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Physico-chemical characterization and transfection efficacy of cationic liposomes containing the pEGFP plasmid

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Abstract

Cationic liposomes–DNA complexes (lipoplexes) are largely used in gene delivery. Deciphering specific chemical and physical properties of lipoplexes is a necessary step to unravel the mechanisms underlying transfection and to improve transfection efficacy in each experimental model. In the present paper we investigated the physico-chemical features of lipoplexes containing a plasmid encoding for the GFP protein, in order to correlate these results with transfection efficacy. Cationic unilamellar vesicles (mean diameter 100 nm) were prepared, from the cationic DC-Chol lipid and the zwitterionic phospholipid DOPE. The two components of the liposome bilayer were used at molar ratio close to unity. ESR spectra were recorded and zeta potential ζ was measured on liposomes complexed with the plasmid. One of the main points of interest in this paper resided in the fact that both kinds of measurements were carried out in the same conditions (i.e. lipid concentration, medium composition, and pH) employed for cell transfection experiments. Transfection was performed on CHO cells; the percentage of fluorescent cells was evaluated and compared with the above physico-chemical features. It emerged that the composition and pH of the medium, the lipoplex / cell ratio, as well as the amount of lipoplex added to the cell culture were critical parameters for transfection efficacy. Finally, lipoplex surface charge played a fundamental role to achieve a high transfection level.

Keywords: Transfection efficacy; Lipoplex; Gene therapy; Zeta potential; ESR

1. Introduction

Cationic liposomes are self-assembled nanosystems. They can be prepared by mixing cationic and neutral surfactants (typically lipids, henceforth simply called lipid$^+$ and lipid, respectively). Typical cationic lipids are 1,2-dioleoyl-3-trimethylammoniopropane (DOTAP) and [*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]-cholesterol (DC-Chol.). Typical zwitterionic lipids are 1,2-dioleoyl-*sn*-glycerol-phosphoethanolamine (DOPE) and, less commonly, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC).

Cationic vectors are currently investigated as possible carriers of nucleic acids in gene delivery. Particular attention is given to complexes of DNA with block cationic copolymers (polyplexes), such as polymethacrylates, poly(ethylene oxide) or poly(propylene oxide) [1–5], and to the stable systems (lipoplexes) which form from cationic liposomes and nucleic acids, possibly in the form of lipid-DNA particles [6–11]. Both kinds of systems are non-viral, and their formation processes are entropically favored because of the release of counterions to the solution [6,7]. In this work our attention was dedicated to lipoplexes that contained natural DNA. Although in the last decades liposomes loaded with different genetic material have been used for in vivo and in vitro experiments, the differences between in vitro and in vivo transfection are still unclear [4]. The results reported here for the in vitro transfection cannot be

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automatically transferred to the in vivo process. Anyway, many physical and biological studies of cationic lipid formulations have been performed [12,13] and the goal of these studies is to improve our understanding of lipoplex structure and lipofection processes.

The positively charged structure binds to the negatively charged outer membrane of most cells. Adsorptive endocytosis has been claimed to be the main mechanism for entering cells [14,15].

The use of the zwitterionic lipid DOPE is justified by the reported observations that DOPE itself, as a liposome component, induces high levels of transfection in several cell lines, promotes fusion of the plasma membrane, and facilitates intracellular DNA delivery [16–19].

A better knowledge of some physico-chemical properties of lipoplexes is necessary to shed light on the transfection mechanism, which has been shown to markedly depend on parameters such as the total lipid/DNA ratio [20], the nature of the surfactant [21], the architecture and charge of cationic liposomes, and the size of the complex [22,23]. These properties can dramatically change the lifetime, the in vivo distribution, and the transfection efficacy of lipoplexes. Several techniques have been employed in recent years, including microelectrophoresis, which yields the zeta potential (ζ), i.e. the surface charge of the moving aggregates [24,25]. In the case of liposomes, ζ is sensitive to the lipid phase transitions, to the adsorption of amphiphiles, proteins or nucleic acids, to steric stabilization, and to surface modifications. This parameter is therefore useful to monitor liposome and lipoplex stability, and to check the reproducibility of different preparations. S-shaped ζ titration curves have been obtained, whose inversion point depends on the system under study.

Electron Spin Resonance (ESR) spectroscopy of inserted nitroxides has been used for the first time by Ciani et al. [26] in the characterization of lipoplexes built up with oligonucleotides (ODN) in Tris/HCl buffer solution. This has allowed to extract interesting insights from the comparison between ζ potential values and ESR spectra of stable radicals inserted either in cationic liposomes or in their lipoplexes with single-strand and double-strand ODN.

