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Characterization of Monoolein-Based Lipoplexes Using Fluorescence Spectroscopy

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Abstract Lipoplexes are commonly used as delivery systems *in vitro* and *in vivo*, the role of a neutral lipid as helper being of extreme importance in these systems. Cationic liposomes composed of dioctadecyldimethylammonium bromide (DODAB) with monoolein (MO) as a helper, at different molar ratios (1:2; 1:1 and 1:0.5) were prepared, and subsequently titrated to DNA. The structural and physicochemical properties of the lipid/DNA complexes were assessed by ethidium bromide (EtBr) exclusion, 90° static light scattering (90° SLS) assays and fluorescence resonance energy transfer (FRET). In EtBr exclusion assays, the steady-state fluorescence spectra of EtBr were decomposed into the sum of two lognormal emissions, emanating from two different environments – H_2O and DNA, and the effect of charge ratio (+/-) was observed. 90° SLS assays gave an important contribution, detecting size variations in systems with different MO fractions on the lipoplexes. In FRET assays, 2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine (DPH-HPC) was used as donor and EtBr as acceptor. The DNA component previously calculated by EtBr exclusion, was used to determine the energy transfer efficiency, as an indirect measurement of the lipoplexes structural and physicochemical properties. Our results demonstrate that the inclusion of monoolein in the cationic liposomes formulation significantly modifies the rate of DNA complexation, being DODAB:MO (1:1) the system with higher DNA condensation efficiency.

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Introduction

The absence of vectors, viral or non-viral, that efficiently condense and release DNA in the target-cells is presently considered the major limitation for the expansion of gene therapy [1]. DNA, when complexed with cationic liposomes, can be used as a non-viral vector [2]. Due to electrostatic interaction, cationic lipids spontaneously associate with DNA by a process of self-assembly, resulting in the formation of cationic lipid-DNA complexes, so-called lipoplexes [3]. These membranous structures are capable of transducing genes into cells, eventually leading to their expression by a process called transfection [4]. Despite of the intensive interest in these systems, namely in the relationship between their biophysical features and the mode of interaction with the cells, it is still not possible today to predict clearly the implication of the physical properties on the efficiency of gene transfer, and the factors involved in this process [5]. Apart from their low transfection efficiencies, lipoplexes are non-imunogenic structures that present low citotoxicity and high potential for large-scale production [6].

In the last two decades, a high number of studies has been published regarding the optimization of lipid formulations and the measurement of the transfection efficiency as a function of several parameters such as the nature of the cationic surfactant [7], the lipid/DNA charge ratio (+/-) [8, 9], the electrostatic properties of the lipoplexes during and after their formation [10], the size of the systems [11, 12], the efficiency of complexation of the DNA [13, 14], and the effect of the presence of additives such as serum [15]. More recently, it has been stated that the inclusion of certain

neutral lipids (also known as *adjuvants* or *helper*) in the liposomal formulation facilitates the fusion of the complexes with the cell membranes, due to its propensity to form nonbilayer structures like hexagonal or cubic phases, which are akin to membrane fusion intermediates [16–17].

In this study, it is explored the effect of monoolein (MO) on the structural and physicochemical properties of dioctadecyldimethylammonium bromide (DODAB)/DNA complexes. The long-chain cationic surfactant DODAB is a synthetic lipid, which in the presence of excess water and above the phase transition temperature $L_\alpha - L_\beta$, tends to form large unilamellar vesicles (LUV's) [18]. The structural organization of these vesicles depends on the lipid concentration, the method of preparation, the solvent composition, the temperature and the presence of other substances [18]. Monoolein, 1-monooleoyl-rac-glycerol, is an amphiphilic neutral lipid of natural origin that has the particularity of presenting two inverted bicontinuous cubic phases, even in excess water (<90% w/w) [19]. Although monoolein has never been used before for gene therapy purposes, its extremely rich diversity of non-lamellar phases represents, *per se*, a significant motive for the study of the influence of this molecule on the lipoplexes final features.

Fluorescence spectroscopy remains as the preferential technique for evaluating the lipoplexes physicochemical properties, since it allows the monitoring of several structural changes in the lipoplexes, namely the ones caused by phenomenon's such as vesicle leakage, membrane fusion and hydrophobic exposure [20].

