Journal of
Medicinal Chemistry

Subscriber access provided by American Chemical Society

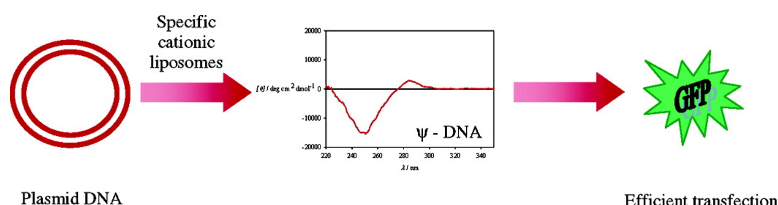
Brief Article

Efficient Transfection of DNA by Liposomes Formulated with Cationic Gemini Amphiphiles

Cecilia Bombelli, Francesca Faggioli, Paola Luciani, Giovanna Mancini, and Maria Grazia Sacco

J. Med. Chem., **2005**, 48 (16), 5378-5382• DOI: 10.1021/jm050477r • Publication Date (Web): 06 July 2005

Downloaded from http://pubs.acs.org on March 28, 2009



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 11 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

ACS Publications
High quality. High impact.

Journal of Medicinal Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Efficient Transfection of DNA by Liposomes Formulated with Cationic Gemini Amphiphiles

Cecilia Bombelli,[†,‡] Francesca Faggioli,[§] Paola Luciani,[†,‡] Giovanna Mancini,*[,†,‡] and Maria Grazia Sacco[§,||]

Istituto di Metodologie Chimiche del CNR and Dipartimento di Chimica, Università degli Studi di Roma "La Sapienza", P.le Aldo Moro 5, 00185 Roma, Italy, Centro di Eccellenza Materiali Innovativi Nanostrutturati per Applicazioni Chimiche, Fisiche e Biomediche, and Istituto di Tecnologie Biomediche del CNR, via Fratelli Cervi, 93, 20090 Segrate - Milano, Italy

Received May 20, 2005

Cationic liposomes formulated with neutral 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and cationic gemini surfactants were used for transfecting different cell lines with a reporter gene. The efficiency in the transfection has been correlated to the high extent of DNA condensation observed by circular dichroism, condensation shown to depend heavily on the gemini spacer structure. Transfection efficiency was better than that obtained with a commercial lipofection kit.

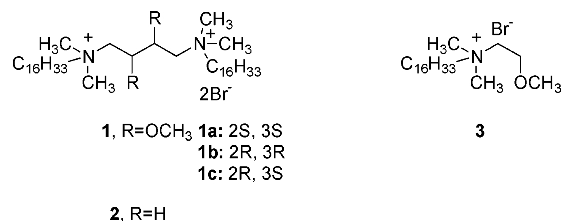
Introduction

The challenge of modern medicine is to fight the causes of diseases and not just to treat symptoms. One of the most promising tools to achieve this goal is represented by gene therapy. The aim of gene therapy is to provide specific cells of a patient with the genetic information necessary to produce therapeutic proteins for correction or modulation of diseases. The therapeutic gene has to reach the target cells via an appropriate delivery system, but a major obstacle in gene therapy has been indeed represented by exploiting proper vectors.[1] The ideal delivery system should be safe, be stable to degradation, reach selectively the target cell, cross the plasma cell membrane efficiently, allow an effective release of the nucleic acid in the cytosol, and result in a substantial production of the therapeutic protein. The development of vectors capable to address as much as possible all these features could render gene therapy a general treatment for many diseases.

Liposomes have the potential of fulfilling the characteristics of the ideal vectors because they are safe, being formulated with natural lipids, and feature a remarkable versatility at the molecular and formulation level.[2] The use of cationic components in the formulations to obtain an overall positive charge of the vesicles can satisfy both the need of the electrostatic interactions with the phosphate backbone with the possibility of condensation of nucleic acids, with the implications of size reduction and higher resistance to degradation, and the need of similar interactions with the plasma membrane.

In the panorama of new amphiphiles, gemini surfactants,[3] featuring two hydrophobic chains and two polar headgroups linked by a spacer, seem quite promising.

