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Evaluation of lipid-based reagents to mediate intracellular gene delivery

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Abstract

We characterized different cationic lipid-based gene delivery systems consisting of both liposomes and nonliposomal structures, in terms of their in vitro transfection activity, resistance to the presence of serum, protective effect against nuclease degradation and stability under different storage conditions. The effect of lipid/DNA charge ratio of the resulting complexes on these properties was also evaluated. Our results indicate that the highest levels of transfection activity were observed for complexes prepared from nonliposomal structures composed of FuGENE 6. However, their DNA protective effect was shown to be lower than that observed for cationic liposome formulations when prepared at the optimal (+/−) charge ratio. Our results suggest that lipoplexes are resistant to serum up to 30% when prepared at a 2:1 lipid/DNA charge ratio. However, when they were prepared at higher (+/−) charge ratios, they become sensitive to serum for even lower concentrations (10%). Replacement of dioleoyl-phosphatidylethanolamine (DOPE) by cholesterol enhanced the resistance of the complexes to the inhibitory effect of serum. This different biological activity in the presence of serum was attributed to different extents of binding of serum proteins to the complexes, as evaluated by the immunoblotting assay. Studies on the stability under storage show that lipoplexes maintain most of their biological activity when stored at −80 °C, following their fast freezing in liquid nitrogen.

Keywords: Cationic liposome; Transfection; Gene delivery; Plasma interaction; DNA protection; Lipoplex stability

1. Introdution

The development observed in molecular biology, genomics and medical genetics has promoted the emergence of novel therapeutic approaches, such as gene therapy, addressed to several diseases including metabolic genetic diseases, viral infections and cancer [1,2]. Nevertheless, the successful application of these strategies is strongly dependent on the ability to transfer therapeutic genes into target cells. Therefore, research in somatic gene therapy has been focused on the development of suitable carriers that, while exhibiting adequate features for in vivo use, would also mediate efficient intracellular delivery of genetic material [3].

So far, the majority of clinical trials have been based on the use of viral vectors. In fact, these vectors have certain advantages, including high levels of transduction, or efficient and stable integration of foreign DNA into host genome. The usefulness of viral vectors is limited, however, by host immune and inflammatory reactions (in the case of adenovirus), difficulty of large-scale production, size limit of the exogenous DNA (in the case of adeno-associated virus), random integration into the host genome (in the case of retroviruses), and the risks of inducing tumorigenic mutations and generating active viral particles through recombination [4,5]. The drawbacks associated with the use of viral vectors, namely those related to safety problems, have prompted investigators to develop alternative methods for gene delivery, cationic lipid-based systems being the most representative. Ideally, it would be desirable to produce carriers of small size, which ensure protection of DNA, prevent nonspecific interactions with blood components, exhibit specific targeting, achieve high levels of transgene expression without causing cytotoxicity and that can be

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easily generated in large scale and stored [6–8]. Although cationic liposomes fulfill some of these requirements, they have a limited efficiency of delivery and gene expression as compared to viral vectors, toxicity at higher concentrations and inability to reach tissues beyond the vasculature unless directly injected into the tissue [9–12]. In addition, the positive charge harboured by cationic lipid-based systems may promote their nonspecific binding to cells such as erythrocytes, lymphocytes and endothelial cells, as well as to extracellular matrix proteins [13,14]. On the other hand, the interaction with serum proteins, such as lipoproteins or immunoglobulins, may result in their coating, thus leading to an increase in their clearance rate from blood circulation [15–18]. Both processes will limit the ability of the complexes to reach target tissues and cells. The colloidal stability of cationic liposome/DNA complexes has also been considered a critical aspect, specially when neutral net charges or high concentrations are required. Although various studies on the stabilization of lipoplexes have been reported [19,20], the use of freshly prepared complexes is still a requirement in therapeutic protocols.

In this work, we have characterized various lipid-based gene delivery systems aiming at identifying critical parameters for their biological activity and stability. In particular, we evaluated the physicochemical properties of these systems in terms of their size and zeta potential and attempts were made to relate such properties to their ability to protect DNA and to prevent their interaction with serum proteins. In addition, lipid-based carriers exhibiting different features were tested for their biological activity and stability.

2. Materials and methods

2.1. Preparation of cationic liposomes and their complexes with DNA

Small unilamellar cationic liposomes (SUV) were prepared from a 1:1 (mol ratio) mixture of 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) and dioleoylphosphatidylethanolamine (DOPE) or cholesterol (Chol) and from a 2:1:1 (mol ratio) mixture of DOTAP, DOPE and Chol, by extrusion of multilamellar liposomes (MLV). Briefly, lipids (Avanti Polar Lipids, Alabaster, AL) dissolved in $CHCl_3$ were mixed at the desired molar ratio and dried under vacuum in a rotatory evaporator. The dried lipid films were hydrated with deionized water to a final lipid concentration of 6 mM and 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 then diluted five times with deionized water and filter-sterilized utilizing 0.22 μm pore-diameter filters (Schleicher & Schuell). Complexes were prepared by sequentially mixing 100 μl of a Hepes-buffered saline solution (HBS) (100 mM NaCl, 20 mM HEPES, pH 7.4), with liposomes (volume was dependent on the (+/−) charge ratio) and with 100 μl of HBS solution containing 1 μg of pCMVluc (VR-1216; a gift of Dr. P. Felgner (Vical, San Diego, CA)). The mixture was further incubated for 15 min at room temperature. Complexes prepared from the commercially available formulations (DOTAP, FuGENE 6 and 1,3-dioleoyloxy-2-(6-carboxy-spermyl)-propylamid (DOSPER) (Boehringer Mannheim, Mannheim, Germany)) were obtained in a similar manner to that described above.