The use of DNA intercalating agents such as ethidium bromide (EtBr) offers a rapid and sensitive way of analysing the lipoplex formation, since they allow the determination of the efficiency of DNA condensation. On DNA complexation at increasing cationic lipid concentration, an overall decrease in EtBr fluorescence intensity is measured, since the intercalating probe is progressively expelled to the external media, where its fluorescence quantum yield is significantly inferior (this decrease is accompanied by a red shift in the probe emission spectra) [21]. Fluorescence resonance energy transfer (FRET) is another technique that is increasingly gaining importance as a method for studying the mechanisms and barriers involved in non-viral gene therapy.

A change in conformation on condensing DNA leads to a change in the distance between two fluorophores, which can be demonstrated through the random double labelling of DNA for FRET [22] or double labelling of the DNA and carriers [23].

As a complementary approach, it have also been used in this study 90° static light scattering (90° SLS) assays, with the purpose of determining size variations during the formation of the lipoplexes. The light scattering is weighted by the intensity of scattered light that varies according to diameter of the particle, thus providing direct information about the structure of the complexes [24].

Materials and methods

Reagents

Monoolein, 1-monooleoyl-rac-glycerol (MO) was purchased from Sigma-Aldrich. DODAB was purchased from Tokyo Kasei. Salmom sperm DNA was purchased from Invitrogen. The intercalating probe ethidium bromide (EtBr) and the lipid probe 2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine (DPH-HPC) were purchased from Molecular Probes. All reagents were used in the same conditions as received.

Liposomes preparation

For preparing the liposome solutions, defined volumes from the stock solutions of DODAB and MO in ethanol (20 mM) were injected under vigorous vortexing to an aqueous buffer solution at 70 °C (30 mM Trizma), so that the final lipid concentration ([DODAB more MO]) was 1 mM and the different DODAB: MO molar ratios (1:2, 1:1, and 1:0.5) were obtained.

Lipoplexes preparation

Lipoplexes were prepared in a cuvette by adding increasing volumes of the cationic liposome solutions into 2.5 ml of salmon sperm DNA solution (20 μg/ml).

The concentration of nucleotide bases (determined by the DNA absorption at wavelength 260 nm [25]), was held constant at 4.2×10^{-5} M in all experiments. The charge ratio (+/-) is an indicator of balance between positive charges (given by the concentration of ammonium groups present in DODAB) and negative charges (given by the concentration of phosphate groups in DNA, which corresponds to nucleotide concentration):

$$C.R. = \frac{[+]}{[-]} = \frac{[DODAB]}{[phosphate]} \quad (1)$$

Throughout the titration, charge ratio (+/-) varied constantly, not only due to the addition of the cationic vesicles to the DNA, but also due to the dilution effect originated by it.

For EtBr exclusion assays, an EtBr aqueous solution ([EtBr]=7.0×10^{-6} M) was added to the DNA solution, leading to the intercalation of the probe. EtBr concentration was kept six times lower than that of the DNA, in order to guaranty a directly proportional decrease in the probe fluorescence to the amount of cationic lipid at a given nucleotide base concentration [26].

In case of FRET assays, for which the presence of the lipid probe DPH-HPC was required ([DPH-HPC]=1/200×[lipid]), the probe was initially inserted within the lipid solution, and only then injected under vigorous vortexing to an aqueous buffer solution at 70 °C (30 mM Trizma), in order to form the

marked liposome solutions. The preparation of the lipoplexes then followed the same steps described above, that included their addition to the initial DNA solution.

