Intracellular Delivery of Nanometric DNA Particles via the Folate Receptor

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The size of condensed DNA particles is a key determinant for both diffusion to target cells in vivo and intracellular trafficking. The smallest complexes are obtained when each DNA molecule collapses individually. This was achieved using a designed cationic thiol-detergent, tetradecyl-cysteinyl-ornithine (C_{14}COrn). The resulting particles were subsequently stabilized by air-induced dimerization of the detergent into a disulfide lipid on the DNA template. Particles are anionic (zeta potential = −45 mV), and their size (30 nm) corresponds to the volume of a single plasmid DNA molecule. The electrophoretic mobility of the condensed DNA, though quasi-neutralized, was found higher than that of the extended DNA. Moreover, the dimerized $(C_{14}COrn)_2$ lipid was found to be an efficient transfection reagent for various cell lines. In an attempt to achieve extended circulation times and to target tumors by systemic delivery, we have coated the particles with PEG−folate residues. Plasmid DNA was condensed into monomolecular particles as described above and coated by simple mixing with DPPE−PEG−folate. Physicochemical measurements showed particles coated with 2% of DPPE−PEG_{3400}−folate remain monomolecular and are stable in the cell-culture medium. Caveolae-mediated cell entry was demonstrated by ligand-dependence, by competition with excess folic acid as well as by confocal microscopy.

INTRODUCTION

Gene delivery with synthetic DNA vehicles remains an attractive approach to gene therapy. Nonviral systems are essentially based on gene compaction by cooperative electrostatic interaction between DNA phosphates and cationic lipids or polymers (*1*−*4*). Their efficacy depends on their ability to overcome several extra- and intracellular barriers the particles encounter between the site of delivery and the nucleus of the target cells. An ideal synthetic vector should (i) condense DNA into neutral or negatively charged complexes, thus avoiding interaction with anionic plasma and extracellular matrix proteins, (ii) form the smallest possible particles to facilitate diffusion, (iii) mediate cell-specific internalization, and (iv) provide vectorial intracellular trafficking to the nucleus.

A small particle size is considered to be a critical parameter for delivery because of known physiological barriers, such as extravasation through blood vessels (*5*), diffusion through mucus (*6*, *7*) or a tumor mass (*8*). Ligand-mediated cellular uptake may be size-dependent as well (*9*, *10*). It was for instance shown that glycolipid-containing liposomes with a size of 30−70 nm were effectively taken up by the asialoglycoprotein receptor of hepatocytes in vitro and in vivo, whereas 90 nm particles were not (*11*). The next step, intracellular trafficking, is also a size-restricted process. Cytoplasmic diffusion of microinjected fluorescent DNA was measured by fluorescence recovery after photobleaching (*12*). It was found that DNA molecules with size > 1 kbp are essentially immobile. Finally, active nuclear pore crossing of nucleoplasmin-coated gold particles was reported to be restricted to particles with size < 25 nm (*13*). These multiple observations highlight the prime importance of controlling the size of the DNA complexes.

Unfortunately, DNA condensation by cationic lipids or polymers generally leads to large polydisperse aggregates containing many DNA molecules. We therefore developed an alternative two-step technique where plasmid DNA molecules were individually condensed with a designed cationic thiol-detergent. The nanometric particles were then stabilized by air oxidation of the detergent into a disulfide lipid on the DNA template (Figure 1) (*14*, *15*). Clean monomolecular DNA collapse was shown to occur for detergent concentrations below the detergent critical micelle concentration (cmc). Recently, we investigated the properties of a series of novel dimerizable cationic detergents made of various combinations of aliphatic chains and headgroups. The tetradecyl-ornithinyl-cysteine (C_{14}COrn) derivative was found capable of both monomolecular DNA condensation and efficient 3T3 cells transfection (*16*). Here, we present additional physical and biological properties of C_{14}COrn, and we explore surface modification of monomolecular DNA particles as a means to increase both intravenous half-life and cell targeting. It is well-known that grafting of flexible water-soluble poly(ethylene glycol) (PEG) chains to the surface of liposomes increases their intravenous circulation time (*17*, *18*). This is thought to be mainly a consequence of the conformational flexibility of PEG chains. Rather than derivatizing our detergent directly with a cell-specific ligand, we attached the ligand to the distal end of a lipid-conjugated PEG molecule. In this way, surface modification of the particle is simply achieved by mixing with the lipid, and so the ligand extends flexibly away from the particle where it can randomly probe the cell surface (*19*). We chose folic acid as the targeting moiety (Figure 1),

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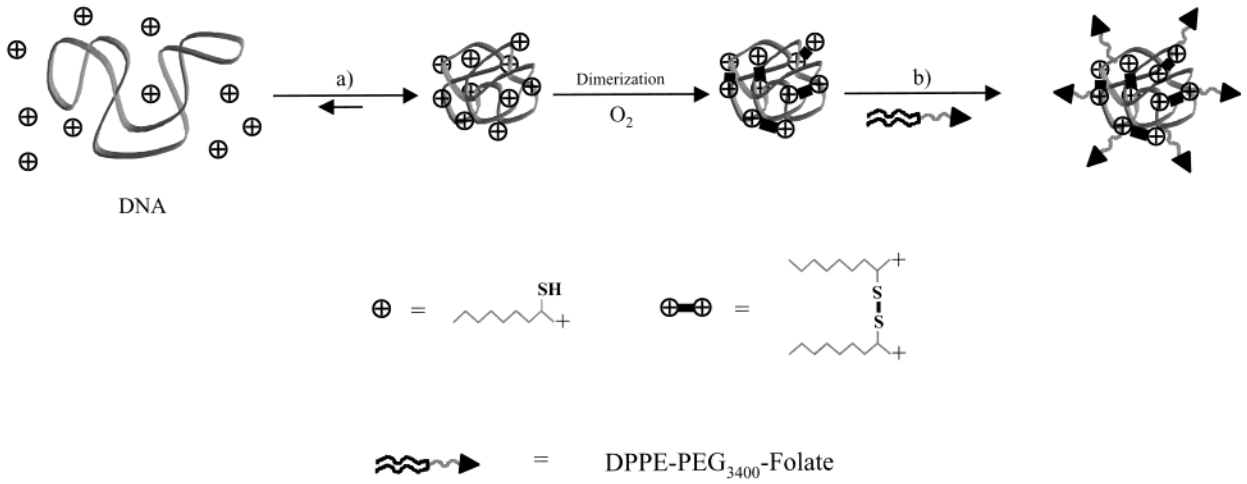