2.2. Physicochemical characterization of complexes

Complexes were characterized with respect to their size and zeta potential using a Coulter N4 Plus and a Coulter DELSA 440 (Coulter Corporation, Miami, FL), respectively. The Coulter N4 Plus is a PCS (photon correlation spectroscopy)-based technique, which uses autocorrelation spectroscopy of scattered laser light to determine its time-dependent fluctuations resulting from the Brownian motion of particles in suspension. The light intensity scattered at a given angle is detected by a photo-multiplier whose output current is passed to an autocorrelator, which analyses time dependence, determining the rate of diffusion or Brownian motion of the particles and hence their size. The detection angle is fixed at 90°. The DELSA 440 is a laser-based multiangle particle electrophoresis analyser that measures the electrophoretic mobility and zeta potential distribution simultaneously with the hydrodynamic size of particles in suspension. Cationic liposome/DNA complexes were prepared immediately before analysis. Samples of the prepared complexes were placed in the measuring cell, whose position was adjusted to cover a previously determined stationary layer, and an electric current of 3.0 mA was applied. Measurements were recorded and the zeta potential was calculated for each scattering angle (8.6°, 17.1°, 25.6° and 34.2°). Data represent the mean ± S.D. obtained for the different angles of three measurements. All complexes showed a unimodal distribution for zeta potential, while a polymodal distribution was observed for size measurements.

2.3. Cells

COS-7 cells (American Type Culture Collection, Rockville, MD) were maintained at 37 °C, under 5% CO_2, in Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) (Irvine Scientific, Santa Ana, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO), penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (4 mM). For transfection, 0.4×10^5 COS-7 cells were seeded in 1 ml of medium in 48-well culture plates and used at 50–70% confluence.

2.4. Transfection activity

Cells were rinsed twice with serum-free medium and then covered with 0.3 ml of DMEM-HG (without serum,

unless indicated otherwise) before lipid/DNA complexes were added. Lipid/DNA complexes 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 containing 10% FBS, 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 a Mediators PhL luminometer (Mediators Diagnostika, Vienna, Austria) and using a standard curve for luciferase activity. 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 nanograms of luciferase per milligram of total cell protein.

2.5. Interaction of cationic liposome/DNA complexes with plasma proteins

The assay for determining the interaction of cationic liposome/DNA complexes with plasma proteins is based on the retention of the complexes by a 200 nm Anopore membrane (Whatman, Kent, UK) and using the protocol described by Ogris et al. [15]. Following their preparation, the complexes were incubated with 2% of human plasma for 30 min at 37 °C. The membrane of microcentrifuge tube filters (Whatman; 200 nm Anopore membrane) was saturated with 500 μl bovine serum albumin (1 mg/ml) to reduce unspecific interaction of plasma proteins with the membrane and was washed three times with 500 μl of HBS solution. The mixture of complexes and plasma was applied on to the membrane, filtered by centrifugation at $10\,000 \times g$ and washed three times with 500 μl HBS. The residue (complex-bound plasma proteins) was eluted with 100 μl HBS containing 5% (w/v) SDS. Samples (eluate) and control (HBS solution treated in the same way) were diluted 10 times with HBS and two times with sample buffer (100 mM Tris–HCl (pH = 6.8); 4% (w/v) SDS; 20% (v/v) glycerol; 0.2% (w/v) bromphenol blue), and the associated proteins denaturated in boiling water for 3 min. Aliquots of the treated samples and control were then applied on to a 7.5% SDS polyacrylamide gel and separated at 180 V for 1 h in a Mini-PROTEAN II electrophoresis cell (Bio-Rad). For immunological identification of the proteins, a gel was blotted on to a Hybond P (PVDF) membrane via semidry blotting (Bio-Rad) at 15 V for 10 min. Unspecific binding was blocked by incubating the blot with 5% milk powder in TBST (2.5 mM Tris–HCl (pH = 7.6); 15 mM NaCl; 0.1% Tween 20) for 2 h at room temperature (RT). The blots were incubated for 1 h at RT with the antibody solutions (antibodies were diluted in TBST) containing rabbit anti-human complement C_3 (Serotec, Oxford, England) 1:4000 or rabbit anti-human albumin (Rockland, Gilbertsville, PA) 1:4000. As second antibody, an alkaline phosphatase (AP) conjugate with goat-anti-rabbit IgG (Amersham Pharmacia Biotech) 1:20 000, was used. After 1-h incubation at RT, the blots were washed several times with TBST and incubated with ECF (alkaline phosphatase substrate) (20 μl of ECF/cm^2 of membrane) for 5 min at RT and then submitted to fluorescence detection at 570 nm using a Storm-860 (Molecular Dynamics, Sunnyvale, CA).

2.6. Ethidium bromide intercalation assay

The accessibility of ethidium bromide to the DNA associated with the complexes was monitored at 37 °C, for 2 min, in a SPEX Fluorolog 2 fluorometer (SPEX Industries, Edison, NJ). The fluorescence was read at excitation and emission wavelengths of 518 and 605 nm, respectively, using 1 mm excitation and 2 mm emission slits. The sample chamber was equipped with a magnetic stirring device, and the temperature was controlled with a thermostatted circulating water bath. The fluorescence scale was calibrated such that the initial fluorescence of EtBr (20 μl of a 2.5 mM solution added to a cuvette containing 2 ml HBS solution) was set at residual fluorescence. The value of fluorescence obtained upon addition of 1 μg DNA (control) was set as 100%. Cationic liposome/DNA complexes (1 μg DNA) were added to the cuvette containing 2 ml HBS solution followed by addition of 20 μl of EtBr. The amount of DNA available to interact with the probe was calculated by subtracting the values of residual fluorescence from those obtained for the samples and expressed as the percentage of the control.

2.7. Resistance to DNase I action

Resistance of cationic liposome/DNA complexes to DNase I (Sigma) was determined by electrophoresis and spectrofluorimetry. DNase I was maintained in a buffer solution (50 mM Tris–HCl (pH = 7.5); 10 mM $MnCl_2$; 50 μg/ml BSA). Complexes were submitted to DNase I action (5 units DNase I/μg of DNA), during 30 min to 37 °C, followed by inactivation of the enzyme upon incubation with 0.5 M EDTA (1 μl/unit of DNase I). Parallel experiments were performed by incubating samples under the same experimental conditions, except that DNase I was previously inactivated. Electrophoresis was performed in 1% agarose gel prepared in TBE solution (89 mM Tris-buffer (pH = 8.6); 89 mM boric acid; 2.5 mM EDTA) and containing 1 μg/ml of EtBr. Following incubation of the complexes with DNase I (active or inactive), aliquots corresponding to 200 ng of DNA, to which 5 μl of loading buffer (15% (v/v) ficoll 400; 0.05% (w/v) bromphenol blue; 1% (w/v) SDS; 0.1 M EDTA; pH = 7.8) have been previously added, were placed in the gel. The electrophoresis elapsed, for 1 h and 45 min, applying a voltage of 100 V. In some experiments, lipid extraction was performed following incubation of the complexes with DNase I (active or inactive). Briefly, a phenol, chloroform and alcohol isoamyl mixture (25:24:1) was

added to the complexes in a volume ratio of 1:1 and subsequently shaken and centrifuged at 10 000 × *g* for 10 s. 2-Propanol was added to the aqueous phase containing DNA 1:2 (v/v) and the solution was shaken and maintained at − 20 °C, for 30 min, to facilitate DNA precipitation. After centrifugation (14 000 × *g*, 15 min, 4 °C), the supernatant was aspirated and the pellet washed again with 1 ml of 2-propanol. Finally, the pellet was let to dry off, dissolved in HBS, and analysed by electrophoresis.

