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DOTAP/DOPE and DC-Chol/DOPE lipoplexes for gene delivery: zeta potential measurements and electron spin resonance spectra

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Abstract

Non-viral vectors represent an important alternative in gene delivery. Among these vectors, cationic liposomes are widely studied, because of their ability to form stable complexes with DNA fragments (lipoplexes). In the present work, we report on the characterization by electron spin resonance (ESR) spectroscopy and zeta potential measurements of cationic liposomes and of their complexes with oligonucleotides. Liposomes were made with a zwitterionic lipid, DOPE, and a cationic lipid, either DOTAP or DC-Chol. Oligonucleotides were the 20-base single strand polyA, the 20-base single strand polyT, and the corresponding double strand dsAT. The zeta potential as a function of the oligonucleotide/lipid$^+$ ratio gave an S-shaped titration curve. Well-defined surface potential changes took place upon charge compensation between the cationic lipid heads and the phosphate groups on the oligonucleotides. The inversion point depended on the specific system under study. The bilayer properties and the changes that occurred with the incorporation of DNA fragments were also monitored by ESR spectroscopy of appropriately tailored spin probes. For all the systems investigated, the ESR spectra showed that no major alteration took place after lipoplex formation and molecular packing remained substantially unchanged. Both zeta potential and ESR measurements were in favor of an external mode of packing of the lipoplexes.
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Keywords: ESR; Gene delivery; Lipoplex; Vesicle; Zeta potential

1. Introduction

For more than 20 years, gene therapy has represented a useful approach to treat acquired and inherited diseases by transfection, which ferries correct copies of defective genes into the cell. Typical agents for this technique are cationic liposomes and the complexes (*lipoplexes*) that they form with negatively charged natural and synthetic oligo-deoxy-nucleotides (ODN) [1,2]. The positive charge on liposomes fabricated with cationic lipids (henceforth indicated as lipid$^+$) ensures the binding to the cell membrane because of the negative charge on most of the latter [3]. Lipoplexes then enter the target cells mainly by absorptive endocytosis [4]. Thus, liposomes can act as vectors able to carry anticancer agents for preferential delivery to distal tumor sites following intravenous injection [5–7], although some controversy exists on the advantage of lipoplexes with respect to viral vectors in gene therapy protocols [8].

The use of synthetic cationic surfactants in the form of liposomes represents about 1/5 of the current clinical trials in gene therapy [2,9–11]. Cationic liposomes spontaneously react with negatively charged ODN, such as plasmid DNA, single-strand and double-strand polynucleotides, with the formation of self-assembled complexes, where 100% of negatively charged molecules are involved in the condensation reaction [12].

Abbreviations: DC-Chol, 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]-cholesterol; DOTAP, 1,2-dioleoyl-3-trimethylammoniopropane; DOPE, 1,2-dioleoyl-*sn*-glycerol-phosphoethanolamine; 5-, and 16-DSA, 5- and 16-doxylstearic acid; dsAT, double strand adenosine-thymidine polynucleotide; EDTA, ethylenediamino-tetraacetate; ESR, electron spin resonance; LUV, large unilamellar vesicle; ODN, oligo-deoxy-nucleotide; polyA, poly-deoxy-adenosine; polyT, poly-deoxy-thymidine

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Much work has been done to determinate the lipoplex microstructure and stability with different experimental techniques, as well as on their transfection efficiency [13–22]. One critical element for efficient gene delivery is the lipid composition of cationic liposomes. Since the first studies in the early years of the field, many quaternary ammonium surfactants have been used, including compounds with alkyl, ether, and ester bonds. The cationic lipids used as transfection agents are indeed readily and easily metabolized by the target tissues [23–25]. Most of the cationic carriers used from the beginning have been fabricated with monocationic lipids [2,23,25,26]. 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP), a double chain quaternary ammonium surfactant, is the most popular cationic lipid used in lipoplex formation. Polycationic carriers have also been used in the last few years in order to elucidate whether the combination of polycations, such as polyethyleneimines [27] or spermin derivatives [8], and cationic liposomes is able to enhance the transfection efficiency.

When cationic lipids are used as carriers of nucleic acids (DNA), periodic multilayer structures are often formed with DNA chains adsorbed between lipid membranes, as suggested by X-ray scattering techniques [15,28]. Most cationic liposomes have a neutral phospholipid component in addition to the lipid$^+$. 1,2-Dioleoyl-*sn*-glycerol-3-phosphoethanolamine (DOPE) is commonly used as a helper lipid [29]. Improved DNA translocation and transfection efficiency in the presence of DOPE were demonstrated by several authors [8,30,31]. DOPE is also chosen because of its ability to reduce the cytotoxicity of cationic liposomes. It is fusogenic and shows a strong destabilizing effect towards lipid bilayers, as suggested by Litzinger and Huang [32].

Despite important advances in both in vivo and in vitro studies on the lipid$^+$–DNA complexes, many questions still remain unsolved, such as the physical mechanisms of the lipoplex formation, the localization of the ODN strands, and the relationship existing between delivery efficiency and molecular structure of the complex. Several recent thermodynamic studies have shown that the process of DNA binding to cationic liposomes is entropy-driven, and proceeds through the release of bound counter-ions from the surface of both components upon complexation [33–35]. Both theoretical [36–40] and experimental papers [41–43] have been published on this subject.

