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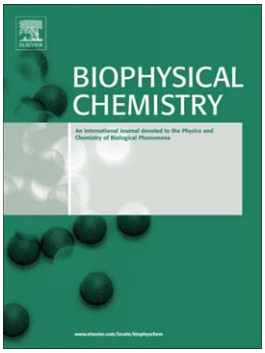

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EFFECT OF THE PREPARATION PROCEDURE ON THE STRUCTURAL PROPERTIES OF OLIGONUCLEOTIDE/CATIONIC LIPOSOME COMPLEXES (LIPOPLEXES) STUDIED BY ELECTRON SPIN RESONANCE AND ZETA POTENTIAL

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Abstract

Lipoplexes with different surface charge were prepared from a short oligonucleotide (20 mer, dsAT) inserted into liposomes of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phospho-ethanolamine (DOPE). The starting liposomes were prepared by two different procedures, i.e. progressive dsAT addition starting from plain liposomes (titration) and direct mixing of dsAT with pure liposomes (point to point preparation). Lipoplexes were characterized from a molecular point of view by Electron Spin Resonance (ESR) of a cationic spin probe and by Nuclear Magnetic Resonance. Structural and surface features were analysed by Zeta Potential (ζ) measurements and Cryo-TEM micrographs. The complete set of results allowed to demonstrate that: i) the interactions between dsAT and cationic lipids were strong and occurred at the liposome surface; ii) the overall shape and physicochemical properties of liposomes did not change when short nucleic acid fragments were added before surface charge neutralization; iii) the bilayer structure of the lipids in lipoplexes was substantially preserved at all charge ratios; iv) the physical status of lipoplexes with electrical charge far from neutrality did not depend on the preparation method.

Key words: Lipoplexes, ESR, Zeta Potential, NMR, Cryo-TEM

Introduction

Cationic liposomes were first introduced as DNA transfection agents by Felgner et al. thirty years ago [1] and they are still extensively studied as a valid alternative to viral vectors in gene therapy.

Though generally less efficient than viral-based delivery agents, the complexes formed between liposomes and DNA (lipoplexes) exhibit a number of attractive properties, such as low toxicity, lack of immunogenicity and highly tunable physico-chemical properties (composition, size and surface charge). In this context, the studies of DNA-liposome interactions represent important issues, since knowledge of the structural changes and of the energy involved in lipoplex formation is a pre-requisite for the optimisation of transfection protocols.

Many papers have appeared on this subject [2-12]. For obvious reasons, particular attention has been dedicated to lipoplexes built up with medium/large linear DNA fragments or plasmids (30-200 kbp). In the majority of cases it has been observed that the preparation procedure induces large variations in the lipoplex features [2,4,13,14]. In this paper we turned our attention to lipoplexes formed with a short oligonucleotide (a 20 mer, dsAT) and DOTAP/DOPE (1,2-dioleoyl-3-trimethylammonium-propane and 1,2-dioleoyl-sn-glycero-3-phospho-ethanolamine, respectively), which were prepared by two different procedures. The first procedure was simply based on progressive addition of dsAT to pre-formed lipoplexes in order to change dsAT concentration up to the desired dsAT content. In the second procedure, each lipoplex was separately prepared, by addition of the components in the appropriated ratio. Therefore, in this latter case the interaction occurred between DNA and unmodified liposomes in each sample, whereas in the case of titration DNA interacted with a preformed lipoplex.

The oligonucleotide used in this work was chosen as a representative example of short nucleic acid. Indeed, the importance of short nuclei acid fragments in gene therapy is demonstrated by outstanding examples such as antisense DNA and small interference RNA (siRNA), recently come into focus as potent inhibitor of disease promoters [15,16]. Here, we present the results

