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Articles

Dimerizable Cationic Detergents with a Low cmc Condense Plasmid DNA into Nanometric Particles and Transfect Cells in Culture

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Abstract: The size of condensed DNA particles is a key determinant for in vivo diffusion and gene delivery to cells. Gene molecules can be individually compacted by cationic thiol detergents into nanometric particles that are stabilized by oxidative conversion of the detergent into a gemini lipid. To reach the other goal, gene delivery, a series of cationic thiol detergents with various chain lengths (C_{12}−C_{16}) and headgroups (ornithine or spermine) was prepared, using a versatile polymer-supported synthetic strategy. Critical micelle concentrations and thiol oxidation rates of the detergents were measured. The formation and stability of complexes formed with plasmid DNA, as well as the size, ξ-potential, morphology, and transfection efficiency of the particles were investigated. Using the tetradecane/ornithine detergent, a solution of 5.5 Kpb plasmid DNA molecules was converted into a homogeneous population of 35 nm particles. The same detergent, once oxidized, exhibited a typical lipid phase internal structure and was capable of effective cell transfection. The particle size did not increase with time. Surprisingly, the gel electrophoretic mobility of the DNA complexes was found to be higher than that of plasmid DNA itself. Favorable in vivo diffusion and intracellular trafficking properties may thus be expected for these complexes.

Introduction

Gene therapy relies on DNA-carrying vehicles. The course of a gene to the cell nucleus is a complex multistage process that requires a multicomponent particulate vector. Unfortunately, in vivo diffusion of such particles, whether nonreplicating viruses or simple DNA complexes, is severely restricted by their size. Synthetic supramolecular vectors are based on the DNA condensing property of cationic polymers or lipids.[1,2] Co-condensation of oppositely charged polymers is a quasi-irreversible process that leads to microprecipitates (hence, a convenient DNA isolation technique).[3] Particles are heterogeneous with respect to composition, size (50−500 nm), and shape (toroids, rods, and their aggregates).[4] Additional aggregation occurs at physiological salt concentration, because of the fact that Coulombic repulsions become shielded by ions, thus making van der Waals attraction between particles predominant. Such

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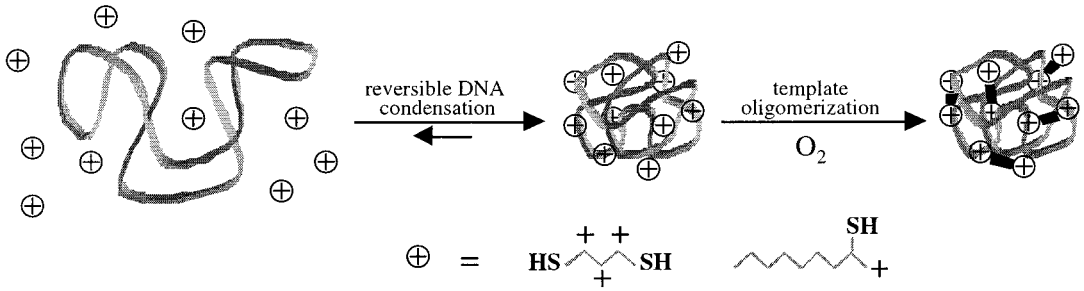

Figure 1. DNA is condensed by thiol-containing oligocations presenting a low binding cooperativity. After equilibration, the particles are "frozen" by DNA template-assisted oxidation of thiols into disulfides.

DNA precipitates are effective at transfecting cells in culture dishes, as sedimentation favors contact with the cell surface. In vivo gene delivery, however, is poor, because of the lack of diffusion within tissues. Even organs with fenestrated blood vessels remain poorly transfected following injection of the complexes in the blood stream, the major event being capture by liver macrophages.[5]

In contrast to cationic polymers or lipid phases, *oligo*cations such as spermine or cationic detergent micelles interact with DNA reversibly. Equilibration ensures a homogeneous particle population. Because entropy tends to direct the system toward the largest number of particles, each particle should ultimately be made of a single molecule of condensed plasmid DNA. This tendency has been observed experimentally.[6,7] Unluckily, the other consequence of reversible binding is that complexes do not withstand dilution in physiologic fluids and cannot be used as DNA vectors per se.[8]

We recently described a general method for "freezing" such small DNA particles. It makes use of polymerizable α,ω-bisthiol oligocations or of dimerizable cationic thiol detergents for DNA condensation (Figure 1). Subsequent oxidation to disulfides converts the reversible condensing agent into a polymer or a lipid, respectively. Stable particles formed with oligoamines[9,10] or with oligolysines[11] were eventually small but not monomolecular with respect to plasmid DNA. Solutions of particles resulting from the DNA template-assisted dimerization of detergents into gemini surfactants,[12] however, were remarkably homogeneous.[9,13,14] Laser light scattering and electron microscopy showed them to be made of individually condensed DNA molecules. We therefore concentrated our efforts on the thiol amphiphiles. The short-chain cysteine-based detergents that were made initially proved to be poor transfection agents. Here, we describe a general synthesis of thiol-based dimerizable detergents that allowed us to explore several other headgroup and hydrocarbon combinations. Some derivatives exhibit both monomolecular plasmid DNA condensation *and* efficient cell transfection properties.

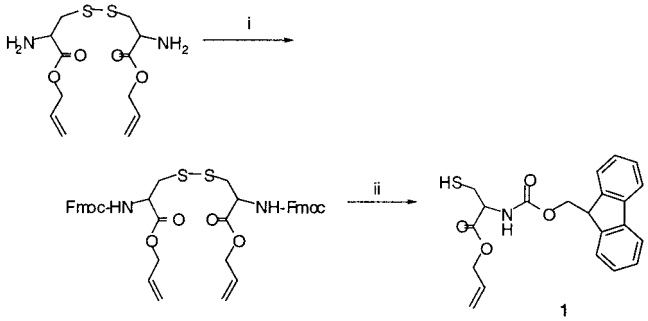

Figure 2. Synthesis of (*N*,*O*)-protected cysteine from L-cystine-bis-allyl ester. (i) FMOC-ONSu, TEA, THF; (ii) DTT, TEA, DCM.