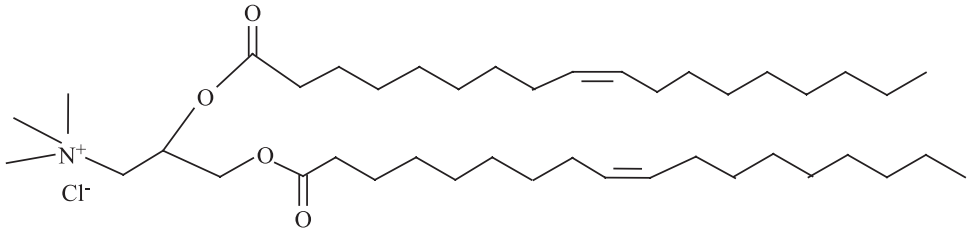
However, too many clinical trials of gene delivery, based on the use of lipoplexes, have been done without sufficient understanding of all the physical and chemical properties responsible for their action. In our opinion, any additional information about these systems will be of help for their complete molecular and/or supramolecular characterization.

In this paper, we report an investigation of liposomes formed by DOTAP or by DC-Chol, a cholestane derivative (3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol, DC-Chol); in both cases DOPE was added as a helper lipid. Single strand homopolynucleotides with purine or pyrimidine ring (poly-deoxy-adenosine (polyA), and poly-deoxy-thymidine (polyT), respectively) or double strand polynucleotides (dsAT) were added to form lipoplexes. This study was carried out by measuring the zeta potential changes with titration of liposomes by ODN addition. The analysis of the electron spin resonance (ESR) spectra of inserted nitroxides was also used to characterize lipoplexes. This is the first time ESR was used in the study of lipoplexes and this technique has proven to be very useful in clarifying the structure of liposomes in the presence or in the absence of ODNs. The data obtained in this work allowed us to establish that: (i) the electrostatic interactions depended on the nature of the base forming in the nucleotides (polyA and polyT showed a different behavior on DC-Chol/DOPE liposomes); (ii) the oligonucleotide, independent of its nature and the number of strands, localized externally to the liposome surface, so that only surface charges were involved.

2. Materials and methods

2.1. Materials

DOTAP (purity >99%, **1**), DOPE (purity >99%, **2**), and DC-Chol (purity >99%, **3**), were purchased from Avanti Polar Lipids, Inc., Alabaster, AL, and used without further purification.



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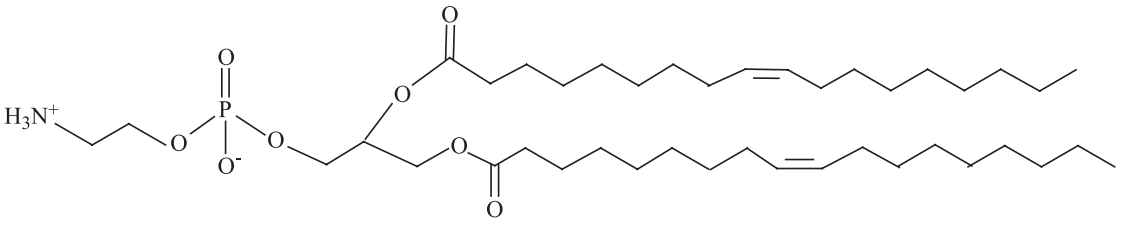

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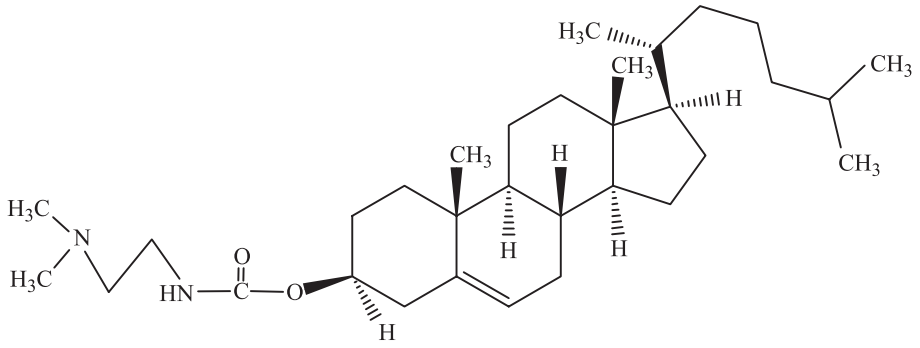

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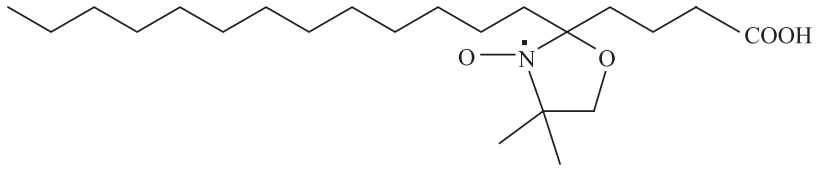
Tris(hydroxymethyl)-aminomethane hydrochloride (Tris, TRIZMA–HCl) was purchased from Sigma, St. Louis, MO, USA. The 20-mer oligonucleotides (ODNs), polyA, and polyT were obtained from QIAGEN Operon, Alameda, CA. The annealing temperature of the two polynucleotides was 312.9 K, as indicated by the manufacturer. 5-Doxyl-stearic acid and 16-doxyl-stearic acid spin probes (5-DSA and 16-DSA, **4**, and **5**, respectively) were purchased from Sigma Chemicals, München, Germany, and used as received.