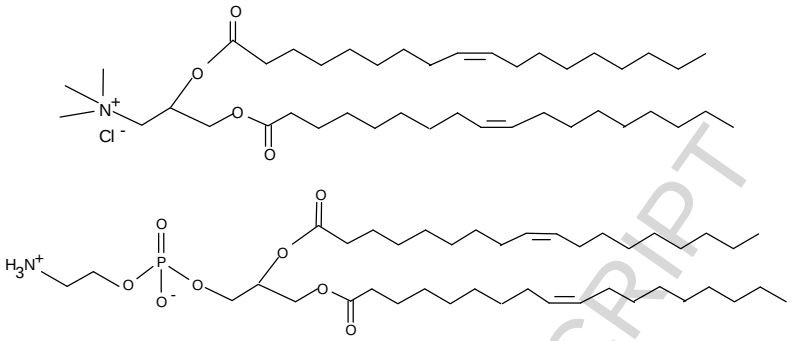
relative to molecular properties obtained by Electron Spin Resonance (ESR) spectroscopy of a cationic spin probe inserted into dsAT/DOTAP/DOPE lipoplexes as compared with the results relative to surface charge (i.e. a bulk property) obtained with Zeta Potential (ZP) measurements in the same systems. The ESR spectra of spin probes inserted in molecular assemblies are sensitive to changes in their shape, local polarity, and mobility of neighbor molecules [17-36]. Thus, any change in the ESR line shape observed by passing from free liposomes to lipoplexes reported on the interaction between nucleic material and liposome bilayers at the molecular level. Moreover, the mobility of the spin labels in fluid lipid membranes was close to the optimal range of motional sensitivity in ESR of nitroxides. Zeta Potential (henceforth indicated as ζ) directly gave the surface charge, i.e. a key parameter to study the interaction with cells membranes, which in most cases bear a negative charge. When necessary, Nuclear Magnetic Resonance spectroscopy (NMR) and electron microscopy at low temperature (Cryo-TEM) measurements were carried out on selected samples and added to clarify particular points of the discussion.

The data presented here gave a picture of the formation mechanism in both procedures. The local structure and ordering of lipid molecules in the bilayer were not drastically modified upon interaction with the negatively charged dsAT, although strong binding of dsAT to the liposome surface was found to take place.

Materials and Methods

Materials.

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



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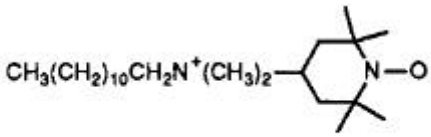
The 20-mer oligonucleotides polyA and polyT were obtained from QIAGEN Operon, Alameda, CA. The annealing temperature of the two nucleotides was 312.9 K, as indicated by the manufacturer. The double strand polyA-polyT (henceforth called dsAT) was obtained by mixing equal amounts of polyA and polyT solutions at the same concentration and then annealing in a vial at room temperature for 24 h. All samples were stored at 253 K. The dsAT concentration was determined by measuring the absorbance at 260 nm, taking into account that Abs_{260} = 1 for a double strand oligonucleotides corresponded to 50 µg/ml, that is $1.5x10^{-4}$ M.

The cationic spin probe 4-(dimethyldodecylammonio)-2,2,6,6- tetramethyl-1-piperidinyloxy (iodide salt, CAT12) was purchased from Molecular Probe, Inc., Eugene, OR, and used without further purification:



CAT12

Phosphate Buffer Saline (PBS) was purchased from Sigma as tablets. After thawing, the pH value was tested and set to 7.4 with 0.1 M NaOH or 0.1 M HCl solutions.

Sample Preparation.

Stock solutions of DOTAP/DOPE liposomes were prepared at 1:1 mole ratio, with total lipid concentration of 1.4×10^{-2} M (10 mg/ml) in PBS. Mixtures of dry lipid powders were dissolved in chloroform. After solvent evaporation, the lipid film was swollen at room temperature with PBS buffer (pH = 7.4). Multilamellar vesicles were obtained upon vortexing. These were then submitted to eight freeze/thaw cycles and extruded through 100 nm polycarbonate membranes (27 passages, LiposoFast apparatus, Avestin, Ottawa, Canada) to obtain monolamellar, monodisperse structures. Samples were stored at 255 K.

Lipoplexes were prepared according to two different procedures:

a) Samples were prepared, as in a typical titration experiments, by progressively adding the desired amount of a 5 mM (in nucleotides) dsAT stock solution to a lipoplex solution. The starting lipid concentration 1.4×10^{-3} M). We simply called titration this preparation procedure. In the case of ESR experiments, 20 μL of lipoplex suspension were collected for the analysis after each addition of dsAT.

b) In a second preparation procedure, each sample was obtained by adding dsAT to a fresh liposome solution. This procedure is indicated in the following as single (point to point) preparation.

In both procedures stock ethanol solutions (1.1×10^{-3} M) of CAT-12 probe were added to preformed liposomes. In practice, the required amount of probe solution was let to evaporate in the bottom of a vial and, after complete ethanol removal, liposomes were added. The system was then equilibrated at room

temperature for 1 h, prior to ESR spectra recording, as described in ref. [22]. In order to avoid inhomogeneous line broadening and marked changes in the properties of the liposome bilayer, the ESR probe content was never higher than 1% of total lipids.