Chart 1. Structures of the Cationic Amphiphiles Used



Their physicochemical features are different from those of conventional surfactants, and recent studies pointed out that opportunely designed cationic geminis gave high transfection efficiency.[4,5]

Fundamental to the success of lipid systems as nucleic acid vehicles is the knowledge, on one hand, of their physicochemical properties and, on the other, of the modes by which such properties influence the behavior of the liposome/nucleic acid complex (lipoplex) in the biological milieu. The correlation between these two aspects is a central point in the development of new efficient lipid vectors for gene therapy. Despite several investigations directed toward an extensive rationalization of the parameters controlling the complexation of DNA and therefore transfection,[6−10] many issues are still under debate.

The work described below is aimed at correlating the molecular structure of the cationic component of liposomes, to the liposome capability of condensing DNA and to the transfection efficiency of the lipoplexes; in particular, we investigated the role of the stereochemistry of the spacer of a cationic gemini surfactant.

Circular dichroism (CD) experiments allowed us to correlate the capability of condensing a plasmid carrying a reporter gene (GFP) under the control of an ubiquitous promoter (CMV) by liposomes formulated with a neutral phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and any of the cationic amphiphiles (CA) reported in Chart 1 to the molecular structure of the cationic component. In vitro experiments carried out on COS-7, LA7, and human fibroblast cell lines demonstrated a remarkable correlation between the condensa-

* Corresponding author. Tel. 00390649913078; Fax 003906490421; E-mail: giovanna.mancini@uniroma1.it.
† Università degli Studi di Roma "La Sapienza".
‡ Centro di Eccellenza Materiali Innovativi Nanostrutturati per Applicazioni Chimiche.
§ Istituto di Tecnologie Biomediche del CNR.
|| Present address: European Commission - Joint Research Centre, Institute for Health and Consumer Protection, Physical and Chemical Exposure Unit T. P. 460, 21020 Ispra (VA), Italy.

10.1021/jm050477r CCC: $30.25 © 2005 American Chemical Society
Published on Web 07/06/2005

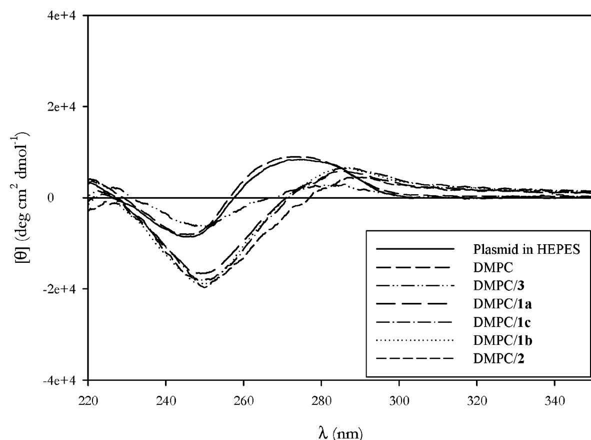


Figure 1. CD spectra of DMPC/CA/plasmid lipoplexes (CA: **1a**, long dash; **1b**, dotted; **1c**, dash-dot-dash; **2**, short dash; **3**, dash-dot-dot) at a charge ratio +/− = 2, $t = 0$. The solid line is the CD spectrum of uncondensed DNA (plasmid in HEPES buffer). The medium dashed line is the CD spectrum of DNA in mere DMPC.

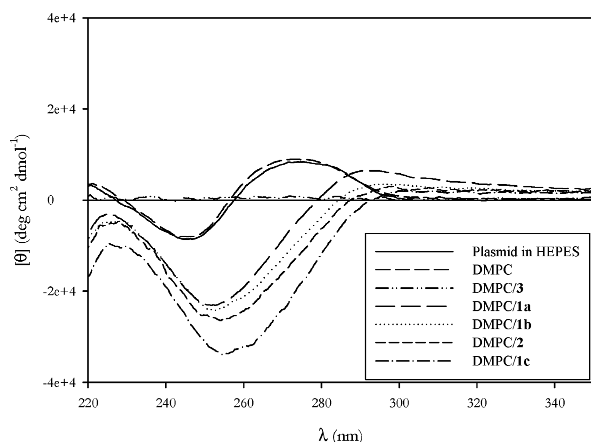


Figure 2. CD spectra of DMPC/CA/plasmid lipoplexes. (CA: **1a**, long dash; **1b**, dotted; **1c**, dash-dot-dash; **2**, short dash; **3**, dash-dot-dot) at a charge ratio +/− = 2, $t = 24$ h. The solid line is the CD spectrum of uncondensed DNA (plasmid in HEPES buffer). The medium dashed line is the CD spectrum of DNA in mere DMPC.

tion of DNA observed by CD and the transfection efficiency. Transfection efficiency of CMV/GFP DNA complexed by different liposome formulations was compared with that obtained using a commercial transfection agent; a formulation more effective than the commercial kit was individuated on all the cell lines tested.