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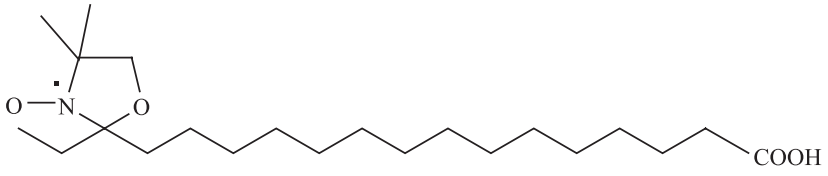

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2.2. Sample preparation

Two types of large unilamellar cationic vesicles (LUVs) were used in this work: DOTAP/DOPE and DC-Chol/DOPE, at 1.1 ($\pm$ 0.1) mole ratio, with a total lipid concentration of 1.4×10^{-2} mol/l. Samples were prepared as follows. Mixtures of dry lipid powders were dissolved in chloroform and the solvent was then dried under vacuum overnight. The resulting mixed lipid film was allowed to swell at room temperature with 10^{-2} mol/l Tris/HCl buffer (pH = 7.4). Upon vortexing, multilamellar vesicles were obtained, which were then submitted to eight freeze/thaw

cycles. This method improved the homogeneity of the size distribution in the final suspension. Liposomes were subsequently reduced in size and converted to unilamellar vesicles by extrusion through 100 nm polycarbonate membranes. Twenty-seven extrusions were performed with the LiposoFast apparatus, Avestin, Ottawa, Canada. LUVs were stored at 277 K. The buffer described above was used most often as the solvent for DNA, but ethylenediamino-tetraacetate (EDTA) was not included in the formulation because it caused liposome and lipoplex aggregation [44].

Each oligonucleotide was solubilized in 10^{-2} mol/l Tris/HCl at pH = 7.4 in order to obtain a negative charge concentration of 0.7×10^{-2} mol/l, corresponding to the positive charge of liposomes prepared as described above. The double strand polyadenosine-polythymidine, henceforth called dsAT, was obtained by mixing equal amounts of polyA and polyT solutions and then annealing at room temperature for 24 h. All samples were stored at 253 K. The actual ODN concentration was determined by measuring the absorbance at 260 nm, taking into account that $Abs_{260} = 1$ for a single strand corresponded to 33 μg/ml, that is 10^{-4} mol/l nucleotides. In the case of a double strand, the correspondence was $Abs_{260} = 1$ for 50 μg/ml, that is 1.5×10^{-4} mol/l.

Lipoplexes were formed by mixing appropriate volumes of extruded vesicles and ODN buffered solutions. The nominal $ODNs^-/lipid^+$ charge ratio was allowed to vary in the range 0–1. In each sample, the lipid concentration was fixed, and the ODN concentration was varied in order to obtain the desired charge ratio. Starting lipid solutions were diluted 1:1 with ODN solution in all samples, so that the working lipid concentration was 0.7×10^{-2} mol/l. For all complexes, DNA was always injected into the cationic liposome suspension to ensure reproducibility [45]. Samples were analyzed not earlier then 10 min and not later than 4 h after preparation, because some samples showed precipitation effects after that time.

2.3. Methods

Measurements of the zeta potential (ζ) and of the sizes of liposomes and lipoplexes were performed with a Coulter DELSA 440 SX (Coulter Corporation, Miami, FL, USA). The zeta potential was automatically calculated from the electrophoretic mobility based on the Hemholtz–Smoluchowski relationship [46]. Homemade hemispherical electrodes, covered by a thin gold layer, were used as the measurement cell. This allowed us to reduce the oxidation that partially affects the silver electrodes, which are typically used in this kind of instrument. The measurement cell was sterilized by dipping in H_2O_2 for 10 min before each set of experiments.

Cationic liposomes and their complexes with ODNs were diluted 8 times, because of instrumental sensitivity; dsAT complexes were diluted 16 times. Runs were performed using an emf of 10 mV and the zeta potential was calculated for the following scattering angles: 8.6°, 17.1°, 25.6° and 34.2°. All data reported in this work represented the average obtained at the four angles and at least two different measurements were carried out for each sample. All lipoplexes showed unimodal distribution of ζ values. Sizes were calculated by assuming light scattering modeling on Brownian diffusion [47]. During the size measurements, the frequency was changed according to the size of the sample. Size measurements were only performed at the largest angle, i.e. 34.2°, in order to minimize artifacts due to possible inter-particle interactions.

ESR spectra were recorded in continuous wave mode with a Bruker ESR spectrometer model 200D, operating at X-band (~ 9.5 GHz). Data acquisition and handling were carried out with the ESR software commercialized by STELAR (Meda, Italy). The temperature was controlled with Bruker VT 3000 apparatus (accuracy ± 1 K). Stock ethanol solutions (10^{-3} mol/l) of 5-DSA and 16-DSA probes were added to liposomes as described in Ref. [48] (*n*-DSA/DOPE molar ratio = 10^{-2} were always used). This method did not affect the overall electrical characteristics of the bilayer (data not shown). Then the desired amount of ODNs was added to the label-containing liposomes. The samples were transferred in a glass capillary and sealed for ESR runs.