The composition r of lipoplexes is expressed as the ratio between the nominal negative charges of phosphate groups in dsAT and the nominal positive charges of DOTAP, i.e.:

$$r = \frac{[\text{phosphate groups of DNA}]}{[\text{DOTAP molecules in liposomes}]}$$

In samples prepared for ESR measurements the charges of CAT-12 were not included in the calculation of r, since their concentration was less than 1%, as mentioned above.

Pure liposomes (r = 0) and samples with r up to 1.5 were investigated. All samples were prepared with the same lipoplex concentration usually tested on cell cultures [20].

Methods.

Electron Spin Resonance Spectroscopy. 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 °C).

ESR line shapes were analyzed and computed with the procedure given by Freed and coworkers, as described in refs. [37,38]. A double-site simulation was used for all the systems investigated.

Zeta potential runs. Measurements of Zeta potential were performed with a Coulter DELSA 440 SX (Coulter Corporation, Miami, FL, USA). Home made

hemispherical electrodes, covered by a thin gold layer, were used as measure cell. This allowed to reduce the oxidation that partially affects the silver electrodes currently used in this kind of instruments. Runs were performed as detailed in ref. [11]. Size were also calculated in the same experiments according to the procedure described by Langley [39].

Nuclear Magnetic Resonance Spectroscopy. Mono and bidimensional (NOESY) NMR experiments were performed with a Bruker DRX-600 Avance spectrometer operating at 600.13 MHz for ^{1}H. A reverse triple resonance (^{1}H, ^{13}C, BB) with xyz gradients for two-dimensional experiments was used as probe head. NOESY spectra were acquired with 2048 complex points for 256 experiments with a recycle delay of 5 s and a TPPI phase cycle.

All spectra were processed using the Bruker Software XWINNMR, version 2.5. Bidimensional spectra were analyzed with the SPARKY software [40].

Cryogenic Transmission Electron Microscopy (Cryo-TEM). A Philips CM 120 bio TWIN cryo electron microscope equipped with a post-column energy filter and an Oxford CT3500 cryo-holder and its working station was used as a courtesy of the Department of Physical Chemistry, University of Lund, Sweden. Samples for cryo-TEM analysis were prepared at room temperature and treated as described in previous papers [41,42].

Results and discussion

Amphiphilic probes, bearing the paramagnetic unit along the alkyl chain, have been extensively employed to obtain information on the dynamics and ordering at the molecular level within aggregates such as micelles and natural or synthetic lipid membranes [18,19,21-25,29-36,43-48]. Here, we used CAT-12, i.e. a positively charged amphiphilic nitroxide, with the aim of: i) mimicking the behavior of the cationic component in the bilayer, and ii) obtaining information on

the liposome outer surface, where interactions with DNA occur. The probe insertion in the bilayer was demonstrated by the change observed in the ESR spectrum, as reported for similar systems described in the literature [49].

Figure 1 shows the ESR spectrum of CAT-12 (1% of lipid concentration) incorporated into DOTAP/DOPE membranes. Two components were identified, which accounted for the presence of fast and slowly moving species. This finding is quite usual in similar systems. The first absorption corresponded to spin probe molecules almost free to move in the buffer solution (fast component). CAT-12 in pure PBS solution gave exactly the same spectrum. The second absorption was due to nitroxides inserted into the polar head region of the bilayer (slow component). Different CAT-12/lipid ratios were tested in order to minimize the presence of spin probes free in solution. However, in all the investigated systems a spin probe partition between fast and slow moving regions was found.

The best-fit simulation of the ESR spectrum with the NLSL software [17,37,38] shown in Figure 1 was obtained with the magnetic and motional parameters reported in table 1.

The same g values were used for both components. The variation due to the different probe environment in these parameters was within the error range that could be appreciated with the NLSL simulation method.

Both species underwent isotropic motion, but, as expected, the ^{14}N-hyperfine coupling constant decreased in a more hydrophobic environment: $\langle A_N \rangle$ =1.68 ± 0.01 mT for free CAT-12 and $\langle A_N \rangle$=1.61 ± 0.01 mT for the inserted radicals.

Figure 2 shows the experimental (black line) and simulated (red line) 298 K ESR spectra of CAT-12 in dsAT/DOTAP/DOPE lipoplexes at increasing r, as prepared by the two methods described in the experimental section. The fitting of ESR absorptions showed that all spectra were the sum of two contributions. These were simulated using the same best-fit parameters of Table 1. It is important to note that the slow motion ESR component had the same line shape in all the spectra collected in this work. Therefore, there was no difference in the

local lipid arrangement, at least in the region close to the polar heads were the CAT-12 probes are localized.