For spectrofluorimetric measurements, following treatment of the complexes with DNase I (active or inactive), the same experimental procedure as described for the EtBr access to the complexes was carried out. The extent of DNA degradation was determined according to the following equation:

$$\text{DNA degradation}(\%) = (F_i - F_a)/(F_{100} - F_0) \times 100 \quad (1)$$

where F_i is the fluorescence value emitted by EtBr in the presence of the complexes treated with the inactive enzyme, F_a is the fluorescence value emitted by EtBr in the presence of the complexes treated with the active enzyme, F_{100} is the fluorescence value emitted by EtBr in the presence of 1 μg of DNA and F_0 is the residual fluorescence of EtBr.

3. Results

3.1. Physicochemical characterization of the lipid-based complexes

The successful application of gene therapy depends on available carriers to efficiently deliver genetic material into target cells. Such efficacy is strongly dependent on their physicochemical properties [6,21].

Fig. 1 illustrates the values of the zeta potential and size (mean diameter) of DOTAP:DOPE/DNA complexes prepared at different lipid/DNA (+/−) charge ratios. As can be observed, increasing the amount of cationic liposomes with respect to a fixed amount of DNA resulted in an increase of the overall charge of the complexes as evaluated by zeta potential measurements. Complexes prepared at 1/2, 1/1 and 4/1 (+/−) charge ratios exhibited a mean diameter ranging from 200 to 400 nm, while lipoplexes prepared at 2/1, which were only slightly positive, exhibited large mean diameters (between 1300 and 2000 nm).

Table 1 summarizes the values for zeta potential and mean diameter obtained for the various complexes including, those prepared from different cationic liposome formulations and those prepared with the FuGENE 6 transfection reagent. As can be observed, the values for the zeta potential and mean diameter of the complexes did not differ significantly among the different cationic liposome formulations. However, complexes prepared from FuGENE 6 (3/1 (v/w)) presented an overall charge slightly positive (close to those obtained for the liposome-based complexes when prepared at a 2/1 (+/−) charge ratio) and a mean diameter double that obtained for the liposome-based complexes prepared at the other charge ratios.

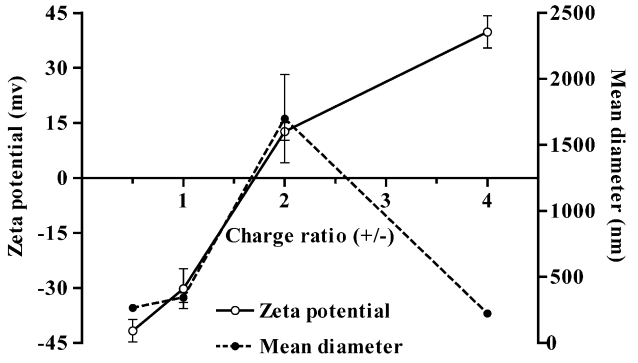

Fig. 1. Dependence of zeta potential and mean diameter of DOTAP:DOPE/DNA complexes on their lipid/DNA charge ratio. DOTAP:DOPE/DNA complexes were prepared at the lipid/DNA charge ratios (+/−) (1/2, 1/1, 2/1 and 4/1) as described in Materials and methods. Zeta potential measurements of the different complexes were performed using a Coulter® DELSA 440 Instrument. Data represent the mean ± S.D. of at least three independent experiments.

3.2. Biological activity of the complexes

The biological activity mediated by cationic liposome/DNA complexes prepared from DOTAP:DOPE (1:1), DOTAP:Chol (1:1) and DOTAP:DOPE:Chol (2:1:1) depends on their (+/−) charge ratio, those prepared at 2/1 (+/−) charge ratio being the most active independently of the liposome composition. Among the different formulations tested, DOTAP:Chol/DNA complexes were those exhibiting the highest levels of transfection (Fig. 2).

A comparison of the biological activity of the various lipoplexes obtained from cationic liposomes prepared in our laboratory and those using commercially available transfection reagents (DOTAP, DOSPER and FuGENE 6) is shown in Fig. 3. The levels of transfection mediated by DOTAP/DNA complexes were of the same order of magnitude as those obtained for complexes prepared from cationic liposomes produced in our laboratory, under the same experimental conditions (compare to Fig. 2). On the other hand, DOSPER/DNA complexes prepared at 8/1 (+/−) charge ratio exhibited a biological activity approximately five times higher than that observed for the best condition presented in Fig. 2 (DOTAP:Chol/DNA at 2/1 charge ratio). Nevertheless, the highest levels of transfection were obtained for FuGENE 6/DNA complexes, these being approximately 50 times higher than those obtained for DOTAP:Chol/DNA at 2/1 charge ratio.

