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Gemini surfactant dimethylene-1,2-bis(tetradecyldimethylammonium bromide)-based gene vectors: A biophysical approach to transfection efficiency

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ABSTRACT

Cationic liposomes have been proposed as biocompatible gene delivery vectors, able to overcome the barriers imposed by cell membranes. Besides lipids, other surfactant molecules have been successfully used in the composition of gene carriers. In the present work, we used a Gemini surfactant, represented by the general structure $[C_{14}H_{29}(CH_3)_2N^+(CH_2)_2N^+(CH_3)_2C_{14}H_{29}]2Br^-$ and herein designated 14–2–14, to prepare cationic gene carriers, both as the sole component and in combination with neutral helper lipids, cholesterol and DOPE. The effectiveness of three Gemini-based formulations, namely neat 14–2–14, 14–2–14:Chol (1:1 molar ratio) and 14–2–14:Chol:DOPE (2:1:1 molar ratio), to mediate gene delivery was evaluated in DNA mixtures of +/− charge ratios ranging from 1/1 to 12/1. After ruling out cytotoxicity as responsible for the differences observed in the transfection competence, structural and physical properties of the vector were investigated, using several techniques. The size and surface charge density (zeta potential) of surfactant-based structures were determined by conventional techniques and the thermotropic behaviour of aqueous dispersions of surfactant/lipid/DNA formulations was monitored by fluorescence polarization of DPH and DPH-PA probes. The capacity of lipoplexes to interact with membrane-mimicking lipid bilayers was evaluated, using the PicoGreen assay and a FRET technique. Our data indicate inefficiency of the neat 14–2–14 formulation for gene delivery, which could result from the large dimensions of the particles and/or from its relative incompetence to release DNA upon interaction with anionic lipids. The addition of cholesterol or cholesterol and DOPE conferred to Gemini-based gene carrier transfection activity at specific ranges of +/− charge ratios. Fluorescence polarization data suggest that an order parameter within a specific range was apparently needed for complexes to display maximal transfection efficiency. The transfection-competent formulations showed to be efficiently destabilized by interaction with different anionic and zwitterionic bilayers, including those containing PS and cardiolipin. These data are discussed in terms of the potential of these formulations to address different intracellular targets.

1. Introduction

Ever since Felgner and co-workers [1] found that cationic lipids have a strong potential to mediate intracellular delivery of genetic material, the development of new lipid-like molecules has become one of the main goals of lipid-based technologies. Lipoplexes, i.e. complexes composed of cationic lipid-based nanostructures and DNA, have been developed as vehicles to deliver DNA into cells of different tissues, providing the possibility to reach specific target cells and to overcome enzymatic degradation, and cell and nuclear membrane barriers [2,3].

Cationic lipid-like amphiphiles not only package DNA, thereby protecting it from DNAase degradation, but also mediate the translocation across negatively charged membrane barriers, in order to guarantee the delivery of the gene cargo into the nucleus [4] or mitochondria [5].

Although the molecular details on the mechanism by which cells internalize particles are still poorly understood, the current evidence supports the hypothesis that cationic amphiphile-based vectors enter cells through the endocytic pathway [4]. In this regard, the endosome

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPH-PA, 3-(p-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid; FRET, Förster Resonance Energy Transfer; MLV, multillamelar vesicles; NBD-PE, N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)-phosphatidylethanolamine; PA, phosphatidic acid; PS, phosphatidylserine; Rho-PE, rhodamine-phosphatidylethanolamine; SUV, small unillamelar vesicles; CL, cardiolipin

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escape plays an important role, and a good delivery system should have the ability to promote release of the carried DNA into the cytosol, eventually leading to nuclear translocation [6]. On the other hand, if the mitochondria are the target, successful gene delivery requires carrier interaction with specific mitochondrial lipids, such as cardiolipin [5].

To improve the properties needed for efficient gene delivery and expression, additional lipid components (helper lipids), such as 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and cholesterol, have been included in the Gemini formulations [7–9]. These helper components may favour the formation of an inverted hexagonal phase (H_{II}) under physiologically relevant conditions [7,9], an event that has been referred to promote membrane fusion [10]. Besides having the propensity to be incorporated in structures of high negative curvature, cholesterol has been shown to enhance the stiffness of cationic lipid bilayers, since it increases lipid order and minimises thermotropic phase transitions [11]. This effect seems also to improve the transfection efficiency, by mechanisms probably independent of the ability to release DNA from the complexes [8]. The capacity of cholesterol for stabilizing a lamellar fluid, yet ordered, phase – liquid-ordered phase, L_o [12] – may lead to a lipid/DNA arrangement that combines stability with plasticity, two essential features of an efficient gene delivery system.

Gemini surfactants have been used as a cationic component of gene carriers [13], having the advantage of being easily prepared and offering a wide range of possibilities for structure modulation [14–16]. This feature allows the design of Gemini surfactants showing low toxicity, very low immunogenicity, high stability in biological fluids and biodegrability, which are essential requirements for a gene delivery system [13]. These surfactants are composed of a long hydrocarbon chain, an ionic group, a spacer, another ionic group and another long hydrocarbon chain, in sequence [15]. Due to the presence of a multivalent positively charged polar group [14,16], Gemini surfactants may efficiently compact the negatively charged molecule of DNA, allowing the formation of a particle small enough to be endocytosed by the cell [13]. Other Gemini surfactant features, such as the conformational flexibility provided by the spacer [17] and the double hydrocarbon chains [16], have been related with the ability of Gemini-based systems to mediate transfection. Gemini surfactants with propensity to form vesicle structures have revealed advantages in terms of transfection activity, when compared to those forming micelles, the former excluding the need of helper lipid addition [16]. The alkanediyl-α,ω-bis(alkyldimethylammonium bromide) dimeric surfactant family has been one of the most investigated with respect to its physicochemical properties [14,16] and several elements of this family have been reported as efficient transfection agents [13].

In this work, we used the Gemini surfactant dimethylene-1,2-bis (tetradecyldimethylammonium bromide) or $[C_{14}H_{29}(CH_3)_2N^+(CH_2)_2N^+(CH_3)_2C_{14}H_{29}]2Br^-$, with 14 carbon atoms in the hydrocarbon chains and 2 carbon atoms in the spacer, referred to as 14–2–14, alone or in combination with the helper lipids cholesterol and DOPE, to compare the biophysical properties of transfecting and non-transfecting formulations based on the same surfactant molecule. In previous studies performed in our laboratory, this surfactant revealed ability to mix with zwitterionic lipids forming liposomes, presenting a phase transition that undergoes modulation by the other components of the mixture, in a temperature range easily followed in laboratory.

The aim of the present work is two-fold: firstly, to explore the potential of the Gemini surfactant 14–2–14, combined or not with the helper lipids cholesterol and DOPE, as an agent for transfection; secondly, to establish a relationship between the biophysical properties of surfactant/lipid-based DNA vectors and their efficiency to promote gene expression, in order to contribute for the generation of meaningful structure–activity relationships.

2. Materials and methods

2.1. Cells

TSA cells (BALB/c female mouse mammary adenocarcinoma cell line) were maintained at 37 °C, under 5% CO_2, in Dulbecco's modified Eagle's medium–high glucose (DMEM–HG; Sigma, St. Louis, MO), supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS; Sigma, St. Louis, MO), penicillin (100 U/ml) and streptomycin (100 μg/ml). TSA cells grown in monolayer were detached by treatment with 0.25% trypsin solution (Sigma, St. Louis, MO). For *in vitro* transfection studies, 5×10^4 TSA cells were seeded in 1 ml of medium in 48-well culture plates, 24 h before transfection, and used at 50–70% confluence.