Results and Discussion

Synthesis of Dimerizable Cationic Detergents. Cysteine was chosen as the polyfunctional core, as it possesses a thiol group for mild dimer formation in aqueous DNA solutions, a carboxylic acid residue for conjugation of lipophilic alcohols or amines, and a protonable primary amine function. Structure−activity studies aimed at improving cell transfection while retaining monomolecular condensation properties required the synthesis of detergents with various headgroups and hydrocarbon tails. Solid-phase synthesis was well adapted to this goal, provided cysteine was bound to the resin via its thiol group. To do so, *N*-fluorenylmethoxycarbonyl-cysteine-allyl ester **1** was prepared from the commercially available L-cystine-bis-allyl ester according to Figure 2. Preliminary experiments were performed with a NOVASYN-MMT alcohol resin having a PEG 3000 spacer. A flexible spacer allows high-resolution NMR spectra of resin-bound synthons to be recorded.[15,16] Reaction conditions for the various steps could be optimized, but the extent of grafting (0.075 mmol/g) was too low for preparative purposes. Instead, **1** was reacted with NOVASYN-MMT chloride (1.7 mmol/g) to yield the starting bead-grafted cysteine (Figure 3). The methoxytrityl thioether linkage was compatible with Fmoc [(fluorenylmethoxy)carbonyl] coupling chemistry and Boc (butoxycarbonyl) protecting groups. Because the α-amino group of cysteine was insufficiently protonated at neutral pH, it was derivatized with ornithine (Orn) and carboxyspermine[17] (Sper) headgroups which widened the range of cmc's and DNA

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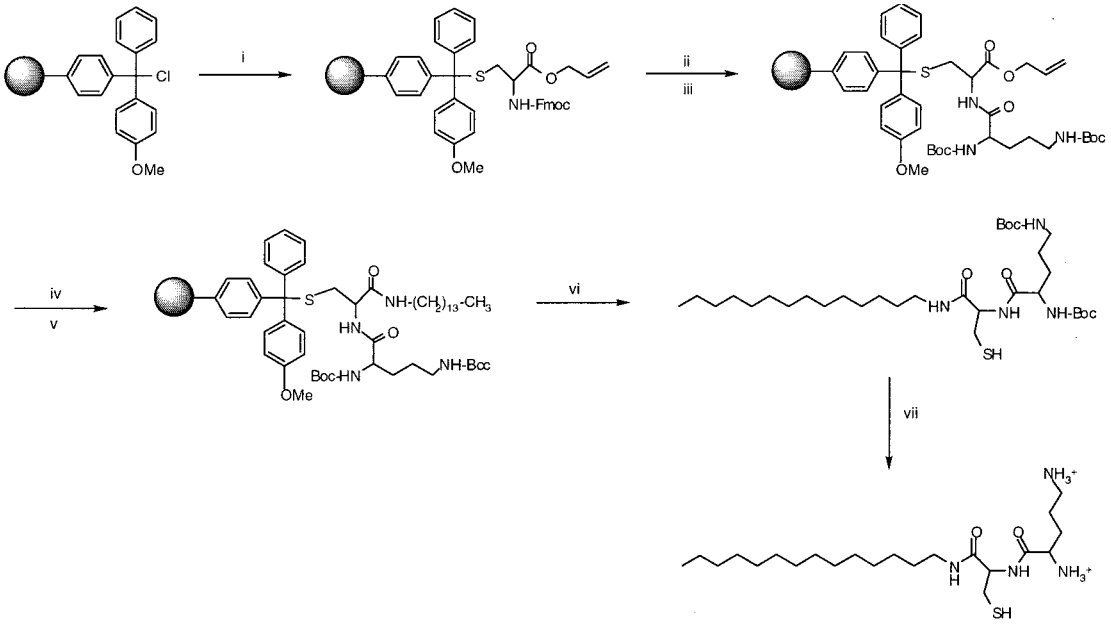
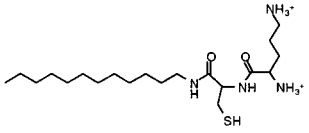
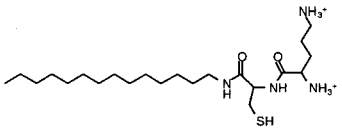
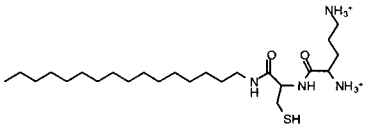
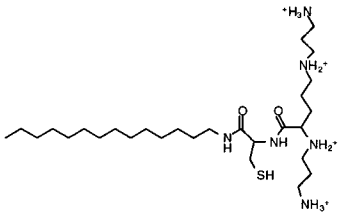


Figure 3. Solid support synthesis of the cationic detergent C_{14}COrn. (i) **1**, DIEA, DCM; (ii) 5−20% piperidine/DMF; (iii) *N*-δ,*N*-β-di-*t*-Boc-L-ornithine, PyBOP, DIEA, DMF; (iv) $Pd(PPh_3)_4$, $CHCl_3$/AcOH/*N*-methylmorpholine (37:2:1); (v) tetradecylamine, PyBOP, DIEA, DMF; (vi) 5% TFA/DCM; (vii) 100% TFA.

Table 1. Dependence of Alkyl Chain Length and Head Group Structure on Critical Micelle Concentration and 3T3 Fibroblasts Transfection

Compound Name	Structure	Overall Yield	Critical micelle concentration[a] (μM)	Transfection efficiency (RLU/mg protein[b])
C_{12}COrn		34%[c]	100	$(7.5 \pm 2.7)\times10^5$
C_{14}COrn		83%	45[d]	$(2.2 \pm 0.4)\times10^7$
C_{16}COrn		46%	20	$(5.6 \pm 0.3)\times10^7$
C_{14}CSper		52%	150	$(7.0 \pm 1.0)\times10^6$

[a] Determined in Hepes 15 mM pH 7.4, DTT 10 mM. ±15% as estimated from independent measurements. [b] Luciferase activity (expressed as relative light units per milligram of cell proteins ±SD) measured 24 h after cells transfection with 2 μg luciferase plasmid. [c] C_{12}COrn was synthesized in liquid phase: *N*-δ,*N*-β-di-*t*-Boc-L-ornithine was activated as its *N*-hydroxysuccinimide ester and treated with *S*-*t*-Boc-L-cysteine. This compound was activated in the same way and treated with dodecylamine. The Boc-protected compound C_{12}COrn$(Boc)_2$ was deprotected by 100% TFA. ^{1}H NMR (EtOH-d_6) δ 0.89 (t, 3H, $J = 7$ Hz), 1.25−1.38 (m, 18H), 1.45−1.96 (m, 6H), 2.80−3.50 (m, 6H), 3.95−4.0 (m, 1H), 4.42−4.48 (m, 1H). [d] cmc = 100 μM at pH 6 in 15 mM Mes, 10 mM DTT.

binding affinities. Moreover, we previously found that cysteine detergents with decane hydrocarbon chains were ineffective at transfecting cells,[13] presumably because the dimeric molecules were still exchanging between the DNA complexes and the medium or the cell membrane. Detergents with longer hydrocarbon chain length (up to 16 carbon atoms) were thus synthesized in seven steps with reasonable yields (46−83%, i.e., 88−97% per step, Table 1) according to the synthetic pathway outlined in Figure 3. Interestingly, mild TFA-mediated resin cleavage in the second-to-last step led to recovery of the fully Boc-protected compound, which allowed straightforward chromatographic purification followed by quantitative depro-

tection. All compounds were kept under argon as concentrated stock solutions (50−100 mM) in deuterated ethanol at −80 °C. This allowed us to check by ^{1}H NMR for stability toward hydrolysis or oxidation during storage.