Figure 1. Formation of folate-bearing DNA particles. (a) Plasmid DNA is condensed by a thiol-containing detergent, and the resulting particles are stabilized by template-assisted oxidation of the thiol functions into disulfides. (b) Monomolecular DNA complexes are subsequently coated with PEG−folate residues by means of hydrophobic anchoring.

because certain cancer cells express large amounts of the folic acid receptor.

MATERIALS AND METHODS

Materials. *N*-Hydroxysuccinimidyl-poly(ethyleneglycol)-maleimide (NHS−PEG$_{3400}$−Mal) was purchased from Shearwater Polymers (Huntsville, USA). Dipalmitoylphosphatidylethanolamine (DPPE) and triethylamine (TEA) were from Fluka (St Quentin Fallavier, France). Acetonitrile (ACN), dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF), ethanol (EtOH), methanol (MeOH), and chloroform ($CHCl_3$) were from Carlo Erba (Val de Reuil, France). YOYO-1 and Lissamine rhodamine B-DPPE (DPPE−rhodamine) were from Molecular Probes (Eugene, USA). Transfectam was from Promega (Madison, USA), and JetPEI was from Polyplus (Illkirch, France). The pCMV−Luc plasmid (5.5 kbp) was propagated and purified as described (*20*, *21*). Three additional purification steps were carried out by ultrafiltration (Centricon-100 filter devices, Millipore). Matrix-assisted, laser desorption/ionization time-of-flight spectroscopy (MALDI-TOF-MS) was performed in the positive ion mode using α-cyano-4-hydroxycinnamic acid matrix.

SYNTHESIS

DPPE−PEG$_{3400}$−Mal. DPPE (11.4 mg, 17 μmol) was added to a solution of NHS−PEG$_{3400}$−Mal (50 mg, 14 μmol) in $CHCl_3$ (500 μL), followed by TEA (11 μL, 85 μmol). The resulting suspension was vigorously stirred and maintained at 40 °C for 1 h. The solvent was rotatory evaporated, and the residue was taken up in ACN (2 mL). After being kept for 1 h at −20 °C, the solution was centrifuged to remove traces of insoluble unreacted DPPE and evaporated. The ACN precipitation step was repeated once, and the product was dried in vacuo. Yield: 55.5 mg (96%). TLC ($CHCl_3$/MeOH 85:15) R_f: 0.55. ^{1}H NMR ($CDCl_3$) ∂ 0.88 (t, J = 6 Hz, CH_3, 6H); 1.21−1.35 (s, 48H); 1.57−1.59 (m, CH_2CH_2C=O, 4H); 2.26−2.32 (m, CH_2CH_2C=O, 4H); 3.39−3.48 (m, CH_2NH, 4H); 3.64 (s, PEG, ≈ 300H); 3.82−3.99 (overlapping m, CH_2O−P, $CHCH_2OP$, CH_2−NMal, 6H); 4.13−4.4 (dd, CH_2OC=O, 2H); 5.22 (m, CH, 1H); 6.7 (s, CH_{Mal}, 2H); mass spectrum (MALDI-TOF-MS) The spectrum exhibited a distribution of 44 Da-spaced lines centered at 4345 Da (calculated molecular mass of 4375 Da).

DPPE−PEG$_{3400}$−Folate. Cysteamine-folate (*22*) (2.6 mg, 5.5 μmol) was added to a solution of DPPE−PEG$_{3400}$−Mal (11 mg, 2.5 μmol) in DMF (100 μL), followed by TEA (3.8 μL, 27.5 μmol). The resulting suspension was stirred and maintained at 40 °C for 24 h. The solvent was rotary evaporated, and the residue was taken up in $CHCl_3$ (1 mL). Mass spectrum (MALDI-TOF-MS) The spectrum exhibited a distribution of 44 Da-spaced lines centered at 4825 Da (calculated molecular mass of 4875 Da).

Particle Formation. C_{14}COrn/DNA complexes were prepared by adding the desired amount of detergent (from a 5 mM EtOH stock solution) to 20 μg of pCMV−Luc plasmid DNA in 1 mL of Hepes buffer (15 mM, pH 7.4) under constant stirring. C_{14}COrn/DNA/DPPE−rhodamine complexes were prepared by mixing 36 nmol of C_{14}COrn (N/P = 1.2) and 0.72 nmol (2%) of DPPE−rhodamine in EtOH prior to addition to 20 μg of pCMV−Luc plasmid DNA (60 nmol of phosphates) in 1 mL of Hepes buffer (15 mM, pH 7.4). The DNA concentration was checked by measuring the absorbance at 260 nm before addition of the detergent. The complexes were kept at room temperature to allow cross-linking to occur prior to further characterization. Complexes that were subsequently PEGylated were prepared in advance. Twenty-four hours after mixing plasmid DNA and C_{14}COrn (N/P = 1.2), the desired amount of DPPE−PEG$_{3400}$−folate (from a 0.18 mM solution in EtOH) was added to the complexes. For flow cytometry experiments, plasmid DNA was labeled with YOYO (1 molecule/150 base pairs) before condensation with C_{14}COrn and PEGylation.