Line shape analysis was carried out by using the NLSL program developed by Freed et al. [46,49]. The parameters of the molecular motion were obtained by a nonlinear least-squares fit of a calculation based on the stochastic Liouville model [46,49,50]. This allowed us to obtain motional and structural parameters [48]. In particular, the perpendicular component of the reorientation time ($\tau_\perp$) was used to calculate the activation energy (E_a) of the viscous wagging process for the lipid chains, according to the following relationship:

$$\tau_\perp = Ae^{-E_a/RT} \quad (1)$$

3. Results and discussion

Figs. 1–3 show the zeta potential values as a function of the lipoplex composition in the systems analyzed in this work. ODN-free DOTAP/DOPE liposomes and ODN-free DC-Chol/DOPE liposomes had ζ potential of +48 ± 5 and +39 ± 4 mV, respectively. The interaction between ODNs and liposomes was electrostatic between the negatively charged phosphate groups of ODNs and the positive charges of DOTAP or DC-Chol units. This kind of interaction was convincingly established as the main interaction [45,51]. Apparently, as suggested by several authors, besides the electrostatic interactions, packing properties of the lipoplex components could be important for the condensation of ODNs. This concept has found support in several studies in the past [52,53], although Campbell et al. [54] do not agree with this approach. It must be considered that the

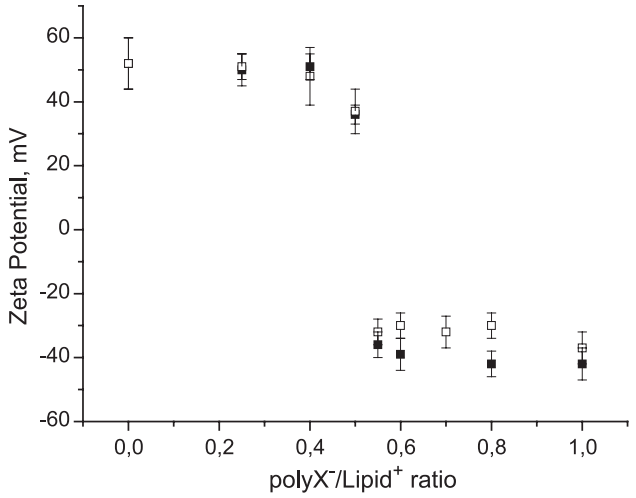

Fig. 1. Zeta potential of DOTAP/DOPE liposomes at increasing values of the polyX$^-$/lipid$^+$ ratios: polyA (full squares) and polyT (empty squares).

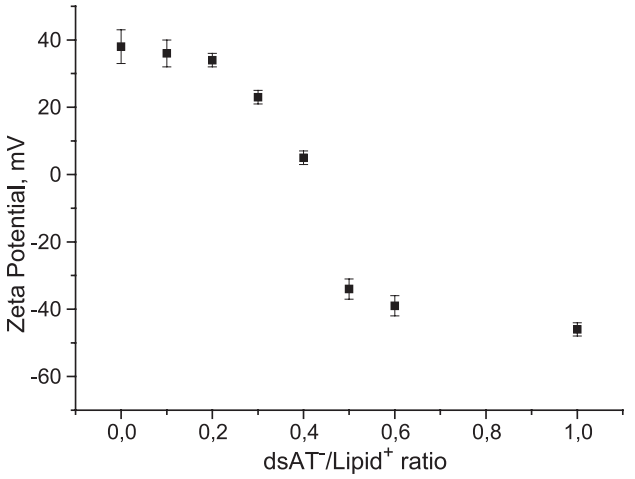

Fig. 3. Zeta potential of DC-Chol/DOPE liposomes at increasing concentration of ds(AT).

electrostatic potential refers to an object built up from the charged particle together with an enclosing reference shell. Thus, the absolute value of the ζ potential could depend considerably on several factors such as the ionic charge, the ion size, etc.

In all the investigated systems, except for dsAT/DOTAP/DOPE (data not shown), the zeta potential versus ODN$^-$/lipid$^+$ ratio had a marked sigmoid shape. The ζ values of the positive lipoplexes were close to the values obtained for pure liposomes and the values of the negative complexes were close to one another. Thus the sigmoid inflection showed a very rapid slope in the cases of single-strand ODNs (Figs. 1 and 2). No differences were found in the electrokinetic behavior of lipoplexes formed by DOTAP/DOPE liposomes containing polyA or polyT (Fig. 1). In both cases, the inversion point was at the charge ratio ODN$^-$/lipid$^+ = 0.5 \pm 0.05$, that corresponded just to half of the cationic molecules in the liposomes. Since $\zeta = 0$ was reached at the neutralization of all the accessible positive charges in liposomes with the negative charges of the ODN phosphate groups, this value suggested that only the external lipids were involved in the interaction. In turn, this meant that liposomes remained most probably intact after wrapping with negative ODNs. This was a very important result, that, to the best of our knowledge, has never been reported in the literature.

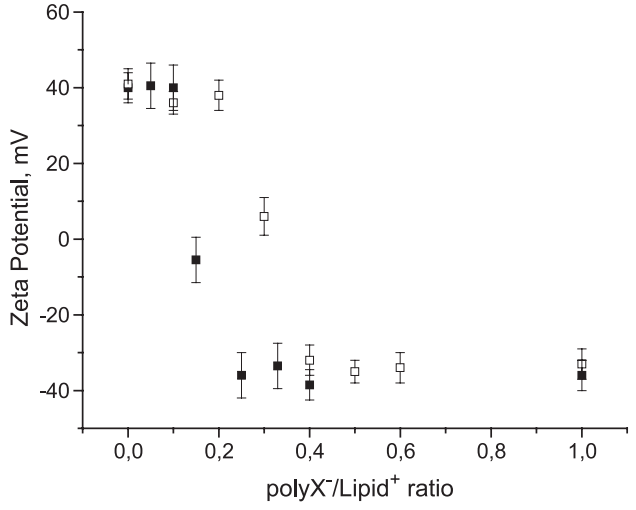

Fig. 2. Zeta potential of DC-Chol/DOPE liposomes at increasing values of the polyX$^-$/lipid$^+$ ratios: polyA (full squares) and polyT (empty squares).