3.3. Effect of serum of transfection activity

Aiming at predicting the in vivo behaviour of the complexes upon their intravenous administration, transfection studies were performed in the presence of different

Table 1
The zeta potential and mean diameter of lipid-based complexes

Complexes		Zeta potential (mV)	Mean diameter (nm)
DOTAP:DOPE/DNA	(1:2)	− 41.7 (± 3.1)	266.3 (± 15.37)
charge ratio (+/ −)	(1:1)	− 30.2 (± 5.45)	344.7 (± 37.5)
	(2:1)	12.7 (± 2.4)	1609 (± 333.8)
	(4:1)	39.8 (± 4.3)	223.8 (± 23.9)
DOTAP:Chol/DNA	(1:2)	− 35.6 (± 3.5)	220.7 (± 17.9)
charge ratio (+/ −)	(1:1)	− 30.3 (± 2.5)	245 (± 15)
	(2:1)	10.3 (± 3.1)	1864.5 (± 450.9)
	(4:1)	38.9 (± 2.2)	272.5 (± 20.4)
DOTAP:DOPE:Chol/DNA	(1:1)	− 30.2 (± 3.6)	267.5 (± 24.7)
charge ratio (+/ −)	(2:1)	10.7 (± 4.1)	1676.7 (± 315.6)
FuGENE 6/DNA (v/w)	(3:1)	11.3 (± 3.3)	578.7 (± 42.9)

DOTAP:DOPE (1:1), DOTAP:Chol (1:1) and DOTAP:DOPE:Chol (2:1:1) liposomes were prepared in our laboratory as described in Materials and methods. The results represent the mean ± S.D. of at least three independent experiments.

amounts of serum. As shown in Fig. 4, the effect of serum on transfection was dependent on the formulation and on the charge ratio tested. DOTAP:DOPE/DNA (4/1), DOTAP:Chol/DNA (4/1) and FuGENE 6/DNA were the complexes where the most significant inhibition was observed, the reduction with the respect to the control being approximately 90%, 95% and 60%, respectively. The transfection activity of DOTAP:DOPE/DNA (2/1) was inhibited only by 25%, while that of DOTAP:Chol/DNA (2/1) was not significantly affected by the presence of serum. It should be noted that the inhibitory effect of serum was essentially independent of its final concentration in cell culture medium (10% and 30%).

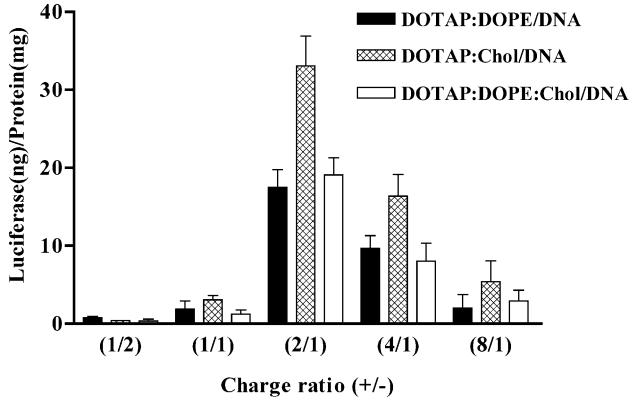

Fig. 2. Effect of lipoplex charge ratio (+/ −) on luciferase gene expression in COS-7 cells. Cells were rinsed twice with serum-free medium and then covered with 0.3 ml of DME-HG before cationic liposome/DNA complexes were added. The liposomes (prepared in our laboratory) were complexed with 1 μg of pCMVluc at the indicated theoretical lipid/DNA charge ratios. After incubation for 4 h, the medium was replaced with DME-HG containing 10% FBS and the cells were further incubated for 48 h. The level of gene expression was evaluated as described in Materials and methods. The data are expressed as nanograms of luciferase per milligram of total cell protein (mean ± S.D. obtained from triplicates), and are representative of three independent experiments.

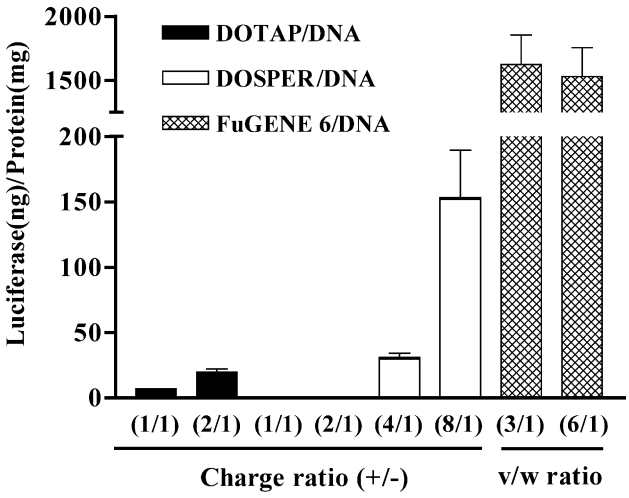

Fig. 3. Effect of lipoplex charge ratio (+/ −) and volume/weight ratio on the levels of transfection of COS-7 cells. Complexes were prepared from commercially available cationic liposome formulations (DOTAP and DOSPER) and from the transfection reagent (FuGENE 6). Experiments were performed as described in the legend to Fig. 2. The data, expressed as nanograms of luciferase per milligram of total cell protein, indicate the mean ± S.D. obtained from triplicates, and are representative of three independent experiments.

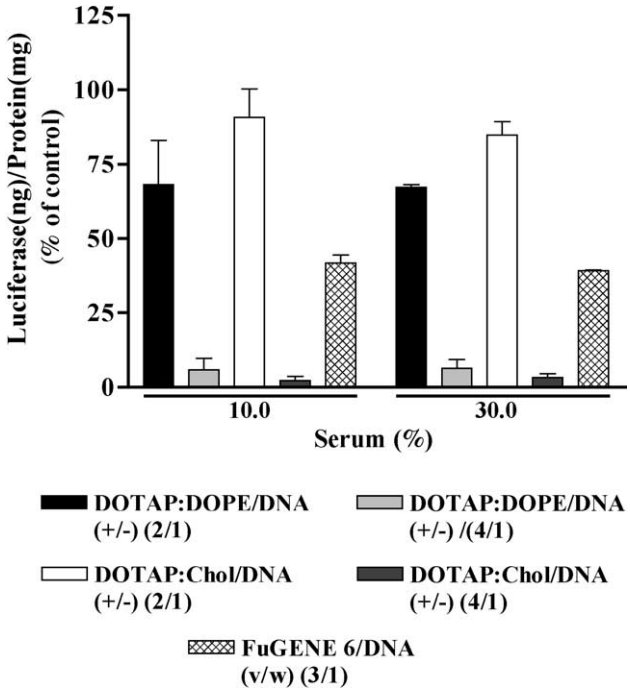

Fig. 4. Effect of presence of serum on luciferase gene expression in COS-7 cells. Cells were covered with 0.3 ml of DME-HG enriched with FBS, in order to obtain a final concentration of 10% or 30%, before lipid/DNA complexes were added. After incubation for 4 h, the medium was replaced with 1 ml of medium containing 10% FBS and the cells were further incubated for 48 h. The level of gene expression was evaluated as described in Materials and methods. The data are expressed as nanograms of luciferase per milligram of total cell protein. The results correspond to the mean ± S.D. obtained from triplicates, and are representative of at least two independent experiments.