Results

CD Investigations. The CD spectra of the lipoplex suspensions (liposome/DNA at a [cationic headgroup]/[DNA single base] = 2, that is +/− = 2 charge ratio) formulated with the three diastereomers of gemini 2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonium)-butane dibromide, **1**, and 1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonium)butane dibromide, **2**, and with the conventional single headgroup surfactant *N*,*N*-dimethyl-N-hexadecyl-*N*-(2-methoxyethyl)ammonium bromide, **3**, are reported in Figures 1 and 2, respectively. The spectra were recorded immediately after the addition of the plasmid-buffered solution to the liposome suspensions and after 24 h from the addition. It can be observed that the conservative CD spectrum of the plasmid in HEPES buffer (solid line), characteristic of DNA-B, changes after the addition of DNA to the suspension formulated with geminis (DMPC/**1** and DMPC/**2**, 1/1) whereas no variation is observed in the presence of liposomes formulated with the single headgroup surfactant **3** (DMPC/**3**, 1/2) and with only DMPC. In particular the spectra of plasmid complexed by the formulations containing geminis show an enhanced negative ellipticity, an overall shift of the bands toward higher values of wavelengths, a flattening of the positive bands, and the appearance of tails above 300 nm. The potential contribution of differential scattering was investigated by collecting different spectra at various distances between the sample and PMT detector.[11] The CD spectra did not show any variation as a function of the distance, ruling out any scattering effect.

Table 1. Percentage of the Survived Cells (LA7 cell line) after Trypan Blue Test[a]

samples	CA concentration					
	425 μM	213 μM	85 μM	42.5 μM	21.3 μM	8.5 μM
DMPC/**1a**	-	-	-	10%	90%	100%
DMPC/**1b**	-	-	-	-	-	100%
DMPC/**1c**	-	-	-	30%	80%	100%
DMPC/**2**	-	50%	80%	100%	100%	100%
DMPC/**3**	-	50%	80%	100%	100%	100%
standard	100%	100%	100%	100%	90%	100%

[a] In the case of liposomes containing surfactant **3**, the total lipid concentration has to be varied, by doubling the concentration of CA.

Biological Evaluation. The toxicity of different preparations was tested on three different cell lines: Cos-7,[12] LA7,[13] and human fibroblasts.[14] Cells were treated as described below. The survival was evaluated by Trypan blue exclusion method by counting cells which did not incorporate the dye (an example of the protocol used is reported in Table 1).[15] The lower toxicity and the maintenance of morphology were obtained with liposomes formulated with **1c**, while the worst conditions were obtained with **1b**.

The ability of these liposome formulations to complex DNA was evaluated by running the lipoplexes (prepared as described below) on 1% agarose gel.[16] Most of the DNA added to liposomes formulated with **3** was detected on the gel, though delayed; a very tiny amount of the DNA added to liposomes containing **1a** and **1b** was observed on the gel, whereas an almost complete complexation was obtained with liposomes containing **1c** and **2** (Figure 3).

The transfection efficiency of the various formulations was then evaluated on three cell lines and compared with that of Fugene (Roche), which is known as one of the most efficient commercial lipofecting agents.