Ethidium bromide exclusion assays

The steady-state fluorescence measurements were performed in a Horiba Jobin Yvon Spex Fluorolog-3 spectrofluorimeter, after each addition of cationic liposomes to the DNA solutions, and a 5 min agitation period with a magnetic stirrer. The fluorescence intensities were determined at λ_{exc}=510 nm, because this wavelength is known to be an isobestic point for EtBr/DNA solutions [27]. All emission spectra were integrated, and the ratio of the areas for the dye solutions and the standard was determined, after subtraction of the solvent background. Each fluorescence emission spectrum was fitted to a sum of two log-normal functions [28], corresponding to different environment states (DNA and H_2O), and had the following form:

$$I_F = \left(\frac{I_F^{\max} \cdot b}{\lambda - a}\right) \cdot e^{-c^2 - \frac{(\ln(x-a)-\ln(b))^2}{2\cdot c^2}} \tag{2}$$

where $I_F^{\max}$ is the maximum emission intensity at wavelength $\lambda_{\max}$, and the parameters a, b, and c are related to $\lambda_{\max}$, half-width (H), and skewness (ρ) by the expressions:

$$a = \lambda_{\max} - \left(\frac{H \cdot \rho}{\rho^2 - 1}\right) \quad b = \left(\frac{H \cdot \rho}{\rho^2 - 1}\right) \cdot e^{\left(\frac{\ln(\rho)}{\sqrt{2\cdot\ln(2)}}\right)^2}$$
$$c = \frac{\ln(\rho)}{\sqrt{2 \cdot \ln(2)}} \tag{3}$$

The area (A) of each curve is given by:

$$A = I_F^{\max} \cdot H \cdot \left[\left(\sqrt{2\cdot\pi}\right) \cdot \left(\frac{\rho}{\rho^2 - 1}\right) \cdot \left(\frac{\ln(\rho)}{\sqrt{2\cdot\ln(2)}}\right) \cdot e^{\frac{\left(\frac{\ln(\rho)}{\sqrt{2\cdot\ln(2)}}\right)^2}{2}}\right] \tag{4}$$

Assuming that the quantum yield of EtBr in the lipoplex remains approximately constant throughout the titration, the percentage of complexed DNA (α) can be determined from the spectral decomposition previously made:

$$\alpha = \frac{\int I_F^{DNA\ band}(C.R.(+/-) = 0.0) - \int I_F^{DNA\ band}(C.R.(+/-) = x)}{\int I_F^{DNA\ band}(C.R.(+/-) = 0.0)} \cdot 100 \tag{5}$$

Fluorescence resonance energy transfer assays

The steady-state fluorescence measurements were performed in a Horiba Jobin Yvon Spex Fluorolog-3 spectrofluorimeter, after each addition of cationic liposomes to the DNA solutions, and a 5 min agitation period with a magnetic stirrer. The fluorescence intensities of the lipoplexes labeled with DPH-HPC were determined at λ_{exc}=390 nm with spectral bandwidths of 1 nm, in the presence and absence of EtBr. All emission spectra were integrated, and the ratio of the areas for the dye solutions and the standard was determined, after subtraction of the solvent background. The fluorescence resonance energy transfer efficiency (Φ_{FRET}) was determined from the following expression:

$$\Phi_{FRET} = 1 - \left(\frac{\Phi_{DA}}{\Phi_D}\right) \Leftrightarrow\Leftrightarrow \Phi_{FRET} = 1 - \frac{\left(\frac{I_{F(DA)}}{I_{F(D)}}\right) - \alpha}{1-\alpha} \Leftrightarrow \Phi_{FRET} = \frac{1 - \left(\frac{I_{F(DA)}}{I_{F(D)}}\right)}{1-\alpha} \tag{6}$$

Where $I_{F(DA)}$ and $I_{F(D)}$ are the fluorescence emission of donor (DPH-HPC) in the presence and absence of acceptor (EtBr), respectively. α is the efficiency of complexation of DNA previously determined in EtBr exclusion assays (Eq. 5), which determines the amount of EtBr (acceptor) that remains in the lipoplex and is able to receive excitation energy from the DPH-PC donor.

90° static light scattering assays

90° SLS due to the formation of complexes by the cationic liposomes and DNA was measured with a Horiba Jobin Yvon Spex Fluorolog-3 spectrofluorimeter with excitation and emission monochromators set respectively at 600 and 601 nm, at which there is neither absorbance, nor fluorescence emission. Scattering intensities were measured 5 min after the addition of cationic liposomes in different conditions (presence (D) and absence (D_0) of DNA) and remained constant after this period. The ratio D/D_0 was determined.