The relative contributions of the two spectral components clearly depended on the r parameter. The slow motion contribution (as % intensity of the total spectrum) is reported in Figure 3 for both methods of preparation.

Surprisingly enough, the behavior of the slow-motion component intensity as a function of r was almost the same for the two preparation procedures. In particular, after an initial decrease, the slow-motion component passed thorough a minimum at r values which were different for samples prepared by titration and by point to point preparation. Beyond the minimum, the intensity of the slow-motion component recovered a high intensity value, which was only slightly dependent on the preparation procedure. This trend agreed with the ζ behavior shown in Figure 4, in which the evolution of ζ for the DOTAP/DOPE liposomes-dsAT system, as a function of charge ratio is reported. The inflections points of ζ for the two methods of lipoplex preparation almost exactly corresponded to the minimum points of the slow moving species in Figure 3. Complexes with charge ratio well beyond the isoelectric point prepared by both procedures had the same ζ within the limits of experimental error (≈-40 mV). This was a further proof that the local lipid arrangement did not change with the preparation procedure. This finding was in contrast with the behavior of lipoplexes of large DNA [2,4,50-52] and suggested that short nucleic acid fragments follow a simpler mechanism of complexation.

As a possible explanation for the ESR intensity recover after the ζ inversion points, we suggest that addition of low amount of the negative dsAT to liposome dispersion in the proximity of this point, that is almost at the neutrality of the aggregated surface charge, to which the positive CAT-12 probe was loosely bound, favored the formation of CAT-12-dsAT ion-pairs, almost free to move in solution. The nature of this adduct remained ill defined; anyway the reorientational mobility of the >NO unit maintained the same physical features that it had in the dsAT-free paramagnet. Only when the dsAT concentration increased up to negative surface potential, CAT-12 was progressively embedded

down to the lipoplex double layer with resultant decrease of its mobility. This way, the negatively charged lipoplexes were not able to expel the spin probe, and the total amount of the slow motional component recovered the high percentage held of pure liposomes.

Further proofs of the fact that the interactions between dsAT and lipid bilayer not drastically modified the structure and the order of liposome bilayer came from NMR of selected samples. Figures 5a and 5b show the ^{1}H-NMR spectrum of the DOTAP/DOPE lipoplexes at dsAT concentrations below and above the change of the surface charge, i.e. at r = 0.25 (spectrum a) and 0.75 (spectrum b). The NMR spectra of the two reference systems, i.e. pure liposomes and dsAT in PBS solution, are also reported for comparison (Figures 5c and 5d, respectively). Peak assignment for pure DOTAP/DOPE liposomes is reported in a previous paper [52].

The proton signals of phospholipids polar heads at 3.3 ppm, as well as those of alkyl chains at 1.4 and 0.9 ppm in the lipoplex spectra were broadened to a greater extent than the corresponding peaks in dsAT free-liposomes (Figure 5c). This was not unexpected because proton signals of most aggregated systems are broadened beyond detection. This occurs because of longer diffusion times and because the molecular order in the bilayer prevented complete averaging out of dipolar interactions. In the present system line broadening was not severe, and most of the relevant signals were distinct and clearly detectable. The observed broadening was therefore due to the interactions with dsAT. Since it was not possible to detect preferential broadening of the polar heads or of the alkyl chain signals, this effect was attributed to growth of the overall aggregates. The practical disappearance of the dsAT peaks in the r = 0.25 lipoplex (Figure 5a) suggested that in this system the oligonucleotide was completely attached to the liposomes surface. This did not contrast with the above presented interpretation of ESR spectra, since the paramagnetic probe, which reported on its specific behavior in the bilayer environment, was not present in the samples prepared for NMR experiments.

When in the presence of a higher amount of dsAT (r = 0.75,), the dsAT peaks were again detectable (Figure 5b). In order to have comparable results, the r = 0.75 lipoplexes were prepared at the same dsAT concentration as in the sample in Figure 5a. This allowed to avoid artifacts due to different dsAT content, which also reduced the intensity of the NMR signals. In all cases, the ratio between the intensity of free dsAT and the liposome peaks remained constant, and only the absolute intensity varied. These observation agreed with the information obtained from Cryo-TEM data on the morphology of the dsAT- containing liposomes in the same conditions where NMR spectra were registered, that is far from the neutrality (Figure 6). As it is known [53] Cryo-TEM micrographs of plain liposomes deposited on a positive grid indicate the coexistence of unilamellar, bilamellar and invaginated vesicles. After addition of dsAT a drastic change in the vesicle morphology occurred. Specifically, a wealth of large, mainly multilamellar, complexes were observed both in excess of lipids or in excess of dsAT as it is shown in the Figures 6A and 6B, where micrographs of samples with r = 0.25 and r = 0.75 are reported. Liposomes and broken liposomal membranes coexisted with novel assemblies of dense multilamellar structures [53-56]. The observed vesicle fusion, twisting and wrapping on one another was due to the presence of short nuclei acid molecules, that induced strong deformation of the original liposomes. This findings were in good agreement with the external model proposed by Felgner and Ringold [57] and with ZP and ESR results reported in this work.