Liposomes formulated with **3** were not able to transfer DNA, whereas transfection was observed with all liposomes formulated with gemini amphiphiles (**1** and **2**). In particular the transfection efficiency was modest with formulations containing **1a** and **1b**, higher with that containing **2** (data not shown) and highest with that containing **1c**. This last formulation yielded a transfection efficiency higher than that obtained using Fugene, in the experiments performed on the three cell lines tested (see Figure 4); in particular, we were able to observe GFP expression even on human fibroblasts,

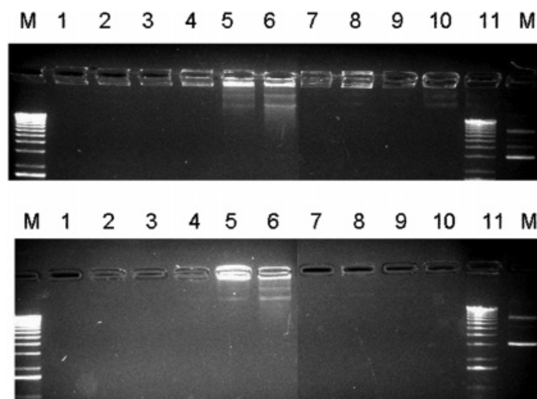


Figure 3. Lipid/DNA complexation assessed by gel electrophoresis. M: 1kb Marker; Lanes 1, 3, 5, 7, 9: liposomes formulated with DMPC/**1c**, DMPC/**2**, DMPC/**3**, DMPC/**1a**, DMPC/**1b**, respectively, incubated for 15 min with 500 ng of CMV-GFP plasmid DNA; Lanes 2, 4, 6, 8, 10: the same formulations incubated for 24 h with DNA. Lane 11: Nude CMV-GFP plasmid DNA (500 ng). Samples were run for 1 h at 60 V on 1% agarose gel.

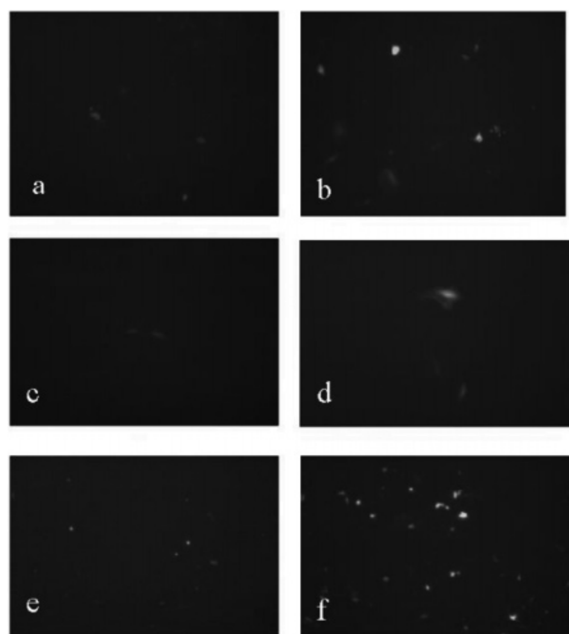


Figure 4. Comparison of transfection efficiency between a commercial lipofecting agent (Fugene) and DMPC/1c liposomes. Left side: Fugene; Right side: DMPC/**1c** liposomes; Panel a, b: Cos-7 cell line; c, d: human fibroblast cell line; e, f: LA7 cell line. 100× magnification, Nikon inverted fluorescence microscope.

which are very difficult to be transfected (Musio et al., personal communication).

Discussion

An effective complexation between DNA and liposomes should yield compaction of the nucleic acid; this will promote the penetration of the nucleic acid into the target cell because of the reduced size of the polymer and will protect the genetic exogenous material from nuclease degradation. A crucial point to obtain a high transfection efficiency is, in fact, to maintain the therapeutic genes undamaged.

The conformational changes of the DNA due to complexation and condensation can be followed by variation of the conservative CD spectrum of the native B form. For example, a secondary structural transition from the native B-DNA to the C motif, as the one induced by a lipid system such as DOTAP or DOTAP-DOPE, is characterized by a very small positive CD band and a negative short-wavelength band whose shape, location, and magnitude are essentially identical to those of the B-motif.[17] The condensation of DNA into a chiral ψ-phase is instead indicated by an overall shift of the spectrum to higher values of wavelengths, an enhanced negative band and an almost complete flattening of the positive CD signal.[18] It has been suggested that cationic liposomes are able to promote the transition of DNA to a ψ-phase where the nucleic acid molecules are suggested to be partially embedded in a lipid columnar inverted-hexagonal assembly which provides DNA with a given spatial organization and a fixed directionality.[19]

The results of CD investigations reported in Figures 1 and 2 show that the liposomes containing gemini amphiphiles induce a structural transition from a B-form of the plasmid to a typical ψ-phase; CD spectra show in fact the ψ anomalies diagnostic of condensation. The conformational changes are more pronounced after 24 h incubation, especially for formulations containing gemini surfactants **1c** and **2**. Interestingly, the formulation containing **3** does not induce any conformational change, demonstrating the difference between gemini surfactants and the analogous single headgroup surfactant.