3.4. Interaction of the complexes with plasma proteins

As illustrated above, the presence of serum decreased the transfection activity of the complexes, most likely through the establishment of their interaction with serum proteins. In an attempt to clarify this issue, the dependence of this interaction on the composition and (+/−) charge ratio of the complexes was investigated by Western blotting. For this purpose, antibodies against human albumin and C3 human complement protein were used. These proteins were investigated because albumin is the most abundant protein in serum and C3 is known to play a major role in opsonization [22,23].

Fig. 5 shows the bands corresponding to albumin (A) and C3 (B) bound to DOTAP:Chol/DNA and DOTAP:DOPE/DNA complexes upon their incubation with 2% human plasma. As shown, increasing the (+/−) charge ratio of the complexes prepared from both formulations tested resulted in an increase in the extent of protein binding, the highest levels being observed for the positively charged complexes (4/1 (+/−) charge ratio). Independently of the lipid/DNA charge ratio, DOTAP:DOPE/DNA complexes bound to albumin more extensively than DOTAP:Chol/DNA complexes. A similar pattern was observed for binding of the complexes to C3 complement protein when the complexes were prepared at 4/1 (+/−) charge ratio, while for 2/1 charge ratio, no significant differences for the extent of binding were observed between DOTAP:Chol/DNA and DOTAP:DOPE/DNA complexes.

3.5. Protection of DNA associated to the complexes

Aiming at evaluating the ability of the complexes to condense and protect DNA, the complexes were tested in terms of the access of EtBr to DNA and of resistance to DNase degradation.

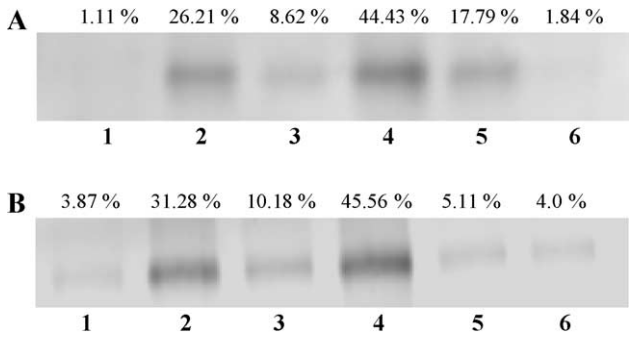

Fig. 5. Interaction of cationic liposome/DNA complexes with human albumin (A) and C3 protein of the human complement (B). Control (HBS) (1); DOTAP:Chol/DNA (+/−) (4/1) (2); DOTAP:Chol/DNA (+/−) (2/1) (3); DOTAP:DOPE/DNA (+/−) (4/1) (4); DOTAP:DOPE/DNA (+/−) (2/1) (5); DOTAP:DOPE/DNA (+/−) (1/1) (6). Complexes were incubated at 37 °C, for 30 min, with human plasma and both albumin and C3 were identified by immunoblotting as described in Materials and methods. Extent of interaction (%), for each experimental condition, was calculated with respect to the sum of the intensities of the bands for each one of the proteins (considered as 100%).

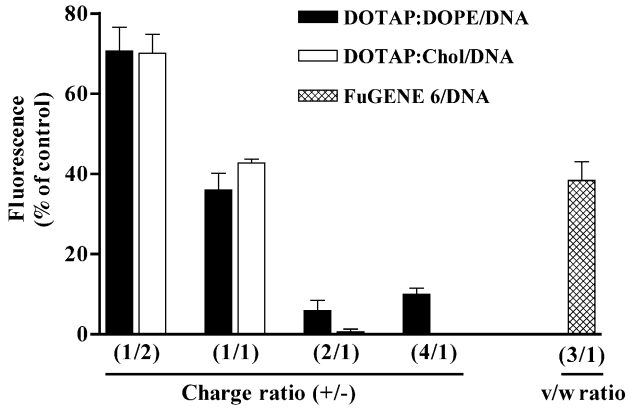

Fig. 6. Accessibility of ethidium bromide to DNA associated with the complexes. Complexes (DOTAP:DOPE/DNA, DOTAP:Chol/DNA and FuGENE 6/DNA) containing 1 μg DNA were incubated with EtBr as described in Materials and methods. The amount of DNA available to interact with the probe was calculated by subtracting the values of residual fluorescence from those obtained for the samples and expressed as the percentage of the control. Control corresponds to free DNA, but in the same amount as that associated with the complexes (100% of EtBr accessibility).

3.5.1. Access of ethidium bromide to DNA associated to the complexes

Ethidium bromide is a monovalent DNA-intercalating agent whose fluorescence is dramatically enhanced upon binding to DNA and quenched when displayed by higher affinity compounds or by condensation of the DNA structure. Therefore, this probe has been used to examine the ability of cationic lipids to protect DNA [24]. Fig. 6 shows that EtBr fluorescence decreased with increasing the (+/−) charge ratio of the complexes, independently of the cationic liposome formulation used in their preparation. These results clearly indicate that an increase in the amount of cationic liposomes led to a higher degree of DNA condensation and protection as compared to that observed when complexes are prepared at low lipid/DNA charge ratios. DOTAP:Chol/DNA complexes prepared at either 2/1 or 4/1 charge ratio allowed a complete protection of DNA. Regarding the FuGENE 6/DNA complexes (at a 3/1 v/w), the access of EtBr to DNA indicates that a great amount of DNA was still exposed (approximately 40% of the total DNA), similar to what was observed for cationic liposome/DNA complexes when prepared at a 1/1 (+/−) charge ratio.

3.5.2. Resistance to DNase I action

In an attempt to demonstrate the degree of DNA protection mediated by cationic liposomes from attack by nucleases in vivo, an in vitro DNase I protection assay was performed.