2.2. Preparation of cationic liposomes and their complexes with DNA

Small unilamellar vesicles (SUV) were prepared either from Gemini surfactant 14–2–14, surfactant plus cholesterol (1:1 mol ratio), or surfactant plus cholesterol and DOPE (2:1:1 mol ratio), by extrusion of multilamellar vesicles (MLV). Briefly, DOPE (Avanti Polar Lipids, Alabaster, AL) and/or cholesterol (Sigma, St. Louis, MO) and Gemini (synthesized in the Chemistry Department, University of Porto, according to an established procedure [15]) were dissolved in $CHCl_3$:CH_3OH (1:1) and then mixed at the desired molar ratio and dried under vacuum in a rotatory evaporator. The dried lipid films were hydrated with deionised water, unless stated otherwise, to a final lipid concentration of 2 mM. The resulting MLV were then sonicated for 3 min and extruded 21 times through two stacked polycarbonate filters of 50 nm pore diameter using a Liposofast device (Avestin, Toronto, Canada). The resulting liposomes (SUV) were subsequently diluted three times with deionised water and, for the transfection studies, filter-sterilized utilizing 0.22 μm pore-diameter filters (Schleicher & Schuell, BioScience, Germany). Lipoplexes were prepared by sequentially mixing 100 μl of a HEPES-buffered saline solution (HBS; 100 mM NaCl, 20 mM HEPES, pH 7.4) with a volume of liposomes (dependent on the desired +/− lipid/DNA charge ratio) and 100 μl of HBS solution containing 1 μg of pCMVluc encoding luciferase (a gift from Dr. P. Felgner; Vical, San Diego, CA). The mixture was further incubated for 15 min at room temperature. Lipoplexes were used immediately after being prepared.

2.3. In vitro transfection activity

TSA cells were covered with 0.3 ml of DMEM–HG without serum before lipid/DNA complexes were added. The complexes (containing 1 μg of DNA) were added gently to cells in a volume of 0.2 ml per well. After 4 h incubation (in 5% CO_2 at 37 °C) the medium was replaced with DMEM–HG and the cells were further incubated for 48 h. The cells were then washed twice with phosphate-buffered saline solution (PBS) and 100 μl of lysis buffer (1 mM DTT; 1 mM EDTA; 25 mM Tris-phosphate, pH 7.8; 8 mM $MgCl_2$; 15% glycerol; 1% v/v Triton X-100) were added to each well. The level of gene expression in the lysates was evaluated by measuring light production by luciferase in an L Max II 384 luminometer (Molecular Devices, Union City, CA). The protein content of the lysates was measured by the Dc Protein Assay reagent (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard. The data were expressed as relative light units (RLU) of luciferase per mg of total cell protein.

2.4. Cell viability

Cell viability was assessed by a modified Alamar Blue assay, as previously described [18]. Briefly, 0.4 ml of 10% (V/V) Alamar Blue dye in complete DMEM–HG medium was added to each well, 45 h after the initial transfection period (4 h). After 45 min of incubation at

37 °C, 200 μl of the supernatant was collected from each well and transferred to 96-well plates. The absorbance at 570 and 600 nm (supplier indication) was measured in a SPECTRAmax PLUS 384 spectrophotometer (Molecular Devices, Union City, CA). Cell viability (as a percentage of control cells) was calculated according to the equation

$$\frac{(A_{570}-A_{600})\text{of treated cells}}{(A_{570}-A_{600})\text{of control cells}} \times 100.$$

2.5. Physical properties (particle size, zeta potential and colloidal stability)

The size of the surfactant-based structures was assessed using a Submicron Particle Size Analyzer, Beckman Coulter N4 Plus. The colloidal suspension of the complexes was diluted with HBS, and the particle size analysis was carried out at a scattering angle of 90° and a temperature of 25 °C.

The zeta potential of the surfactant-based structures was measured using ZetaPals (Brookhaven Instruments Corporation, New York, U.S.A). The measurements were performed in the aqueous buffer HBS, at 25 °C, using disposable zeta cells and the protocol for general purposes. The zeta potential was calculated by the device software, using the Smoluchowski equation.

The turbidity of liposome and lipoplex dispersions, providing a measure of their colloidal stability, was monitored as a function of time on a SPECTRAmax PLUS 384 spectrophotometer (Molecular Devices, Union City, CA) at a wavelength of 550 nm. The final concentration of lipid plus Gemini surfactant was 0.1 mM in HBS buffer, pH 7.4. Charge ratios +/− ranging from 2/1 to 12/1 were tested and the turbidity values were registered over time.

2.6. Fluorescence polarization measurements

The probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 3-(p-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA) in dimethylformamide were injected (a few microliters) into surfactant/lipid aqueous dispersions (final surfactant/lipid concentration of 200 μM) to give a final surfactant or surfactant plus lipid/diphenylhexatriene probe molar ratio of about 200/1, as previously described [19]. A suspension of dimyristoylphosphatidylcholine (DMPC; Avanti Polar Lipids, Alabaster, AL) vesicles (SUV) was also prepared as described above, at a final concentration of 200 μM, and labelled with DPH or DPH-PA (lipid:probe molar ratio of 200:1).

Probe addition was performed at 55 °C, a temperature above the phase transition temperature of all the surfactant/lipid systems tested. The mixture was incubated at room temperature, in the dark for probe protection, for a period of 15 h to reach equilibrium. In order to correct fluorescence measurements for the contribution of light scattering, appropriate blanks with equivalent volumes of dimethylformamide were prepared, although these corrections were found to be negligible.

The fluorimetric measurements of the DPH- or DPH-PA-labelled surfactant/lipid systems, in the absence or presence of plasmid DNA at different cationic surfactant/DNA charge ratios, were performed in a Perkin Elmer LS 55B fluorescence spectrophotometer (Perkin Elmer, U.S.A), equipped with polarization filters. The excitation wavelength was set at 336 nm and the emission wavelength at 450 nm (5 nm excitation and 6 nm emission band pass). The fluorescence polarization (P) was calculated according to Shinitzky and Barenholz [20], from the equation

$$P = \frac{I_{II} - GI_{\perp}}{I_{II} + GI_{\perp}},$$

where I_{II} and $I_{\perp}$ are the intensities of the light emitted with its polarization plan parallel and perpendicular to that of the exciting beam, respectively. G, the instrumental grating factor, is given by the ratio of vertically to horizontally polarized emission components where the excitation light is polarized in the horizontal plane.

2.7. DNA protection provided by cationic surfactant/lipid formulations and lipoplex destabilization induced by anionic lipid vesicles

A PicoGreen solution was prepared in HBS (dilution, 1:200), according to the manufacturer's instructions (Molecular Probes, Eugene, OR). Cationic SUV were prepared as described above, but hydrated with HBS saline buffer. Lipoplexes containing 2 μg of pDNA, prepared in a total volume of 200 μl of HBS, at room temperature, were added to the PicoGreen solution and lipoplex destabilization induced by anionic/zwitterionic lipid vesicles was evaluated. The anionic or zwitterionic SUV were prepared, as described above, from mixtures of dioleoylphosphatidylethanolamine (PE)/dioleoylphosphatidylcholine (PC)/egg phosphatidic acid (PA) at a 2:1:1 molar ratio; PE/PC/palmitoyloleoylphosphatidylserine (PS) at 2:1:1 molar ratio; PE/PC/heart cardiolipin (CL)/Ca^{2+} at 2:1:1:1 molar ratio or PE/PC at 1:1 molar ratio and added in a two-fold molar excess. The fluorescence of PicoGreen, directly proportional to the presence of accessible/free DNA, was monitored in a Spex Fluorolog Spectrometer. The excitation wavelength was set at 485 nm and the emission wavelength at 520 nm (1 nm excitation and 2 nm emission band pass).

The amount of DNA protected by cationic lipids was calculated as follows:

$$P_{DNA} = 1 - \frac{F - F_{100}}{F_0 - F_{100}}$$

where F is the fluorescence measured after adding the complexes to the PicoGreen solution, F_0 is the maximum fluorescence of a positive control which accounts for 0% of DNA protection, and F_{100}, the minimum fluorescence of a negative control which is taken as 100% of protection. For the positive control, free DNA, in the same amount of that associated with the complexes, was used and a PicoGreen solution without DNA was used as the negative control.