The results obtained in the lipofection tests on different cell lines allowed us to correlate the high extent of condensation to the transfection efficiency. In fact, the best transfecting agent resulted from the liposome suspension formulated with gemini **1c**, and a fairly good transfection was obtained with gemini **2**. The GFP expression obtained after the treatment of the three cell lines with **1c** liposomes was superior to that obtained after the standard treatment with a commercial kit, as shown in Figure 4. Formulations containing a smaller amount of total lipids for a CA/DNA charge ratio +/− = 1 did not show an appreciable condensation by CD and were not efficient in transfecting any of the cell lines. This was probably due to both the absence of condensation and a poor electrostatic interaction with the cell membrane.

The comparison between all the geminis and surfactant **3** confirms the outstanding features of geminis as compared to conventional surfactants.[3] Some geminis have been previously proposed, in fact, as efficient nonviral transfecting agents in gene therapy,[4] but the comparison with conventional amphiphiles has not been studied in this specific field; moreover, their efficiency as condensing agents has not been investigated.

This investigation shows that the structure and stereochemistry of the spacer play a crucial role in the complexation capability and in the transfection efficiency of the liposomes. In fact, among the three diastereoisomers, the meso form results the most efficient both in the DNA condensation and in the transfection. The presence of methoxy groups on C2 and C3 position of the spacer improves the condensation and transfection capability of **1c** with respect to **2**, whereas it decreases the corresponding features of **1a** and **1b** with respect to **2**.

The comparison of the results obtained in the CD experiments (Figures 1 and 2) with those obtained in a preliminary investigation carried out on calf thymus DNA[20] demonstrates that the condensation properties of the cationic formulations depend on the molecular structure of the cationic amphiphile as well as on the nature of DNA. Under the same experimental conditions, in fact, only the CD spectra of calf thymus DNA complexed by **1a** and **1c** showed the transition to a ψ-phase.

The reported results show the importance of a correlation between physicochemical properties and biological features and confirm the unique properties of geminis with respect to "conventional" amphiphiles. According to this investigation, the condensation capability seems to be an important property for transfection.

Materials and Methods

Materials. A plasmid DNA containing GFP reporter gene under the control of CMV ubiquitous promoter was purified with Endo free maxi plasmid kit (Qiagen) and resuspended in H_2O. Geminis amphiphiles were prepared by quaternization in acetone at room temperature of the corresponding terziary amine by 1-bromoehexadecane and crystallized from acetone. *N*,*N*-Dimethyl-*N*-hexadecyl-*N*-(2-methoxyethyl)ammonium bromide was prepared by quaternization of *N*,*N*-dimethyl-*N*-hexadecylammine by 2-bromethyl methyl ether.

Lipoplex Preparation. Monodispersed 100 nm liposomes were prepared according to the extrusion protocol.[21] Liposomes at 1/1 DMPC/CA ratios were prepared at constant total lipid concentrations (170 μM). In the case of liposomes containing surfactant **3**, the total lipid concentration was varied, by doubling the concentration of CA, to keep constant the total number of headgroups and tails.

DNA/DMPC/CA complexes (lipoplexes) were prepared by addition, at room temperature, of known volumes of an aqueous 2 mM solution of plasmid in HEPES buffer (5 mM HEPES, 0.1 mM EDTA, at pH 7.4) to monodispersed suspensions of liposomes composed by equimolar amounts of DMPC and CA in HEPES buffer; formulations containing lower concentration of CA did not yield appreciable condensation, whereas those containing higher concentrations yield condensation analogous to DMPC/CA 1/1. Concentration of plasmid was dictated by the concentration of CA in order to obtain a $+/- = 2$ charge ratio.