Fig. 7 illustrates the electrophoretic resulted obtained upon incubation of the complexes with active or inactive DNase I. As shown, similar patterns can be observed for the

DNA degradation and for the access of EtBr (compare to Fig. 6). In fact, increasing the lipid/DNA (+/−) charge ratio of the complexes results in a decrease of DNA digestion, as reflected by the difference between the intensity of the bands corresponding to the complexes exposed to active and inactive DNase I (lanes 2, 4, 6, 8, 10 and 3, 5, 7, 9, respectively). DNA protection against DNase I was essentially achieved for the complexes prepared at the 2/1 and 4/1 (+/−) charge ratios. Surprisingly, no bands were observed for the FuGENE 6/DNA complexes irrespective of whether DNase I was active or inactive. Therefore, these results were not in accordance with those obtained when the DNA protection was evaluated in terms of access of EtBr, where approximately 60% of protection was observed. These intriguing results were most likely attributed to the chemical nature of FuGENE 6 which, despite being unknown, may interfere with the electrophoretic mobility of DNA. In order to circumvent this difficulty, the lipid component of the complexes was extracted before their incubation with DNase I. As Fig. 8 illustrates, the bands corresponding to FuGENE 6/DNA complexes exposed to inactive DNase I are now visible, exhibiting higher intensity than that observed upon treatment with active DNase I (lanes 3 and 4, respectively). Therefore, it can be concluded that FuGENE 6 was not able to ensure an effective protection against DNase I, in contrast to the cationic liposome-based complexes particularly at high lipid/DNA (+/−) charge ratio. It should be noted that for the latter complexes, no significant differences, in term of band intensities, were obtained upon extraction of the lipid components (data not shown).

Despite lipid extraction, the results obtained for FuGENE 6 were still inconclusive since the agarose gel assay did not have enough sensitivity to allow a quantitative evaluation of DNA protection, as clearly suggested by the low intensity of the bands as compared to the control (Fig. 8, lanes 3 and 1, respectively). Therefore, the access of EtBr to the genetic material following its degradation by DNase I was evaluated by spectrofluorimetry. Assuming that a complete DNA degradation corresponds to 0% EtBr fluorescence and that intact DNA (obtained upon incubation with inactive DNase I) corresponds to 100% fluorescence, it was possible to quantitate the degree of DNA protection conferred by FuGENE 6 (Section 2.7, Eq. (1)). Our results indicate that approximately 36% of DNA degradation occurred for this transfection reagent, while only 3% of degradation was observed for the DOTAP:DOPE/DNA complexes prepared at 2/1 (+/−) charge ratio (data not shown).

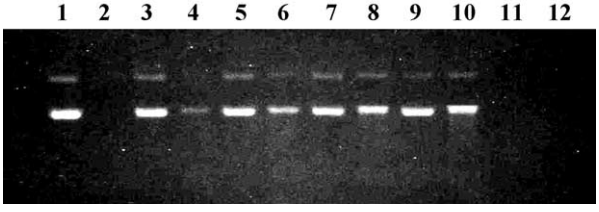

Fig. 7. Resistance of cationic liposome/DNA complexes to DNase I. Control (1 and 2); DOTAP:DOPE/DNA (+/−) (1/2) (3 and 4); DOTAP:DOPE/DNA (+/−) (1/1) (5 and 6); DOTAP:DOPE/DNA (+/−) (2/1) (7 and 8); DOTAP:DOPE/DNA (+/−) (4/1) (9 and 10) and FuGENE/DNA (v/w) (3/1) (11 and 12). Incubations with inactive DNase I (odd numbers) or with active DNase I (even numbers) were performed at 37 °C, for 30 min, using 10 units DNase I per microgram of DNA. Control corresponds to the free DNA, but in the same amount as that associated with the complexes. Electrophoresis was performed in 1% agarose gel prepared in TBE solution and containing 1 μg/ml of EtBr as described in Materials and methods.

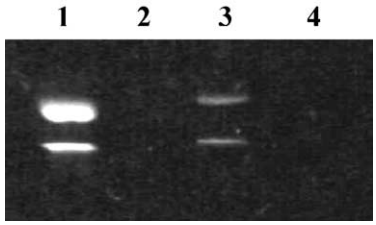

Fig. 8. DNase I resistance of the complexes following lipid extraction. Control + inactive DNase I (1); control + active DNase I (2); FuGENE/DNA (v/w) (3/1) + inactive DNase I (3) and FuGENE/DNA (v/w) (3/1) + active DNase I (4). Experiments were carried out as described in the legend to Fig. 7, except that before the electrophoresis, lipid extraction was performed.

3.6. Biological stability of the complexes upon storage

The development and production of carriers for in vivo gene delivery should fulfill a number of requirements including the ability to be stored, without losing their biological stability. This issue is particularly relevant when repeated administration is needed. Therefore, in this work, we investigated the effect of different storage conditions, namely duration and temperature, on the maintenance of the transfection activity of the DOTAP:Chol/DNA complexes prepared at a 2/1 (+/−) lipid/DNA charge ratio. For this purpose, complexes prepared immediately before the transfection experiments were used as controls. The results presented in Fig. 9 indicate that the storage temperature strongly affects the transfection activity. Complexes stored for 1 day at both 25 and 4 °C lose about 70% of their activity, this reduction being even more pronounced for longer periods of incubation. Storage of the complexes at −20 °C resulted in a 50% decrease of their ability to transfect COS-7 cells, independent of the duration of storage. Nevertheless, when fresh complexes were immediately frozen in liquid nitrogen and stored at −80 °C, no significant effect on their transfection activity was observed over the time range between 1 and 20 days. The maintenance of the biological activity of the complexes under these conditions clearly suggests that no major changes in their physicochemical properties occurred during the freezing, storage and thawing processes. Zeta potential and size measurements of the complexes upon their storage support this hypothesis, since no significant differences were observed as compared to freshly prepared complexes (data not shown).

