was directly mixed with the oxidized disulfide lipid $(C_{14}COrn)_2$, the precipitated complexes remain in the well (lanes 7–10). Twenty microliter samples containing 0.4 μg pCMV-Luc plasmid DNA in Hepes buffer (15 mM pH 7.4) and concentrations of $C_{14}COrn$ or $(C_{14}COrn)_2$ detergent as indicated were loaded on to the gel after 3 h incubation. The 0.8% agarose gel was run for 90 min at 8 V/cm in Tris-acetate buffer (40 mM, pH 8).*

detergent in the presence of the template DNA. At low charge ratios (N/P <1), coexistence of free DNA with fully formed particles shows that dimerizable cationic detergent-mediated DNA condensation is an all-or-nothing process, ie the folding transition is discrete. For a stoichiometric charge ratio (N/P = 1.0), complete condensation occurs and particles move in the gel faster than free plasmid DNA, albeit most DNA phosphate residues are neutralized by the cationic amphiphile. In order to fully characterize the nature of this band, its lipidic component was revealed by addition of a 2% rhodamine-labeled lipid to the initial mixture. The position of lipid tracer was examined under fluorescence illumination. As shown in Figure 2 (lane 6), the fluorescence of rhodamine colocalizes with that of ethidium bromide-intercalated DNA. On the contrary, the cationic lipid $(C_{14}COrn)_2$ obtained by oxidation of the detergent in the absence of DNA forms large particles with DNA that remain in the loading well (Figure 2, lanes 7–10). Stokes diffusion of $(detergent)_2$/DNA particles through the agarose gel thus appears to be easier than the De Gennes crawling of the extended DNA polymer.

The DNA complexes formed at equimolar amine to phosphate ratio remained monomolecular within a 20–400 μg/ml DNA concentration range and over a period of 2 weeks in 15 mM Hepes buffer. More interestingly, no aggregation was noticed following 4-h incubation in DMEM cell-culture medium. The size of the $(C_{14}COrn)_2$/DNA nanoparticles was determined by dynamic light scattering. Particles prepared with a 5.5 kb plasmid DNA at a N/P ratio of 1.2 were found to have a diameter of 39.5 ± 3.6 nm. In order to confirm their size and dispersal and to visualize their morphology, the particles were examined by transmission electron microscopy. A single population of particles of 35 ± 5 nm (n = 10) was found (Figure 3a). The population appeared homogeneous with respect to size, but not to shape. At a higher magnification it became obvious that $(C_{14}COrn)_2$ was forming a tubular phase with repeating distance of approximately 6 nm. The irregular particle shapes thus appeared to be a consequence of tube bending induced by close packing with DNA. This type of packing was previously observed with Transfectam, a potent transfection agent which also displays a tubular phase into which DNA can be intertwined.[11] According to typical DNA and amphiphile molecular dimensions, the calculated volume of a single condensed 5.5 kb plasmid is equivalent to a sphere of 31 nm. Taking into account that inter-