2.8. Interaction of lipoplexes with anionic vesicles (lipid mixing)

We have conducted Förster Resonance Energy Transfer (FRET) experiments, in order to assess the propensity for occurrence of lipid mixing upon interaction of cationic surfactant-based vectors with anionic vesicles. For this purpose, cationic liposomes were labelled with 1% N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)-Phosphatidylethanolamine (NBD-PE) and 1% rhodamine-phosphatidylethanolamine (Rho-PE) (Molecular Probes, Eugene, OR). Aliquots of both labelled lipids in chloroform solution were added to a chloroform/methanol solution of the cationic surfactant/lipid components. The dried lipid film was hydrated in a HBS saline buffer (pH 7.4) to obtain SUV. The concentration of surfactant, or surfactant plus lipid, in the resulting liposome suspensions was 0.1 mM. Unlabelled zwitterionic or anionic SUV with the same composition as those used in the PicoGreen assay (see Section 2.7) were prepared as described above and hydrated in HBS saline buffer (pH 7.4), at a concentration of 0.4 mM. Labelled cationic liposomes and the respective lipoplexes were transferred to the cuvette of an LS 55B fluorescence spectrophotometer (Perkin Elmer, U.S.A) and treated with the unlabelled vesicles at 37 °C, in a 1:4 molar ratio. Fluorescence intensity was recorded at 535 nm (5 nm band pass), as a function of time, with excitation at 470 nm (5 nm band pass). At the concentration at which the probes were incorporated in the liposomes (1%), Rho-PE efficiently suppresses the emission of NBD-PE at 535 nm, when the excitation wavelength

was set at 470 nm. Due to the energy transfer dependence on the distance between the donor and acceptor probes, lipid mixing between fluorescently-labelled preparations and non-labelled anionic/zwitterionic vesicles is signalled by an increase of NBD-fluorescence as a consequence of probe distribution in a larger lipid environment. Control vesicles of 14–2–14:Chol:DOPE (2:1:1) or 14–2–14:Chol (1:1) were labelled with 0.2 mol.% NBD-PE and 0.2 mol.% Rh-PE, taken as the probe concentrations which are expected to be achieved when the lipid mixing of liposomes in the sample reaches its theoretical maximum. The fluorescence intensity obtained with the control vesicles was used for the normalization of measurements. All the fluorescence intensities were corrected for the scattered light intensities and for the dilution factor. The percent lipid mixing was calculated as follows:

$$\%\ \text{Lipid Mixing} = \left(\frac{R_t - R_0}{R_\infty - R_0}\right) \times 100 \qquad 1$$

where, R_t, R_0, and R_∞ are the fluorescence intensities measured at 535 nm at a given time t (R_t), prior to addition of anionic vesicles (R_0), and at infinite probe dilution obtained with control vesicles (R_∞), respectively.

2.9. Statistical analysis

Data are presented as mean result ± S.D. The significance of the results was statistically analysed by a one-way analysis of variance (ANOVA) with Tukey's multiple comparison for pairwise comparison. Statistical significance was set at $P<0.05$.

3. Results

3.1. Transfection efficiency and cytotoxicity

The gene delivery efficacy of aqueous dispersions prepared from a) the Gemini surfactant 14–2–14, b) equimolar concentrations of 14–2–14 and cholesterol or c) 14–2–14, cholesterol and DOPE at 2:1:1 molar ratio was assessed by measuring luciferase activity. Lipoplexes were prepared by simple addition of the plasmid DNA encoding luciferase to the surfactant/lipid aqueous dispersion, in order to obtain +/− charge ratios ranging from 1/1 to 12/1, which includes the theoretically neutral combination of cationic surfactant and DNA.

Fig. 1 displays the effect of the lipid composition and +/− charge ratio of the surfactant plus lipid-based lipoplexes on transfection in TSA cells. These cells were chosen on the basis of their high propensity for transfection [21]. Although essentially no gene expression was observed with lipoplexes prepared from the neat surfactant aqueous dispersion at +/− charge ratios ranging from 1/1 to 12/1 (data not shown), the incorporation of helper lipids in the formulation induced a significant increase of the biological activity, which showed a strict dependence on the lipoplex +/− charge ratio. Lipoplexes composed of 14–2–14:Chol/DNA displayed the highest transfection efficiency at a +/− charge ratio of 4/1, which progressively decreased with increasing +/− charge ratio. No significant gene expression was observed for lipoplexes prepared at charge ratios of 1/1 or 2/1. With DOPE-containing lipoplexes, a progressive increase of transfection capacity was achieved with the increase of the +/− charge ratio (from 1/1 to 8/1). However, at the highest charge ratio tested (12/1) transfection was negligible.

The most efficient formulation (14–2–14:Chol:DOPE/DNA at a +/− charge ratio of 8/1) and the current bench marks of transfection agents, Lipofectamine and Fugene, were assayed in parallel to comparatively evaluate their transfection activity in TSA cells. Although exhibiting lower transfection efficiency than lipofectamine, our formulation is significantly more efficient than Fugene in transfecting TSA cells (42% for our formulation and 25% for Fugene, with respect to lipofectamine; data not shown).

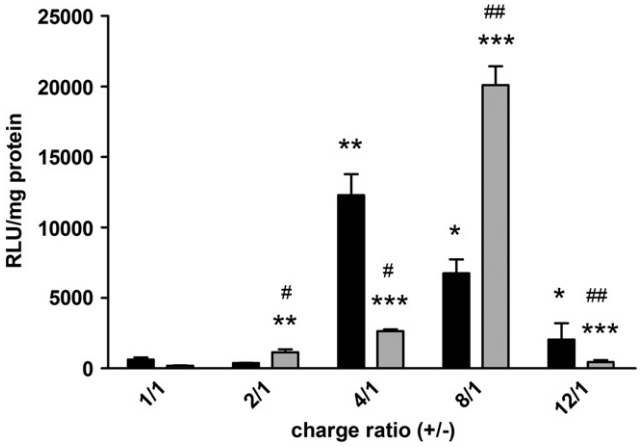

Fig. 1. Effect of lipoplex composition and +/− charge ratio on luciferase gene expression in TSA cells. Cells were covered with 0.3 ml of DMEM–HG without serum prior to the addition of cationic liposome/DNA complexes. 14–2–14:Chol (1:1) (■) and 14–2–14:Chol:DOPE (2:1:1) (■) liposomes were complexed with 1 μg of pCMVluc at the indicated lipid/DNA charge ratios. After 4 h incubation, the medium was replaced with DMEM–HG and the cells were further incubated for 48 h. The level of luciferase gene expression was evaluated as described in Materials and methods. The data are expressed as relative light units (RLU) of luciferase per mg of total cell protein (mean ± standard deviation obtained from triplicates), and are representative of at least three independent experiments. Data comparisons were performed for the following paired observations: lipoplexes at different +/− charge ratios vs the same lipoplex formulation at the immediately precedent +/− charge ratio (*$p<0.01$, **$p<0.005$, ***$p<0.001$) and DOPE-containing lipoplexes vs DOPE-free lipoplexes at the same +/− charge ratio (#$p<0.01$, ##$p<0.001$).

Fig. 2 shows how the viability of TSA cells was affected upon transfection with 14–2–14/DNA, 14–2–14:Chol/DNA or 14–2–14:Chol:DOPE/DNA complexes at the different +/− charge ratios, as assessed by a modified Alamar Blue assay [18]. The results were expressed as the percentage of cell viability with respect to a control corresponding to untreated cells. As observed, all the complexes caused relatively low toxicity (with a minimum viability observed of ca. 73% for 12/1 complexes) in TSA transfected cells, thus suggesting that the different activities of transfection observed for the various formulations are not related to any cytotoxic effect. No significant differences in the cytotoxicity between our formulation 14–2–14:

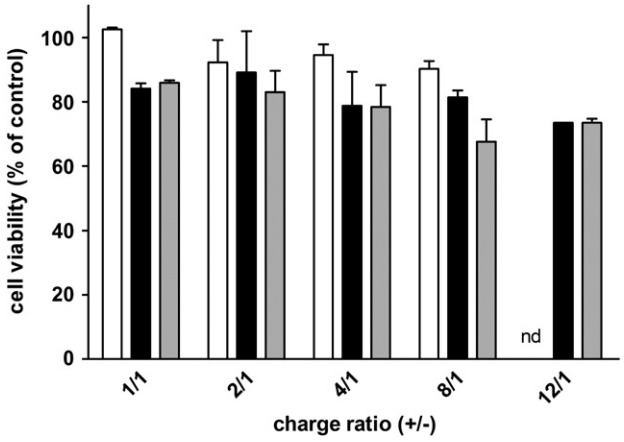

Fig. 2. Effect of lipoplex composition and +/− charge ratio on viability of TSA cells. Liposomes composed of 14–2–14 (□), 14–2–14:Chol (1:1) (■) and 14–2–14:Chol:DOPE (2:1:1) (■) were complexed with pCMVluc at the indicated +/− charge ratios. Values of cell viability measured by the Alamar Blue assay are expressed as a percentage of the untreated control cells (mean ± standard deviation obtained from triplicates) and are representative of at least three independent experiments.