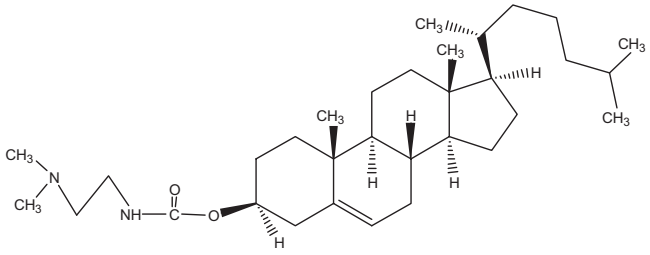
The ESR spectra of spin probes inserted into aggregates is indeed sensitive to changes in the aggregate structure, in the environmental polarity and in the mobility of neighboring molecules [27]. Thus, any change in the ESR line shape by passing from ODN-free to ODN-loaded liposomes should suggest the incorporation of the polynucleotide into the liposome bilayers.

In the present work we discuss the ESR and ζ potential data obtained on lipoplexes formed by DC-Chol/DOPE liposomes and a plasmid encoding for the Enhanced Green Fluorescent Protein (pEGFP). A comparison is also made with ODN-loaded liposomes in Tris/HCl buffer and in the same culture medium used for plasmid transfection experiments. Lipoplex structure and charge were analyzed in the same experimental conditions used for transfection, and were correlated to transfection efficacy.

2. Experimental procedures

2.1. Materials

3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]-cholesterol (DC-Chol, purity > 99%, **1**) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, purity > 99%, **2**) were purchased from Avanti Polar Lipids, Inc., Alabaster, AL, and used without further purification.



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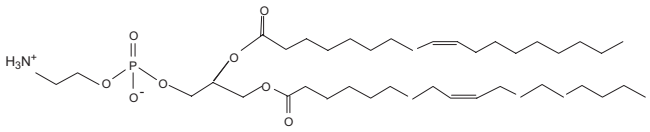

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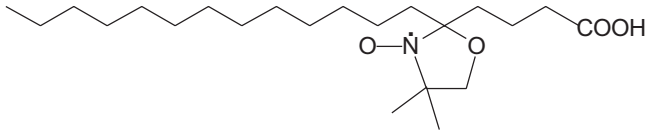
5-Doxyl-stearic acid spin probe (5-DSA, **3**) was purchased from Sigma Chemicals, München, Germany, and used as received.



3

The plasmid used to form lipoplexes was pEGFP-pcDNA 3.1 (Invitrogen, insert length 4.7 kb). Plasmid extraction from *E. Coli* DH5α cells was performed using the Qiagen MIDI kit, according to the manufacturer's instructions.

Tris(hydroxymethyl)aminomethane hydrochloride (Tris, TRIZMA-HCl) was purchased from Sigma, St. Louis, MO, USA. Culture medium was DMEM/F12 (1:1) (Euroclon) containing 25 mM Hepes and 10% FCS (HyClone). The DMEM/F12 medium, which is commonly used in transfection experiments for its low toxicity toward cells, will be indicated as "transfection medium" throughout this work.

The 20-mer oligonucleotides (ODNs) polyadenine (polyA) and polythymidine (polyT) were purchased from Qiagen Operon, Alameda, CA, and were solubilized in 10^{-2} mol/dm^3 Tris/HCl at pH = 7.4 to obtain a total phosphate concentration of 7×10^{-3} mol/dm^3. The ODN solutions were diluted to the desired concentration with Tris/HCl or DMEM/F12 containing no serum and 25 mM Hepes as internal buffer.

The double strand polyA–polyT, in the following called dsAT, was obtained by mixing equal amounts of polyA and

polyT solutions at the same concentration and then annealing at room temperature for 24 h. All samples were stored at 253 K.

Chinese Hamster Ovary (CHO) cells were cultured in 100-mm Petri dishes in DMEM/F12 (1:1) medium (Euroclone) containing 25 mM Hepes and 10% FCS (Fetal Calf Serum, HyClone). This medium will be henceforth referred to as "culture medium". Cells were kept in a humidified incubator at 310 K, 5% CO_2 and split 1 to 5 twice a week by treating them with a 0.05% Trypsin, 0.02% EDTA solution in PBS (Euroclone).

2.2. Sample preparation

The large unilamellar cationic vesicles (LUVs) used in this work were made by DC-Chol and DOPE at molar ratio 1.1 (±0.1). Liposomes and ODN lipoplexes were prepared as described in Ref. [26]. LUVs were stored at 277 K.

pEGFP plasmid solutions at the required concentration were added to an equivalent volume of liposomes to form lipoplexes. The DNA concentration was determined by measuring the absorbance at 260 nm.