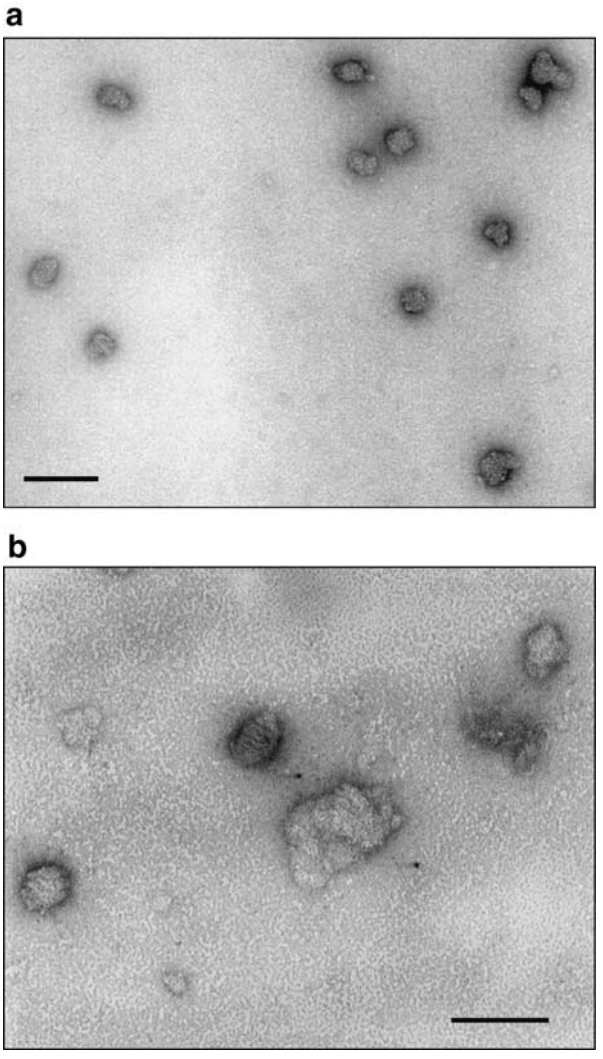

Figure 3 *Transmission electron microscopy of $(C_{14}COrn)_2$/DNA particles at N/P = 1 in Tris-HCl buffer (15 mM pH 8.4). When DNA is mixed with the detergent and then oxidized according to the procedure outlined in Figure 1, the resulting particles reveal an homogeneous population of quasi-spherical complexes with a diameter of 35 ± 5 nm (a). When DNA is directly mixed with the oxidized $(C_{14}COrn)_2$ lipid, the aggregating particles are large (50–200 nm) and heterogeneous in shape (b). Bar = 100 nm.*

digitated packing of the lipid and nucleic acid phases may be less perfect, the measured size of the particles provides strong evidence for monomolecular condensation of plasmid DNA. When plasmid DNA is directly mixed with the oxidized $(C_{14}COrn)_2$ lipid, the particles are large (50–200 nm) and polydisperse (Figure 3b). Moreover, additional precipitation occurs in physiological conditions.

Condensation of oligonucleotides

Natural oligonucleotides are very quickly degraded by nucleases present in blood and do not efficiently enter cells, highlighting the need for delivery vectors. Smaller being again better, we therefore condensed 31-mer oligodeoxynucleotides with $C_{14}COrn$, at various N/P. Electron microscopy showed small spherical particles with an average diameter of 18.9 ± 1.2 nm (n = 8), at N/P = 1 (Figure 4). Other ratios led to more heterogeneous populations, in terms of size and shape (data not shown). Due to their very small size, attempts to measure their zeta potential and size in solution by light scattering failed under our experimental conditions. We could not determine their electrophoretic mobility either, as oligonucleotides were released from the particles in the electric field. As compared with plasmid DNA (see above), electrostatic interaction of a small oligonucleotide with cationic amphiphiles is much weaker, hence their reduced stability in the electric field. However, this does not preclude their stability in physiological conditions.

Gene delivery to cells in culture

The cell-surface receptors for cationic lipid/DNA complexes are anionic heparan sulfate proteoglycans (HSPG).[11,12] The transfection efficiency of the monomolecular complexes was only slightly above that of naked DNA. Indeed, being devoid of a cationic surface charge (the measured zeta potential (approximately −45 mV) was even indicative of a strong anionic surface charge), these particles are unable to bind to cell-surface heparan sulfate polyanions. Larger cationic particles were therefore prepared with higher N/P ratio and tested *in vitro* using the luciferase reporter gene. The magnitude of gene expression varied with the structure of the detergent, the N/P ratio and the cell line. The large transfection increase observed between decane, dodecane and tetradecane gemini lipids tended to culminate for tetra- and hexadecane lipids.[10] In order to further explore the structure–activity relationship, we introduced some variations in the structure of the detergent, using another DNA-binding head group (spermine; $C_{14}CSper$) or an unstable ester bond in place of an amide ($C_{14}oCOrn$). The highest transfection activity was found with the $C_{14}oCOrn$ derivative (Table 1) and correlates with the apparition of a supramolecular organization similar to that of Transfectam. Indeed the C_{10} and C_{12} homologs, which do not form supramolecular structures do not transfect cells either. $(C_{14}CSper)_2$/DNA particles gave 10-fold lower transfection values than $(C_{14}COrn)_2$/DNA and showed signs of

