Brief Articles

PEGylated Lipoplexes: Preparation Protocols Affecting DNA Condensation and Cell Transfection Efficiency

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Received October 27, 2006

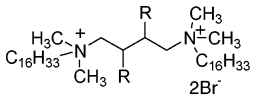
The inclusion of poly(ethylene glycol) monolaurate in liposomes formulated with dimyristoyl-*sn*-glycero-3-phosphocholine and certain cationic gemini surfactants improves their capability of condensing DNA into a ψ phase and transfecting it into cells. Both the condensation, observed by circular dichroism, and the transfection efficiency are strongly effected by the protocol of inclusion of the polymer in the formulations. The highest transfection efficiency is observed in correspondence of the highest extent of DNA condensation.

Introduction

A major problem with colloidal particles, such as liposomes, for drug and gene delivery is that therapeutic activity may be completely lost due to capture and digestion of liposomes by the reticuloendothelial system (RES), chiefly macrophages in the liver and spleen.[1] It has been suggested that liposome uptake by macrophages takes place via adsorption of plasma macromolecules, mostly proteins of the immune system, called opsonins;[2] this is why this process is commonly known as opsonization. A modification of the liposomes' surface by attaching poly(ethylene glycol) (PEG) polymers[3] was therefore introduced to reduce opsonization. The reduction in the extent of uptake by macrophages is the result of a decreased association of opsonins with the liposomes because the inert polymer prevents close approach of macromolecules.[4]

We have recently reported that liposomes formulated with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and cationic gemini surfactants **1** and **2** (Chart 1) are able to efficiently transfect DNA in various cell lines, and the transfection efficiency was correlated to their capability of condensing DNA related to the spacer stereochemistry, liposome formulated with gemini **1c** being the most efficient.[5] In perspective, the investigation in vivo of these liposomes requests the inclusion in the formulation of a PEG polymer. The inclusion of a new component in a liposome formulation modifies its organization and hence its physicochemical and biological features, and we believe that a gradual increase of the complexity of the formulation may help to clarify the role of each component in the organization of the lipid vesicles. Circular dichroism investigations reported herein are aimed at inquiring if, and to what extent, the condensation of plasmid DNA is affected by the inclusion, and by the procedure of inclusion, of poly(ethylene glycol) monolaurate (PEG-ML) in the liposomes formulated with DMPC and any of the cationic amphiphiles (CA) reported in Chart 1, (2*S*,3*S*)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonium)butane dibromide (**1a**), (2*R*,3*R*)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonium)butane dibromide (**1b**), (2*R*,3*S*)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonium)butane dibromide (**1c**), 1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonium)butane dibromide (**2**), and *N*,*N*-dimethyl-*N*-hexadecyl-*N*-(2-methoxy)ethylammonium bromide (**3**). The transfection efficiency of the DNA/liposomes complexes, lipoplexes, was evaluated on a COS-7 cell line and correlated to the extent of condensation of DNA observed by CD.

Chart 1. Structures of the Cationic Amphiphiles



1, R=OCH_3 **1a:** 2S, 3S
1b: 2R, 3R
1c: 2R, 3S

2, R=H

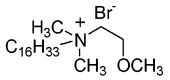


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Table 1. Concentrations of the Components of PEGylated DMPC/CA/DNA Lipoplexes

lipid lipoplex	DMPC μM	**1** and **2** μM	**3** μM	PEG-ML μM
CA/DNA = 1, DNA = 85 μM	42.5	42.5	85	4.25
CA/DNA = 2, DNA = 85 μM	85	85	170	8.5

Results

All CD and biological evaluation experiments were carried out on both PEGylated and non-PEGylated lipoplexes, prepared at two [cationic head group]/[DNA single base] ratios ([cationic head group]/[DNA single base] = 1 and 2, i.e., +/− = 1 and

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10.1021/jm061260f CCC: $37.00
Published on Web 10/31/2007