Since our best results were obtained when the complexes are stored at −80 °C, we investigated whether the other

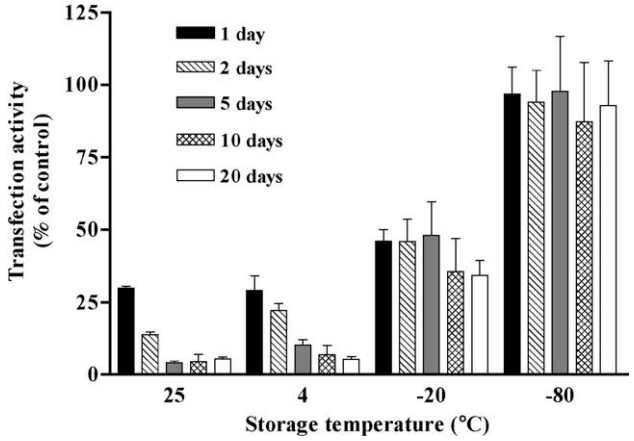

Fig. 9. Effect of storage conditions (time and temperature) on the biological activity of DOTAP:Chol/DNA complexes. Complexes, prepared at the (+/−) (2/1) charge ratio were stored at different temperatures and for different times immediately after their preparation. Transfection activity is expressed in terms of percentage of control (levels of transfection mediated by complexes prepared immediately before use). Lipoplexes stored at −80 °C were previously frozen in liquid nitrogen. The results correspond to the mean ± S.D. obtained from triplicates, and are representative of at least two independent experiments.

formulations also exhibited a similar behaviour. As expected, for DOTAP:DOPE/DNA and DOTAP:DOPE:Chol/DNA complexes, both prepared at a 2/1 (+/−) charge ratio, no decrease of their transfection activity was observed following 20 days of storage at −80 °C. Surprisingly, however, the high ability of FuGENE 6/DNA to mediate transfection was rapidly reduced to approximately 30% (after 1 day of storage), being maintained at this level during 20 days (data not shown).

4. Discussion

The variations in size, structure and surface charge observed for complexes prepared at different lipid/DNA ratios and even for those prepared at the same ratio are experimental evidences of the heterogeneous and dynamic nature of the complexes [25–27]. This heterogeneity can be attributed to variation of several parameters involved in the preparation of the complexes, namely the order and rate of the addition of the components, the maturation time of the lipoplexes and the type and composition of the cationic lipid-based gene delivery systems. The control of such parameters is, therefore, of highest importance in the final physicochemical properties of the complexes [1,28]. In this work, we investigated how these properties affect different cationic lipid-based gene delivery systems, consisting of both liposomes and nonliposomal structures, in terms of their biological activity, resistance to the presence of serum, protective effect against nuclease degradation and stability under different storage conditions.

The negative zeta potential determined for the complexes prepared at the 1/2 (+/−) charge ratio indicates that a significant amount of DNA is noncondensed and exposed, thus contributing to the negative surface charge exhibited by the complexes. In contrast, complexes prepared at the 2/1 (+/−) charge ratio contain an excess of cationic lipid capable of fully condensing DNA, thus neutralizing the negative charges of its phosphate groups [29].

The low values obtained for the mean diameters of the complexes exhibiting positive or negative surface charge are most likely due to electrostatic repulsive forces established between them, thus preventing their aggregation. On the other hand, for slightly positive complexes (prepared at a 2/1 (+/−) charge ratio), these repulsive forces are minimal, thus favouring the formation of large aggregates. It has been reported that during the maturation process (15 min), Van der Waals interactions can occur and overcome the existing repulsive forces, thereby facilitating complex aggregation [29–32].

As illustrated in Table 1, complexes prepared at a 2/1 (+/−) charge ratio are those exhibiting the largest sizes, which favours their sedimentation over the cells, thus promoting cell internalisation. Besides the enhanced interaction of large complexes with cells, it should be considered that these complexes carry a large number of DNA copies, which can also explain the high levels of transfection observed [6,8,33,34].

In addition to the size, the surface charge of the complexes also plays an important role in determining lipoplex–cell interaction. The comparison of the results presented in Table 1 and Fig. 2 shows that complexes prepared at different lipid/DNA charge ratios (1/1 and 4/1), although exhibiting similar sizes, mediate different transfection activities, which can be due to the extensive interaction of the positively charged complexes (4/1) with the negatively charged cell membrane [24,35]. Another important aspect that should be considered is the degree of DNA condensation, which increases with increasing the cationic lipid/DNA charge ratio [36,37]. In fact, an extensive condensation of DNA may render difficult the dissociation of the complex, thus preventing intracellular delivery of DNA. This may explain the lower levels of luciferase gene expression mediated by the 8/1 (+/−) complexes as compared to 4/1 (+/−) complexes.

The results obtained using different liposome formulations (DOTAP:Chol (1/1); DOTAP:DOPE (1/1); DOTAP:DOPE:Chol (2/1/1)) are in agreement with previous reports showing that the composition of the cationic liposomes is also relevant for the biological activity of the resulting complexes [38,39]. This aspect is reinforced by comparing the transfection activity mediated by the referred formulations to that observed for those obtained from commercially available transfection reagents (DOTAP; DOSPER and FuGENE 6). DOSPER is a multivalent cationic lipid bearing four positive charges and thus the 8/1 charge ratio of the resulting complexes corresponds, in terms of the amount of cationic lipid, to the 2/1 charge ratio of the complexes containing DOTAP. Complexes prepared from DOSPER at

the 8/1 charge ratio exhibit a transfection activity approximately five times higher than that obtained for the best condition using liposomes prepared in our laboratory (DOTAP:Chol/DNA at a 2/1 (+/−) charge ratio). Nevertheless, this high level of transfection is accompanied by a significant cell toxicity, in contrast to the absence of cytotoxicity observed for all the other formulation tested. In the case of FuGENE 6/DNA complexes, the high transfection activity may be attributed to the chemical nature of this transfection reagent, which while not being disclosed, may allow the complexes to overcome more efficiently the different biological barriers faced by the complexes.

Serum was shown to inhibit transfection activity depending on the charge and composition of the complexes. The significant inhibition of the biological activity mediated by lipoplexes prepared at the 4/1 (+/−) charge ratio in the presence of serum is attributed to their extensive interaction with the negatively charged proteins. These interactions result either in the dissociation of the complexes and/or in the formation of large aggregates, which, above a certain size limit, may be prevented from being internalized by the cells [39,40]. These same reasons may justify the low inhibition of transfection in the presence of serum observed for the approximately neutral complexes (2/1 (+/−) complexes). Regarding the composition of the formulations, our results show that the biological activity of complexes containing cholesterol is less inhibited by the presence of serum than for those containing DOPE. This observation is consistent with recent reports that formulations containing cholesterol are more active in vivo than those containing DOPE, which has been attributed to their higher stability in biological fluids [38,41,42]. This higher stability in the presence of serum is most likely due to the ability of cholesterol to confer membrane stability in contrast to DOPE, which is known to have an inverted cone shape that is associated with membrane fluidity, thus facilitating binding and/or incorporation of serum proteins [39,40,43]. In this regard, it is reasonable to assume that the significant decrease in transfection activity mediated by complexes prepared from FuGENE 6 in the presence of serum results from interactions established between them and serum proteins.