$DNA^-/lipid^+$ charge ratio was in the range 0–1. In each sample the lipid concentration was taken as constant, and the DNA concentration was varied in order to obtain the desired charge ratio. For all the complexes, DNA was injected into the cationic liposome suspension and not vice versa, to ensure reproducibility [28]. Samples were analyzed not earlier than 10 min and not later than 24 h after the preparation.

The final lipid concentration was 1.4×10^{-3} mol/dm^3, and it was diluted to 7×10^{-4} mol/dm^3 by adding an equal volume of DNA solution at the required concentration.

2.3. Transfection experiments

The day before transfection experiment, CHO cells, cultured as described above, were seeded in a 24 multi-well plate (Corning) at 1.5×10^5 cells/well in 500 μl of culture medium. Six hours before transfection, the culture medium was replaced by the transfection medium. In the meanwhile, 50 μl of plasmid solution (at different DNA concentrations) were mixed with 50 μl of DC-Chol/DOPE liposomes (1.4×10^{-3} mol/dm^3) and incubated at room temperature for 10 min, to ensure the stabilization of the complex. The plasmid/liposome mixture was then added to the cells. After a 6-h incubation (in typical culture conditions), the transfection medium was replaced by 500 μl of the culture medium.

Experiments were usually stopped 24 h later. Cells were fixed with 4% PFA (paraformaldehyde) in PBS and analyzed under an inverted microscope (Nikon TS100) equipped with a fluorescence apparatus. Three fluorescence and three phase contrast photos were acquired with a digital Nikon Coolpix 5000, for each well. The ratio of fluorescent and total cells was used as a parameter to estimate transfection efficacy of the lipoplexes.

2.4. Methods

The ζ potential, as well as the size of liposomes and lipoplexes, were measured with the ζ-meter Coulter DELSA 440 SX (Coulter Corporation, Miami, FL, USA). In this apparatus the ζ potential is automatically calculated from the electrophoretic mobility. Home made hemispherical electrodes, covered by a thin gold layer, were used as the measure cell. This reduced oxidation that partially affects the silver electrodes typically used in this kind of instruments. The measure cell was sterilized by dipping in H_2O_2 for 10 min before each set of experiments. Runs were performed as detailed in Ref. [26]. Sizes were calculated by assuming light scattering due to Brownian diffusion [29].

ESR spectra were obtained with a Bruker ESR spectrometer model 200D, working in the continuous wave mode at X-band (~9.5 GHz). Samples were inserted in the typical rectangular cavity. Data acquisition and handling were carried out with the ESR software commercialized by STELAR (Meda, Italy). Temperature was controlled with the Bruker VT 3000 apparatus (accuracy ±0.5 K). Stock ethanol solutions (10^{-3} mol/dm^3) of 5-DSA probe were used as described in Refs. [26,30]. ESR spectra were recorded from samples obtained by adding the spin label to preformed lipoplexes. In order to avoid inhomogeneously lined broadening and marked changes in the electric properties of the liposome bilayer, the ratio between ESR probe and lipid molecules was always kept $\leq 1/100$.

ESR spectra line shapes were analyzed and computed with the procedure given by Budil et al. as described in Ref. [31].

3. Results

3.1. Physico-chemical characterization

This section provides in depth details on the lipoplex structure, mean size and overall charge, which were extracted from ESR, ζ potential and light scattering, respectively. Such information was subsequently used to guide our choice of the systems to be used in transfection experiments.

Fig. 1 compares the 298 K ESR spectra of 5-DSA (7×10^{-6} mol/dm^3) inserted into the bilayer of plasmid-free DC-Chol/DOPE liposomes (absorption A) and into DC-Chol/DOPE liposomes containing pEGFP at two different $DNA^-/lipid^+$ ratios, i.e. 0.15 and 0.5 (absorption B and C, respectively), dispersed in the culture medium. Spectra A and B were consistent with 5-DSA spin probes incorporated into structure with partially averaged magnetic anisotropies. The same interpretation was given to the broad component in spectrum C of Fig. 1, whereas the narrow triplet marked with * in the same spectrum C was attributed to fast moving free nitroxides in solution. From double integration of the ESR spectrum in Fig. 1C the contribution of the free moving radical was estimated as 7–10% of the total intensity. The superposition of two different signals in the same ESR spectrum could be