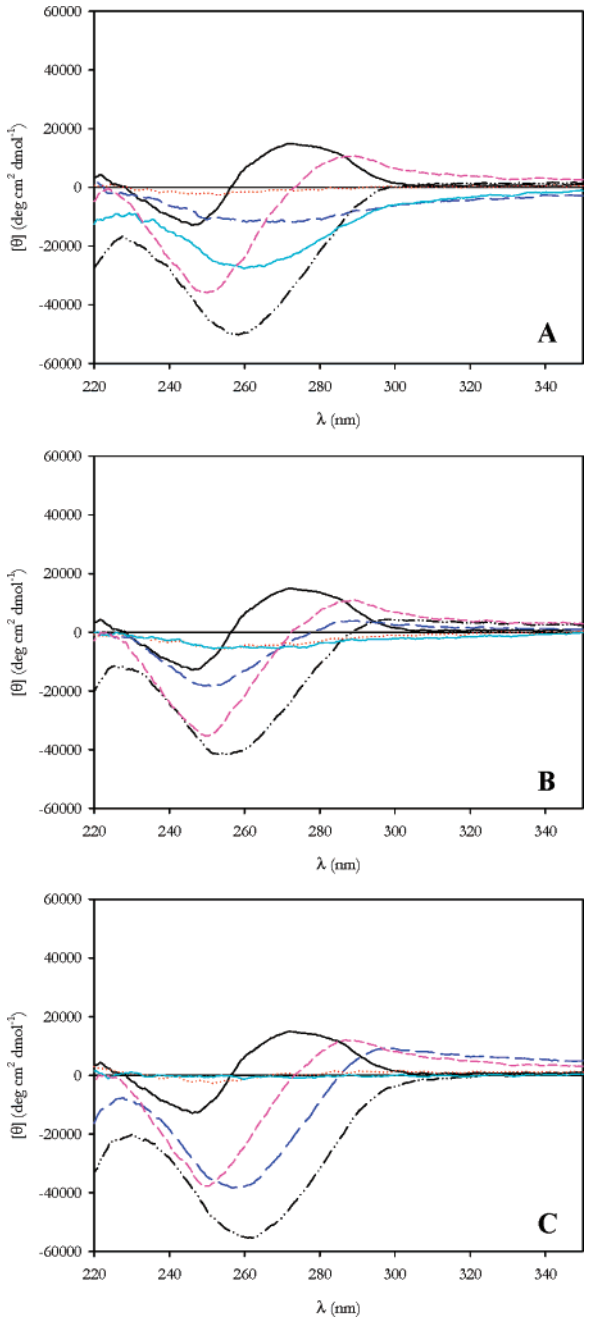

Figure 1. CD spectra of DMPC/CA/PEG-ML/plasmid DNA lipoplexes (**1a**, blue long dash; **1b**, red dotted; **1c**, black dash−dot−dot; **2**, cyan solid; **3**, pink short dash) at a charge ratio +/− = 2, performed at t = 24 h and prepared according to (A) protocol **A**, (B) protocol **B**, and (C) protocol **C**. The black solid line is the CD spectrum of uncondensed DNA (DNA/DMPC).

2 charge ratio, respectively), as described in the experimental details reported as Supporting Information (SI), at the concentrations reported in Table 1.

PEGylated lipoplexes were prepared by addition of known volumes of an aqueous 2 mM solution of plasmid in HEPES buffer to liposome suspensions obtained either by dissolving PEG-ML in chloroform together with lipids (protocol **A**) or by hydrating standard dried lipid films with HEPES buffer containing PEG-ML (protocol **B**). In a third protocol (protocol **C**), PEG-ML was added only after the formation of lipoplexes.

CD Experiments. Circular dichroism allows detection of DNA condensation through the appearance of the so-called ψ