Chol:DOPE/DNA at a +/− charge ratio of 8:1 and Lipofectamine or Fugene were observed in these cells (data not shown).

3.2. Physical properties (zeta potential, particle size and colloidal stability)

Fig. 3 presents the zeta potential values obtained for DNA-complexes prepared with the Gemini surfactant alone, and in combination with cholesterol (1:1) or with cholesterol and DOPE (2:1:1), at different +/− charge ratios. These three formulations showed a very similar behaviour. Complexes prepared at +/− charge ratios of 1/1 and 2/1 displayed a surface negative charge, while positive values were obtained at the 4/1 and 8/1 charge ratios.

The mean diameter of the lipoplexes, prepared from the different formulations at +/− charge ratios ranging from 1/1 to 8/1, and of the corresponding particles without DNA was determined by light scattering (Table 1). Noteworthy, the particles composed of surfactant and DNA, especially those with higher amount of surfactant, exhibited much larger size than the helper lipid-containing complexes, at the same +/− charge ratio. The lipoplexes prepared from 14–2–14:Chol and 14–2–14:Chol:DOPE dispersions showed the expected behaviour with respect to size vs +/− charge ratio [18]. That is, at charge ratios putatively closer to the neutrality the particle size observed is much larger than at more extreme charge ratios, at which lipoplexes exhibited an excess of negative and positive charges. At the +/− charge ratios of 4/1 for the 14–2–14 plus cholesterol formulation and 2/1 for the 14–2–14:Chol:DOPE formulation, sizes of ca. 700 and 1600 nm were detected respectively, while for the remaining charge ratios, smaller sizes (ca. 150–190 nm) were observed.

The colloidal stability of surfactant/lipid dispersions and corresponding lipoplexes was determined by measuring the turbidity at 550 nm, as a function of time, as illustrated in Fig. 4 for the 14–2–14:Chol:DOPE formulation. As expected, liposomes and lipoplexes with higher amounts of lipid showed the lowest turbidity (light scattering) values. For all the formulations assayed, a high stability was found within 24 h.

3.3. Characterization of the physical properties of liposomes and lipoplexes

In search of an explanation for the impressive differences in transfection ability of surfactant vs surfactant plus helper lipid formulations, a more extensive characterization of surfactant and surfactant plus lipid aqueous dispersions, as well as their respective DNA-complexes, was performed using a biophysical approach. For this purpose, we used two fluorescent probes, DPH, buried in the hydrophobic core of an amphiphilic structure, occupying the middle of a lipid bilayer, in cases where a lamellar structure is favoured [20], and DPH-PA, interacting with the headgroups of the amphiphilic molecules through its charged group [22]. Fig. 5 displays the fluorescence polarization vs temperature of DPH (A) and DPH-PA (B) embedded in preparations obtained from aqueous dispersion of surfactant Gemini or surfactant plus cholesterol or cholesterol and DOPE. For comparison, a typical thermotropic profile of DMPC is also represented, showing a characteristic sigmoidal curve with an abrupt decrease of fluorescence polarization within a very narrow temperature range, centred at 24 °C, the transition temperature (T_m). This behaviour, reflecting a phase transition from a gel or solid-ordered phase (s_o) to a liquid-crystalline or liquid-disordered (l_d) phase, is observed in pure lipids as well as in some lipid mixtures, although extended along a broader temperature range [19]. A similar thermotropic profile was obtained with the surfactant dispersion, when DPH was used as fluorescent probe (Fig. 5A). DPH fluorescence polarization derivative curves for DMPC and surfactant Gemini preparations, determined in GraphPad Prism program (Fig. 5, inset), allowed for the identification of the transition temperature midpoint (T_m) corresponding to the peak of those curves. It is noticeable the similarity of the T_m values for the aqueous dispersions of the synthetic lipid (DMPC) and the surfactant (14–2–14), which have in common the length (14 carbon atoms) of the respective hydrocarbon chains.

In contrast to the surfactant dispersion, the surfactant plus lipid formulations, namely the equimolar mixture of surfactant plus cholesterol or the mixture of surfactant plus cholesterol and DOPE (2:1:1), showed a distinct behaviour, exhibiting a monotonic temperature-dependent decrease of fluorescence polarization of DPH (Fig. 5A). Fluorescence polarization of DPH-PA decreases monotonically for all the formulations studied (Fig. 5B).

A comparison of the lipid order (taken as proportional to the P values) for the different formulations, at temperatures above 30 °C, reveals the lowest order values in surfactant dispersions and the highest in the surfactant plus cholesterol (1:1) mixture; the formulation containing surfactant, cholesterol and DOPE (2:1:1) presented intermediate P values when compared to the other formulations (Fig. 5 and Table 2).

The addition of DNA resulted in significant alterations of the physical properties of all the surfactant/lipid systems tested. As observed, DNA induces a significant increase of fluidity, in the core of the neat surfactant-based structure, at temperatures below 37 °C. In contrast, at 37 °C and temperatures above, DNA promoted an increase of lipid order, in the inner as well as in the outer regions of the surfactant structure (Table 2). The thermotropic profiles of these lipoplexes did not show any significant dependence on the surfactant/DNA charge ratio. For the formulation containing the surfactant and cholesterol, an upward trend of fluidity at lower temperatures (below 45 °C) and a downward trend at higher temperatures, upon the addition of DNA, were also observed, being particularly evident in DPH thermograms (Table 2). However, a distinct dependence of fluorescence polarization values on the surfactant/DNA charge ratio was found. The lowest value of fluorescence polarization reflecting the highest level of fluidity was achieved at the surfactant/DNA charge ratio of 4/1, in the outer as well as in the inner regions of Gemini: cholesterol structures. In contrast, the DNA-induced decrease of fluidity at 45 °C, also affecting the environments of both fluorescent probes, was more evident for the surfactant/DNA charge ratios of 2/1 and 12/1.

A very significant effect of the presence of cholesterol in lipoplexes is, as previously referred for liposomes, the increase of lipid order. As illustrated in Table 2, values of fluorescence polarization of DPH of 0.107 and 0.309 were obtained at 45 °C for lipoplexes at the +/−

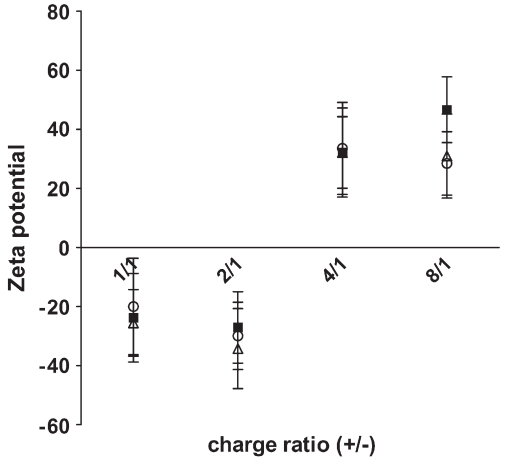

Fig. 3. Zeta potential of 14–2–14 (Δ) 14–2–14:Chol (■) and 14–2–14:Chol:DOPE (o) lipoplex dispersions in HBS buffer (pH 7.4). Values were obtained in a Brookhaven Zeta Pals, Zeta Potential Analyser, by software analysis of electrophoretic particle mobility, from which zeta potential distribution is calculated. The values represented are mean ± standard deviation, obtained for quintiplicates, in three independent experiments.