Results & discussion

Ethidium bromide exclusion assays

Ethidium bromide (EtBr) is a well-known fluorescence fluorophore that, when intercalated between base-pairs of DNA double helix, presents a high fluorescence emission at 610 nm (excitation at 500 nm), compared to its fluorescence in water [21]. During condensation of DNA after the addition of cationic lipid, varying amounts of EtBr are forced to dissociate from DNA and go into the aqueous phase resulting in a decrease in the total fluorescence intensity [21].

Figure 1 shows the fluorescence spectra of EtBr for the system DNA-DODAB:MO (1:1), at the charge ratios (+/-) of 0.0, 0.5., 1.1 and 1.8. With charge ratio (+/-) increase, it is visible a decrease in the DNA band as opposed to the

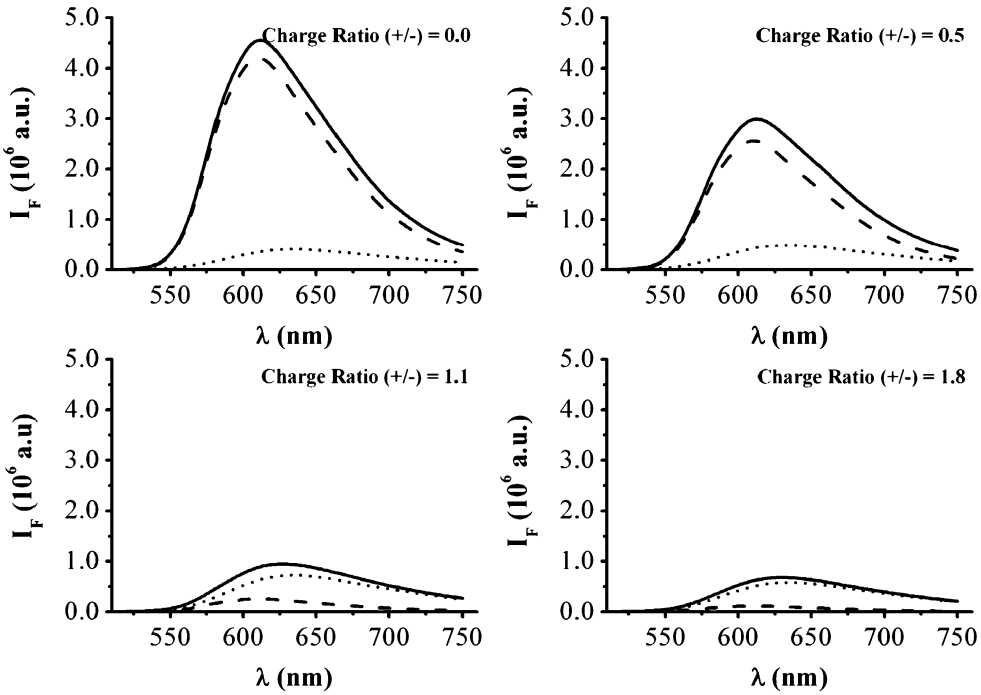

Fig. 1 Fluorescence emission spectra of EtBr (λ_{exc}=510 nm) in a salmon sperm DNA solution ($[phosphate]_{initial}=4.2\times10^{-5}$ M), with increasing concentrations of DODAB:MO (1:1) vesicles, showing the spectral decomposition into DNA (*dashed line*) and H_2O bands (*pointed line*)

enhancement of the H_2O band, confirming the condensation of DNA and the exclusion of EtBr to aqueous environment.

Figure 2 shows the evolution in the fluorescence intensities of the two fluorescence bands of EtBr in H_20 environment and intercalated in DNA for pure DODAB and for different molar ratios of DODAB:MO (1:0.5, 1:1 and 1:2), at different charge ratios (+/-).