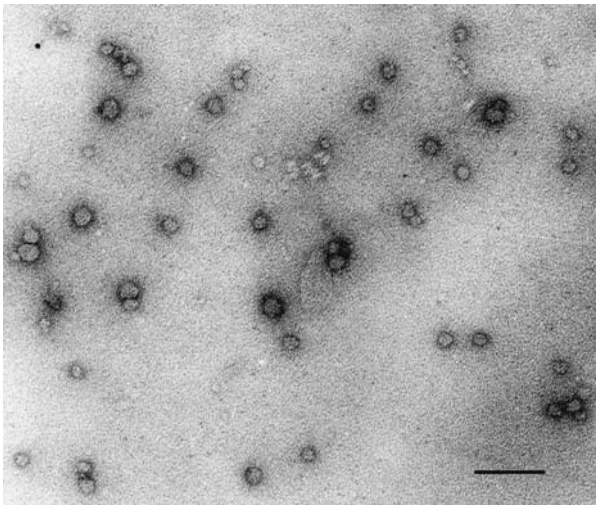

Figure 4 *Transmission electron microscopy of $(C_{14}COrn)_2$/oligonucleotides particles at N/P = 1.0 in Hepes buffer (15 mM pH 7.4) reveals a population of spherical particles with a diameter of 18.9 ± 1.2 nm. Bar = 100 nm.*

Table 1 Transfection efficiency of various $(detergent)_2$/DNA complexes on several cell lines

	Highest efficiency obtained		
Detergent	*Cell line*	*N/P*	*RLU × 10^6/mg protein*
C_{14}COrn	BNL CL2	3	529.4 ± 23
	3T3	5	146.6 ± 10
	BHK	4	3674.0 ± 669
	BHK + SVF[a]	4	4053.3 ± 745
C_{14}CSper	BNL CL2	3	69.2 ± 3
	3T3	5	55.2 ± 7
C_{14}oCOrn	BNL CL2	4	15.5 ± 6
	3T3	6	32.2 ± 9
C_{16}COrn	BNL CL2	3	82.3 ± 9

Luciferase activity measured 24 h after cells transfection with 2 μg luciferase plasmid.
[a]Transfection was performed in presence of 10% SVF.

toxicity with increasing N/P. Both effects may be related to the higher cmc of the spermine relative to the ornithine derivative.[10] $(C_{14}oCOrn)_2$/DNA particles showed only modest transfection efficiencies. Results obtained with several cell lines show that $(C_{14}COrn)_2$ presents transfection properties which compare favorably with those of potent transfection reagents such as Transfectam and JetPEI (data not shown).[13,14] Taken together, these results show that, provided the cationic $(C_{14}COrn)_2$/DNA complexes are able to bind to the cell membrane, they follow the typical fate of synthetic gene delivery vectors.

Poly(ethylene glycol)-coated monomolecular plasmid DNA particles

An attractive application of monomolecular DNA particles would be targeting of non-localized tumor metastases by way of systemic delivery. Poly(ethylene glycol) has been extensively used for its ability to enhance the stability and the life span of liposomes in the bloodstream, via the so-called 'steric-stabilization' mechanism.[15,16] For the same purpose, PEG has been grafted to polyplexes where it was shown to reduce nonspecific interaction with plasma proteins, as well as complement system activation.[17,18] This beneficial effect is due to the inertness of the polyether skeleton, as well as to brush-type polymer crowding. Towards increasing their intravenous half-life, the monomolecular particles were equipped with a poly(ethylene glycol) moiety. We therefore synthesized a lipid-PEG_{3400} molecule that would anchor via its lipidic moiety to the particle. We expected plasmid DNA and detergent to form a mixed hydrophobic and ionic core particle, while the PEG chains would form a hydrophilic corona (Figure 1). The lipid-PEG amphiphile was anchored to the surface of the preformed particles by simply adding it to the solution. Binding was examined by electrophoresis as a function of the lipid-PEG/C_{14}COrn stoichiometry (Figure 5). Agarose gel electrophoresis showed that particles with 0.5–2% lipid-PEG were stable and still able to migrate through the gel. However, their mobility decreased as the amount of lipid-PEG increased, suggesting insertion of the lipid into the particle and some diffusional hindrance brought about by the poly(ethylene glycol) calix (Figure 5). Transmission electron microscopy of $(C_{14}COrn)_2$/DNA/2% lipid-PEG showed a population of 37 ± 6 nm spherical particles (Figure 5).