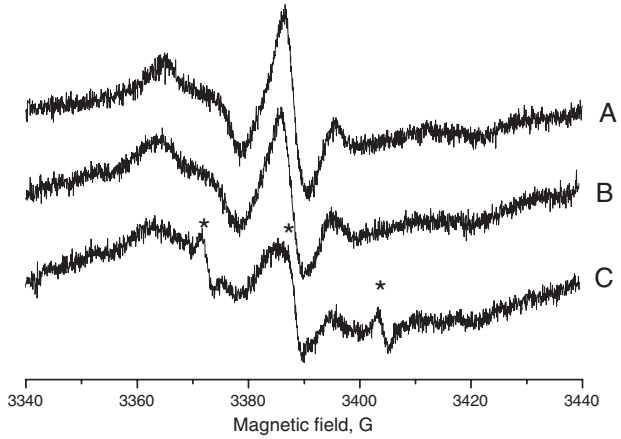


Fig. 1. Experimental 298 K spectra of 5-DSA (7×10^{-6} mol/dm^3) in preformed DC-Chol/DOPE lipoplexes (total lipid concentration 7×10^{-4} mol/dm^3) in DMEM/F12 medium containing different pEGFP concentrations. Line A: $pEGFP^-/lipid^+=0$; line B: $pEGFP^-/lipid^+=0.15$; line C: $pEGFP^-/lipid^+=0.50$; * indicates the three-line signal of the spin probes free in solution.

interpreted on the basis that, with high amounts of plasmid ($DNA^-/lipid^+\geq0.5$), the negative DNA-covered surface of the lipoplex hindered the probe insertion above a certain threshold. As it can be evidenced from the overall line shape of the ESR spectra reported in Fig. 1, the probe did not sense large differences due to the presence of the plasmid. Therefore, the bilayer structure was not drastically modified by the DNA interactions with the liposome surface, at least in the molecular range probed by isolated 5-DSA molecules (2–3 nm lateral width). These results are in agreement with those reported by Ciani et al. [26], who show that the ESR spectra of 5-DSA and 16-DSA inserted in the same type of liposomes inserted in Tris/HCl buffer do not change when different amounts of single and double stranded oligo-deoxy-nucleotides are added to vesicles.

As mentioned above, the broad ESR signal of 5-DSA in lipoplexes was the well known spectrum typically attributed to species that slowly move in restricted-motional conditions, such as they are paramagnetic probes inserted into large, slowly moving aggregates. In these conditions, the **g** and **A** tensor components are not completely averaged and the ESR line shape is dominated by relaxation effects due to not-averaged magnetic anisotropies [26,30].

Spectra were calculated according to the procedure given by Freed and coworkers [31–33] with the following ESR magnetic parameters that gave the best fit for 5-DSA:

g_{xx}	2.0087±0.0005
g_{yy}	2.0067±0.0005
g_{zz}	2.0028±0.003
A_{xx}	0.60±0.01 mT
A_{yy}	0.56±0.01 mT
A_{zz}	3.35±0.02 mT

The motional parameters obtained from the best fit calculation of the 5-DSA ESR spectra in DC-Chol/DOPE liposomes dispersed in the culture medium and in Tris/HCl buffer are summarized below:

5-DSA in DC-Chol/DOPE in DMEM/F12

$\tau_\perp$	$45(\pm2)\times10^{-10}$ s
$\tau_{\|\|}$	$0.81(\pm0.05)\times10^{-10}$ s
S_{20}	0.22(±0.02)

5-DSA in pEGFP/DC-Chol/DOPE in DMEM/F12

$\tau_\perp$	$45(\pm2)\times10^{-10}$ s
$\tau_{\|\|}$	$0.81(\pm0.05)\times10^{-10}$ s
S_{20}	0.22(±0.02)

5-DSA in DC-Chol/DOPE in Tris/HCl [from Ref. [26]]

$\tau_\perp$	$26(\pm2)\times10^{-10}$ s
$\tau_{\|\|}$	$1.1(\pm0.2)\times10^{-10}$ s
S_{20}	0.55(±0.02)

5-DSA in dsAT /DC-Chol/DOPE in Tris/HCl [from Ref. [26]]

$\tau_\perp$	$26(\pm2)\times10^{-10}$ s
$\tau_{\|\|}$	$1.1(\pm0.2)\times10^{-10}$ s
S_{20}	0.55(±0.02)

where $\tau_\perp$ and $\tau_{||}$ are the correlation times describing the probe anisotropic motion, and S_{20} was the order parameter [31–33].

From the above data, it emerged that the effect of the suspension medium was relevant. In fact, 5DSA probes experienced a more restricted motion when inserted in the bilayer of liposomes suspended in the culture medium rather than in Tris/HCl buffer. On the other hand, ESR results from liposomes and lipoplexes, dispersed either in Tris/HCl buffer or in the culture medium, completely agreed with wrapping of the plasmid on liposome surface, as it is suggested in Ref. [26] for polyA, polyT and dsAT on DC-Chol/DOPE liposomes in Tris/HCl. This suggested that mobility, order and polarity of the plasmid containing lipoplexes, as revealed by ESR data, were not modified with respect to the plasmid-free liposomes and only depended on the environmental medium. This corresponded to the external model proposed by Felgner and Ringold [34] for the adsorption of nucleic acid on cationic liposomes, and meant that the lipoplexes under study were intact liposomes wrapped by DNA, that bridged together lipoplexes to form larger aggregates.