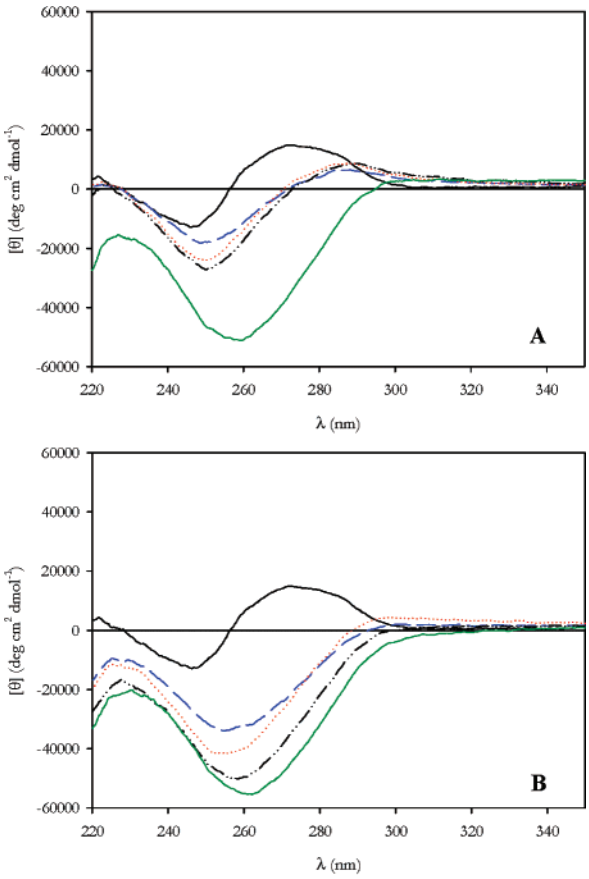

Figure 2. CD spectra of DMPC/**1c**/PEG-ML/plasmid DNA lipoplexes (standard liposomes without PEG, blue long dash; protocol **A**, black dash−dot−dot; protocol **B**, red dotted; protocol **C**, green solid line) at a +/− = 2 charge ratio, registered at (A) t = 0 and (B) 24 h. The black solid line is the CD spectrum of uncondensed plasmid DNA.

anomalies, that is, 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.[6] It is, in fact, hypothesized that the high negative ellipticity showed by ψ-DNA is due to a high extent of compaction of the macromolecule into a cholesteric-like phase,[6b] though, to the best of our knowledge, there is no definite evidence of the structure of ψ-DNA.

We observed the features of condensed DNA only in the CD spectra of some of the cationic lipoplexes (at +/− = 2 ratio); therefore, we report here only results relative to the cationic lipoplexes. Results relative to neutral lipoplexes are available as SI.

The CD spectra of the considered cationic lipoplex suspensions formulated with 5% PEG-ML, included according to protocols **A**, **B**, and **C** (described in the SI), performed at t = 24 h, are reported in Figure 1.

All liposomes formulated with gemini surfactant **1c** exhibit an extraordinary condensing capability, as the CD spectra of the corresponding lipoplexes show ψ anomalies; however, the extent of condensation depends on the protocol of inclusion of PEG-ML according to the following order, **C** > **A** > **B**. The formation of ψ-DNA is observed also in lipoplexes formulated with **1a** and prepared according to protocols **B** and **C** and in those formulated with **2** and prepared according to protocol **A**. Strong variations with respect to the conservative spectrum of B-DNA (black solid line) are also observed in all lipoplexes formulated with **3**, though the shape of the bands is not

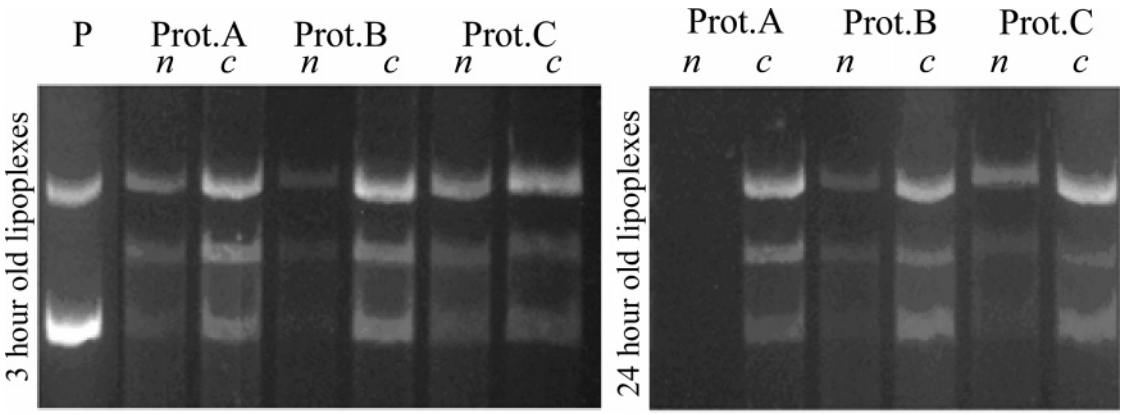

Figure 3. Neutral (n) and cationic (c) lipoplexes formulated with DMPC/**1c**, prepared either after 3 (upper panel) or 24 h and PEGylated according to the described protocols (Prot. A., Prot. B, and Prot. C) treated with DNase I. Lane P (plasmid) corresponds to untreated DNA.