In conclusion, we have succeeded in developing a two-step chemical technique for condensation of individual DNA molecules. Earlier work had shown DNA to be protected from degradation in serum.[9] Here we show that the condensed nucleic acid has also improved diffusion properties as compared with the extended form, a property of prime interest for both distribution through an organism and intracellular trafficking. Moreover, the size of particles formed with a typical 5.5 kb gene remains compatible with extravasation from the blood stream in the liver, the spleen and tumors, as well as with nuclear pore crossing once in the cell. Finally, after PEG grafting, which makes them invisible to the immune system, these particles will now be equipped with ligands for *in vivo* cell-specific gene delivery.

Materials and methods

Materials

C_{14}COrn, C_{14}CSper and C_{16}COrn were synthesized as previously described.[10] The syntheses of C_{14}oCOrn and DPPE-PEG_{3400} (lipid-PEG) will be reported elsewhere. Lissamine rhodamine B-DPPE (lipid-Rhodamine) was from Molecular Probes (Eugene, OR, USA). JetPEI was from Polyplus (Illkirch, France).

The pCMV-Luc (5.5 kb) plasmids was propagated and purified as described.[19] Three additional purification steps were carried out by ultrafiltration (Centricon-100 filter devices, Millipore, Bedford, MA, USA).

Formation of DNA/detergent complexes

DNA complexes were prepared by adding the desired amount of detergent from a 5 mM EtOH stock solution to 20 μg pCMV-Luc plasmid DNA or 10 nmoles of 31-mer oligonucleotides in 1 ml Hepes buffer (15 mM pH 7.4) under constant stirring. C_{14}COrn/DNA/lipid-Rhodamine complexes were prepared by mixing 36 nmoles of C_{14}COrn (N/P = 1.2) and 0.72 nmoles (2%) lipid-Rhodamine in EtOH before addition to 20 μg pCMV-Luc plasmid DNA (60 nmoles phosphates) in 1 ml Hepes buffer (15 mM pH 7.4). The DNA concentration was checked by measuring the absorbance at 260 nm and complexes were kept at room temperature to allow cross-linking to occur before further characterization. Complexes that were subsequently PEGylated were prepared

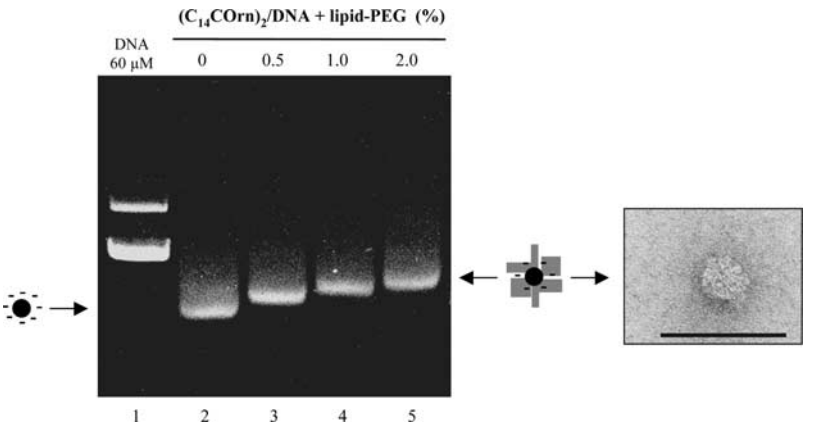

Figure 5 *Characterization of $(C_{14}COrn)_2$/DNA/lipid-PEG_{3400} complexes by gel electrophoresis. Agarose gel electrophoresis of free DNA (lane 1) and its complexes with $C_{14}COrn$ 1.2 eq (N/P) with various amounts of lipid-PEG_{3400} (%): 0, 0.5, 1, and 2 (lanes 2–5). Twenty microliter samples containing 0.4 μg pCMV-Luc plasmid DNA in Hepes buffer (15 mM pH 7.4) were loaded on to the gel after 24 h incubation. The 0.8% agarose gel was run for 90 min at 8 V/cm in Tris-acetate buffer (40 mM, pH 8). Transmission electron microscopy showed particles coated with 2% lipid-PEG to be compact and monomolecular. Bar = 100 nm.*

in advance. Twenty-four hours after mixing plasmid DNA and $C_{14}COrn$ (N/P = 1.2), the desired amounts of DPPE-PEG_{3400} (0.5 mM in EtOH) were added to the complexes.