Monitoring Complex Formation by Gel Electrophoresis. Twenty microliter samples containing 0.4 μg of pCMV−Luc plasmid DNA in Hepes buffer (15 mM, pH 7.4) were loaded onto 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 DPPE−rhodamine was assessed by fluorescence spectroscopy (λ_{ex} = 532 nm, λ_{em} = 580 nm) with a Typhoon 8600 apparatus (Amersham Pharmacia Biotech, Orsay, France), and the gel was stained with ethidium bromide to reveal DNA.

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. 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. Samples were prepared by adding 36 nmol of C_{14}COrn (N/P = 1.2) to 20 μg of pCMV−Luc plasmid DNA (60 nmol of phosphates) in Hepes buffer (15 mM pH 7.4) and leaving the solution in aerobic condition during 24 h. Carbon films were prepared by sublimation on freshly cleaved mica and recovered by flotation on Cu/Rh grids (300 mesh, Touzard & Matignon, Courtaboeuf, France). After overnight drying, grids were kept on blotting paper in a Petri dish. Immediately before sample addition, grids were glow-discharged (110 mV, 25 s, 25 μA). A drop (5 μL) of sample was left on the grid for 1 min. Complexes were stained with 30 μL of aqueous uranyl acetate (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. Size was determined taking into account the length (L) and the width (l) of each particle (($L + l$)/2).

Cell Culture and Transfection. 3T3 murine fibroblast were grown in Dulbecco's modified Eagles medium (DMEM) (Gibco BRL, Paris, France), BNL CL2 murine hepatocytes in DMEM high glucose (4.5 g/L), HeLa human cervix epitheloid carcinoma in modified Eagles medium (MEM) with Earle's salt (PolyLabo, Strasbourg, France), CHO hamster ovary cells in Ham F-12 (Gibco BRL, Paris, France), and BHK-21 hamster kidney in BHK-21 medium (Gibco BRL). All cell culture 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 μg/mL streptomycin (Gibco BRL). Cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere. Complexes were formed by adding at once 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 to be completed. Complexes (100 μL of solution corresponding to 2 μg of plasmid per well) were added to the cells maintained in serum-free medium. FCS was added to a final concentration of 10% v/v 3 h later. Transfectam and JetPEI were used as positive controls (*23*, *24*). Cells were lysed 24 h post-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 of cell protein lysate (RLU/mg of protein) using the bicinchoninic acid assay (Pierce, Paris, France). The errors bars represent standard deviation derived from triplicate transfection experiments (± S.D., $n = 3$).

Flow Cytometry. KB cells, a human nasopharyngeal cancer cell line that expresses elevated levels of folate receptors, were cultured in MEM containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin, at 37 °C in a 5% CO_2 humidified atmosphere. As described by others (*25*), before each experiment, the cells were grown for two passages in folate-deficient modified Eagle's medium (FDMEM) containing the above-mentioned supplements. Cells were seeded in 24-well plates 24 h before incubation. Immediately prior to incubation with the fluorescent complexes (see "particle formation" paragraph), cells were washed with 1 mL of serum-free FDMEM and incubated for 4 h at 37 °C with particles in 300 μL of serum-free FDMEM (20 μg of plasmid DNA/mL). For the inhibition experiment, incubation was carried out in the presence of 1 mM of free folic acid. After incubation, cells were washed with 1 mL of serum-free FDMEM and incubated 15 min with 100 μg of calf thymus DNA in 0.5 mL of serum-free DMEM to account for any nonspecific electrostatic binding (I. Kopatz, unpublished result). After incubation, the cells were harvested by trypsinization, washed with phosphate-buffer saline (PBS) (centrifugation, 5 min, 500*g*) and suspended in PBS 1% BSA.

Flow cytometric analysis was performed with a FACStar^{Plus} (Becton Dickinson, San Jose, USA) using an argon laser (400 molecular mass power at a wavelength of 488 nm). Sorting windows were used on forward and side scatter to eliminate debris. Granulation, size, and fluorescence (λ_{em} 515 nm) intensity were recorded at a rate of 300 cells/s. Data were analyzed using CellQuest software (Becton Dickinson) and were expressed as mean fluorescence.

Confocal Microscopy. For the confocal microscopy experiments, the cells were cultured on 4-well-chambered cover glass dishes (Lab-Tek, Merck, France) at room temperature. Transfections were done as described above, and the images were taken 24 h post-transfection from living cells on a A MRC-1024ES (Bio-Rad/Elexience, Verrières-le-Buisson, France) confocal laser scanning imaging system equipped with an Eclipse TE 300 (Nikon) inverted microscope, using a 60× water immersion objective. YOYO-labeled DNA was imaged using the 488 nm excitation line of an argon/krypton laser. Confocal sections were taken every 0.8 μm. Digital image recording was performed using the LaserSharp 2.3 software (Bio-Rad).