Table 1
Particle size values, in nanometers, obtained at 25 °C for 14–2–14, 14–2–14:Chol (1:1) and 14–2–14:Chol:DOPE (2:1:1) aqueous dispersions (no DNA) and corresponding lipoplexes at the indicated +/− charge ratios.[a]

Formulations	No DNA	+/− Charge ratios of lipoplexes			
		8/1	4/1	2/1	1/1
14–2–14	2737 (±270)	2168 (±467)	1694* (±321)	236 (±36)	260 (±86)
14–2–14:Chol (1:1)	139 (±28)	188 (±71)	674* (±150)	186** (±3)	172 (±8)
14–2–14:Chol:DOPE (2:1:1)	100 (±8)	173 (±36)	191* (±12)	1634** (±156)	146 (±10)

[a] Particle size values represent mean ± standard deviation of three independent experiments. Data for each formulation at the different charge ratios were compared to those for the same formulation at the immediately precedent +/− charge ratio (*$p<0.01$, **$p<0.05$).

charge ratio of 4/1 prepared from surfactant and surfactant plus cholesterol, respectively. At the same temperature and +/− charge ratio, *P* values for DPH-PA of 0.179 and 0.333 were registered for the same structures, respectively (Table 2).

For structures prepared from a mixture of surfactant, cholesterol and DOPE (2:1:1), the addition of DNA promoted an increase of lipid order over the temperature range under study, in the inner as well as in the outer regions of the lipoplex structure (Table 2). Interestingly, at 37 °C, at which temperature transfection was performed, the highest *P* values of both DPH and DPH-PA were obtained for the lipoplexes exhibiting higher transfection competence, i.e., at a +/− charge ratio of 8/1 (Table 2).

3.4. DNA protection and release

A high level of DNA protection is an important feature of gene carriers. However, efficient gene delivery also requires that genetic cargo will be released into the intracellular medium. Therefore, in order to further characterize Gemini-based systems in terms of their ability to mediate transfection, the PicoGreen fluorescence assay was used to assess both their efficiency to protect DNA and to promote its release. Since PicoGreen fluorescence is favoured by the presence of double helix DNA, an increase of PicoGreen fluorescence is interpreted as an increase of DNA exposure.

The results on the level of DNA access provided by the Gemini/lipid systems, 14–2–14, 14–2–14:Chol (1:1) and 14–2–14:Chol:DOPE (2:1:1), at several lipid/DNA +/− charge ratios are presented in Fig. 6. A very similar degree of DNA protection was conferred by the different formulations, at the same +/− charge ratios. An increase from 1/1 to 4/1 +/− charge ratio induced a significant increase of DNA protection for all the formulations assayed. A maximum DNA protection was achieved at the 4/1 +/− charge ratio, this being maintained at the +/− charge ratio of 8/1 and, predictably, for higher values as well.

The same technique was used to assess the efficiency of negatively charged or zwitterionic vesicles (see Materials and methods for vesicle composition) to destabilize lipoplexes. Anionic lipids were chosen as components of these vesicles based on their presence in endosome (PA and PS) and mitochondria (CL) membranes. PE was

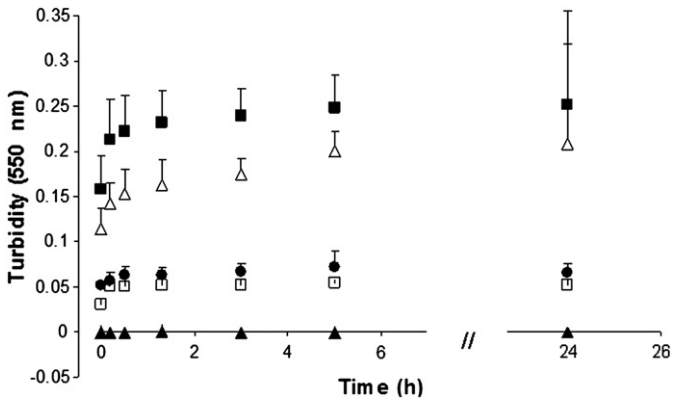

Fig. 4. Colloidal stability of 14–2–14:Chol:DOPE, 2:1:1 in aqueous dispersions (▲) and of the respective lipoplexes at +/− charge ratios of 2/1 (■), 4/1 (Δ), 8/1 (●) and 12/1 (□), monitored by turbidity measurements, at 550 nm, at different time periods. The values represent the mean ± standard deviation, obtained for triplicates, in three independent experiments.

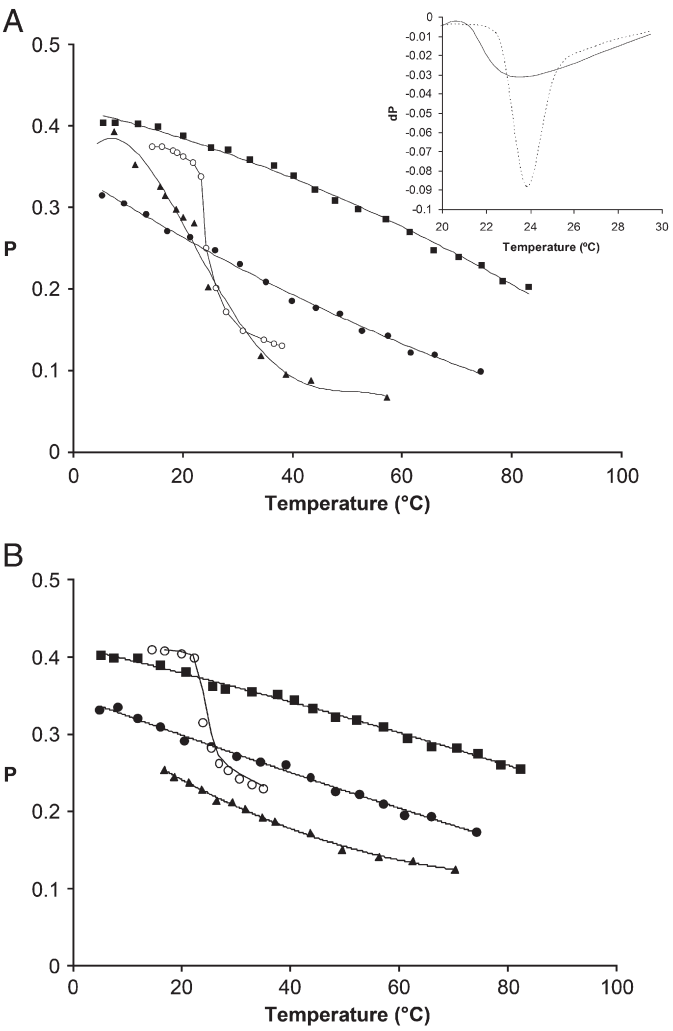

Fig. 5. Thermogram of fluorescence polarization (P) of DPH (A) and DPH-PA (B) incorporated in 14–2–14 (▲), 14–2–14:Chol (1:1) (■), 14–2–14:Chol:DOPE (2:1:1) (●) and DMPC (○) aqueous dispersions. The inset in A represents the fluorescence polarization derivative curves for DMPC (○) and surfactant Gemini (▲) preparations, determined in GraphPad Prism program, allowing to identify the transition temperature midpoint (T_m) corresponding to the peak of those curves.