The fact that dsAT NMR peaks were detectable in the r = 0.75 sample meant that a fraction of the oligonucleotide did not appreciably interact with the large, slowly tumbling, aggregates. The NOESY spectra recorded for free dsAT and for the r = 0.75 lipoplex, could be exactly superimposed and showed cross-peaks with the same sign of diagonal peaks. It is known that these relative signs depend on the rotational correlation time τ_c of the molecule embedded in the aggregates [58]. In particular, if the rotational correlation time of the molecules is long (i.e. $\tau_c > 3x10^{-10}$ s), the diagonal and cross-peaks have same sign. This meant that dsAT molecules whose peaks were recovered in the NMR spectrum

after the neutralization point were in slow motion conditions alike free dsAT, and showed the same dipolar coupling of this latter. This is a valuable piece of information, which is not easily attainable in solution, especially for large DNA fragments.

Conclusions

In this paper ESR and ζ measurements were performed on DOTAP/DOPE/dsAT lipoplexes prepared by two different procedures. This allowed to demonstrate the following four main points:

a) the interactions between dsAT and cationic lipids were strong and occurred at the liposome surface;
b) the general shape and physicochemical properties of the lipoplexes did not change when short fragments of nucleic acid were introduced into the lipid bilayer of cationic liposomes before the charge neutralization point;
c) the bilayer structure of the liposomal carrier in the lipoplexes was substantially preserved at all charge ratio;
d) the physical status of the lipoplexes at electrical charge far from the neutrality did not depend on the preparation method.

Concerning the second point, ESR measurements did not detect any disruption of the bilayer into small lamellar fragments. This finding has also proved by NMR analysis and by Cryo-TEM micrographs of selected samples. Cryo-TEM showed that fusion among different vesicles took place.

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Table1. Best fit ESR parameters.

	Fast	slow
g_{xx}	2.0087	2.0087
g_{yy}	2.0067	2.0067
g_{zz}	2.0029	2.0029
A_{xx}, mT	0.7	0.67
A_{yy}, mT	0.7	0.67
A_{zz}, mT	3.65	3.5
τ_{iso}, sx10^{11}	3.33	2.64

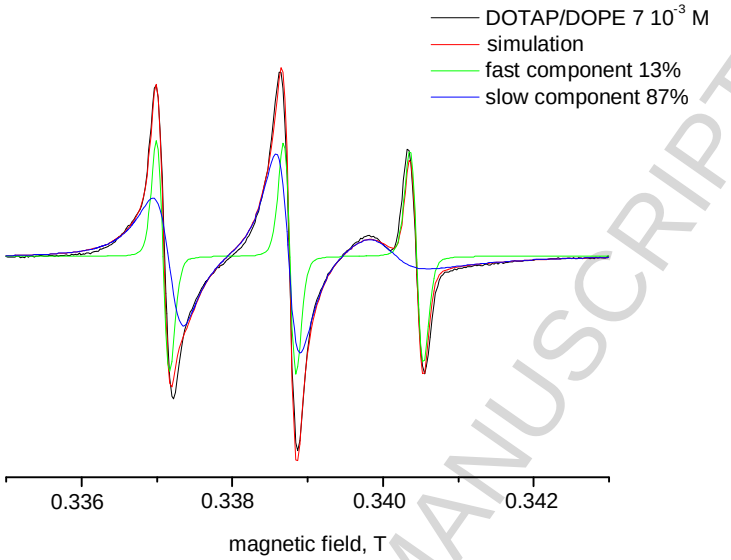

Figure 1. Experimental (black line) and computed (red line) 298 K ESR spectra of CAT-12 nitroxide inserted into DOTAP/DOPE liposomes. The computed signal was the result of a sum of 13% fast moving species (green line) and 87% slow moving species (blue line).