RESULTS AND DISCUSSION

Synthesis of the Amphiphiles. C_{14}COrn was synthesized as previously described (*16*). To equip the DNA particles with a targeting ligand, we first synthesized a lipid−PEG conjugate capable of inserting within the particle and bearing a maleimide group at its distal end. DPPE−PEG_{3400}−Mal was synthesized from DPPE and the commercially available bifunctional reagent *N*-hydroxysuccinimidyl-poly(ethylene glycol)-maleimide with average molecular mass of 3400 Da. The cysteamine-folate ligand (*22*) was covalently attached to the maleimide group to give DPPE−PEG_{3400}−folate. At the end of the reaction, the desired product was solubilized in $CHCl_3$ and the unreacted cysteamine-folate was simply removed by centrifugation. Sample purity was checked by TLC, and MALDI-TOF analysis of DPPE−PEG_{3400}−folate showed a broad peak centered at $m/z = 4825$ that was consistent with formation of the conjugate (Figure 2).

Monomolecular DNA Condensation. As schematically depicted in Figure 1 path a), anionic DNA molecules were individually condensed with the cationic detergent. Detergents are water-soluble and upon addition of the complexes to cells, their fast exchange with the medium or cell membranes would lead to DNA unfolding (*26*), hence their poor transfection properties (*27*). The particles were therefore stabilized by chemically converting the thiol detergent to a gemini lipid on the template DNA (*14*). In practice, complexes were prepared by adding the desired amount of dimerizable cationic detergent to plasmid DNA, under constant stirring. For each condition, the DNA concentration was determined by measur-

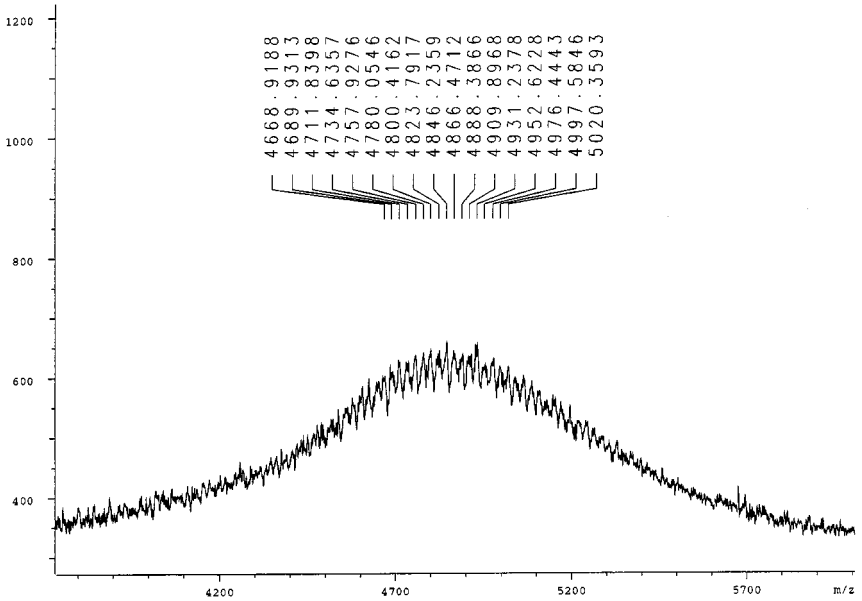

Figure 2. MALDI-TOF spectrum of DPPE−PEG_{3400}−folate.

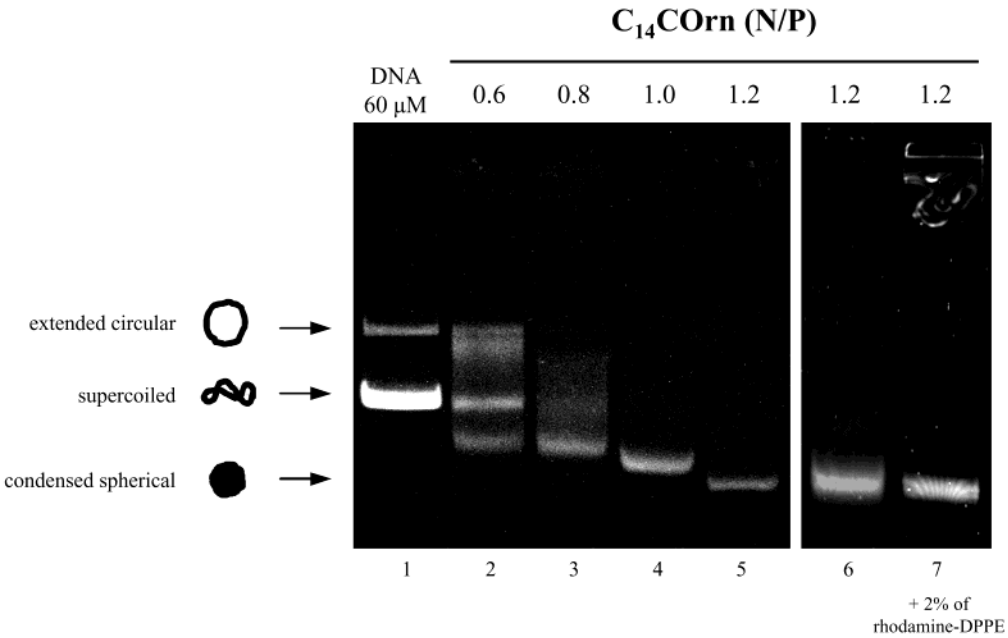

Figure 3. Complex formation as visualized by gel electrophoresis. Free DNA (lane 1) and its complexes with C_{14}COrn for various N/P ratios (lanes 2−5). The gel was stained with ethidium bromide to visualize DNA. The presence of lipid in the particles (N/P 1.2, lane 6) was revealed by addition of 2% of rhodamine-labeled DPPE to the initial mixture (lane 7; fluorescence $\lambda_{ex/em}$: 538/580 nm). Twenty microliter samples containing 0.4 μg of pCMV−Luc plasmid DNA in Hepes buffer (15 mM, pH 7.4), and concentrations of C_{14}COrn detergent as indicated were loaded onto the gel after 6 h incubation.