CD Experiments. CD measurements were carried out with a Jasco spectropolarimeter J-715 (Jasco, Easton, MD). Spectra were acquired in a 0.5-cm path length quartz cuvette. Spectra were measured as the average of three scans from 220 to 350 nm at a scan rate of 50 nm/min.

CD spectra were registered at $T = 303$ K immediately after the addition of DNA to the liposome suspension ($t = 0$), after 30 min, 12 h, and 24 h ($t = 24$ h). At time 30 min, no significant change was observed with respect to $t = 0$; no variation was observed between 12 and 24 h, and most measurements were consequently taken for opportunity at 24 h.

Biological Evaluation. Three cell lines were used for in vitro experiments: COS-7 cells and human fibroblasts were grown in DMEM (Dulbecco Modified Eagle's Medium, GYBCO-BRL) with 10% FCS (fetal calf serum, Sigma), antibiotics, (penicillin/streptomycin, GYBCO-BRL). LA7 (rat mammary adenocarcinoma cell line) were grown in the same medium supplemented with insulin and hydrocortisone (50 ng/mL each).

The toxicity of different liposome formulations was first tested in vitro at different concentrations ranging from 425 μM to 8.5 μM as reported in Table 1, where results of a typical experiment are reported. After 6 h of incubation with liposomes, the medium was substituted with fresh medium. After 24 h, cell viability was determined with Trypan blue exclusion dye. Experiments were carried out on duplicate at least five times. The nontoxic concentrations were then chosen for further experiments.

CMVGFP plasmid DNA (500 ng) was condensed with different lipoplex for 15 min or 24 h and run on 1% agarose gel in order to evaluated the ability of liposomes to condense DNA. COS-7, LA7, and human fibroblast cell lines were transfected with different preparations of liposomes and compared with a commercial kit (Fugene, Roche). The same amount of plasmid DNA (500 ng) was used for all the preparations. Experiments were carried out six times in duplicate on the formulation that showed no toxicity ([DMPC] = 8.5 μM, [**1**] or [**2**] = 8.5 μM, [plasmid DNA] = 8.5 μM for charge ratio CA/DNA $+/- = 2$; [DMPC] = 4.25 μM, [**1**] or [**2**] = 4.25 μM, [plasmid DNA] = 8.5 μM for charge ratio $+/- = 1$; [DMPC] = 8.5 μM, [**3**] = 17 μM, [plasmid DNA] = 8.5 μM for charge ratio $+/- = 2$; [DMPC] = 4.25 μM, [**3**] = 4.25 μM, [plasmid DNA] = 8.5 μM for charge ratio $+/- = 1$). Transfection efficiency was evaluated by observation of GFP positive cells with a fluorescence inverted microscope (Nikon).

Acknowledgment. The technical assistance of E. Mira Catò is acknowledged. This is manuscript No. 82 of Genoma 2000 Project funded by Cariplo. Funded in part by FIRB-MIUR No. RBNE019J9W and by Progetto Nazionale Cellule Staminali No CS3.

References

(1) Pedroso de Lima, M. C.; Simoes, S.; Pires, P.; Faneca, H.; Duzgunes, N. Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications. *Adv. Drug Delivery Rev.* **2001**, *47*, 277−294.

(2) Lasic, D. D. Novel applications of liposomes. *Trends Biotechnol.* **1998**, *16*, 307−321.

(3) Menger, F. M.; Keiper, J. S. Gemini Surfactants. *Angew. Chem., Int. Ed.* **2000**, *39*, 1906−1020.

(4) Camilleri, P.; Kremer, A.; Edwards, A.; Jennings, K. H.; Jenkins, O.; Marshall, I.; McGregor, C.; Nevelle, W.; Rice, S. Q.; Smith, R. J.; Wilkison, M. J.; Kirby A. J. A novel class of cationic Gemini surfactants showing efficient in vitro gene transfection properties. *Chem. Commun.* **2000**, 1252−1254.

(5) Kirby, A. J.; Camilleri, P.; Engberts, J. B. F. N.; Feiters, M. C.; Nolte, R. J. M.; Söderman, O.; Bergsma, M.; Bell, P. C.; Fielden, M. L.; García Rodríguez, C. L.; Guédat, P.; Kremer, A.; McGregor, C.; Perrin, C.; Ronsin, G.; van Eijk, M. C. P. Gemini surfactants: New synthetic vectors for gene transfection. *Angew. Chem., Int. Ed.* **2003**, *42*, 1448−1457.