The effect of the presence of DMEM/F12 culture medium with respect to the simple Tris/HCl buffer clearly appeared also from ζ potential measurements. Fig. 2 shows the ζ values as a function of the lipoplex composition: part A compares the ζ values of polyT-containing DC-Chol/DOPE lipoplexes in both Tris/HCl (filled squares) and in transfection DMEM/F12 medium (empty squares) while part B compares the ζ values of dsAT-containing DC-Chol/DOPE lipoplexes in Tris/HCl (filled squares) and in DMEM/F12 (empty squares).

DNA-free DC-Chol/DOPE liposomes had ζ=+30 (5) mV when measured in the culture medium. This value differed from that of the same liposomes in Tris/HCl (ζ=+39±2 mV). As expected on the basis of previously reported results on similar systems, ζ values as a function of the $ODN^-/lipid^+$ ratio had

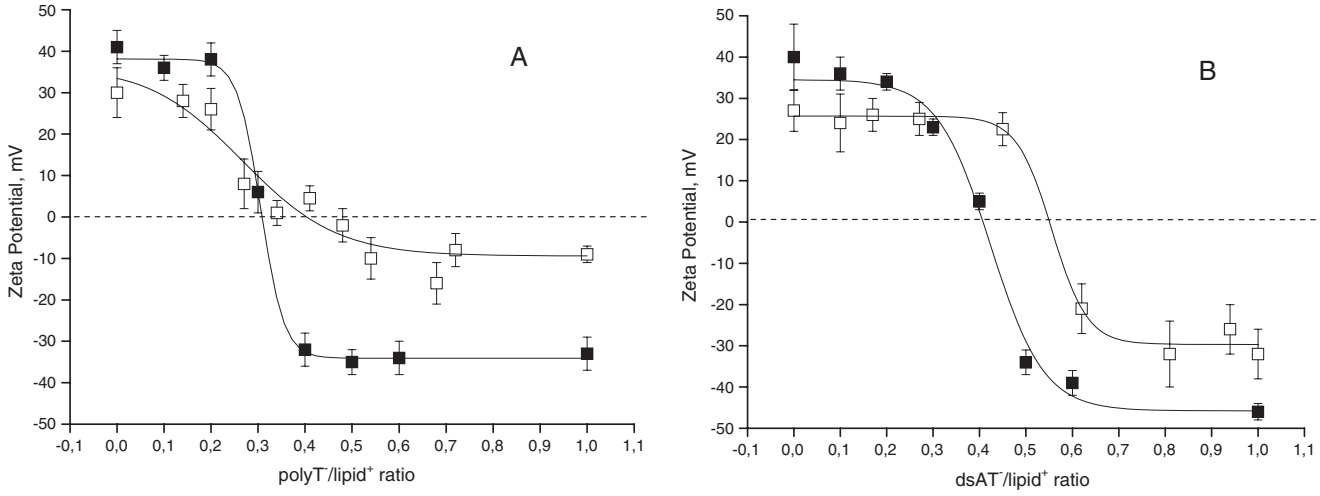


Fig. 2. Zeta potential of DC-Chol/DOPE liposomes at increasing values of the polyX$^-$/lipid$^+$ratios: A): polyT/DC-Chol/DOPE lipoplexes in Tris/HCl, total lipid concentration 8.75×10^{-4} mol/dm^3 (full squares) and in DMEM/F12, total lipid concentration 7×10^{-4} mol/dm^3 (empty squares). B): dsAT/DC-Chol/DOPE lipoplexes in Tris/HCl, total lipid concentration 8.75×10^{-4} mol/dm^3 (full squares) and in DMEM/F12, total lipid concentration 7×10^{-4} mol/dm^3 (empty squares). All curves underlying data simply represent a guide to the eye and were drawn to better evidence trend variations.

sigmoidal shapes, which markedly depended on the nature of the suspension medium. In Tris/HCl the ζ potential curves showed a larger vertical drop, which corresponded to a larger variation of the of lipoplexes surface charge as a function of DNA$^-$/lipid$^+$ ratio. In the different suspension media the value ζ=0 was reached at different polyT$^-$/lipid$^+$ and dsAT$^-$/lipid$^+$ ratios, namely 0.30 and 0.40 for polyT, and 0.40 and 0.54 for dsAT, in Tris/HCl and in the transfection medium, respectively.