ing the absorbance at 260 nm before addition of the detergent and complexes were kept at room temperature for at least 4 h to allow cross-linking to occur prior gel electrophoretic analysis. Figure 3 shows the electrophoretic migration pattern observed for plasmid DNA mixed with increasing initial concentrations of C_{14}COrn. For cationic lipids, it is well-known that DNA complexes are too large to migrate into the gel. Here, we found lipid/DNA particles to be fully mobile when formed by oxidation of the detergent in the presence of DNA. At low charge ratios (N/P < 1), the coexistence of free DNA with the complexes showed dimerizable cationic detergent-mediated DNA condensation to be an all-or-nothing process. For a stoichiometric ratio (N/P = 1.0), condensation was completed and particles moved in the gel faster than free plasmid DNA, albeit most DNA phosphate residues were neutralized by the cationic amphiphile. To fully characterize the nature of this surprisingly fast-moving band, its lipidic component was revealed by addition of a 2% rhodamine-labeled DPPE to the initial mixture. The position of rhodamine-labeled DPPE was examined under fluorescence illumination ($\lambda_{ex/em}$: 538/580 nm) before the gel was stained with ethidium bromide to visualize DNA. As shown in Figure 3, the fluorescence of rhodamine colocalized with that of ethidium bromide-intercalated DNA. Stokes diffusion of condensed DNA particles through the agarose gel thus appears to be easier than reptation of the extended micrometric DNA polymer. As already discussed in the introduction, favorable in vivo diffusion and intracellular trafficking properties may thus be expected from the fast migration of the complexes.

The size and surface charge of the $(C_{14}COrn)_2$/DNA particles (N/P = 1.2) were measured by laser light

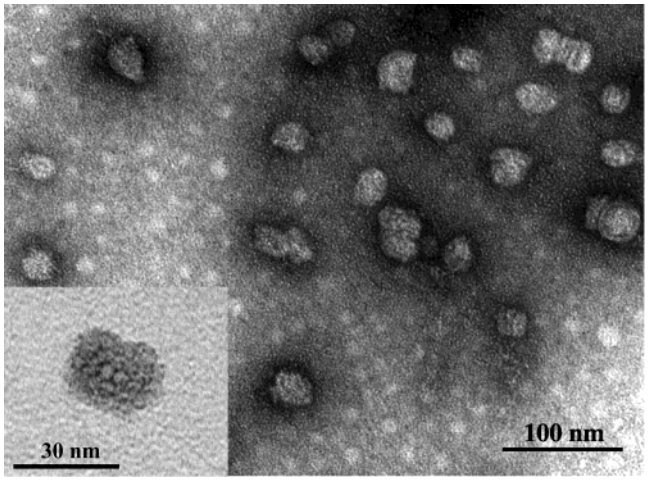

Figure 4. Transmission electron microscopy of $(C_{14}COrn)_2$/DNA particles at N/P = 1.2 in Hepes buffer (15 mM, pH 7.4).

scattering. The average size of the particles and their zeta potential were found to be 39 ± 4 nm and −45 ± 5 mV, respectively. To visualize their morphology and to confirm their size and dispersity, the particles were observed by transmission electron microscopy. A single population of particles with size of 28 ± 4 nm (n = 20) was found (Figure 4). The significantly smaller size found by transmission electronic microscopy may be due to the dehydration process which occurs during sample preparation. According to typical plasmid DNA and amphiphile molecular dimensions, the volume of a monomolecular DNA particle should correspond to a 31 nm sphere. The actual size of the particles thus provides strong evidence for monomolecular collapse of plasmid DNA. Uranyl acetate staining also suggested an internal supramolecular organization consistent with a compacted tubular or micellar phase (Figure 4, inset). This type of packing was previously observed with complexes prepared with Transfectam, a potent cationic lipid transfection reagent (*28*). The similarity of both structures was a good sign that $C_{14}COrn$ would be able to transfect cells.

Cell Transfection Properties of $(C_{14}COrn)_2$. The gene delivery properties of $(C_{14}COrn)_2$/DNA particles were tested on cells of various origins. The transfection