Table 2
Fluorescence polarization (P) of DPH and DPH-PA, at 15, 37 and 45 °C, in 14–2–14, 14–2–14:Chol (1:1) and 14–2–14:Chol:DOPE (2:1:1) aqueous dispersions (no DNA) and respective lipoplexes at different +/− charge ratios.[a]

Probe	T (°C)	14–2–14				14–2–14:Chol (1:1)				14–2–14:Chol:DOPE (2:1:1)			
		No DNA	12/1	8/1	4/1	No DNA	12/1	4/1	2/1	No DNA	12/1	8/1	2/1
DPH	15	0.326	0.230	0.206	0.238	0.399	0.388	0.338	0.374	0.271	0.300	0.312	0.306
	37	0.095	0.118	0.124	0.152	0.351	0.350	0.327	0.340	0.209	0.224	0.236	0.236
	45	0.088	0.105	0.107	0.107	0.308	0.332	0.309	0.324	0.177	0.196	0.206	0.208
DPH-PA	15	0.253	0.261	0.272	0.238	0.389	0.383	0.341	0.381	0.309	0.314	0.334	0.320
	37	0.186	0.194	0.205	0.191	0.351	0.352	0.339	0.352	0.261	0.274	0.290	0.271
	45	0.171	0.181	0.192	0.179	0.334	0.338	0.333	0.340	0.226	0.272	0.272	0.271

[a] Lipoplexes of each formulation at different +/− charge ratios were prepared from the same pool of surfactant/lipid aqueous dispersion. Data shown for each formulation were obtained in one fluorescence polarization assay taken as typical of three independent experiments.

chosen due to its fusagenic properties and Ca^{2+} was added to CL-containing vesicles (in a 1:1 molar ratio) to increase CL propensity for H_{II} phases, hence favouring fusion. Finally, PC was included in the composition of all anionic lipid formulations, as a typical bilayer lipid (DOPC). Zwitterionic vesicles of PE and PC were also used to evaluate the importance of the anionic character of membrane lipids for DNA release.

Lipoplexes prepared from dispersions of the Gemini surfactant 14–2–14 did not exhibit significant levels of DNA exposure at any of the +/− charge ratios tested upon interaction with anionic liposomes (Fig. 7). On the other hand, helper lipids-containing lipoplexes exposed their DNA cargo at significant levels for the highest +/− charge ratios (4/1 and 8/1). For statistical purposes, results from these experiments were compared to those obtained with the respective formulation at the 1/1 (+/−) charge ratio, under which conditions DNA exposure was negligible for all the formulations. The addition of neutral vesicles (PE:PC 1:1) did not induce any significant destabilization of lipoplexes, independently of the formulation or the +/− charge ratio.

3.5. Interaction of lipoplexes with anionic vesicles (lipid mixing)

The interactions between surfactant/lipid structures or lipoplexes and the anionic or zwitterionic vesicles used in the previous section were evaluated using a Förster Resonance Energy Transfer (FRET) technique. For this purpose, the fluorescent probes NBD-PE and Rho-PE were included in the preparation of the surfactant/lipid structures and the occurrence of lipid mixing with unlabeled anionic/zwitterionic vesicles was monitored by an increase in NBD-PE fluorescence at 535 nm (excitation at 470 nm).

As observed in Table 3, both 14–2–14:Chol and 14–2–14:Chol:DOPE formulations, by themselves or after complexation with DNA, showed competence for undergoing significant lipid mixing with vesicles containing PA, PS or CL. An exception was observed with surfactant:Chol/DNA lipoplexes at 12/1 +/− charge ratio in the presence of PS-containing vesicles, in which case the lipid mixing observed was ca. 13%. In contrast, the zwitterionic vesicles prepared from the mixture PE:PC (1:1) were unable to induce high levels of lipid mixing with the cationic liposomes and their respective lipoplexes (maximum lipid mixing was observed for lipoplexes of surfactant plus cholesterol at a +/− charge ratio of 4/1, ca. 32%). Lipoplexes of both formulations displayed different susceptibility to lipid mixing depending on the +/− charge ratio and lipid composition of the destabilizing vesicles. Regarding the transfection-competent formulations (14–2–14:Chol at the +/− charge ratio of 4/1 and 14–2–14:Chol:DOPE at the +/− charge ratio of 8/1), we observed that both were more efficiently destabilized by PA- and PS-containing vesicles than by those containing cardiolipin. On the other hand, in comparison with 14–2–14:Chol formulation, the DOPE-containing formulation showed higher levels of lipid mixing with any of those vesicles.

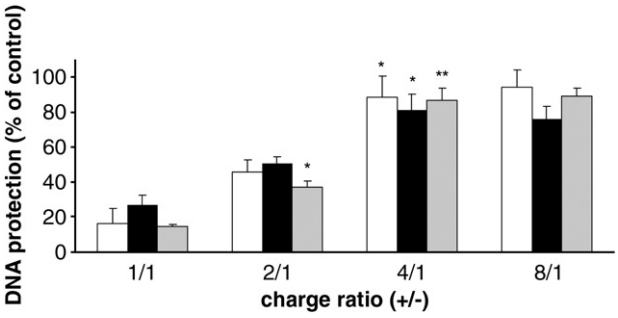

Fig. 6. Effect of lipoplex composition and +/− charge ratio on DNA protection as assessed by measuring the PicoGreen fluorescence. Complexes were prepared upon incubation of (□) 14–2–14, (■) 14–2–14:Chol and (■) 14–2–14:Chol:DOPE aqueous dispersions with 1 μg of DNA and added to a PicoGreen solution, as described in the Material and methods section. The results represent the mean ± standard deviation obtained from triplicates of three independent experiments. DNA protection for each lipoplex formulation was compared to the same lipoplex formulation at the immediately precedent +/− charge ratio (*$p<0.05$, **$p<0.005$).

4. Discussion

In the present work, we attempted to correlate the transfection efficiency of Gemini surfactant 14–2–14-based lipoplexes or the corresponding surfactant/lipid systems with their biophysical properties.

In contrast with other Gemini surfactants, which are able to transfect cells without the addition of helper lipids [14], the Gemini 14–2–14 did not exhibit transfection activity on its own (data not shown). However, the ability of transfection was significantly improved, at certain surfactant/DNA charge ratios, when the Gemini 14–2–14 was used in combination with cholesterol, at a 1:1 molar ratio, or with cholesterol and DOPE, at a 2:1:1 molar ratio.

Cholesterol and DOPE have been widely reported as suitable helper lipids [7,8], and different hypotheses have been suggested to explain their role in promoting gene delivery mediated by cationic liposomes. A body of evidences have demonstrated that helper lipids modify the aggregation morphology of the complexes by promoting the formation of non-lamellar phases under physiologically relevant conditions [2,6]. This property has been associated to a specific ability of helper lipids to induce fusion of the complexes with the endosomal membrane, facilitating DNA release into the cytoplasm [2,6]. This assumption is based on two types of experimental evidence: firstly, non-lamellar phases, especially those identified by ^{31}P-NMR and freeze–fracture electron microscopy as hexagonal II or cubic phases, have been implicated in bilayer destabilization and fusion phenomena [10]; secondly, lipoplexes that adopt the H_{II} phase have shown to strongly facilitate intracellular release of DNA and display the highest transfection efficiency [23,24].

Since different levels of transfection efficiency were observed for 14–2–14-based lipoplex formulations, depending on the nature of their components and the +/− cationic surfactant/DNA charge ratio,

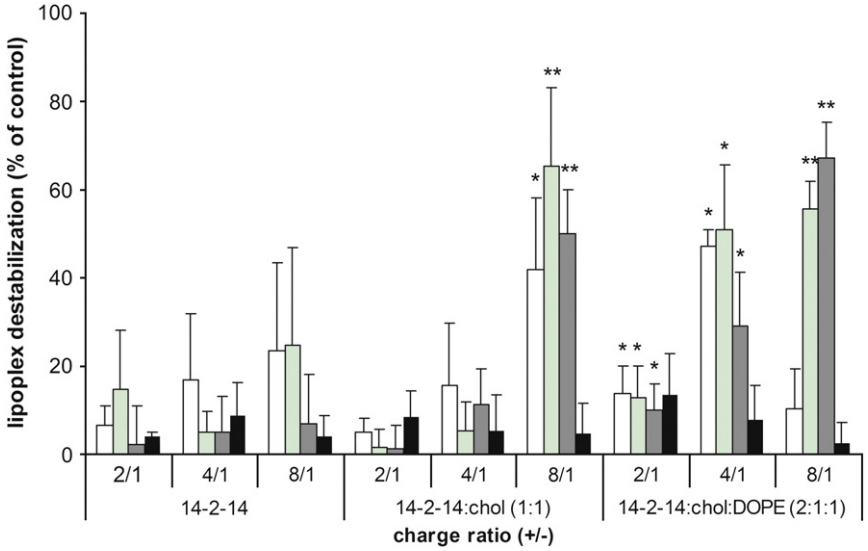

Fig. 7. Effect of the interaction of anionic/zwitterionic liposomes with lipoplexes in terms of DNA accessibility. The complexes made of Gemini surfactant 14–2–14, 14–2–14:Chol (1:1) and 14–2–14:Chol:DOPE (2:1:1), containing plasmid DNA (1 μg), at the indicated +/− charge ratios, were added to a PicoGreen solution (dilution: 1:200). After signal stabilization, the anionic liposomes PE:PC:PS (2:1:1 mole ratio) (□) and PE:PC:PA (2:1:1) (▒) or the zwitterionic liposomes PE:PC:CL:Ca^{2+} (2:1:1:1) (■) and PE:PC (1:1) (■) were then injected in a two-fold excess over the cationic lipids. The fluorescence signal increased and stabilized within 300 s. The results represent the mean ± standard deviation obtained from three independent experiments. Data obtained for each formulation at +/− charge ratios of 2/1, 4/1 and 8/1 were compared to those obtained with the same formulation at the 1/1 charge ratio (*$p<0.05$, **$p<0.005$).