Critical Micelle Concentrations (cmc's). Knowledge of the cmc's is of upmost importance for transfection: the presence of excess cationic micelles of the thiol detergent during the complex formation step may trigger aggregation of anionic condensed DNA particles; a high cmc is thus preferable. On the other hand, once oxidized, the resulting disulfide lipid should have a very low cmc to avoid early extraction from the lipid/DNA complexes during the gene delivery process.

The cmc's of the thiol detergents were determined at neutral pH in 10 mM dithiothreitol (DTT) using the fluorescent probe *N*-phenyl-1-naphthylamine.[18] The observed trends (Table 1) were as expected: cmc's decreased in the order C_{12}COrn > C_{14}COrn > C_{16}COrn with increasing hydrocarbon chain length, and for a given hydrophobic tail, cmc increased with the headgroup polarity (C_{14}COrn < C_{14}CSper). The α-amino group of the ornithine amide is presumably not fully protonated in the isolated detergent molecule at pH 7.4, and even less so in the micellar environment. As a consequence, micelle formation is pH-dependent, and decreasing the pH to 6 increased the cmc of C_{14}COrn twofold (Table 1). The only unexpected finding was the rather small decrease of cmc observed with hydrocarbon chain length increase. For classical series of detergents, a 10-fold decrease was observed following addition of two carbon atoms to the amphiphilic tail.[19] The lower values observed here (a two- to threefold decrease) may be a consequence of the apparent p*K* decrease upon micelle formation.

Coming to our goal, working plasmid DNA concentrations for transfection experiments being typically 10−50 μM, all compounds described in Table 1 were candidates for DNA condensation in aggregation-free conditions, provided the excess free cationic detergent was kept below the cmc, that is, the ratio of total detergent (expressed as amines, N) to DNA (expressed as phosphates, P), N/P < 1.5.

We also attempted to measure cmc values for the dimers, to set the limit below which cationic lipid-mediated transfection can occur. Their aggregation concentration was found far below the limit of the fluorescent technique (10 μM).[18] Unfortunately, measurements performed with the other available technique, the Langmuir balance, failed as well. They gave an upper limit of ∼500 nM for the cmc's of both $(C_{12}COrn)_2$ and $(C_{14}COrn)_2$. Natural bis-hexadecanoylglycerolipids have typical cmc's in the nanomolar range when measured with an ad hoc technique.[20] Assuming the cmc of $(C_{12}COrn)_2$ to be around 500 nM, this would bring the cmc of $(C_{14}COrn)_2$, the compound capable of cell transfection (see below), to 10−100 nM.

Thiol-to-Disulfide Conversion Rates. To be monomolecular, DNA condensation by the cationic detergent and subsequent equilibration must be faster than detergent oxidation (otherwise the process would just be DNA condensation by a cationic lipid). According to Figure 1, DNA then serves as a matrix for dimer formation, and provided kinetic constants remain comparable, the reaction may be accelerated by DNA as a consequence of local concentration increase. This was indeed observed for all compounds. As an example, the spontaneous aerobic oxidation rate of C_{14}CSper is shown in Figure 4. After 4 h, the amount of thiol remaining was ∼20-fold less when DNA was present. Rate constants cannot simply be extracted from the curves in Figure 4, because, as oxidation proceeds, the remaining C_{14}CSper will partition between the aqueous solution and the already formed $(C_{14}CSper)_2$ lipid phase. Apparently, reaction occurred somewhat more slowly in the lipid phase than in solution, yet overnight oxidation of the DNA complexes was sufficient in all cases to ensure full detergent conversion.

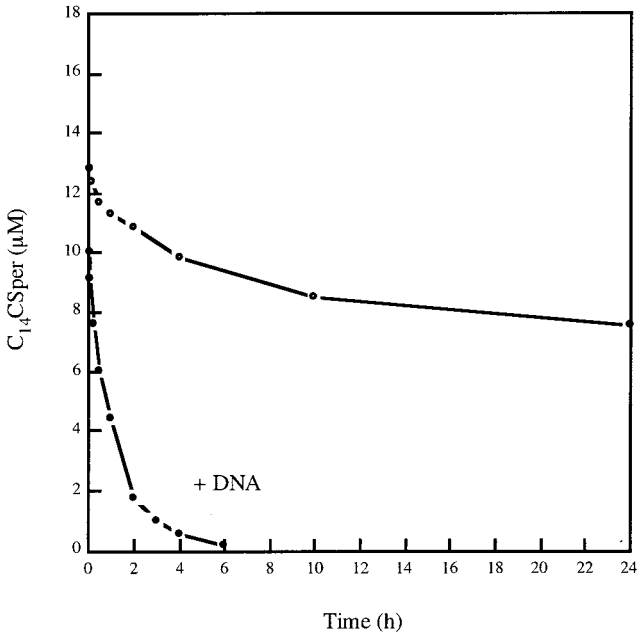


Figure 4. Oxidation of C_{14}CSper occurs faster in the presence of template DNA. C_{14}CSper was diluted to 18 μM in 5 mL of Hepes buffer (15 mM pH 7.4), and time-dependent oxidation was followed by titration of the remaining thiol. For the template experiment, pCMV-Luc plasmid DNA was added to a final concentration of 60 μM phosphate.