Light scattering measurements

After overnight oxidation, the particle size and zeta potential were determined by dynamic light scattering using a Zetamaster 3000 (Malvern Instruments, Paris, France) with the following specifications: sampling time, 30 s; medium viscosity, 1.054 cP; refractive index, 1.45 (typical liposome RI); scattering angle, 90°; temperature, 25°C. Data were analyzed using the multimodal number distribution software included with the instrument. Complexes with measured diameters of less than 500 nm were found to be monodisperse, with size distributions of the order of 30% of their mean value. Those with diameters greater than 500 nm were generally polydisperse, with larger size distributions. Zeta potentials were measured with the following specifications: sampling time, 30 s; 10 measurements per sample; medium viscosity, 1.054 cP; medium dielectric constant, 80; temperature, 25°C; beam mode F(Ka) = 1.5 (Smoluchowsky).

Transmission electron microscopy

Carbon films were prepared by sublimation on freshly cleaved mica and recovered by flotation on Cu/Rh grids (300 mesh, Touzard and Matignon, Courtaboeuf, France). After drying overnight, grids were kept on blotting paper in a Petri dish. Immediately before sample addition, grids were glow-discharged (110 mV, 25 s, 25 μA). DNA particles were prepared according to the afore-mentioned procedure. A drop (5 μl) of sample was left on the grid for 1 min. Complexes were stained with 30 μl aqueous uranylacetate (2% wt/wt) for 20 s and excess liquid was removed with blotting paper. Observations were performed at 80 kV with a Philips EM 410 transmission electron microscope.

Characterization of complex formation by gel electrophoresis

Twenty microliter samples containing 0.4 μg pCMV-Luc plasmid DNA in Hepes buffer (15 mM pH 7.4) were loaded on to a 0.8% agarose gel. The gel was run for 90 min at 8 V/cm in Tris-acetate buffer (40 mM pH 8). The location of lipid-Rhodamine was assessed by fluorescence spectrophotometry (λEx = 532 nm, λEm = 580 nm) with a Typhoon 8600 apparatus (Amersham Pharmacia Biotech, Orsay, France), and the gel was stained with ethidium bromide to locate the DNA.

Transfection

3T3 murine fibroblast were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Paris, France), BNL CL2 murine hepatocytes in DMEM high glucose (4.5 g/l) and BHK-21 hamster kidney in BHK-21 medium (Gibco BRL). All cell media were supplemented with 10% heat-inactivated fetal calf serum (FCS, D Dutcher, Brumath, France), 2 mM glutamine (Gibco BRL), 100 units/ml penicillin (Gibco BRL) and 100 μl/ml streptomycin (Gibco BRL). Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. Complexes were formed by adding at one time the desired amount of detergent from the concentrated ethanolic stock solution to the plasmid solution (60 μM phosphate) in Hepes buffer (15 mM pH 7.4). The solution was kept for 24 h to ensure cross-linking was fully completed. Complexes (100 μl solutions corresponding to 2 μg plasmid per well) were added to the cells maintained in serum-free medium. Fetal calf serum was added to a final concentration of 10% v/v 3 h later. JetPEI was used as positive control.[14] Cells were lysed 24 h after transfection, and luciferase gene expression was quantified using a commercial kit (Promega, Cergy Pontoise, France) and a luminometer (Mediators PhL, Wien, Austria). Results were expressed as relative light units (RLU) integrated over 10 s per milligram cell protein lysate (RLU/mg protein) using the bicinchoninic acid assay (Pierce, Paris, France). The standard deviation was derived from triplicate transfection experiments.

Acknowledgements

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