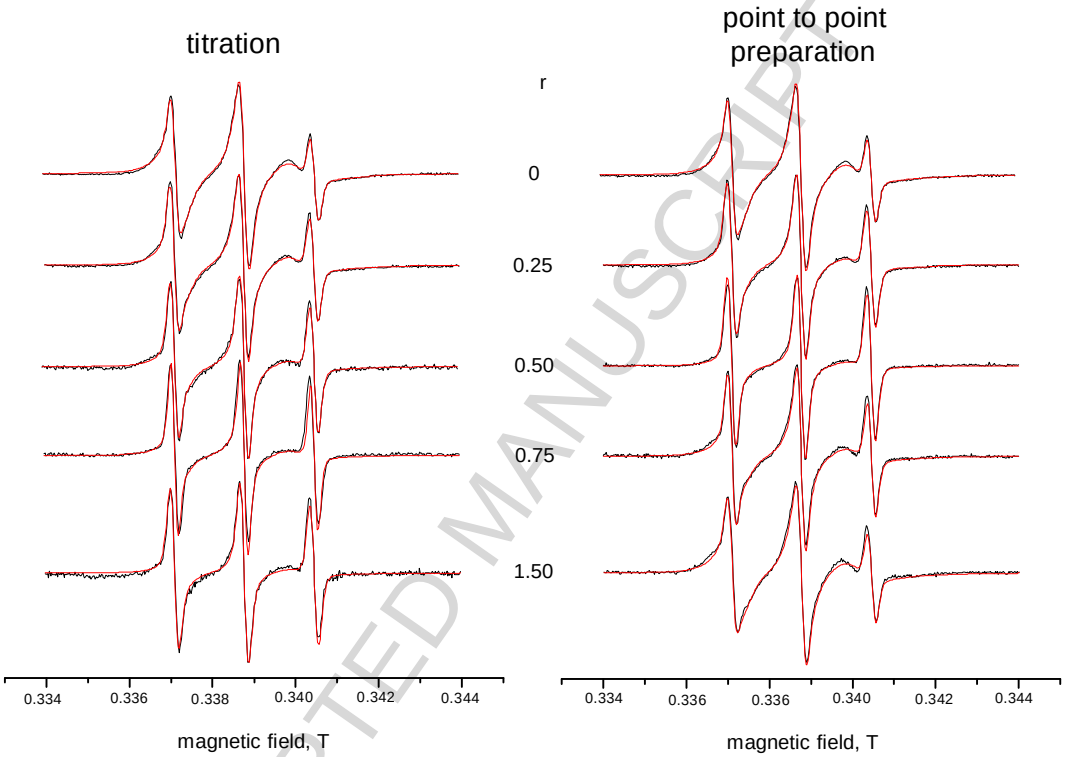

Figure 2. Experimental (black line) and simulated (red line) ESR spectra at 298 K of CAT-12 inserted into dsAT/DOTAP/DOPE lipoplexes prepared by titration (left) and by point-to-point (right) at different r ratios.

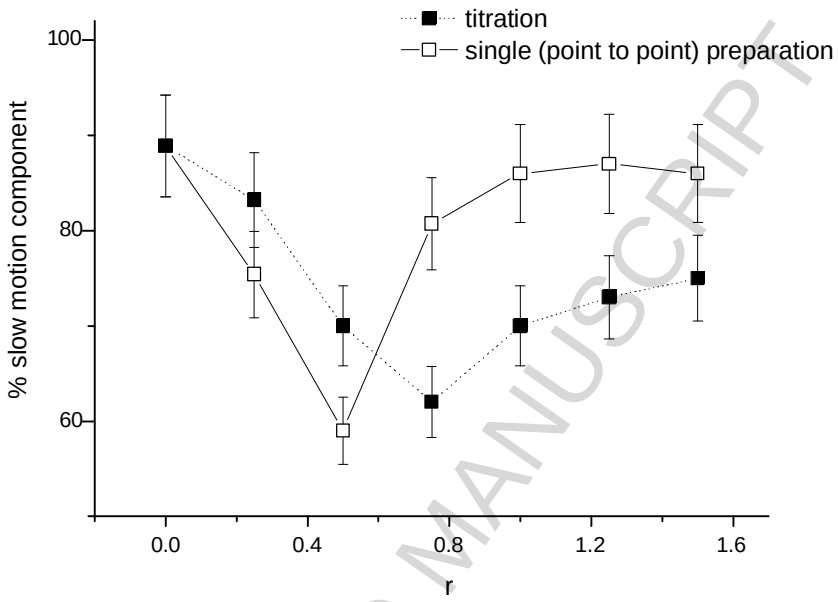

Figure 3. Percentage of the slow moving species intensity used for the simulation of the ESR signals at different r for both preparation procedures described in the text. The error bars were determined by the fitting program.