(6) Zuidam, N. J.; Hirsch-Lerner, D.; Margulies, S.; Barenholz, Y. Lamellarity of cationic liposomes and mode of preparation of lipoplexes affect transfection efficiency. *Biochim. Biophys. Acta* **1999**, *1419*, 207−220.

(7) Cherng, J.-Y.; Schuurmans-Nieuwenbroek, N. M. E.; Jiskoot, W.; Talsma, H.; Zuidam, N. J.; Hennink, W. E.; Crommelin, D. J. A. Effect of DNA topology on the transfection efficiency of poly((2-dimethylamino)ethyl methacrylate)-plasmid complexes. *J. Controlled Release* **1999**, *60*, 343−353.

(8) Kennedy, M. T.; Pozharski, E. V.; Rakhmanova, V. A.; MacDonald, R. C. Factors governing the assembly of cationic phospholipid-DNA complexes. *Biophys. J.* **2000**, *78*, 1620−1633.

(9) Kamiya, H.; Yamazaki, J.; Harashima, H. Size and topology of exogenous DNA as determinant factors of transgene transcription in mammalian cells. *Gene Ther.* **2002**, *9*, 1500−1507.

(10) Karlsson, L.; van Eijk, M. C. P.; Söderman, O. Compaction of DNA by Gemini Surfactants: Effect of Surfactant Architecture. *J. Colloid Interface Sci.* **2002**, *252*, 290−296.

(11) Braun, C. S.; Jas, G. S.; Choosakoonkriang, S.; Koe G. S.; Smith J. G.; Middaugh C. R. The structure of DNA within cationic lipid/DNA complexes. *Biophys. J.* **2001**, *84*, 1114−1123.

(12) Gluzman, Y. SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **1981**, *23*, 175−182.

(13) Musio, A.; Montagna, C.; Zambroni, D.; Indino, E.; Barbieri, O.; Citti, L.; Villa, A.; Ried, T.; Vezzoni, P. Inhibition of BUB1 results in genomic instability and anchorage-independent growth of normal human fibroblasts. *Cancer Res.* **2003**, *63*, 2855−2863.

(14) Dulbecco, R.; Bologna, M.; Unger, M. Role of Thy-1 antigen in the in vitro differentiation of a rat mammary cell line. *Proc Natl Acad Sci U.S.A.* **1979**, *76*, 1848−1852.

(15) Sacco, M. G.; Zecca, L.; Bagnasco, L.; Chiesa, G.; Parolini, C.; Bromley, P.; Cato, E. M.; Roncucci, R.; Clerici, L. A.; Vezzoni, P.A transgenic mouse model for the detection of cellular stress induced by toxic inorganic compounds. *Nat. Biotechnol.* **1997**, *15*, 1392−1397.

(16) Maniatis, T.; Fritsch, E. F.; Sambrook, J. *Molecular cloning: A laboratory manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 1982.
(17) Hanlon, S.; Brudno, S.; Wu, T. T.; Wolf, B. Structural transitions of deoxyribonucleic acid in aqueous electrolyte solutions. I. Reference spectra of conformational limits. *Biochemistry* **1975**, *14*, 1648−1660.
(18) Keller, D.; Bustamante, C. Theory of the interaction of light with large inhomogeneous molecular aggregates. II. Psi-type circular dichroism. *J. Chem. Phys.* **1986**, *84*, 2972−2980.
(19) Zuidam, N. J.; Barenholz, Y.; Minsky, A. Chiral DNA packaging in DNA-cationic liposome assemblies. *FEBS Lett.* **1999**, *457*, 419−422.
(20) Bombelli, C.; Borocci, S.; Diociaiuti, M.; Galantini, L.; Luciani, P.; Mancini, G. Role of the Spacer of Cationic Gemini Amphiphiles in the Condensation of DNA (unpublished results).
(21) Hope, M. J.; Nayar, R.; Mayer, L. D.; Cullis, P. R. In *Liposome Technology*, 2nd ed.; Gregoriadis, G., Ed.; CRC Press: Boca Raton, FL, 1992; Vol. 1, Chapter 8.

JM050477R