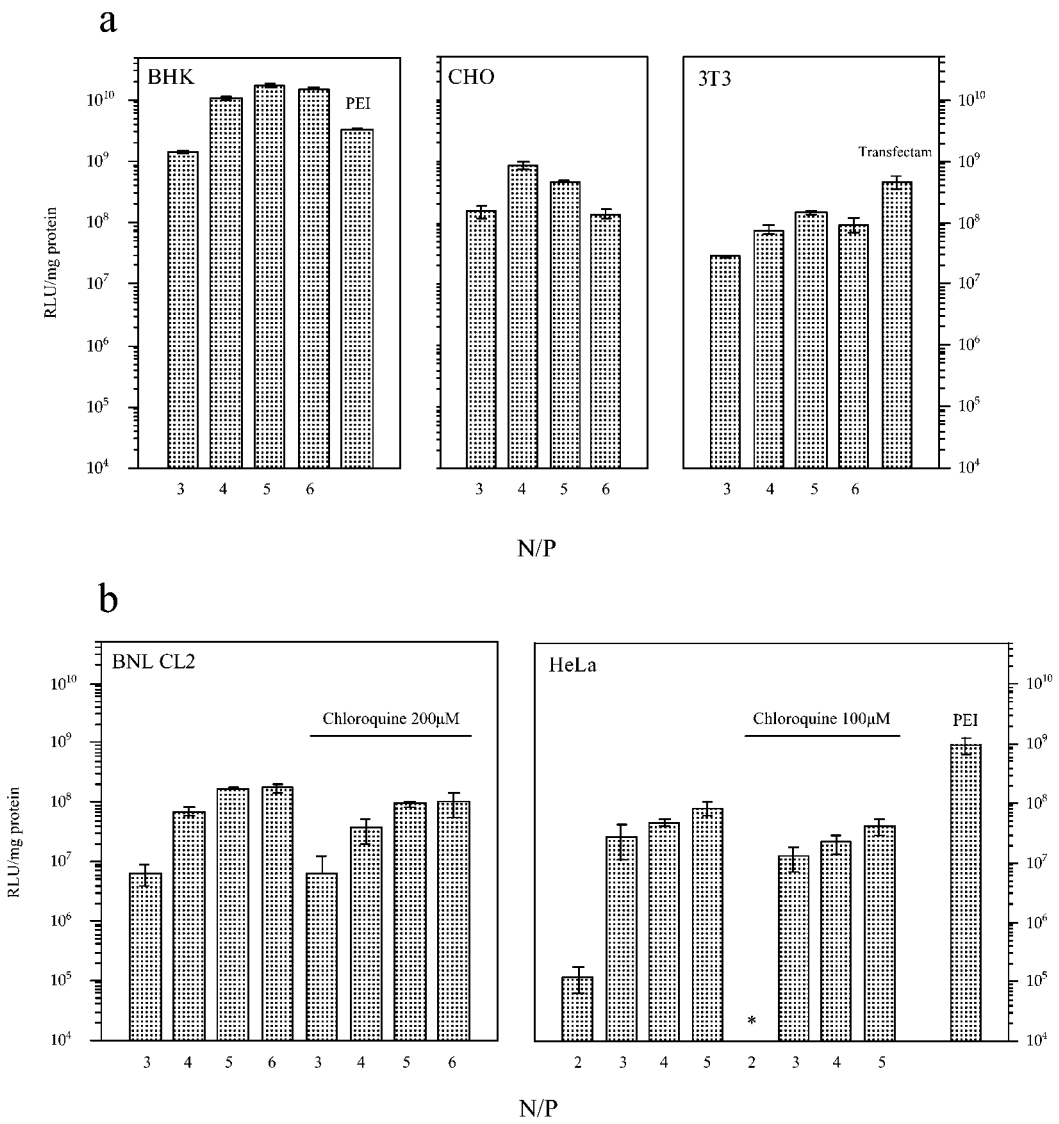

Figure 5. Transfection of BHK, CHO, 3T3, BNL CL2, and HeLa cells. (a) BHK, CHO and 3T3 cells were transfected with pCMV−Luc (2 μg/well) complexed with increasing amounts of $C_{14}COrn$. After 3 h, the transfection medium was completed with 10% FCS, and cells were cultured for an additional 21 h (b) BNL CL-2 and HeLa cells were transfected with pCMV−Luc (2 μg/well) complexed with increasing amounts of $C_{14}COrn$ in the presence or absence of chloroquine. After 3 h, the transfection medium was replaced with fresh culture medium, and cells were cultured for an additional 21 h. Luciferase activity was measured as described in Experimental Procedures. Transfections were carried out in triplicate; error bars indicate standard deviations.

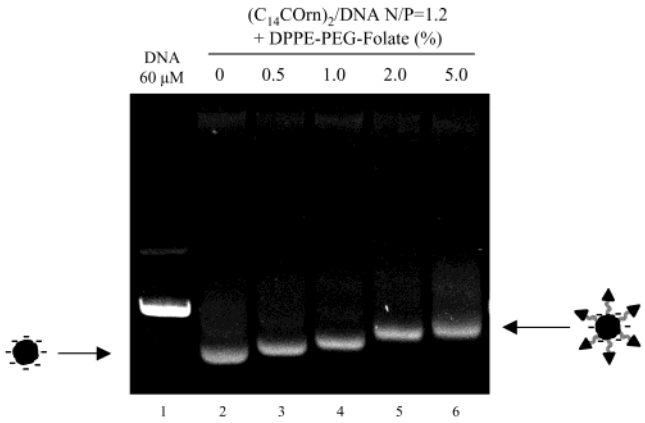

Figure 6. Characterization of $(C_{14}COrn)_2$/DNA/DPPE−PEG_{3400}−folate complexes by agarose gel electrophoresis. The minigel shows plasmid DNA (lane 1) and its complexes with $C_{14}COrn$ 1.2 eq (N/P) with various amounts of DPPE−PEG_{3400}−folate (%): 0, 0.5, 1.0, 2.0, and 5.0 (lanes 2−6). Twenty microliter samples containing 0.4 μg of pCMV−Luc and amphiphile(s) in Hepes buffer (15 mM, pH 7.4) were loaded onto the gel after 24 h incubation.

efficiency of the monomolecular complexes was only slightly above that of naked DNA. Indeed, being devoid of a cationic surface charge (zeta potential of −45 mV), these particles are unable to bind their internalizing receptors, namely, heparan sulfate polyanions (*28*, *29*). Larger cationic particles were therefore prepared with higher N/P ratios, i.e., standard conditions for cationic lipids, and tested in vitro using the luciferase reporter gene. The magnitude of gene expression varied with cell type (Figure 5). However, $(C_{14}COrn)_2$ was always found close to potent transfection reagents such as Transfectam and JetPEI (*23*, *24*). Optimal transfection was observed for N/P = 4−5, which corresponds to the formation of cationic particles. Beyond this optimum, luciferase activity eventually decreased as the N/P ratio was further raised, a possible sign of toxicity. 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 intracellular fate of synthetic gene delivery vectors.