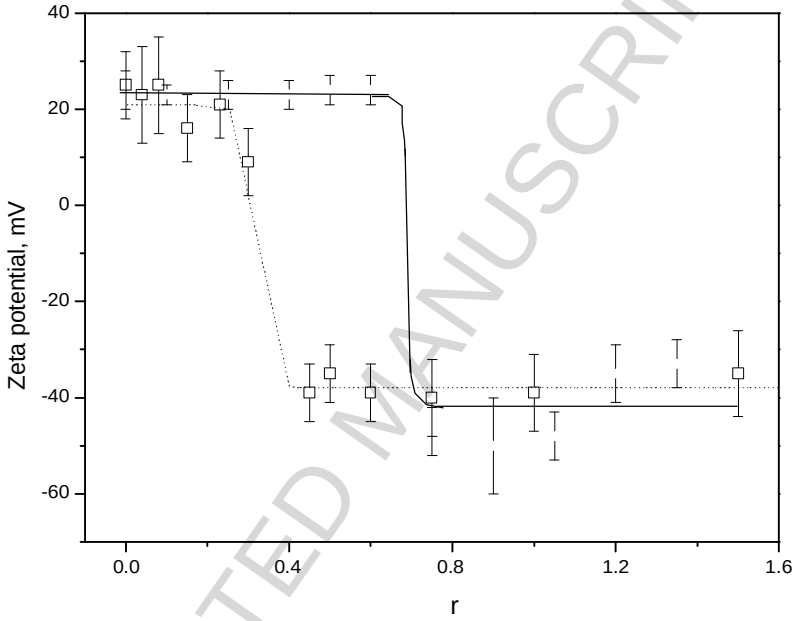

Figure.4. Zeta potential values obtained in the titration of DOTAP/DOPE liposomes with 20-mer dsAT (empty squares) and by point to point preparation (full squares), as described in the text. Both cationic and anionic component were dissolved in 10^{-2} M PBS solution and temperature was 298 K. The dotted and continuous lines have simply been drawn to guide the eye.