Size and Stability of the Condensed DNA Particles. Aggregation properties of the detergents with pCMV-luc plasmid DNA (60 μM phosphate) were determined by dynamic light scattering (DLS) for increasing detergent/DNA ratio. The detergent/plasmid particles did not diffuse sufficient light intensity for accurate measurements to be made. Measurements were thus performed after overnight oxidation. With the initial driving force for complex formation being of electrostatic nature, clean complex formation should occur up to a cationic amphiphile/DNA phosphate charge ratio close to 1. The precise N/P ratio will depend on the apparent amine p*K*'s of ornithine and spermine headgroups in DNA complexes. The lowest p*K* of Transfectam, a structurally related lipospermine derivative used for cell transfection, is 5.5.[8] Neutral complexes should thus form for an N/P ratio slightly above 1 at pH 7. DLS results for N/P = 1−2 are listed in Table 2. Using a multimodal analysis, a single population of particles with size ∼35 nm was found with all compounds for an N/P ratio close to 1. Increasing the amount (N/P ≥ 1.4) or the chain length (C_{16}) of the amphiphile led to an increase of the particle size. Both effects may be related to the presence of excess free cationic micelles able to induce aggregation of anionic condensed DNA particles. Indeed, ζ-potential measurements of the $(C_nCOrn)_2$/DNA complexes gave increasing yet still negative values (−50 to −20 mV) for N/P increasing from 1.2 to 2 (data not shown). This behavior agrees with the view that the driving force for complex formation decreases asymptotically as N/P increases, thus leaving particles still containing phosphate residues that are not neutralized in equilibrium with some detergent molecules free in the solution. In the region where complexes grow large, the

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Table 2. Size[a] (nm) of the (Detergent)$_2$/DNA Particles as Determined by Dynamic Light Scattering in Hepes Buffer (15 mM pH 7.4)

	(N/P)[b]			
detergent	1.0	1.2	1.4	2.0
C_{12}COrn	37.5 ± 3.0 (n = 3)	30.7 ± 3.0 (n = 2)	236 ± 3.4 (n = 3)	>500
C_{14}COrn	32.9 ± 0.0 (n = 2)	39.5 ± 3.6 (n = 3)	>500 (n = 3)	>500
C_{16}COrn	32.7 ± 1.4 (n = 3)	47.7 ± 2.2 (n = 3)	>500 (n = 3)	>500
C_{14}CSper	33.9 ± 2.5 (n = 3)	43.3 ± 2.5 (n = 3)	>500 (n = 3)	>500

[a] Mean diameter from the multimodal distribution analysis; average and standard deviation of n determinations. [b] Ratio of amphiphile amine functions to DNA phosphates.

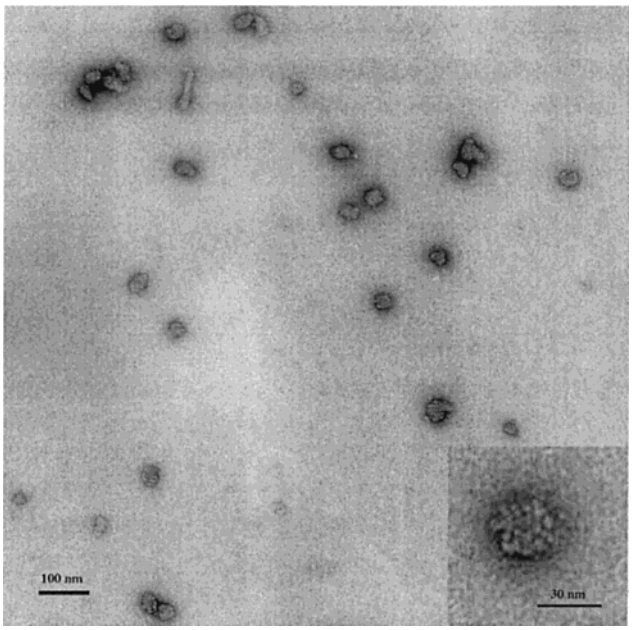

Figure 5. Transmission electron microscopy of (C_{14}COrn)$_2$/DNA particles at N/P = 1 in Tris−HCl buffer (15 mM pH 8.4).

excess free detergent concentration is close to the cmc, and initially small anionic complexes can aggregate through cationic micelle bridges.

The DNA complexes formed at an equimolar amine-to-phosphate ratio remained monomolecular within a 60−1200 μM DNA concentration range and over a period of two weeks in 15 mM Hepes buffer. More interestingly, no aggregation was noticed following a 4 h incubation in DMEM cell-culture medium. The size and morphology of the complexes were examined by transmission electron microscopy (Figure 5). The population appeared homogeneous with respect to size but not to shapes. At a higher magnification (inset in Figure 5), it became obvious that (C_{14}COrn)$_2$ was forming a tubular phase with repeating distance of ~6 nm. The irregular particles' shapes thus appeared to be a consequence of tube bending induced by close packing with DNA. A similar ultrastructure was observed previously with DNA complexes of another polycationic lipid (Transfectam).[21] The size of the complexes (35 ± 5 nm, n = 25) was in good agreement with the light scattering measurements. Assuming hexagonal packing of DNA with interaxis

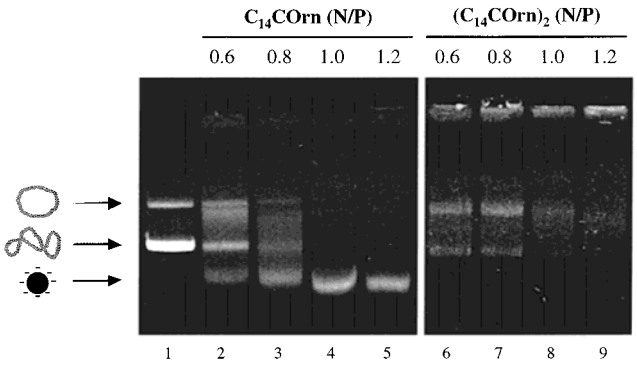

Figure 6. Agarose gel electrophoresis of (C_{14}COrn)$_2$/DNA complexes. When DNA was mixed with the detergent and then oxidized according to the procedure outlined in Figure 1, the resulting complexes migrate in the gel (lanes 2−5). When DNA was directly mixed with the oxidized gemini lipid, the precipitated complexes remain in the well (lanes 6−9). 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 onto 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).

distances of 2.7 nm,[22] the volume of a 5.5 Kbp plasmid is calculated to be 11 800 nm^3. Assuming 6 nm-wide hexagonal packing of a tubular phase containing 2700 lipid molecules (N/P ≈ 1) with a surface area per molecule of 0.7 nm^2 gives an additional volume of 3200 nm^3. The volume of a particle containing the plasmid and 2700 lipid molecules with separate packing is thus equivalent to a sphere with a 31 nm diameter. Taking into account that interdigited 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.