At lower charge ratio (+/-) (<1.0), the relative proportion of MO seems to have an important role in the evolution process of DNA condensation, as seen by the different DNA condensation efficiencies (α) observed at charge ratios (+/-) 0.5 and 1.1 (Table 1). The system DODAB:MO (1:1) seems to be the one that more efficiently condenses DNA at charge ratio (+/-) 1.0 when compared with pure DODAB (at 25°). Oppositely, the similarity of the DNA complexation efficiencies (α) for the different systems at charge ratio (+/-)=1.8 (Table 1), suggests that the amount of MO is not determinant in the final DNA condensation

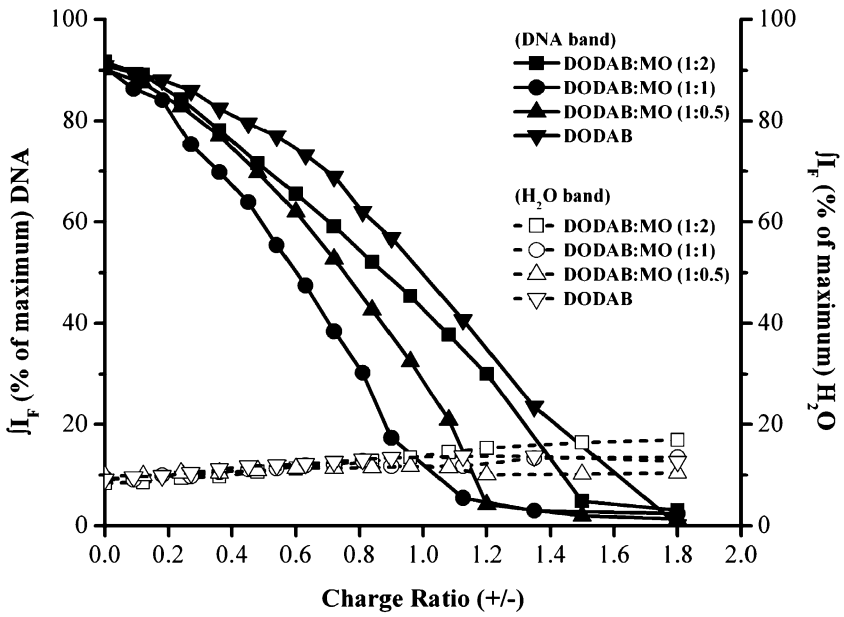

Fig. 2 Variation of the decomposed fluorescence intensities of EtBr in DNA and H_2O bands with charge ratio (+/-) increase, for the titration of different DODAB:MO systems presenting different molar ratios (*squares* (1:2), *circles* (1:1) and *triangles* (1:0.5)) and pure DODAB systems (*upside-down triangles*) to salmon sperm DNA solution (20 μg/ml)

Table 1 Percentages of complexed DNA (α) at charge ratios (+/-) 0.0, 0.5, 1.1 and 1.8, for the titration of different DODAB:MO systems presenting different molar ratios (1:2, 1:1, and 1:0.5) and pure DODAB systems (25 and 55 °C) to salmon sperm DNA solution (20 μg/ml)

Liposomes	% of Complexed DNA (α)			
	R.C. (+/-) 0.0	R.C. (+/-) 0.5	R.C. (+/-) 1.1	R.C. (+/-) 1.8
DODAB:MO (1:2)	0.0	21.9	58.8	96.6
DODAB:MO (1:1)	0.0	38.8	93.9	97.3
DODAB:MO (1:0.5)	0.0	22.7	76.8	98.5
DODAB (25 °C)	0.0	15.4	55.2	99.8
DODAB (55 °C)	0.0	42.4	94.0	98.0

efficiency leading to the lipoplex formation, but may be important in the morphology of the lipoplexes formed. It is observed that the rate of DNA condensation increases in order DODAB:MO (1:1) > DODAB:MO (1:0.5) > DODAB:MO (1:2) > DODAB.

It is known that an important effect of the mixture of monoacylglycerols with vesicles is the reduction of chain packing constraints by partitioning into the interstices in the hydrocarbon region. Monoacylglycerols also confer instability to DODAB liposomes due to its "curvature loving" properties [20]. This fact may offer the opportunity for MO softening the DODAB lipid bilayer, decreasing its bending rigidity and so increasing the lateral mobility of the chain lipid.

This can explain the increase of DNA condensation efficiency with MO content, but an inversion is observed at higher MO molar ratio DODAB:MO (1:2). In order to gain further insights on the MO role on the lipoplex formation we have performed some 90° SLS experiments and preliminary energy transfer studies.