In Fig. 3 the ζ potential values are reported as a function of pEGFP plasmid concentration in the transfection medium. Apart from the isoelectric point, which occurred at a lower pEGFP$^-$/lipid$^+$ ratio in plasmid-containing liposomes, the shape was almost the same as for dsAT$^-$/lipid$^+$ lipoplexes in the same medium.

Two factors could account for the above reported trends: i) the nature of the suspension medium; ii) the different final surface charge. The occurrence of inorganic salts, electrolytes, and organic compounds in the transfection medium modified both the electric double layer at the liposome surface and the surface charge (that is the ζ value), which remained more positive in Tris/HCl buffer than in the transfection medium.

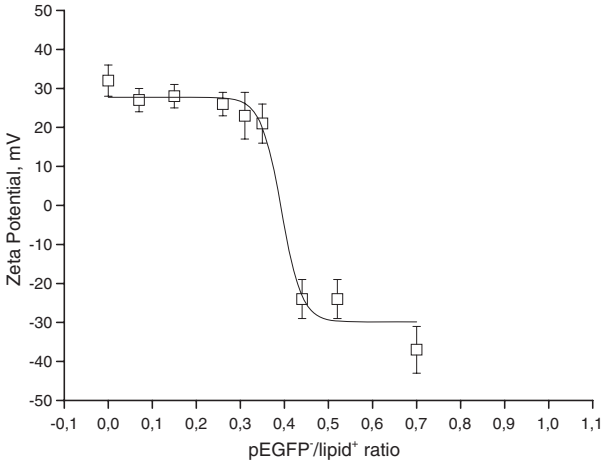


Fig. 3. Zeta potential of DC-Chol/DOPE liposomes in DMEM/F12 (total lipid concentration 7×10^{-4} mol/dm^3) at increasing values of the pEGFP$^-$/lipid$^+$. The curve underlying data simply represents a guide to the eye and was drawn to better evidence the trend.

In the transfection medium, the size of the aggregates, as measured by electrophoretic light scattering, increased from 120–130 nm for pure liposomes to 300–400 nm for lipoplexes in the two plateau regions before and after the inversion point. This strongly pushed towards a partial bridging together of DNA-containing-liposomes to form larger aggregates. This hypothesis was in agreement with the arguments of Bordi et al. [35], who report the time-course aggregation of cationic liposome–polyelectrolyte (mimicking DNA) complexes, studied by dynamic light scattering. The size deformability and the susceptibility to field-induced polarization might also be considered because the mobilities of prolated spheroidal shapes often fit in a better manner the electrophoretic behavior of liposomes in salt-containing medium, as recently shown by Pysher and Hayes [36], who demonstrate that spherical colloid theories typically used to model the electrophoretic behavior of liposomes [37–43], and therefore their ζ potentials, do not adequately depict these systems. So the size value we reported was only an indication, and not a precise value, describing the lipoplex (or complex) growing.

Another important variable in lipoplex preparation was the concentration of lipids in the starting liposome solutions. Liposomes were extruded at the concentration of 1.4×10^{-2} mol/dm^3 total lipids, and diluted to the proper concentration (i.e. 4×10^{-3} mol/dm^3) for ESR and ζ potential measurements. This procedure always ensured the formation of positively charged stable liposomes. The same did not happen when liposomes were prepared at lower lipid concentration. In this latter case, we obtained liposomes with variable, usually very low ζ value (i.e. 0–10 mV). This fact often impaired the formation of stable lipoplexes when liposomes were mixed with high concentrations of plasmid and it was carefully taken into account for transfection experiments.

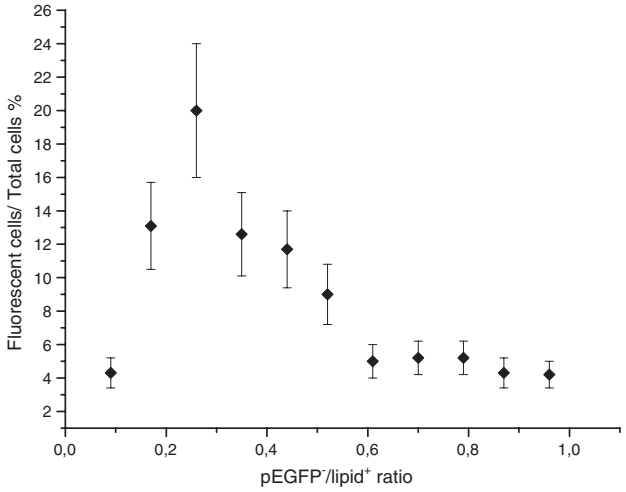


Fig. 4. Transfection efficacy of DC-Chol/DOPE lipoplexes (total lipid concentration 7×10^{-4} mol/dm^3) on CHO cells, reported as percentage of EGFP-positive cells versus pEGFP$^-$/lipid$^+$ charge ratios.