Agarose Gel Electrophoresis of the Complexes. Gel retardation is a widely used technique for assessing complex formation between plasmid DNA and gene delivery vectors. Cationic lipids condense DNA into large particles that remain in the loading well. The cationic lipid (C_{14}COrn)$_2$ obtained by oxidation of the detergent prior to addition to DNA behaved similarly (Figure 6, lanes 6−9). On the contrary, cationic detergents interact with DNA reversibly. Both components fall apart quickly and migrate in opposite directions. As a consequence, the mobility of DNA is practically unaffected.[13] Remarkably, the monomolecular particles formed by oxidation of the detergent in the presence of template DNA were mobile and moved even *faster* than DNA, that is, a gel "acceleration" experiment (Figure 6, lanes 2−5). The lipidic component of the DNA band was revealed by addition of 2% rhodamine-labeled dipalmitoylphosphatidylethanolamine to the initial mixture: The fluorescence of DNA-intercalated ethidium bromide colocalized with that of rhodamine (not shown). Stokes diffusion of a compact nanometric DNA particle through the agarose network thus appears to be easier than De Gennes reptation of the corresponding micrometric DNA polymer.

Mixtures formed at intermediate ratios, N/P = 0.6−0.8, showed coexistence of free DNA and fully formed complexes, which was also confirmed by electron microscopy. This is evidence for a highly cooperative all-or-nothing condensation process. Closer examination of the disappearance of the supercoiled vs circular relaxed forms of the plasmid showed the supercoiled form to be preferentially taken up in the complexes.

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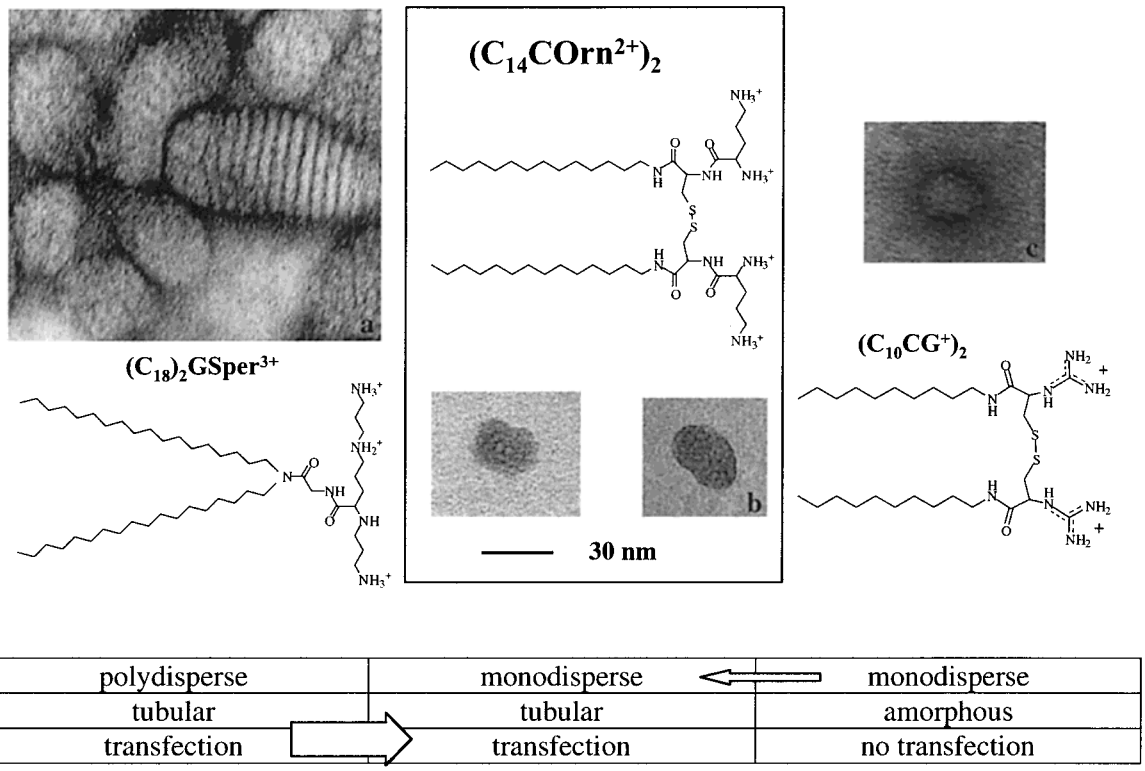

Figure 7. $(C_{14}COrn)_2$/DNA complexes combine the interesting structural and biological properties of lipopolyamines (left) and the morphological properties of dimerizable detergents (right). All electron microscopy pictures are to scale (bar = 30 nm).

Indeed, supercoiled DNA is more compact than the circular form, and hence, easier to condense.

Transfection of Cells in Culture. The cell-surface receptors for cationic lipid/DNA complexes are anionic heparan sulfate proteoglycans (HSPG).[21,23] With binding being of electrostatic nature, particles must contain excess cationic charges to enter cells efficiently; hence, N/P $\gg$ 1, and particles become aggregated (Table 2). Even in these conditions, dimers of a C_{10}-hydrocarbon detergent were unable to transfect cells.[13] The cmc of the dimeric C_{10} amphiphile was probably not low enough to prevent exchange with the extracellular milieu[8] or partition into the cell membrane[24] to occur faster than cell entry. We tested our series of amphiphiles with increased lipophily on 3T3 murine fibroblasts, using luciferase as reporter gene. For all amphiphiles, the best results were obtained for N/P = 3−5 (not shown), which is typical for cationic lipids. Relative transfection efficiencies $(C_{12}COrn)_2$:$(C_{14}COrn)_2$:$(C_{16}COrn)_2$ increased in the order 1:30:75. (Table 1). The large transfection increase observed between decane, dodecane, and tetradecane gemini lipids tended to culminate for tetra- and hexadecane lipids, in agreement with the threshold cmc hypothesis mentioned above. $(C_{14}CSper)_2$ gave lower transfection values than $(C_{14}COrn)_2$ and showed signs of toxicity with increasing N/P. Both effects may be related to the higher cmc of the spermine relative to the ornithine derivative, which highlights again the impact of this factor on the biological properties of the amphiphile.

Although we failed to obtain accurate values for the cmc's of the gemini lipids, effective transfection seems to require a cmc value below 100 nM. Qualitatively similar results were observed for transfection of HeLa human epithelial cells and BNL CL.2 murine hepatocytes. Altogether, in vitro luciferase expression observed using $(C_{14}COrn)_2$ was only 2−10-fold less than using some of the most potent transfection reagents such as Transfectam and PEI (not shown).