90° static light scattering

The 90° SLS is a good measure of relative changes in particle size [24]. It gives important information on size instability, which is one of the major factors in determining transfection efficiency in cell culture [1]. The SLS data were presented under the form D/D_0, where D is the 90° SLS after the addition of mixed cationic liposomes in the presence of DNA, and D_0 is the 90° SLS the in absence of DNA, at corresponding lipid concentration. Figure 3 presents the D/D_0 variation as a function of charge ratio (+/-). The curves have a bell shape, reaching their highest values at charge ratios (+/-) between 0.7 and 1.1, the values being dependent on the liposome lipid composition.

Theoretical studies show that at the charge ratio region (+/-) between 0.7 and 1.1, lipoplexes composed of "curvature-loving" helper lipids can have sandwich type (L_α^C) and inverted aggregates (H_{II}^C) [6]. Only when helper lipid content is higher than 50%, the inverted structures are predicted. In our results, this corresponds to a shift in the 90° SLS peak to lower charge ratios (+/-) and higher mean

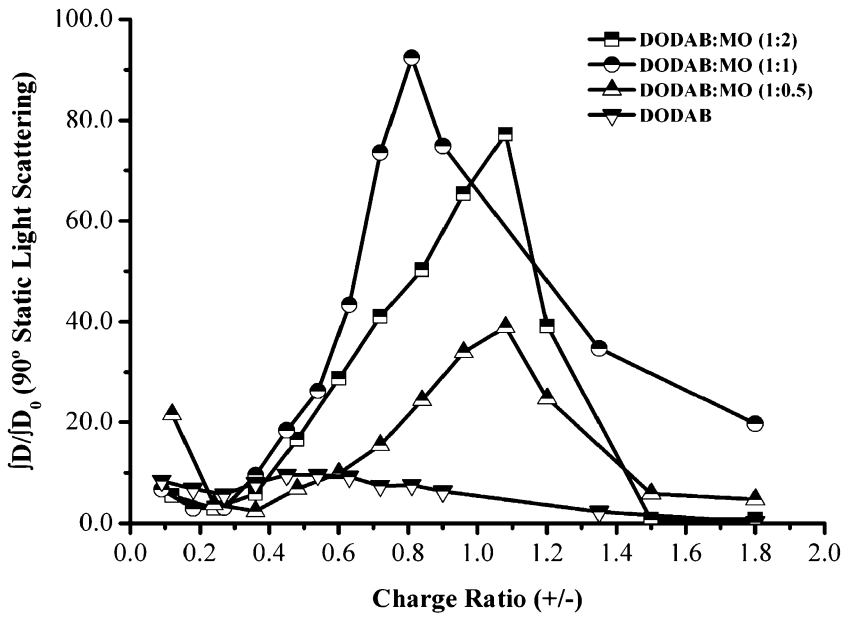

Fig. 3 Variation of scattered light intensity (D/D_0) with charge ratio (+/-) increase, for the titration of DNA with different DODAB:MO systems presenting different molar ratios (*squares* (1:2), *circles* (1:1) and *triangles* (1:0.5)) and pure DODAB systems (*upside-down triangles*). D/D_0 represents the ratio between scattered light intensity (λ_{exc}=600 nm, λ_{emi}= 601 nm) in the presence of DNA (D) and scattered light intensity (λ_{exc}=600 nm, λ_{emi}=601 nm) in the absence of DNA (D_0)