diagnostic of a ψ phase and therefore of condensed DNA. We observed precipitation of all PEGylated lipoplexes formulated with **1b**, of the lipoplexes formulated with **1a** according to protocol **A**, and of those formulated with **2** according to protocols **B** and **C**; consequently, in these cases, CD experiments did not provide information on DNA phase. Note that all non-PEGylated **1**- and **2**-containing lipoplexes show ψ anomalies, as reported previously.[5]

The comparison of the CD spectra of all PEGylated lipoplexes formulated with **1c** with the spectrum of the corresponding lipoplexes formulated in the absence of PEG-ML (Figure 2), taken at $t = 0$ (panel A) and 24 h (panel B), evidences that the inclusion of PEG-ML in the formulation, by each of the protocols, induces a higher extent of condensation of DNA with respect to non-PEGylated lipoplexes. Moreover, the comparison of spectra taken at $t = 0$ and 24 h shows that the organization of lipoplexes is faster when PEG-ML is included after the addition of DNA (protocol **C**). The analogous comparison of PEGylated and non-PEGylated **1a**-containing lipoplexes, reported as SI, shows that only the inclusion of PEG-ML according to protocol **C** induces an extent of condensation higher than that observed in the absence of PEG-ML and that the organization of lipoplexes is slow for all of the protocols.

Enzyme Degradation Evaluation. Condensation of DNA should protect the macromolecule from degradation by nucleases. We carried out a DNase I protection assay in order to verify the extent of protection from nuclease attack conferred to DNA by the compaction mediated by cationic liposomes formulated with PEG-ML.[7] All PEGylated and non-PEGylated lipoplexes were treated with DNase I either 3 or 24 h after complex preparation. A plasmid DNA run on agarose gel showed two or three bright bands corresponding to different forms of DNA; DNA damaged by nuclease attack did not show bright bands. Results are very similar for all formulations. A high protection of the nucleic acid can be better achieved in cationic rather than in neutral lipoplexes, as shown in Figure 3, where we report, as an example, only results relative to the formulations that contain **1c**. The protection cannot be correlated to the length of incubation of lipoplexes (3 or 24 h), to the nature of CA, to the presence of PEG-ML, nor to the protocol of its inclusion.

Transfection Experiments. CMV-GFP plasmid was transfected in the COS-7 cell with PEGylated and non-PEGylated lipoplexes and with two commercial kits (Fugene and Dosper, Roche) as controls. In particular, three Fugene/DNA mixtures, F1, F2, and F3, corresponding to different amounts of DNA (0.2, 0.4, and 0.8 μg, respectively), and a single Dosper/DNA mixture (3/1.5 μg) were used. Cells were observed at 24 and 48 h. In the controls, the best transfection efficiency was observed for F3 and the worst one for Dosper.

Table 2. Number of Green Cells Observed at 48 h by Fluorescence Inverted Microscopy

	green cell number/mm^2	
transfecting agent	3 h old lipoplexes	24 h old lipoplexes
DMPC/**1a**	10	20
DMPC/**1a**/PEG (A)	10	20
DMPC/**1a**/PEG (B)	20	30
DMPC/**1a**/PEG (C)	40	8
DMPC/**1b**	5	10
DMPC/**1b**/PEG (A)	15	20
DMPC/**1b**/PEG (B)	20	30
DMPC/**1b**/PEG (C)	30	40
DMPC/**1c**	80	70
DMPC/**1c**/PEG (A)	80	125
DMPC/**1c**/PEG (B)	100	140
DMPC/**1c**/PEG (C)	100	150
DMPC/**2**	40	70
DMPC/**2**/PEG (A)	60	110
DMPC/**2**/PEG (B)	80	110
DMPC/**2**/PEG (C)	90	120
F2	80	
F3	150	

In the evaluation of lipoplexes, the expression of green fluorescent protein (GFP) was obtained only with cationic complexes, whereas neutral lipoplexes were not able to transfer DNA into cells, this in analogy to what was observed previously in the absence of PEG.[5]

Higher transfection efficiency was observed at 48 h in the cells treated with both the liposomes incubated for 3 and 24 h. As reported previously, non-PEGylated liposomes formulated with **3** were not able to transfer DNA, whereas all of those formulated with a gemini showed the ability to transfer DNA, cationic lipoplexes formulated with **1c** being the best transfecting agent.