Conclusion

In 1989,[25] we described the remarkable transfection properties of lipospermines, of which several members including Transfectam (Promega), Lipofectamine (Life Technologies), and DOSPER (Roche Biochemicals) are still on the molecular biology reagents market. These compounds form stable mesophases with DNA that are visible by electron microscopy (Figure 7, left), but particles are heterogeneous and too large to diffuse in vivo.

A decade later,[13] we took advantage of an in situ dimer formation reaction to direct the DNA condensation process toward the smallest possible particles (Figure 7, right). However, the dimerized detergent $(C_{10}CG^+)_2$-condensed particles displayed no internal structure and failed to transfect cells.

In the present work, the chemical structure of the amphiphile was tuned to a cmc high enough for the monomer to perform monomolecular DNA condensation and low enough for the dimer to form a stable mesophase capable of cell transfection (Figure 7, middle). Favorable diffusion properties can be inferred from the fast migration of the complexes as observed in the gel electrophoresis experiment. Improved in vivo diffusion is expected, yet intracellular trafficking may be favored too, especially as plasmid DNA itself was shown to be essentially immobile in the cytoplasm.[26] Finally, the size of the particles remains compatible with nuclear pore crossing, which may open the way to transfection of post-mitotic cells. These favorable physical properties, however, are incompatible with heparan

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sulfate proteoglycan-mediated cell entry. The next step toward artificial viruses, that is, grafting the monomolecular DNA particles with ligands able to trigger specific cell entry, is in progress.

Experimental Section

Materials. 4-Methoxytrityl chloride resin (1.73 mmol/g) and benzotriazole-1-yl oxytrispyrrolidinophosphonium hexafluorophosphate (PyBOP) were purchased from Novabiochem (Meudon, France). *N*-δ,*N*-β-Di-*t*-Boc-L-ornithine was from Bachem (Voisins-le-Bretonneux, France). Transfectam was synthesized as described.[25] L-Cystine-bis-allyl ester bis(toluene-4-sulfonate), tetradecylamine, dodecylamine, hexadecylamine, *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (FMOC-ONSu), *N*,*N*-diisopropylethyleneamine (DIEA), 4-dithio-DL-threitol (DTT), *N*-methylmorpholine (NMP), piperidine, triethylamine (TEA), and trifluoroacetic acid (TFA) were from Fluka (St Quentin Fallavier, France). Tetrakis(triphenylphosphine)palladium ($Pd(PPh_3)_4$) was from Lancaster (Bischheim, France). Acetonitrile (ACN), dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF), ethanol (EtOH), methanol (MeOH), chloroform ($CHCl_3$), acetic acid (AcOH), and tetrahydrofuran (THF) were from Carlo Erba (Val de Reuil, France). We used a glass column ended with a join connection and a glass frit (porosity n°2) on the upper and lower ends, respectively, as a reaction vessel. Solid-phase reactions were carried out at room temperature. Stirring was obtained by connecting the column to a rotatory evaporator. The Kaiser qualitative ninhydrin test was carried out as described elsewhere.[27] pCMV-Luc plasmid was propagated and purified as described.[28]

Monomer Synthesis (Figure 2). (A) *N*,*N*′-Bis(fluorenylmethoxycarbonyl)-[R]-cystine-bis-allyl Ester. L-Cystine-bis-allyl ester bis(toluene-4-sulfonate) (2 g, 2.75 mmol) was dissolved in 10 mL of THF. TEA (950 μL, 6.9 mmol) was added, followed by *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (2.4 g, 6.9 mmol). The reaction mixture was stirred for 2 h and concentrated. The residue was diluted with DCM (500 mL) and washed with H_2O (250 mL), 5% citric acid (250 mL), and H_2O (250 mL). The organic phase was dried with $MgSO_4$ and concentrated. The resulting product was purified by silica gel column chromatography and eluted with DCM/MeOH (96:4). A white product was obtained (2.7 g, 98%): TLC (98:2 DCM/MeOH) R_f 0.8; ^{1}H NMR ($CDCl_3$) δ 3.22 (d, 4H, $J = 5$ Hz), 4.22 (t, 2H, $J = 7$ Hz), 4.41 (d, 4H, $J = 7$ Hz), 4.60−4.78 (m, 6H), 5.22−5.40 (m, 4H), 5.76 (d, 2H, $J = 8$ Hz), 5.80−6.03 (m, 2H), 7.28−7.47 (m, 8H), 7.60 (d, 4H, $J = 7$ Hz), 7.76 (d, 4H, $J = 7$ Hz).

(B) *N*-Fluorenylmethoxycarbonyl-[R]-cysteine-allyl Ester (1). To a solution of *N*,*N*′-bis(fluorenylmethoxycarbonyl)-[R]-cystine-bis-allyl ester (1.68 g, 2.2 mmol) in 25 mL DCM was added DTT (507 mg, 3.3 mmol) and TEA (460 μL, 3.3 mmol). The reaction was allowed to proceed for 30 min, and the mixture was concentrated. The residue was purified by silica gel column chromatography and eluted with DCM/MeOH (96:4). A white product was obtained (1.25 g, 74%): TLC (99:1 DCM/MeOH) R_f 0.7; ^{1}H NMR ($CDCl_3$) δ 1.38 (t, 1H, $J = 9$ Hz), 3.02 (s, 2H), 4.25 (t, 1H, $J = 7$ Hz), 4.45 (d, 2H, $J = 7$ Hz), 4.7 (s, 3H), 5.28−5.43 (m, 2H), 5.75 (d, 1H, $J = 8$ Hz), 5.87−6.00 (m, 1H), 7.31−7.45 (m, 4H), 7.62 (d, 2H, $J = 7$ Hz), 7.78 (d, 2H, $J = 7$ Hz); FABMS *m*/*e* 384.1 $(M + H)^+$.

Solid-Phase Synthesis (Figure 3). (A) *N*-9-Fluorenylmethyl-allyl-cysteine (1)-Grafted Resin. A sample of 4-methoxytrityl chloride resin (900 mg, 1.56 mmol) was poured into the glass column and washed with DCM. Protected cysteine **1** (1.03 g, 2.68 mmol) was dissolved in DCM (8 mL) with DIEA (1.87 mL, 10.7 mmol) and added to the resin. The suspension was stirred for 2 h. The solvent was filtered off, and the resin beads were washed with DCM/MeOH/DIEA (17:2:1, 3 × 20 mL), DCM (3 × 20 mL), DMF (2 × 20 mL), DCM (2 × 20 mL), and MeOH (3 × 20 mL). The degree of substitution of the resin was estimated by spectrophotometric determination of Fmoc following treatment of a weighed sample with 20% piperidine in DMF. Level of loading versus the manufacturer's: 80% (1.4 mmol/g).