PolyA or polyT in DC-Chol/DOPE liposomes (Fig. 2) showed $\zeta = 0$ at compositions different from each other. Both values were lower than in DOTAP/DOPE lipoplexes. In polyA lipoplexes the inversion point was found at polyA$^-$/lipid$^+$ charge ratio of 0.15 ± 0.05, and it was 0.30 ± 0.05 in polyT lipoplexes. The simplest way to interpret this result was that polyX induced a significantly lower protonation on the surface of DC-Chol/DOPE liposomes, that is a higher surface pH. The effects of the changes in the surface pH have been studied in details by Zuidam and Barenholz [51] and Meidan et al. [55]. In pure DOTAP liposomes Meidan et al. [55] suppose that the approach of the phosphates towards lipid$^+$ is more hindered in polyA than in polyC and polyT because of the presence of the purine ring in polyA which is larger than the pyrimidine ring in polyC and polyT.

The sigmoid curve in the dsAT$^-$/DC-Chol/DOPE system (Fig. 3), where the inversion point appeared at dsAT$^-$/lipid$^+ \cong 0.4$, was smoother than in polyX/DC-Chol/DOPE. Since any steric hindrance might be ruled out in dsAT-containing systems, because of the coupling among ODN nitrogen bases through hydrogen bonds, this value could be explained by considering that only electrostatic interactions between the remaining negatively charged phosphate groups available in the double strand and the positive lipids in liposome occurred.

The zeta potential behavior in dsAT/DOTAP/DOPE lipoplexes was rather peculiar. At $dsAT^{-}/lipid^{+}$ charge ratio below 0.20, lipoplexes were invariantly positive, whereas at higher ratio, the ζ potential showed considerable, irreproducible scatter between positive and negative values. DOTAP/DOPE liposomes containing dsAT at $dsAT^{-}/lipid^{+}$ ratio ≥ 0.2 were therefore not stable under our experimental conditions. The complexes were disrupted and all the lipid charges became available for electrostatic interactions, as known from the literature [56].

Due to bridging effects, non-negligible aggregation occurred among separated lipoplexes to form larger complexes. This was proved by the sizes of the ODN-free and ODN-containing liposomes. Both DOTAP/DOPE and DC-Chol/DOPE pure liposomes diameters were in the range 110–120 nm, in a good agreement with the size of the membrane pores used for the extrusion (100 nm) and with standard light scattering measurements (not reported in this work). In ODN/DOTAP/DOPE lipoplexes, the sizes increased to 500–700 nm. In ODN/DC-Chol/DOPE the mean diameter was in the range 200–250 nm before the inversion point, whereas the sizes of the negatively charged lipoplexes were in the range 300–500. In all cases the polydispersity increased as the equivalent point was reached, and several hours after the measurements precipitation of lipoplexes was often observed. Phase separation effects are also reported by Xu et al. [45], and by Meidan et al. [55].

Two main families of models for the structure of lipoplexes have been proposed [9,19,57–61], which are based on the relative location of cationic lipids and DNA: (i) the *external* model [62], which allows for an electrostatic bond between the negative ODN strand and the outside of the cationic liposome with wrapping of ODNs onto the liposome surface; (ii) the *internal* model, in which the lipids coat on the ODNs, that therefore resides in the lipid shell. Experimental data in favor of both models are given in the literature. The results reported by Felgner and Ringold [62] agree with the external model. Cryo-TEM images, representing multilamellar structures with DNA is trapped between lamellae [14], are also more consistent with the external model of a lipid core surrounded by DNA. The zeta potential measured by Eastman et al. [60] are also interpreted in terms of the external model.

ESR spectroscopy could give additional insights on this matter. The ESR line shape of spin probes inserted into aggregated structures is sensitive to changes in the aggregate shape, in the environmental polarity and in the mobility of the neighboring molecules [63]. Thus, any change in the ESR line shape by passing from ODN-free liposomes to ODN-loaded liposomes should suggest the incorporation of the polynucleotide into the liposome bilayers.

Before analyzing the ESR spectra of DSA probes in ODN/liposomes, the ESR shape of the same probes in ODN-free liposomes must be briefly discussed. Fig. 4 shows the experimental ESR spectra ($T = 298$ K) of 5-DSA (1.4×10^{-4} mol/l) in DOTAP/DOPE liposomes and in DC-Chol/DOPE liposomes (total lipid content 1.4×10^{-2} mol/l), and of 16-DSA in the same experimental conditions. The 5-DSA spectra gave information about the bilayer region immediately below the polar heads (external membrane surface), whereas 16-DSA sensed internal regions. The line shape of the 5-DSA spin-label was consistent with that one of a nitroxide spin label incorporated into a lamellar phase with partially averaged magnetic anisotropies. The $2A'_{\parallel}$ value, as measured from the distance between the lowest and the highest field peaks, was larger in DC-Chol/DOPE liposomes than in DOTAP/DOPE liposomes. This finding suggested that replacement of DOTAP with DC-Chol lead to a significant decrease in membrane fluidity [64–68].