PEGylated lipoplexes formulated with **3** were not able to transfect cells, whereas all PEGylated lipoplexes formulated with geminis transfected cells more efficiently than those in the absence of PEG-ML, according to the same trend observed for non-PEGylated lipoplexes,[5] that is, **1c** > **2** > **1a** = **1b**, as shown in Table 2, where the number of green cells observed by fluorescence-inverted microscopy at 48 h is reported. Lipoplexes formulated with **1c** and **2** were more efficient than F1 and F2, which were comparable with F3. The efficiency of transfection of the Dosper liposomal transfection reagent was lower with respect to our lipoplexes, though the DNA concentration was

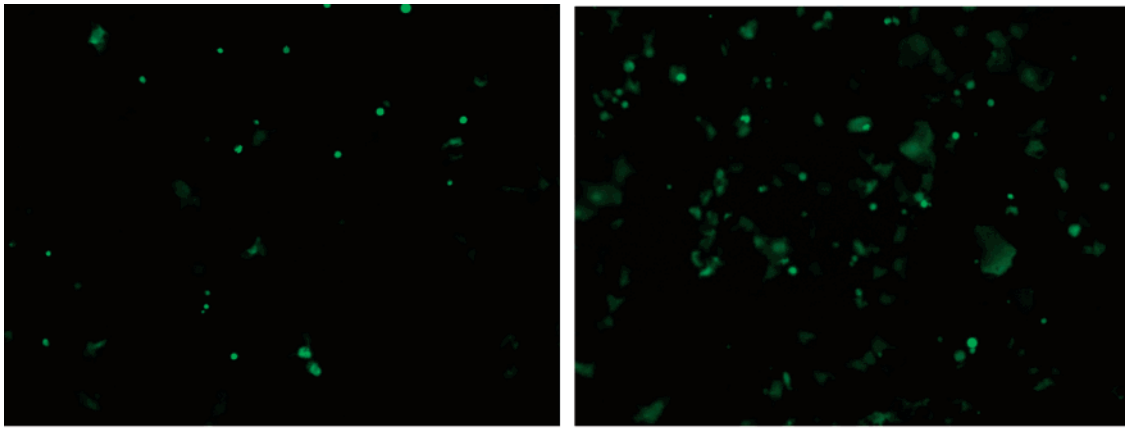

Figure 4. COS-7 cell line observed at 48 h after transfection with 0.5 μg of plasmid DNA included in (A) non-PEGylated liposomes formulated with gemini **1c** and (B) PEGylated lipoplexes formulated with gemini **1c** according to protocol **C** (lipoplexes incubated for 24 h), using a 20× magnification Nikon inverted fluorescence microscope.

higher than that in our formulations (1.5 versus 0.5 μg, as suggested by standard protocol).

The formulations prepared by protocol **C** resulted in being more efficient than those prepared by protocols **A** and **B** so that the most efficient lipoplexes resulted from those formulated with **1c** according to protocol **C**; note that an analogous expression of green fluorescent protein was obtained with control F3, that is, in correspondence with a much higher amount of plasmid (0.8 μg in F3 versus 0.5 μg in DMPC/**1c** liposomes). Figure 4 compares microscope images obtained in the transfection experiments with non-PEGylated and PEGylated lipoplexes (protocol **C**) formulated with gemini **1c**.

Cellular Uptake of *N*-Rh−PE-Labeled Lipoplexes. The cellular uptake of the most efficient formulations, namely those containing **1c**, was evaluated using 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (*N*-Rh−PE)-labeled lipoplexes in order to investigate the role of the presence of PEG-ML in cell internalization. Experiments were performed on lipoplexes prepared after 3 and 24 h. After 6 h of incubation with the cells, nonbound lipoplexes were washed with trypan blue to quench the fluorescence of external lipoplexes, non-quenched fluorescence thus indicating only internalized complexes. The results, reported as SI, show that the cell uptake experiment is scarcely reproducible and that cell uptake is very modest in all cases.

Discussion

In the last years, several in vivo studies[8] pointed out the importance of an appropriate coating on drug delivery systems in order to avoid a quick and undesired digestion of active principles in blood circulation. The addition of poly(ethylene glycol), PEG, functionalized with a hydrophobic anchor, represents one of the most efficient and reliable methods of liposome stabilization and protection. It has been demonstrated that subtle changes in formulations, such as, for example, the configuration of a stereogenic center on the polar head group of one of the formulation components[5] or the length of the hydrophobic chain,[8d] can produce effects on the morphology and/or on the biological activity of the delivered systems and on the active principle nucleic acid in our case. Therefore, the evaluation of the effects of the addition, and of the procedure of inclusion, of PEG-ML on the physicochemical and biological features of lipoplexes can be an important issue in the design of efficient transfecting formulations.