(B) (Allyl-O)-cyst-Grafted Resin. The resin (900 mg, 1.4 mmol/g) was suspended in 5% piperidine in DCM/DMF (1:1) for 10 min and then in 20% piperidine in DMF for 30 min. The vessel was drained, and the resin was washed with DCM (3 × 20 mL), DMF (3 × 20 mL), and DCM (3 × 20 mL).

C_{14}COrn. (A) Elongation with *N*-δ,*N*-β-Di-*t*-Boc-L-ornithine. A solution of *N*-δ,*N*-β-di-*t*-Boc-L-ornithine (287 mg, 855 μmol), PyBOP (450 mg, 855 μmol), and DIEA (300 μL, 1.7 mmol) in 4 mL DMF was added to (allyl-O)-cyst resin (250 mg, 1.4 mmol/g). The suspension was stirred at room temperature for 4 h. The vessel was drained, and the resin was washed with DMF (3 × 5 mL), MeOH (3 × 5 mL), and DCM (3 × 5 mL). The Kaiser test was negative.

(B) Removal of the Allyl Protecting Group. Tetrakis(triphenylphosphine)palladium ($Pd(PPh_3)_4$) (1.22 g, 1.02 mmol) was suspended in a solution of $CHCl_3$/AcOH/*N*-methylmorpholine (37:2:1, 20 mL) under argon. The mixture was poured onto the resin (250 mg, 1.4 mmol/g) and left under argon overnight with gentle stirring. The vessel was drained, and the resin was washed with 0.5% DIEA in DMF (3 × 20 mL), sodium diethyldithiocarbamate (0.5% w/w) in DMF (3 × 20 mL), DMF (3 × 20 mL), and MeOH (3 × 10 mL).

(C) Elongation with Tetradecylamine. A solution of tetradecylamine (64.8 mg, 283 μmol), PyBOP (147 mg, 283 μmol), and DIEA (100 μL, 565 μmol) in 2 mL of DCM was added to the resin (83 mg, 1.4 mmol/g). The suspension was stirred at room temperature for 4 h. The vessel was drained, and the resin was washed with DCM (3 × 5 mL), MeOH (3 × 5 mL), and DCM (3 × 5 mL).

(D) Cleavage of the Protected Detergent from the Resin. The resin was suspended in 3% TFA in DCM (1 mL) for 5 min followed by 5% TFA in DCM (1 mL) for 1 h. The solution was filtered, and the resin was washed as mentioned previously. The combined organic fractions were concentrated after dilution in ACN. The crude product was dissolved in a small volume of DCM and was purified by silica gel column chromatography and eluted with DCM/MeOH (97:3). A solid was recovered (41 mg, 65 μmol): TLC (90:10, DCM/MeOH) R_f 0.6; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, $J = 7$ Hz), 1.25 (m, 22H), 1.44 (s, 9H), 1.45 (s, 9H), 1.48−1.70 (m, 6H), 1.84−1.91 (m, 2H), 3.13−3.30 (m, 4H), 4.1 (t, 1H, $J = 6$ Hz), 4.58 (t, 1H, $J = 6$ Hz), 4.72 (s, 1H), 5.3 (s, 1H), 6.75 (s, 1H), 7.14 (d, 1H, $J = 8$ Hz).

(E) Removal of the Boc Protecting Groups. Boc-protected compound $C_{14}COrn(Boc)_2$ (39 mg, 62 μmol) was deprotected in 100% TFA. After stirring at room temperature for 1 h, the mixture was concentrated in vacuo, and the resulting yellow oil was dissolved in 800 μL of EtOH-d_6. The overall yield was 83% as determined by thiol titration.[29] The stock solution was kept under argon at −80 °C. ^{1}H NMR (EtOH-d_6) δ 0.89 (t, 3H, $J = 7$ Hz), 1.28−1.32 (m, 22H), 1.51−1.56 (m, 2H), 1.71−2.06 (m, 4H), 2.85−3.31 (m, 6H), 4.02−4.08 (t, 1H, $J = 6$ Hz), 4.48 (t, 1H, $J = 6$ Hz); FABMS *m*/*e* 431.2 $(M + H)^+$.

C_{16}COrn. (A) Elongation with *N*-∂,*N*-β-Di-*t*-Boc-L-ornithine. A solution of *N*-δ,*N*-β-di-*t*-Boc-L-ornithine (465 mg, 1.4 mmol), PyBOP (720 mg, 1.4 mmol), and DIEA (500 μL, 2.8 mmol) in 4 mL DMF was added to the (allyl-O)-cyst resin (400 mg, 1.4 mmol/g). The suspension was stirred at room temperature for 4 h. The vessel was drained, and the resin was washed with DMF (3 × 5 mL), MeOH (3 × 5 mL), and DCM (3 × 5 mL). The Kaiser test was negative.

(B) Removal of the Allyl Protecting Group. LiOH (34 mg, 1.4 mmol) was suspended in a solution of THF/MeOH/H_2O (5:2:1, 10 mL). The mixture was poured onto the resin (400 mg, 1.4 mmol/g) and left under argon overnight at 35 °C with gentle stirring. The vessel was drained, and the resin was washed with THF/MeOH (1:1, 3 × 10 mL), THF (3 × 10 mL), and MeOH (3 × 10 mL).

(C) Elongation with Hexadecylamine. A solution of hexadecylamine (113 mg, 490 μmol), PyBOP (240 mg, 490 μmol), and DIEA (160 μL, 980 μmol) in 4 mL of DCM was added to the resin (140 mg, 1.4 mmol/g). The suspension was stirred at room temperature for 4 h. The vessel was drained, and the resin was washed with DCM (3 × 5 mL), MeOH (3 × 5 mL), and DCM (3 × 5 mL).

(D) Cleavage of the Protected Detergent from the Resin. The resin was suspended in 3% TFA in DCM (1 mL) for 5 min followed

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by 5% TFA in DCM (1 mL) for 1 h. The solution was filtered off, and the resin was washed as mentioned above. The combined organic fractions were concentrated after dilution in ACN. An oil was recovered: TLC (90:10, DCM/MeOH) R_f 0.7. The detergent was partially deprotected and was used without chromatografic purification.