3.2. Transfection experiments

The pEGFP-containing lipoplexes characterized in the above described way were tested for in vitro transfection experiments; CHO cells were used due to their propensity to be efficiently transfected.

The occurrence of the transfection process could be easily detected, since the plasmid used in our experiments allowed the cells to acquire a fluorescent signal. We determined the number of fluorescent cells, instead of the amount of fluorescent protein per cell, since we believe that the former parameter better mirrored the transfection efficacy of lipoplexes.

The lipoplex concentration and the ratio between the negative charges of DNA and the positive charges of liposome were investigated as the main variable, which influenced transfection efficacy. As described above, the pEGFP$^-$/lipid$^+$ ratio influenced important physico-chemical properties of the lipoplex, such as its ζ potential value and mean size. These differences actually corresponded to different transfection levels (Fig. 4). In addition, lipoplexes with the same pEGFP$^-$/lipid$^+$ ratio but administered in smaller volume resulted in lower efficacy. An example of this trend is reported in the Table 1 for pEGFP$^-$/lipid$^+$=0.25.

Fig. 4 shows transfection efficacy, calculated as the percentage of fluorescent cells in the culture, as a function of pEGFP$^-$/lipid$^+$ ratio. In this type of experiments, cultures were treated with the same volume of liposomes (100 μl), but contained different plasmid amount. It is evident that the maximum percentage of fluorescent cells was reached at a pEGFP$^-$/lipid$^+$=0.25, which corresponded to positive lipoplexes.

The trend reported in Fig. 4 shows that, as the pEGFP$^-$/lipid$^+$ ratio increased, the fluorescent cell percentage started to grow from 4% to 20%. When the isoelectric point (around 0.4 pEGFP$^-$/lipid$^+$ charge ratio, from Fig. 3) was approached, transfection efficacy started to decline. This could be due to the fact that cationic liposomes covered by the plasmid, aggregated because Van der Waal's attraction overcame electrostatic repulsions. It has been reported that larger aggregates result in a reduced transfection efficacy [44,45]. At negative ζ values (i.e. when the negative charge of the wrapping plasmid masked the positive surface of cationic liposomes) transfection efficacy decreased down to the initial values. In these conditions, the addition of increasing amount of DNA did not turn into higher transfection efficacy. The same trend is found by Congiu et al. [46] in inverted hexagonal $H^C_{//}$ complexes, with the same type of lipids. This suggested that the best conditions were achieved when liposomes bound relatively high amount of DNA, but not enough to completely neutralize the positive charge of the liposome surface.

Fig. 5A shows the morphological appearance of CHO cells transfected in the best experimental conditions of Fig. 4.

Some other experimental variables needed to be taken into account in transfection experiments. The best transfection

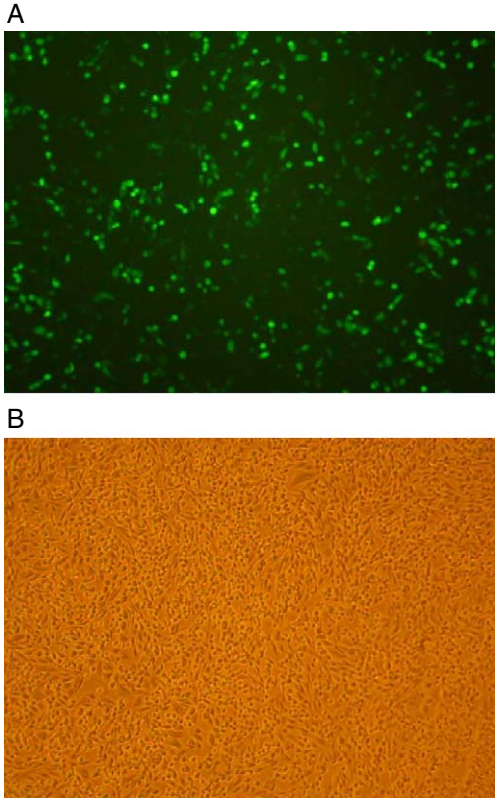


Fig. 5. Fluorescence (A) and contrast phase (B) micrograph of CHO cells treated with DC-Chol/DOPE lipoplexes containing 3 μg of pEGFP, corresponding to a 0.26 pEGFP$^-$/lipid$^+$ charge ratio, and imaged at 10× magnification. Successfully transfected cells are intensely EGFP-positive (green). Cells are imaged as reported in Methods. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Transfection efficacy as a function pf lipoplex volume at pEGFP$^-$/lipid$^+$=0.25