Gene expression was not enhanced in the presence of 100 μM (HeLa) or 200 μM (BNL CL2) chloroquine. The $(C_{14}COrn)_2$/DNA complexes must therefore be released from the endosomal compartment by an intrinsic membrane disrupting activity. Treatment of the complexes with a biologically relevant concentration of glutathione (5−20 mM) (*30*, *31*) caused a partial DNA release as seen by electrophoresis (data not shown). Although the cellular compartments in which glutathione is present are not clearly identified, there is some evidence for the presence of glutathione within endosomes (*32*). The cationic disulfide lipid $(C_{14}COrn)_2$ may thus be reduced back into a cationic detergent that causes endosome disruption (*33*, *34*).

PEG-Coated Monomolecular DNA Particles. Systemic delivery through the blood circulation is the most attractive route for transferring genes to nonlocalized tumor metastases. Poly(ethylene glycol) is being extensively used to enhance the stability and the life span of liposomes in the bloodstream, via the so-called "steric-stabilization" mechanism (*17*). Several publications have also described the synthesis of PEG-containing polymers designed to create an inert hydrophilic layer at the surface of DNA condensates, with the aim of improving their solubility and in vivo performances (*35*−*38*). As PEG will also inhibit the ionic interaction between the

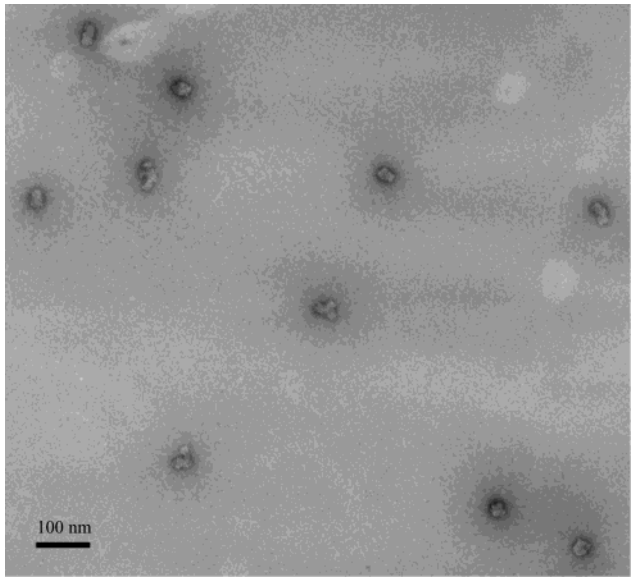

Figure 7. Transmission electron microscopy of $(C_{14}COrn)_2$/DNA/2% DPPE−PEG_{3400}−folate particles at N/P = 1.2 in Hepes buffer (15 mM, pH 7.4).

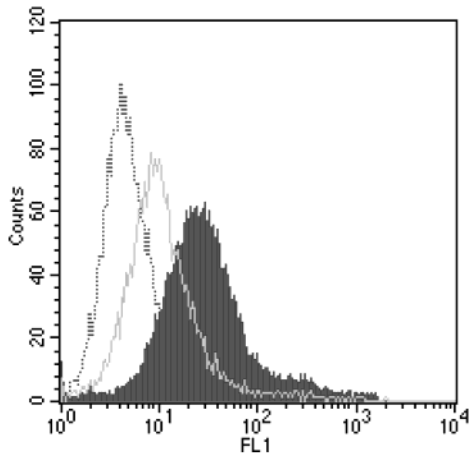

Figure 8. Folate-coated particles adhere to KB cells. Flow cytometry of KB cells exposed for 4 h to 2%-coated DPPE−PEG_{3400}−folate monomolecular YOYO-DNA particles (grey peak). Continuous line: plus 1 mM folic acid. Dotted line: autofluorescence of the cells. *X* axis, fluorescence intensity; *Y* axis, number of cells.

DNA complexes and the cell membrane that leads to cell entry, we coupled folic acid to the remote end of the PEG chain. This vitamin interacts with nanomolar affinity with the folate receptor overexpressed on tumor cells, and is able to trigger receptor-mediated endocytosis of the complexes (*39*). Accordingly, we synthesized a DPPE−PEG_{3400}−folate derivative that may anchor itself into the hydrophobic core particle (Figure 1). Since it was shown that grafting PEG molecules on the condensing agent may interfere with DNA compaction (*40*), we chose a postgrafting strategy. Plasmid DNA was first condensed by the cationic detergent. The monomolecular particles were subsequently coated with the lipid−PEG derivative by simple addition to the aqueous dispersion. The insertion equilibrium was reached within minutes as previously reported by Sou and co-workers in the case of liposomes (*41*). Incorporation was monitored by electrophoresis as a function of the DPPE−PEG_{3400}−folate/C_{14}-COrn ratio (Figure 6). Particles containing 0.5−5% DPPE−PEG_{3400}−folate were stable and able to migrate through the gel. However, their mobility decreased as the