(E) Removal of the Boc Protecting Groups. The compound C_{16}-COrn(Boc)$_2$ was completely deprotected in 100% TFA. After stirring at room temperature for 30 min, the mixture was concentrated in vacuo, and the resulting yellow oil was dissolved in EtOH-d_6 (46% yield as judged by thiol titration). The stock solution was kept under argon at −80 °C. ^{1}H NMR (EtOH-d_6) δ 0.87 (t, 3H, J = 6 Hz), 1.27−1.30 (m, 22H), 1.50 (m, 2H), 1.8−2.03 (m, 4H), 2.81−3.29 (m, 6H), 4.08 (t, 1H, J = 6 Hz), 4.48 (t, 1H, J = 6 Hz); FABMS *m*/*e* 459.1 $(M + H)^+$.

C_{14}CSper. (A) Elongation with Tetrakis(*t*-Boc)spermine-5-carboxylic Acid. A solution of tetrakis(*t*-Boc)spermine-5-carboxylic acid[17] (650 mg, 1 mmol), PyBOP (550 mg, 1 mmol), and DIEA (370 μL, 2 mmol) in 3 mL DMF was added to (allyl-O)-cyst resin (150 mg, 1.4 mmol/g). The suspension was stirred at room temperature for 6 h. The vessel was drained, and the resin was washed with DMF (3 × 5 mL), MeOH (3 × 5 mL), and DCM (3 × 5 mL). The Kaiser test was negative.

(B) Removal of the Allyl Protecting Group. LiOH (2.6 mg, 110 μmol) was suspended in a solution of THF/MeOH/H_2O (5:2:1, 10 mL). The mixture was poured onto the resin (80 mg, 1.4 mmol/g) and left under argon for 1 h at 35 °C with gentle stirring. The vessel was drained, and the resin was washed with THF/MeOH (1:1, 3 × 10 mL), THF (3 × 10 mL), and MeOH (3 × 10 mL).

(C) Elongation with Tetradecylamine. A solution of tetradecylamine (120 mg, 560 μmol), PyBOP (292 mg, 560 μmol), and DIEA (100 μL, 560 μmol) in 2 mL DCM was added to the resin (80 mg, 1.4 mmol/g). The suspension was stirred at room temperature for 6 h. The vessel was drained, and the resin was washed with DCM (3 × 5 mL), MeOH (3 × 5 mL), and DCM (3 × 5 mL).

(D) Cleavage of the Protected Detergent from the Resin. The resin was suspended in 0.5% TFA in DCM (1 mL) for 5 min, followed by 1% TFA in DCM (1 mL) for 1 h. The suspension was filtered off, and the resin was washed as usual. The combined organic fractions were concentrated after dilution in ACN. The crude product was dissolved in a small volume of DCM, purified by silica gel column chromatography, and eluted with DCM/MeOH (95:5). A solid was recovered (47 mg, 49 μmol): TLC (95:5, DCM/MeOH) R_f 0.5; ^{1}H NMR ($CDCl_3$) δ 0.87 (t, 3H, J = 7 Hz), 1.20−1.31 (m, 22H), 1.45 (m, 36H), 1.41 (m, 12H), 3.07−3.20 (m, 14H), 4.14 (m, 1H), 4.54 (m, 1H).

(E) Removal of the Boc Protecting Group. Boc-protected compound C_{14}CSper(Boc)$_4$ (47 mg, 49 μmol) was deprotected using 100% TFA. After stirring at room temperature for 1 h, the mixture was concentrated in vacuo, and the resulting yellow oil was diluted in EtOH-d_6 (52% yield as judged by thiol titration). The stock solution was kept under argon at −80 °C. ^{1}H NMR (EtOH-d_6) δ 0.88 (t, 3H, J = 7 Hz), 1.25 (m, 22H), 1.5 (m, 2H), 1.75−2.25 (m, 6H), 2.8−3.3 (m, 14H), 4.1−4.16 (m, 1H), 4.38−4.58 (m, 1H); ES-MS *m*/*e* 545.5 $(M + H)^+$.

Determination of Critical Micelle Concentrations (cmc's). The cmc's of the detergents were obtained by measuring the fluorescence of a solution of increasing detergent concentration in 2 mL of an argon-saturated Hepes (15 mM pH 7.4) or Mes (15 mM pH 6) buffer, containing 1 μM *N*-phenyl-1-naphthylamine (NPN) and 10 mM DTT to avoid detergent oxidation.[18] Excitation and emission wavelengths were 356 and 410 nm, respectively. A plot of the fluorescence versus the logarithm of the detergent concentration displayed a sharp break, and the corresponding concentration was taken as the cmc.

Kinetics of Detergent Oxidation. The detergent was injected from a stock solution (5 mM in ethanol) to a final concentration of 18 μM in 5 mL of Hepes buffer (15 mM pH 7.4). For each time point, a 0.5 mL aliquot was removed and mixed with 0.5 mL 2X Ellman's reagent.[29] The remaining free thiol concentration was quantitated spectrophotometrically according to Riddles et al.[29] For the template experiment, pCMV-Luc plasmid DNA was added to a final concentration of 60 μM phosphate before addition of the detergent (N/P = 1.2).

Detergent/DNA Complex Formation. 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 in 1 mL Hepes buffer (15 mM pH 7.4) under constant stirring. The DNA concentration was checked by measuring its absorbance at 260 nm. Complexes were kept at room temperature to allow cross-linking to occur prior to further characterization.

Light Scattering Measurements. After overnight oxidation, particle size and ζ-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 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. ζ-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 the detergent (30 μM final concentration) to a 60 μM phosphate pCMV-Luc DNA solution in Tris−HCl buffer (15 mM pH 8.4) and leaving the solution in aerobic conditions for 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 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). A drop (5 μL) of sample was left on the grid for 1 min. Complexes were stained with 30 μL aq uranyl acetate (1 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.

Cell Transfection. 3T3 cells were cultured and plated as described.[28] 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. Complexes (100 μL solutions corresponding to 2 μg plasmid per well) were added to the cells maintained in the serum-free medium. Fetal calf serum was added to a final concentration of 10% after 3 h. Transfectam (Promega, Madison, USA) and jetPEI (Polyplus, Illkirch, France) were used as positive controls according to the manufacturer's protocol. Luciferase gene expression was determined after 24 h using conventional procedures and commercial kits.[28] Results were expressed as light units integrated over 10 s, per milligram of cell protein using the BCA assay (±SD, n = 3).

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Supporting Information Available: NMR spectra (pdf). This material is available free of charge via the Internet at http://pubs.acs.org.

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