studies were performed in order to investigate the molecular mechanisms underlying the different gene carrier biological behaviour. Among all the factors that may be involved in the lack of transfection efficiency displayed by a formulation, the potential toxicity resulting from the presence of a specific component or from an unfavourable mixture of components arises as one of the most important. Our results showed that none of the tested formulations induces a severe toxicity to TSA cells, since, for all of them, cell viability was maintained at levels above 70%, with respect to an untreated sample (control).

Particle size and surface charge density were also investigated because they have revealed to be strongly related with lipoplex ability to mediate gene delivery [25–27]. Accordingly, our results (Table 1) showed that the formulation consisting only of the surfactant 14–2–14, which has shown to be inefficient in gene delivery, presented the highest diameter (one order of magnitude higher when compared with the other formulations). When complexed with DNA, at the highest +/− charge ratios (4/1 and 8/1), the Gemini-structures still display a large diameter (>1500 nm), and only for the lowest +/− charge ratios (1/1 and 2/1), the size of the particles decreased to hundreds of nanometers, a size that is within the range of dimensions usually considered as enabling complexes to be easily internalized by endocytic pathways [9]. This fact may be related to surfactant ability to form elongated (wormlike) micelles instead of liposomes [28], consistent with the non-milky aspect of their aqueous dispersions. In fact, the efficiency of lipoplexes to enter the cell by the endocytic pathway [4], namely by clathrin- and caveolin-mediated endocytosis, which involve the formation of coated vesicles and flask-shaped invaginations, respectively, has been shown to largely depend on particle size [6,9]. Thus, lipoplexes composed of surfactant and DNA seem to have an endocytosis-compatible size only under conditions (at the lowest +/− charge ratios) where a negative zeta potential predictably creates unfavourable interactions with cell membranes, probably justifying the inability of these vectors to accomplish efficient gene delivery.

It is noteworthy that at the +/− charge ratios at which 14–2–14:Chol/DNA and 14–2–14:Chol:DOPE/DNA complexes showed the highest transfection efficiency (4/1 and 8/1, respectively), their sizes are significantly different. Whereas the DOPE-containing particles exhibit diameters (ca. 170 nm) compatible with clathrin-mediated endocytosis [6,9], 14–2–14:Chol/DNA particles have an average diameter of 600 nm (Table 1), thus being more prone to be internalized by macropinocytosis or endocytosed via caveolae. When reaching caveosomes, the cholesterol-containing lipoplexes may be directly transported to the Golgi and/or endoplasmic reticulum (ER), circumventing lysosomal degradation [4]. On the other hand, due to the topological relation of ER and mitochondria, the Gemini–cholesterol formulation may also constitute a good candidate to deliver genetic cargo into mitochondria. Studies are currently in progress to address this possibility.

The surface charge density of the complexes, measured in terms of zeta potential, has also been reported as a critical factor for lipoplex

Table 3
Lipid mixing, in % of the control, obtained at 25 °C for 14–2–14:Chol (1:1) and 14–2–14:Chol:DOPE (2:1:1) aqueous dispersions (no DNA) and lipoplexes at the +/− charge ratios indicated in the table, in the presence of vesicles composed of a mixture of DOPE and DOPC (PE:PC) or this mixture and one of the following phospholipids: phosphatidic acid (PA), phosphatidylserine (PS) and cardiolipin plus Ca^{2+} 1:1 (CL).[a]

	14–2–14:Chol (1:1)				14–2–14:Chol:DOPE (2:1:1)			
	No DNA	12/1	8/1	4/1	No DNA	12/1	8/1	4/1
PE:PC:PA (2:1:1)	47.64 (±0.75)	50.90 (±1.54)	39.56 (±13.05)	46.39 (±12.69)	72.24 (±6.14)	72.83 (±2.10)*	78.61 (±5.31)	43.15 (±11.87)
PE:PC:PS (2:1:1)	36.92 (±9.76)*	13.07 (±7.78)	51.40 (±6.79)*	50.01 (±6.11)**	54.27 (±9.88)**	78.81 (±0.15)	62.60 (±11.41)*	31.08 (±11.93)
PE:PC:CL (2:1:1)	40.57 (1.59)*	56.05 (±16.07)**	59.23 (±17.18)**	31.23 (±7.98)	41.24 (±2.31)*	33.35 (±4.36)	44.77 (±14.45)**	48.69 (±14.63)
PE:PC (1:1)	12.90 (±0.64)	20.51 (±10.33)	18.95 (±10.33)	32.13 (±17.51)	8.54 (±5.09)	11.09 (±4.77)	6.76 (±4.38)	20.15 (±13.27)

[a] Lipid mixing values represent mean ± standard deviation of at least three independent experiments. Data for the lipid mixing with the different anionic vesicles were compared to those obtained with the zwitterionic vesicles prepared from PE:PC mixture (*$p<0.01$, **$p<0.05$).

stability and interaction with cell surface, as well as relevant in preventing interactions with serum proteins and resisting to nuclease degradation *in vivo* [2,25]. All the complexes prepared at 1/1 and 2/1 +/− charge ratios displayed negative zeta potentials, justifying their inability for gene delivery. Consistently, the transfection-competent complexes showed positive zeta potentials, enabling their interaction with the negative cell surface.

Another important lipoplex feature, shared by all the formulations, irrespectively of the lipid composition and charge ratio, is their capacity to preserve the original size (colloidal stability) over 24 h.

In order to explain why the formulations used in this work displayed different transfection activities, an extensive characterization of their biophysical properties was carried out. Fluidity is the physical parameter most frequently assessed in lipid-containing systems to characterize and evaluate the extent of lipid–foreign molecule interactions [29,30]. However, lipid bilayers (and lipoplexes) are highly anisotropic systems, and, therefore, the meaning of the term fluidity applied to these structures differs from the physical concept of fluidity, the latter being only applicable to isotropic media. In this work, we used fluorescent fluidity probes and the fluorescence polarization to characterize the physical properties of the Gemini-containing lipid structures, associated or not with DNA at different +/− charge ratios. The rotational diffusion of the probes, depending on the degree of the molecular packing (order) in the surfactant/lipid system, results in depolarization of fluorescence. Therefore, the term fluidity is used here as being inversely proportional to the degree of fluorescence polarization of DPH/DPH-PA probes. This technique, which is very sensitive and reproducible, has been extensively used to obtain a detailed picture of physical phenomena at a molecular level in the interior of a lipid bilayer [19,29,30]. Additionally, by using different fluorescent probes, the technique offers the possibility of detecting alterations of molecular order at different depths of the surfactant/lipid structure [20,22].