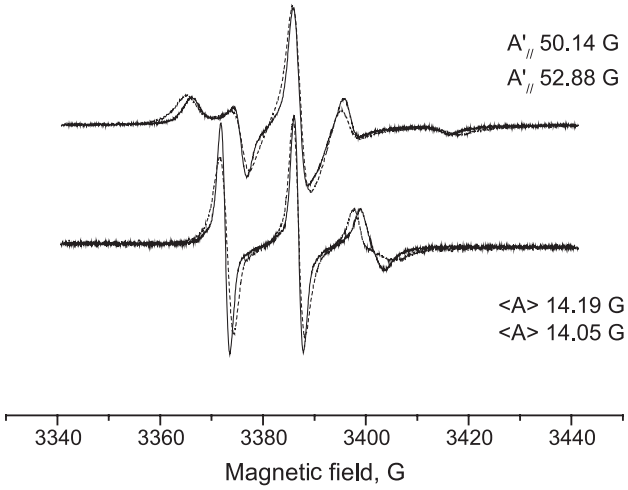

Fig. 4. Above: experimental 298 K ESR spectra of 5-DSA (1.4×10^{-4} mol/l) in DOTAP/DOPE liposomes (full line, $A'_{\parallel} = 50.14$ G) and DC-Chol/DOPE liposomes (dashed line, $A'_{\parallel} = 52.88$ G). Below: experimental 298 K ESR spectra of 16-DSA in the same systems (full line, $A'_{\parallel} = 14.19$ G; dashed line $A'_{\parallel} = 14.05$).

Both 5-DSA spectra were simulated, according to the procedure outlined in Materials and methods, with the same magnetic parameters ($g_{xx} = 2.0087$, $g_{yy} = 2.0067$, $g_{zz} = 2.0028$, $A_{xx} = 6.00$ G, $A_{yy} = 5.60$ G, $A_{zz} = 33.50$ G), and different correlation times ($\tau_{\perp}$ and $\tau_{\parallel}$) and order parameter (S_{20}):

DOTAP/DOPE liposomes	$\tau_{\parallel} = 1.3 \times 10^{-10}$	$S_{20} = 0.52$
$\tau_{\perp} = 1.3 \times 10^{-9}$ s		
DC-Chol/DOPE liposomes	$\tau_{\parallel} = 1.1 \times 10^{-10}$	$S_{20} = 0.55$
$\tau_{\perp} = 2.6 \times 10^{-9}$ s		

The 16-DSA spin probe exhibited a rather different behavior (Fig. 4, bottom). The spectrum of 16-DSA in both types of liposomes was that of a nearby freely tumbling nitroxide, with significant differences in the two liposomes. This finding was consistent with the location of the spin probe, which was deeper in the liposome than in the sites

occupied by 5-DSA [69]. This was clearly evidenced by the simulations of both spectra, which used the following $\tau_\perp$ correlation time and order parameter:

DOTAP/DOPE liposomes	$\tau_\perp = 9.8 \times 10^{-10}$ s	$S_{20} = 0.13$
DC-Chol/DOPE liposomes	$\tau_\perp = 1.66 \times 10^{-9}$ s	$S_{20} = 0.60$

These values argued for a motion of 16-DSA which was more restricted in DC-Chol/DOPE liposomes than in DOTAP/DOPE liposomes.

Since the ESR spectra of 5-DSA probe were more informative, they are discussed here in more detail. The experimental 298 K ESR spectra of 5-DSA in lipoplexes containing polyA or polyT that were observed in DOTAP/DOPE liposomes, at different concentrations, were practically the same as that observed in ODN-free DOTAP/DOPE liposomes, and they were simulated with the same parameters. Fig. 5A shows the ESR spectra obtained from 5-DSA pure liposomes (full line), which is exactly superimposed on the spectrum of the same probe in polyA/DOTAP/DOPE lipoplexes at a $polyA^-/lipid^+ = 0.5$ (dashed line). Other ratios gave the same result. 5-DSA in pure and polyX containing DC-Chol/DOPE liposomes also gave the same results (Fig. 5B). For the sake of clarity only the two $ODN^-/lipid^+$ ratios are reported in the figure. The same behavior was observed with the 16-DSA probe.

Figs. 6 and 7 show experimental (full lines) and simulated (dashed lines) ESR spectra of 5-DSA in polyX/DOTAP/DOPE and in polyX/DC-Chol/DOPE lipoplexes in Tris/HCl buffer (pH = 7.4) at temperatures in the range 253–333 K. As expected, $2A'_\parallel$ decreased at high temperature, which reflected increased mobility in a more fluid bilayer.

Fig. 8 shows the dependence of $\tau_\perp$ on the reciprocal temperature of 5-DSA in ODN-DOTAP/DOPE and in ODN-DC-Chol/DOPE liposomes, which were practically undistinguishable from those of ODN-free liposomes. The value of $\tau_\perp$ reports on the motional regime of the probe. As a result of the ability of superpose the spectra of ODN-free and ODN-loaded liposomes, the $\tau_\perp$ values only depended on the starting liposomes. Perissi et al. [48] have calculated the activation energies of the viscous process from Arrhenius plots of $\ln\tau$ versus T^{-1} of DOTAP/DOPE pure liposomes, which were as follows:

from $T = 253$ to 277 K	$E_a = 80 \pm 5$ kJ/mol
from $T = 317$ to 283 K	$E_a = 40 \pm 3$ kJ/mol
from $T = 313$ to 333 K	$E_a = 20 \pm 2$ kJ/mol

For the reasons given above, these values might be accepted also for ODN-liposomes and the consideration given by Perissi et al. [48] applied for free and loaded liposomes.