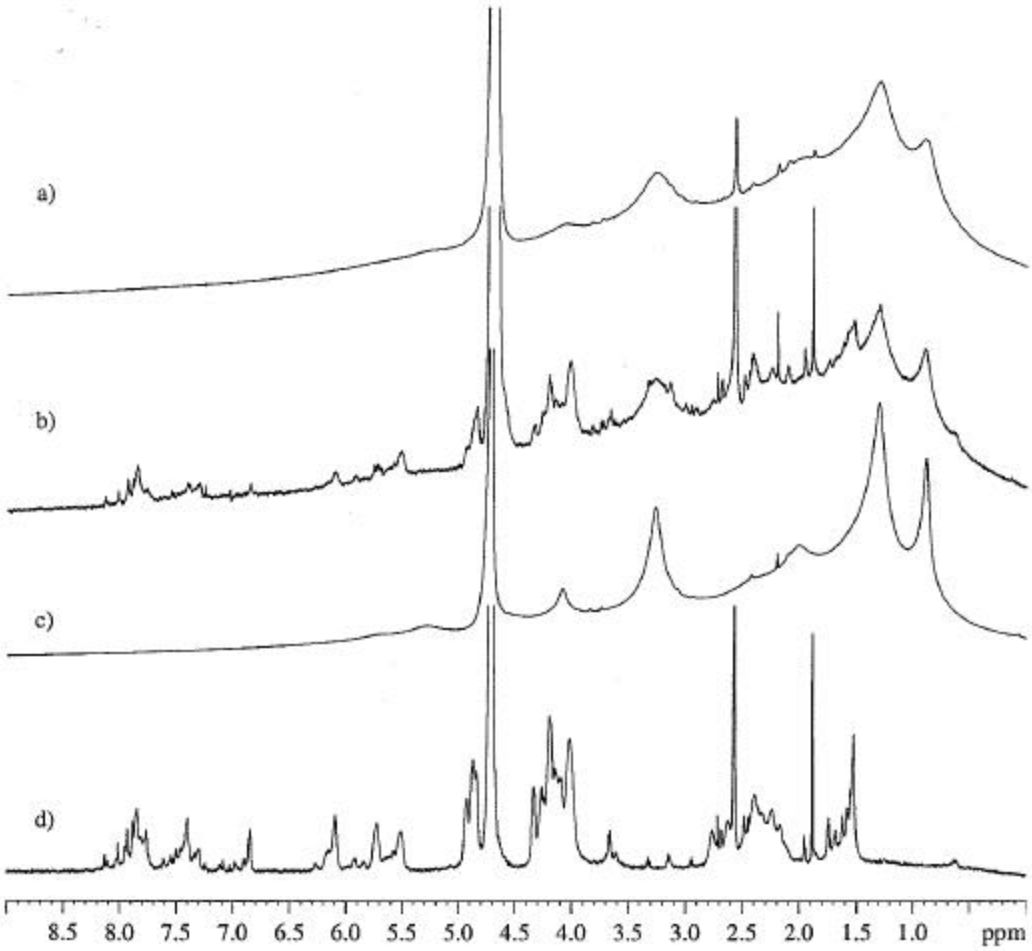

Figure 5. a) ^{1}H NMR proton spectrum of dsAT complexed with DOTAP/DOPE liposomes (r = 0.25, dsAT concentration = 9.45x10^{-3} M); b) dsAT complexed with DOTAP/DOPE liposomes (r = 0.75, dsAT concentration = 9.45x10^{-3} M); c) pure DOTAP/DOPE liposomes (lipid concentration = 1.4 10^{-2} M); d) uncomplexed dsAT (9.45x10^{-3} M). The dissolving medium was 0.1 M PBS solution for all samples and spectra were recorded at 298 K.

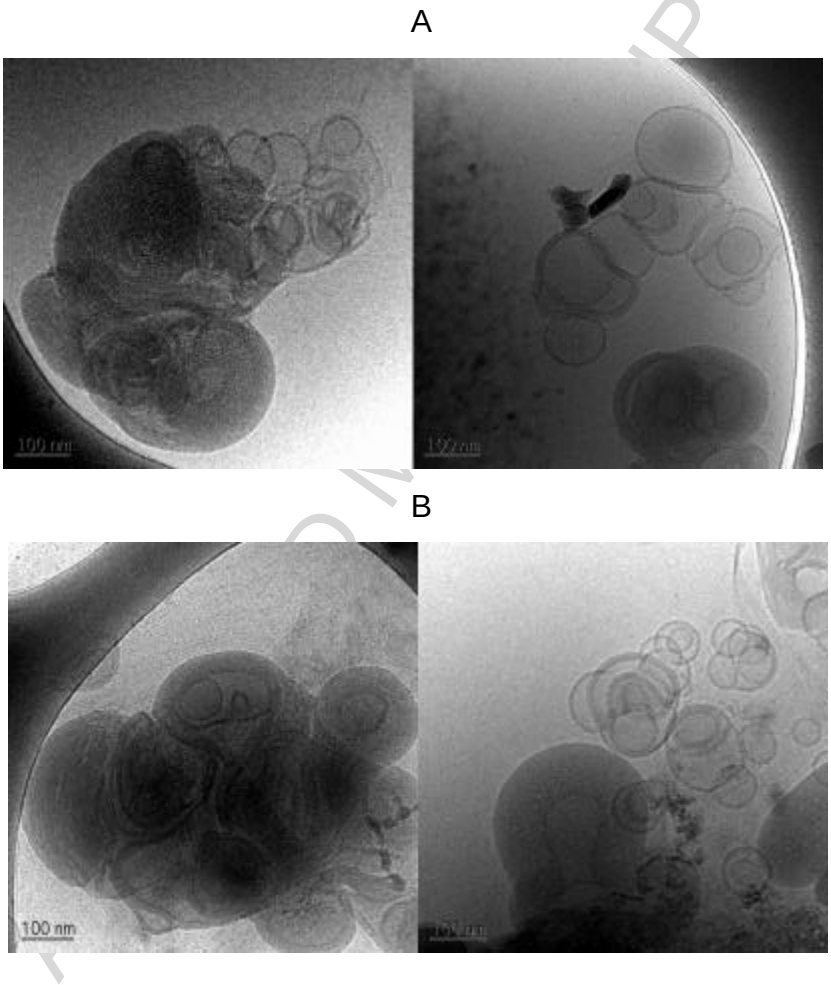

Figure 6. Cryo-electron microscopy images of lipoplexes formed by extruded DOTAP/DOPE vesicles ($7x10^{-3}$ M total lipid), that show the coexistence of different structures both in excess of lipid (A, r = 0.25) and in excess of dsAT (B, r = 0.75). In both cases left and right panels represent different regions of the same sample.