As expected, the range of values within which fluorescence polarization of DPH and DPH-PA varies along the transition of DMPC (from 0.34 to 0.15 and 0.40 to 0.24, respectively), reflects an increase of disorder from the surface to the core of the lipid bilayer, consistent with data provided by this [31] and other spectroscopic techniques, namely EPR and NMR [32]. Interestingly, for the surfactant plus lipid systems, prior or following DNA complexation (Table 2), the fluorescence polarization of DPH-PA reaches higher values than those reported by DPH at the same temperature, with few exceptions. This is a clear indication that DPH and DPH-PA have been efficiently incorporated in all the formulations, in distinct lipid environments. Establishing a parallel to what has been reported in lipid bilayers [29,30], the fluorescent probes DPH and DPH-PA are expected to monitor the structural order of the lipid/DNA systems at two distinct levels: in the hydrophobic core, i.e., far from the aqueous medium, the preferred DPH environment [20], and closer to the water/lipid interface, where electrostatic interactions dominate and DPH-PA prefers to be located [22].

Fig. 5 evidenced two other important aspects, related with the influence of cholesterol incorporation in the surfactant system: a) the disappearance of the phase transition, found in both cholesterol-containing systems, and b) the induction of a higher level of lipid order, in the system at which DOPE was not added. These aspects may reflect a cholesterol-induced increase of the packing of surfactant hydrocarbon chains, producing more compact arrangements, provided with much less void volume in its interior, in agreement with classical reports of cholesterol effects on lipid structures [11,12].

DNA addition to all surfactant-containing aqueous dispersions also showed important physical effects. Because of the DNA electrostatic interaction with the positive charges of the surfactant Gemini, a decrease of the repulsive lateral pressure at the level of surfactant/lipid polar groups could induce a tighter packing of the hydrophobic chains, at high temperatures. Thus, with few exceptions, an increase of the fluorescence polarization of DPH-PA, as well as DPH, was noticed at 37 and 45 °C, in the presence of DNA (Table 2). In the case of the surfactant formulation, DNA complexation could impair the formation of a highly ordered phase at low temperatures, justifying the DNA-induced decrease of fluorescence polarization of DPH and, at a lesser extent, of DPH-PA, at 15 °C (Table 2). Interestingly, at this temperature, a higher level of order was detected in the hydrophobic core of the surfactant structure than at the outer regions. This observation is, as mentioned above, opposed to what is usually found in a typical bilayer, and may reflect a micellar structure adopted by surfactant molecules upon hydration [28], according to what has been reported for other Gemini surfactants [33,34]. Under these conditions, due to steric constraints, it is likely that the repulsive interactions of surfactant cationic groups create a more relaxed structure in the proximity of the micelle surface, the van der Waals interactions being progressively more favoured towards the centre of the micelle. Upon DNA complexation, the surfactant aggregates undergo rearrangement and, similarly to surfactant plus lipid structures, show a typical gradient of fluidity, increasing from the outer to the more hydrophobic inner regions of the complex structure.

The physical properties of cholesterol-containing lipoplexes display a notable dependence on the surfactant/DNA charge ratio. Curiously, at 37 °C, temperature at which transfection was carried out, the fluorescence polarization of DPH and DPH-PA in surfactant plus cholesterol (1:1)/DNA complexes, featured by a high level of order, reaches the lowest values at the 4/1 +/− charge ratio. On the other hand, in DNA complexes containing the surfactant, cholesterol and DOPE (2:1:1), which show a more disordered structure, the fluorescence polarization of both probes attains the highest values at a +/− charge ratio of 8/1 (Table 2). Thus, the values of lipid order in both lipoplex formulations become closer to each other at +/− charge ratios at which lipoplexes exhibited the highest competence for transfection. This fact suggests the existence of a putative range of the surfactant/lipid hydrocarbon chain order within which the formulations present the highest ability for gene delivery.

A very high degree of DNA protection (Fig. 6) was achieved for all the formulations with an excess of surface positive charges (+/− charge ratios above 2/1 as assessed by zeta potential measurements). As expected, both formulations with the highest transfection activity, 14–2–14:Chol at the 4/1 +/− charge ratio and 14–2–14:Chol:DOPE at the 8/1 +/− charge ratio, are included in the group of those which provide maximum DNA protection. The surfactant *per se* seems also to be able to efficiently protect DNA. This fact is consistent with other reports showing that micellar as well as vesicular aggregates can confer high protection to DNA [8]. As expected, such protection was negligible when the cationic amphiphile–DNA complexes were put in the presence of anionic lipid vesicular aggregates (Fig. 7). The purpose of this study was to mimic the situation of DNA release into the cell. In fact, a central step in cationic lipid-mediated transfection is the endosome escape of DNA into the cytoplasm [2,4,35], or into the mitochondrial matrix [5]. As pointed out by some authors [2,4–6,35,36], this event may be preceded by interaction and neutralization of cationic lipids by the endosomal/mitochondrial anionic lipids. As clearly shown in Fig. 7, the highest values of PicoGreen fluorescence were observed for both formulations containing helper lipids, at the highest +/− charge ratios. At +/− charge ratios of 1/1 or 2/1, all the formulations were not or were only slightly destabilized by the addition of anionic vesicles. At these +/− charge ratios, lipoplexes displayed a negative zeta potential, as shown in Fig. 3, hence efficiently repelling anionic lipid vesicles. In contrast to the other formulations, Gemini surfactant/DNA complexes showed a limited ability to expose their genetic cargo upon contact with anionic vesicles, even at the highest +/− charge ratios, under which conditions a favourable interaction with negatively charged phospholipids would be predictable. Therefore, the surfactant structure,

although efficiently protecting DNA, has an apparently low susceptibility for destabilization, justifying its incompetence for gene delivery. In fact, although DNA protection conferred by the gene carriers is of extreme importance to prevent its degradation by the action of nucleases [2,25,35], an excessive condensation of DNA by the cationic lipids may be an obstacle to its release into the intracellular medium. As opposed to anionic lipid-containing mixtures, those containing only zwitterionic lipids showed low ability to compromise DNA protection conferred by all the cationic surfactant/lipid formulations.

To further characterize the interaction of surfactant/lipid carriers with membrane-mimicking vesicles, the propensity for lipid mixing when cationic lipids were confronted with anionic phospholipid-containing vesicles was investigated using a Förster Resonance Energy Transfer (FRET) assay.

Both surfactant/lipid dispersions and respective lipoplexes were able to undergo lipid mixing with PA-, PS- and CL-containing vesicles, and even with PE:PC vesicles, although at a significantly lower level. The competence of these formulations for lipid exchange with membranes of different lipid composition and net charge (negative for PA- and PS-containing membranes and neutral for $CL+Ca^{2+}$-containing membranes and the mixture PE and PC) suggests a high propensity for DNA release upon membrane interaction. From the results obtained from PicoGreen and FRET experiments, we may suggest that, at the highest +/− charge ratios tested, DNA exposure detected by the PicoGreen assay can, apparently, be attributed to the occurrence of a high level of interaction involving the cationic systems and anionic vesicles, accompanied by lipid exchange and membrane fusion.

The endosomal release of DNA from different lipid-based gene vectors [33,35,37] has been proposed as involving the phospholipid PS. Apparently, PS undergoes flip-flop from the outer to the inner leaflet, following interaction of lipoplexes with the endosome membrane, resulting in DNA dissociation and formation of ion-pairs between PS and the cationic lipids [6,9,35]. PS access to lipoplexes has been proposed as being favoured by the presence of DOPE/cationic lipid domains adopting inverted hexagonal phases. Our data indicate that endosomes and mitochondria are potentially good targets for the studied formulations, since they demonstrate a significant level of lipid mixing with PS- as well as with CL-containing vesicles. It is important to note that mitochondria targeting represents a recent focus of interest for gene therapy applications [5].

5. Conclusions

The Gemini surfactant 14–2–14 successfully replaces other cationic lipids in gene carrier formulations, offering a high level of DNA protection. However, Gemini-based gene delivery systems need to be assisted by helper lipids to exhibit competence for transfection. The incorporation of cholesterol or cholesterol and DOPE in the surfactant system promoted significant structural alterations and higher susceptibility for DNA exposure in the presence of anionic lipids.

Since cholesterol- and cholesterol:DOPE-containing lipoplexes show very different average sizes (600 nm and 170 nm, respectively) at the +/− charge ratios they are most efficient (4/1 and 8/1, respectively) in transfection, it is likely that they use distinct pathways for cell internalization. This fact will be taken into account in future work, when considering different cell and intracellular targets for gene delivery.

Acknowledgements

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