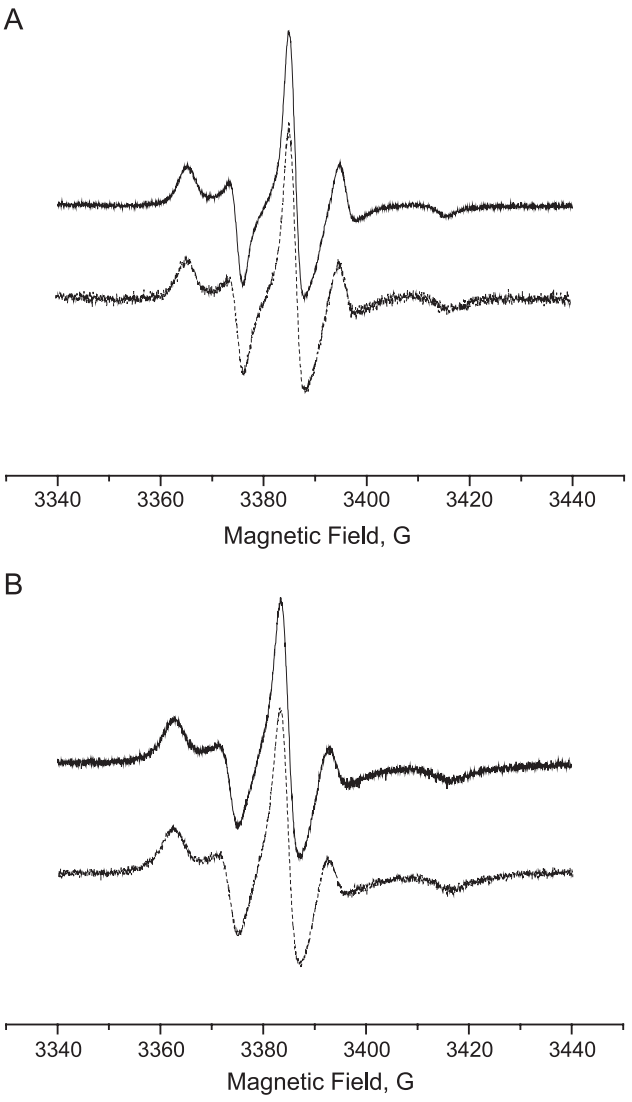

Fig. 5. (A) Experimental 298 K ESR spectra of 5-DSA (0.7×10^{-4} mol/l) in DOTAP/DOPE liposomes (full line) and in $polyA^-/lipid^+ = 0.5$ lipoplexes (dashed line). (B) Experimental 298 K ESR spectra of 5-DSA (0.7×10^{-4} mol/l) in DC-Chol/DOPE liposomes (full line) and in $polyA^-/lipid^+ = 0.15$ lipoplexes (dashed line).

The only way to account for these results was to assume that single or double strands of oligonucleotides almost perfectly wrapped the quasi-spherical liposomes, without substantially altering the mobility, order or polarity of the bilayer. In the specific case of dsAT/DOTAP/DOPE systems, bilayer fragments wrapped by double strands were supposed to exist, in which the local environment sensed by the ESR probes was substantially unaltered. The external model, proposed by Felgner and Ringold [62], perfectly fitted our ESR results.

4. Conclusions

In this paper we characterized lipoplexes by zeta potential measurements and ESR spectroscopy. Our results gave

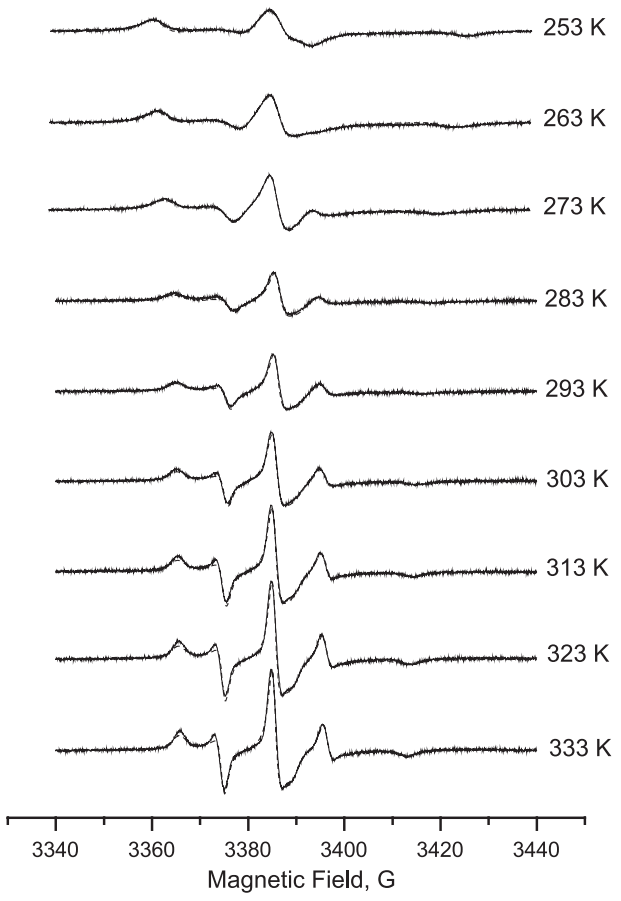

Fig. 6. Experimental (full lines) and computed (dashed lines) ESR spectra of ODN^{-}/DOTAP/DOPE lipoplexes at total lipid concentration 1.4×10^{-2} mol/l containing 5-DSA 1% in mol as a function of the temperature.