Lipoplex volume	Transfection efficacy (%)
100 μl	20±4
50 μl	12±2
25 μl	5±1

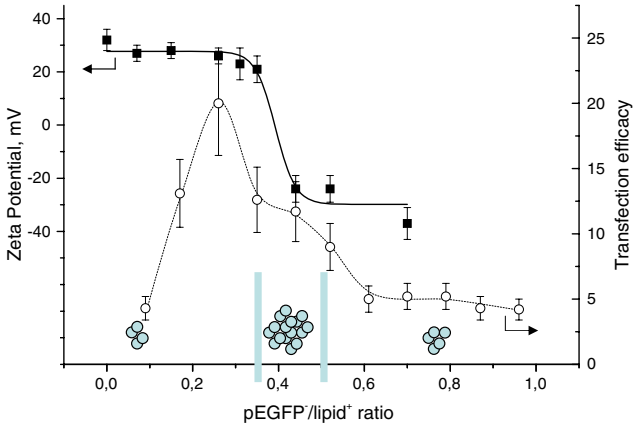


Fig. 6. Comparison between transfection efficacy (right side scale) and zeta potential (left side scale) as a function of $pEGFP^-/lipid^+$ charge ratios. Blue aggregates at the bottom are just a schematic representation of lipoplexes size $pEGFP^-/lipid^+$ charge ratios and are not in scale. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

results were obtained using a cell inoculum of 1.5×10^5 cells per well and culturing cells for 24 h starting from semiconfluent cell culture. Lower cell densities turned in a lower transfection efficacy. On the other hand, the inoculum of a larger number of cells produced an increased cell death after the 24 h culture time. The same occurred if the incubation time after transfection was prolonged to 48 h.

Another important factor for the optimization of transfection was the medium: we used the transfection medium, defined in the experimental section, for both cell culture and sample preparation. On the other hand, when DMEM/F12 without buffer was used, liposomes were more difficult to prepare and the final liposome suspension was not stable.

As far as toxicity of pEGFP/DC–Chol/DOPE lipoplex is concerned, all cell systems described in this work exhibited good viability. To exemplify this point Fig. 5B shows the contrast phase micrograph corresponding to the transfection experiment of Fig. 5A.

In summary, the main variables affecting transfection efficacy and the best conditions emerged in our experimental systems were the following:

- *cell concentration*: 1.5×10^5 cells per well in a 24 multi-well plate
- *transfection medium*: DMEM/F12 (1 : 1) with 25 mM Hepes at pH: 7.4–7.5
- *incubation time after transfection*: 24 h
- *extrusion lipid concentration*: 1.4×10^{-2} mol/dm^3
- *$pEGFP^-/lipid^+$ ratio:* 0.25
- *lipoplex volume:* 100 μl.

4. Discussion and conclusion

In this paper DC-Chol/DOPE liposomes loaded with the plasmid encoding for the EGFP protein were characterized with respect to their physico-chemical structure and transfection efficacy. In particular, the physico-chemical characterization of lipoplexes was carried out in the same experimental conditions used in transfection experiments. Both features turned out to be strictly linked to each other.

ESR experiments allowed to establish that $pEGFP^-/lipid^+$ lipoplexes were formed by intact liposomes wrapped with the DNA. ζ potential and size investigation could be correlated with transfection efficacy, as it is shown in Fig. 6, where the results reported above are summarized in a schematic comparison among: i) ζ potential value, ii) transfection efficacy, and iii) aggregate size, as a function of the $pEGFP^-/lipid^+$ ratio.

When the complexes were positively charged (left side of the Fig. 6), they could easily interact with the cells resulting in efficient transfection and the particle size was not very different from the starting liposomes. When the amount of plasmid loaded into liposomes increased above $pEGFP^-/lipid^+$ ~0.25 (central panel of Fig. 6), the size of lipoplexes increased and penetration across the plasma membrane was hindered, thus overcoming the favorable charge effect. When ζ potential became negative (right side of Fig. 6) transfection efficacy fell down to a basal level. This decrease in transfection efficacy could be related to the repulsive interaction between negative lipoplexes and the negative charge of the plasma membrane.

On the other hand, many experimental variables had to be checked and adjusted to reach a satisfactory transfection protocol. Additional parameters which ensured high transfection level in our system turned out to be neutral pH and semiconfluent cell culture.

All of these variables should be taken into account and adjusted to their optimal value for efficient DNA delivery in different cell lines.

In conclusion, the surface lipoplex charge and its interaction with the cell membrane have to be considered in each experimental model to reach optimum DNA delivery. In fact, each cell to be transfected can display different behaviors on the plasma membrane, which facilitates/impairs transfection [47]. However, also the amount and size of the lipoplex/cell couple involved in each transfection experiment may play a role and have to be investigated as possible important parameters.

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