The CD spectra reported in Figures 1 and 2 show that either the chiroptical features of DNA or the solubility of lipoplexes are effected not only by the inclusion of PEG-ML in the formulation but also by the procedure of inclusion. In particular, the chiroptical features of ψ-DNA in cationic complexes formulated with **1a** and **1c** are remarkably enhanced by the presence of PEG-ML (the broadest effect being observed in lipoplexes prepared according to protocol **C**). It is conceivable that, in general, the inclusion of PEG-ML in the formulation induces a higher compaction of DNA by dehydrating the gemini head groups. The addition of PEG-ML to the formed lipoplexes (protocol **C**) allows a better organization of the polymer on the surface of the lipoplexes, whereas its inclusion according to either protocol **A** or **B** entraps in the internal layer of the vesicles an amount of polymer that does not interact with DNA, probably, requesting a longer time for reorganization. The inclusion of PEG-ML in particles formulated with **3** induces chiroptical variations as well, though these cannot be ascribed to the formation of ψ-DNA, whereas the inclusion in formulations containing either **1b** or **2** induces precipitation of lipoplexes in most instances.

The biological evaluation showed, on the one hand, that the protection of complexed DNA from enzymatic degradation cannot be correlated with the composition of formulations, to their protocol of preparation, or to the extent of DNA condensation. On the other hand, the results of cell transfection showed that, at least in some cases, the biological feature of lipoplexes can be correlated to their physicochemical features. In fact, the highest transfection was obtained in correspondence to the formulation characterized by the highest extent of condensation of DNA, that is, with PEGylated lipoplexes formulated with **1c** and prepared according to protocol **C**. A good transfection efficiency was also observed with lipoplexes formulated with **1a** according to protocol **C**, still in correspondence to a high extent of ψ-DNA observed by CD. Unfortunately, because of precipitation, nothing can be said about the extent of DNA condensation in the other formulations that gave transfection; however, no GFP expression was obtained in the absence of a ψ phase. In fact, the PEGylated liposomes formulated with the single head group−single tail surfactant **3** did not induce the transition of B-DNA to ψ-DNA, nor transfected COS-7 cells, thus confirming the exceptional colloidal features of geminis with respect to the corresponding "conventional" amphiphile.

The cell uptake experiments carried out on PEGylated and non-PEGylated lipoplexes labeled with *N*-Rh−PE did not allow us to make a hypothesis on the mode by which the presence of PEG-ML influences the transfection efficiency. However, the low cell uptake of labeled lipids in the presence of relevant transfection would imply that either DNA internalization or its transcription is independent of lipid internalization.

Summarizing, the addition of PEG-ML improves the transfection efficiency of cationic lipoplexes formulated with gemini surfactants **1** and **2** with respect to the corresponding formulations in the absence of PEG-ML. The efficiency of the transfecting lipoplexes can be correlated to the extent of DNA condensation and to the protocol of inclusion of PEG-ML. In particular, the highest transfection efficiency was observed in correspondence to the addition of PEG after the formation of lipoplexes. Note that, generally, PEGylated lipids are included in the formulation in the phase of film preparation, therefore, before hydration of the liposomes.[9]

Acknowledgment. We acknowledge contributions by CNR, Dipartimento di Progettazione Molecolare, by a EUROSTELLS grant (STELLAR) and by Progetti NOBEL (Cariplo Fondation) to Paolo Vezzoni. We are grateful to Dr. Matteo Guerrini for technical assistance in FACS analysis.

Supporting Information Available: Experimental details (S1); CD spectra of neutral (charge ratio +/− = 1) DMPC/CA/PEG-ML/plasmid DNA lipoplexes prepared according to different protocols, at $t = 0$ (S2) and 24 h (S3); comparison of the CD spectra of cationic (charge ratio +/− = 2) lipoplexes formulated with **1a** in the absence and in the presence of PEG-ML, included according to different protocols (S4); and the percentage of rhodamine positive cells sorted by FACS in cellular uptake experiments carried out with DMPC/**1c** lipoplexes (S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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JM061260F