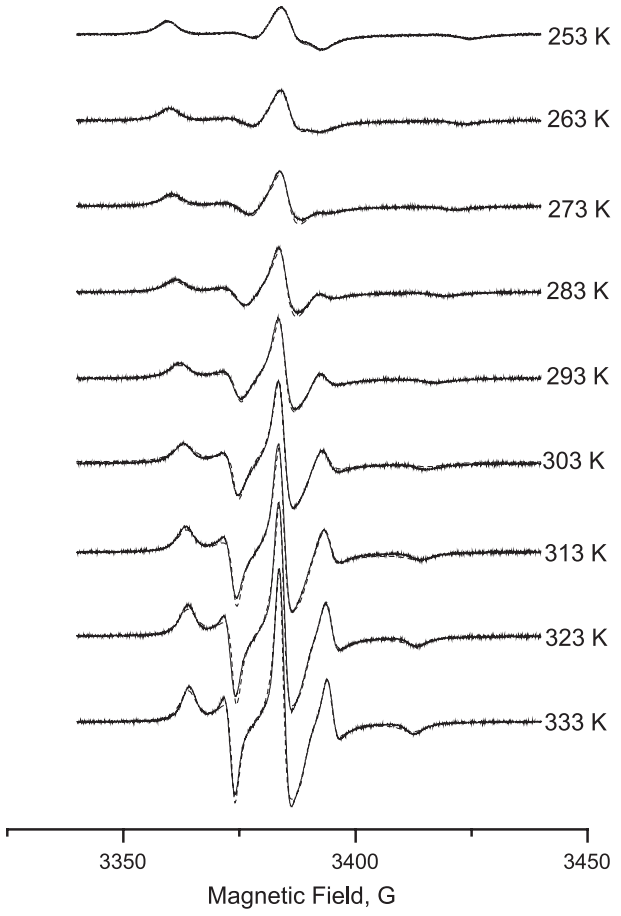

Fig. 7. Experimental (full lines) and computed (dashed lines) ESR spectra of DC-Chol/DOPE liposomes at the total lipid concentration 1.4×10^{-2} mol/l containing 5-DSA 1% in mol as a function of the temperature.

evidence that electrostatic interactions together with steric effects were the driving forces in the association of cationic liposomes and polyA, polyT and the corresponding double strand, dsAT.

Titration curves were performed by adding single or double strand ODNs to the liposome solution and by following the zeta potential change as a function of increasing $polyX^{-}/lipid^{+}$ ratio. In the case of DOTAP/DOPE liposomes, different single strand ODNs behaved in the same manner and the inversion point was found at about 0.50 charge ratio. This meant that the neutralization of the particles in solution occurred when the net negative charges of the ODN phosphate groups were equal to the positive net charges on the liposome surface. Thus, only the lipids located on the external surface interacted with DNA.

In the case of the titration of DC-Chol/DOPE with ODNs, the inversion point shifted to lower charge ratios both for polyA and polyT, being about 0.15 and 0.30, respectively. Several reasons could be invoked to justify this finding, including the different steric hindrance and the different basic strength of purine and pyrimidine rings. In all these cases the liposomes remained intact. The observed behavior was in agreement with the so-called external model, in which the genetic material wraps the charged surface without altering the inner features of the membrane

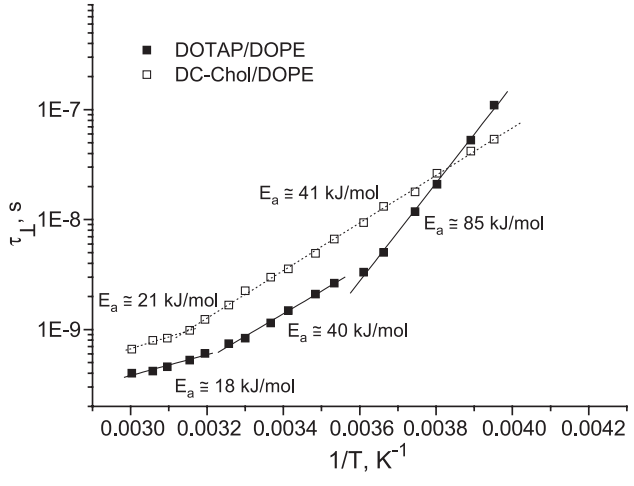

Fig. 8. Dependence of $\tau_\perp$ on the reciprocal temperature for 5-DSA in ODN-loaded DOTAP/DOPE (full symbols) and ODN-loaded DC-Chol/DOPE (empty symbols) liposomes.

bilayer. In particular, ESR spectra confirmed this hypothesis because doxyl stearic probes inserted in the lipid bilayer didn't sense any appreciable difference by passing from pure liposomes to lipoplexes.

In addition, ESR spectra showed that, in the case of dsAT/cationic liposome interaction as well the internal structure of the bilayer was not affected by lipoplex formation.

Lipoplexes formed by dsAT and DC-Chol/DOPE liposomes showed the same trend of the single strand ODNs, whereas with DOTAP/DOPE liposomes a different behavior was observed: in the latter case, at charge ratio beyond 0.20, the liposomes were probably disrupted. This result was in agreement with other data reported in the literature.

ODN-loaded lipoplexes had diameters larger than pure liposomes and this could be due to aggregation of intact liposomes covered by oligonucleotides, possibly by bridging to form larger heterogeneous complexes.

Diameters were smaller in the ODN^-/DC-Chol/DOPE than in ODN^-/DOTAP/DOPE complexes and this was due to the fact that the former liposomes were more rigid. This fact, in turn, could allow a higher probability to form larger aggregates. In fact self-association of cholesterol in membranes is known to be related to a decreased ability to order phospholipids [70], as a result of weak sterol/phospholipid interactions, which explains the lower fluidity of the hydrophobic chains. As discussed above, DOTAP/DOPE liposomes were more fluid and, hence, more easily deformed than DC-Chol/DOPE liposomes. DOTAP/DOPE liposomes probably collapsed in the presence of concentration amounts of double strand oligonucleotides.

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