In support of these observations, the results obtained on the interaction of the complexes with serum proteins show that highly positively charged complexes interact more extensively with albumin and C3 complement protein than complexes whose charge is close to neutrality, stressing that electrostatic forces play a major role in this process [15,16]. Among highly positively charged complexes, DOPE containing complexes were shown to be more prone to bind to serum proteins than cholesterol containing complexes, suggesting that lipid composition also plays a role in this interaction process. For the 4/1 (+/−) complexes, repulsive electrostatic interactions result in smaller complexes containing highly curved lipid bilayers. This high curvature leads to highly unstable lipid bilayers, thus favouring protein incorporation, which becomes more evident for complexes containing DOPE, as would be expected, taking into account the cone shape of this lipid. Nevertheless, our findings provided evidence that for slightly positive (2/1) complexes, no significant differences were observed for the extent of C3 binding, independently of the cationic liposome composition. In contrast, a significant difference in the extent of albumin binding was observed between DOTAP:DOPE/DNA and DOTAP:Chol/DNA complexes. A possible explanation for this differential behaviour is that differences in the molecular weight and structure of the proteins (C3 exhibits a higher molecular weight than albumin) may cause differences in the extent of their interaction with the complexes, especially when the charge of the complexes is attenuated. For albumin, being a smaller protein, besides charge, the lipid composition will determine the extent of interaction, namely through its association with the lipid bilayer, this being favoured by DOPE containing membranes. Surprisingly, however, results on transfection activity indicate that the effect of lipid composition, in the presence of serum, was more pronounced for the 2/1 than for 4/1 (+/−) charge ratio complexes. A possible explanation is that for the 4/1 (+/−) complexes the degree of binding to serum proteins is enough to inhibit transfection activity in such an extent, thus masking the effect of lipid composition. Overall, these results suggest that DOTAP:Chol/DNA complexes constitute good candidates for in vivo gene delivery, since their low ability to interact with serum proteins predicts that they will be stable in circulation for long periods, thus allowing them to reach target organs, while remaining active [42,44].

The studies on the access of EtBr to DNA indicate that for the 1/2 (±) charge ratio complexes, a significant amount of DNA (70%) is exposed, which is in agreement with the highly negative value obtained for their zeta potential. Electron microscopy studies performed with cationic liposome/DNA complexes, for which 65% of DNA was available to EtBr intercalation, indicate that these complexes exhibit a structure where DNA can be detected, suggesting that part of it is free and noncondensed [45]. On the other hand, the absence of EtBr intercalation for DOTAP:Chol/DNA complexes prepared at the 2/1 and 4/1 lipid/DNA charge ratios shows that DNA is condensed and fully protected, in contrast to what was observed for DOTAP:DOPE/DNA complexes (at the same charge ratios). Regarding complexes composed of FuGENE 6, our results show that DNA is not completely condensed. Nevertheless, this lack of condensation cannot be associated with the presence of free DNA, since their zeta potential is positive, suggesting that DNA is covered by FuGENE 6 [45].

Regarding the protection of DNA complexed with the liposomes, the results on the access of EtBr to DNA should be taken with caution since, being a small molecule, it can reach DNA domains that may not be available to DNase I digestion [46]. In an attempt to clarify this issue, DNase I degradation assays were performed for some of the formu-

lations tested. The results obtained indicate that positively charged cationic liposome/DNA complexes ensure a higher degree of DNA protection, this being essentially complete for complexes prepared at 2/1 and 4/1 charge ratios, independent of the lipid composition of the complexes. These results reinforce the importance of performing the DNase I degradation assay, which is more biologically relevant than the EtBr access assay. Nevertheless, for complexes prepared from FuGENE 6 at a 3/1 (v/w) ratio, a significant amount of genetic material was degraded by DNase I, thus confirming the results obtained from studies using EtBr.

Regarding the effect of time and temperature of storage of the complexes on their biological activity, the significant inhibition observed when the cationic liposome/DNA complexes are stored at 25 and 4 °C can be attributed to the formation of large aggregates, which as referred above, may prevent their cell internalisation and consequent intracellular gene delivery [47,48]. The loss of approximately 50% of the transfection activity of the complexes stored for 24 h at − 20 °C is most likely due to structural changes of the complexes during the freezing process, since 50% of their biological activity remains unaltered for longer periods of storage [19,20]. In fact, when the complexes are rapidly frozen and stored at − 80 °C, their ability to mediate transfection is essentially preserved for at least 20 days. This hypothesis was supported by the observation that no major modifications of size and charge of the complexes occurred under these conditions. In contrast to what was observed for the case of cationic liposome/DNA complexes, this process of freezing and storage at − 80 °C is not suitable to maintain the transfection activity of FuGENE 6/DNA complexes.

Taking into account the parameters affecting the formation and structure of lipoplexes, their biological stability, and those affecting their mode of interaction with cells, it can be concluded that the design of a nonviral vector capable of fulfilling the conflicting requirements imposed by each of the different stages involved in the gene delivery process is a challenging task. The results obtained in this work show that the complexes leading to the highest biological activity are those exhibiting high sensitivity to the presence of serum and lower protection against DNase I. On the other hand, lipoplexes that are able to protect DNA against enzymatic degradation and are resistant to the presence of serum are not so efficient in mediating gene delivery. Nevertheless, the ability to protect DNA and to exhibit serum resistance are major requirements for in vivo applications as long as the physicochemical properties of the complexes, namely size and charge, will be suitable for such purpose. Our findings reinforce the importance of fully characterizing cationic liposome/DNA complexes and of comparing different lipid-based gene delivery systems, in order to allow the identification of critical formulations parameters aiming at obtaining a suitable vector for application in gene therapy.

Acknowledgements

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