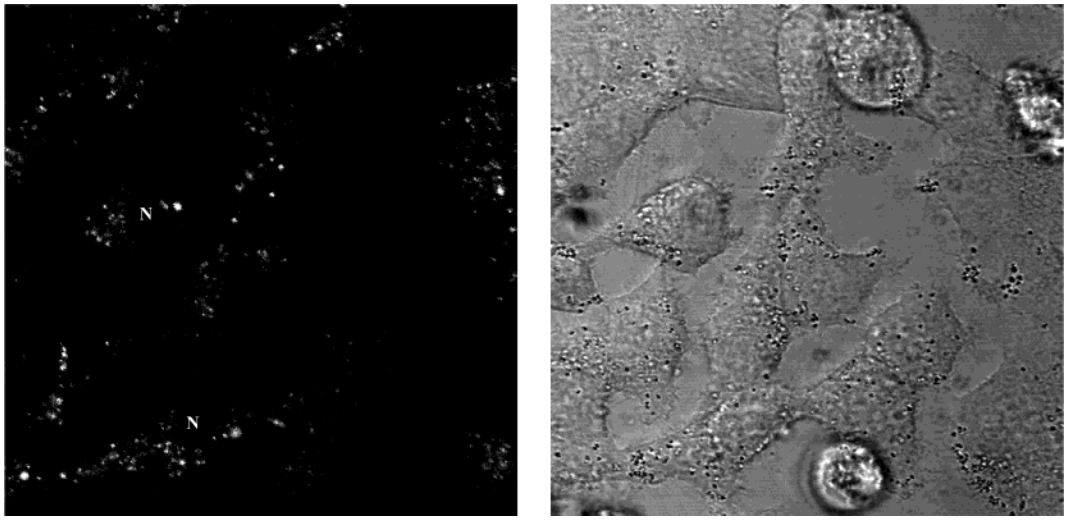

Figure 9. Folate-coated particles are taken up by KB cells. Cells were exposed to 2%-coated DPPE−PEG_{3400}−folate monomolecular YOYO-labeled complexes for 4 h. The medium was subsequently replaced by cell culture medium and cells incubated for an additional 20 h before analysis. Images were taken from living cells. The picture in the left panel corresponds to the fluorescence overlay of z-sections between 2 and 8 μm from the bottom of the cell. The corresponding transmission image is presented in the right panel. N indicates nucleus.

amount of DPPE−PEG_{3400}−folate increased, possibly due to diffusional hindrance brought about by the poly(ethylene glycol) calix (Figure 6).

The optimal in vivo formulation of PEG-grafted liposomes was shown to be reached using 3−7% of DSPE−PEG_{2000} in the lipid mixture (*42*). Since the lipid−PEG was distributed among the two bilayer leaflets, half this percentage should in principle be sufficient to cover a solid particle. The surface of a spherical 35 nm $(C_{14}COrn)_2$/DNA particle is approximately 4000 nm^2. Assuming a PEG_{3400} residue covers roughly 80 nm^2 (*43*), the insertion of ca. 50 molecules DPPE−PEG_{3400}−folate in the particule (i.e., 1%) should be sufficient for effective protection, in rough agreement with the liposome data. Experimentally, particle migration profiles became identical above 2% DPPE−PEG_{3400}−folate. Electron microscopy confirmed that the PEG-coated particles remained compact (Figure 7). Importantly, electrophoresis also showed that coated monomolecular particles did not aggregate in cell-culture medium (data not shown).

Entry into Folate Receptor-Expressing Cells. We finally examined the ability of the particles to carry genes into carcinoma cells overexpressing the folate receptor. The $(C_{14}COrn)_2$/DNA/2%DPPE−PEG_{3400}−folate particles were labeled with the fluorescent DNA-intercalating dye YOYO-1. Particles binding and entry into KB cells was studied by flow cytometry following a 4 h incubation period in folate-free cell culture medium. As shown in Figure 8, virtually all cells took up similar, yet moderate, amounts of the fluorescent complexes. This is to be compared to cationic lipid/DNA complexes, where cellular uptake was shown to be both higher and more heterogeneous (*44*, *45*). Control experiments performed with anionic $(C_{14}COrn)_2$/DNA as well as with $(C_{14}COrn)_2$/DNA/2%DPPE−PEG particles, both lacking the folic acid ligand, confirmed this shift to be significant. Competition experiments in the presence of 1 mM folic acid decreased the amount of particle binding (Figure 8). Effective competition required a large excess of ligand, as a single particle may form multiple tethers with a cluster of receptors on the cell surface. Taken together, these results clearly show that cell binding occurred via the folate receptor.

Unfortunately, transfection results were very disappointing: luciferase activity was found ca. 5 orders of magnitude lower than using JetPEI as a vector. We thus followed the fate of the particles by confocal microscopy (Figure 9). Tiny fluorescent spots were found beneath the cell surface or spread throughout the cytoplasm at 4 h. At 24 h, they were somewhat larger and spread ramdomly. The cytometry (see above) and microscopic observations contrast with typical cationic lipid-mediated transfection, where large clumps of DNA complexes sediment onto the cells, are taken up by heparan sulfate proteoglycan ligation (*46*) and accumulate in a perinuclear compartment (*44*, *45*).

Unsuccessful transfection may thus arise from either (or from a combination) of several factors: (i) 35 nm particles do not sediment well onto the cells; (ii) the number of folate receptors is certainly well below that of ubiquitous anionic proteoglycans; (iii) caveolae are thought to be folic acid-storing organelles and vehicles for transcytosis (*47*). All functions may be nonproductive for gene delivery.

CONCLUSION

This work was meant to be an extension of encouraging results described in the literature that showed that grafting of poly(ethylene glycol)−folic acid residues to DNA complexes led to selective expression of foreign genes in folate-expressing cells in vitro (*35*, *39*, *48*). To our knowledge, these results have had no in vivo developments so far. The size of DNA complexes being a key factor for successful delivery in vivo, we applied our recently developed technique of monomolecular DNA condensation to this goal. The reason we failed up to now is not clear, yet it precludes in vivo experiments. Additional in vitro experiments are being performed.

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NOTE ADDED AFTER ASAP POSTING

The sentence "The pCMV−Luc plasmid was linearized with *Xmn* I, according to standard protocols (*21*)" was deleted from Materials and Methods.

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