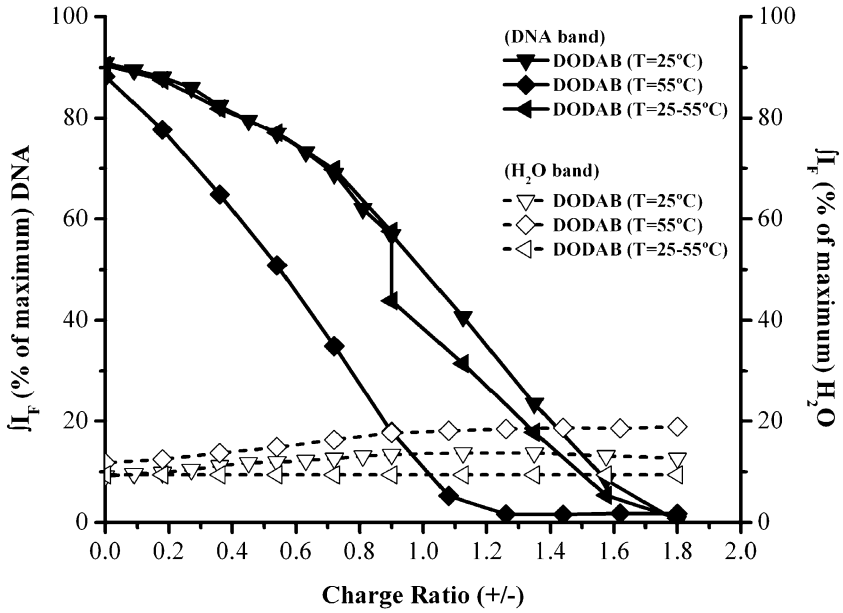

Fig. 4 Variation of the decomposed fluorescence intensities of EtBr in DNA and H_2O bands with charge ratio (+/-) increase, for the titration of pure DODAB systems at different temperatures (*triangles* (25 °C), *diamonds* (55 °C) and *arrowheads* (25 °C (0.0<C.R(+/-)<0.9) to 55 °C (0.0<C.R(+/-)<0.9)) to salmon sperm DNA solution (20 μg/ml)

particle size (Fig. 3). Again an inversion is observed for the DODAB:MO system with the highest MO content, which is not expect for the theoretical calculations [6]. The formation of inverted structures is expected to exclude more efficiently the EtBr, confirming the results in the previous section. This suggests that an excess of MO does not promote inverted DNA structures as much as expected.

A possible explanation for the behavior at higher MO content is the coexistence of DODAB-MO inverted structures without DNA which, by being smaller, originate lower light scattering level. But, we can not rule out the possibility of being away from the thermodynamical equilibrium. To test this hypothesis, we have performed some studies with pure DODAB lipoplexes at two different temperatures, below and above DODAB melting temperature ($T_m \approx 45$ °C) [29–32] (Fig. 4). Initially, it was promoted the DNA complexation at 25 °C until charge ratio (+/-) was 0.9. At this point, the temperature was increased to 55 °C and then maintained throughout the rest of the titration. It was observed that the DNA complexation efficiency did not catch up the one obtained when the titration was performed at 55 °C since the beginning. This behaviour indicates that thermodynamical issues are not the only factors that explain the behaviour of lipolexes formation. There may be energetic barriers involved in the organization of the complexes.

Fluorescence resonance energy transfer

A third approach for assessing the direct effect of the MO as a helper on DODAB-DNA complexes (lipoplexes), was

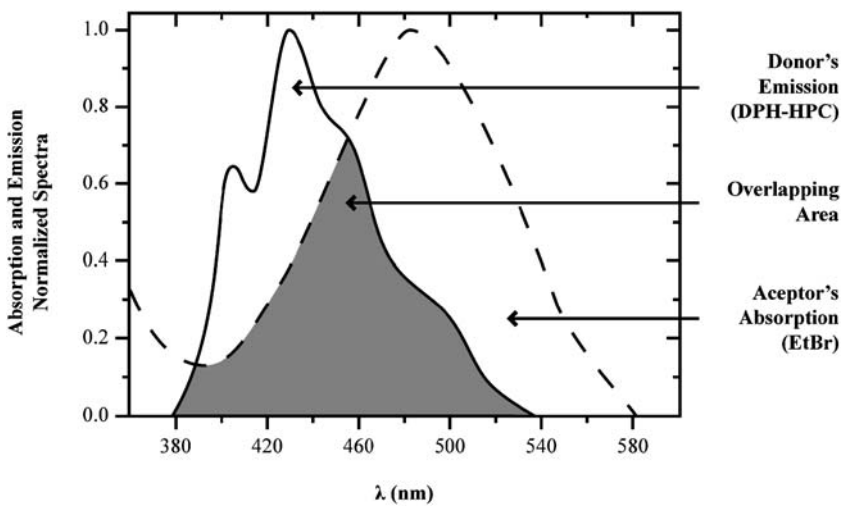

Fig. 5 Normalized spectra of absorption of EtBr (*dashed line*) and fluorescence of DPH-PC (*straight line*), showing the spectral overlapping (*grey area*) between donors emission and acceptors absorbance

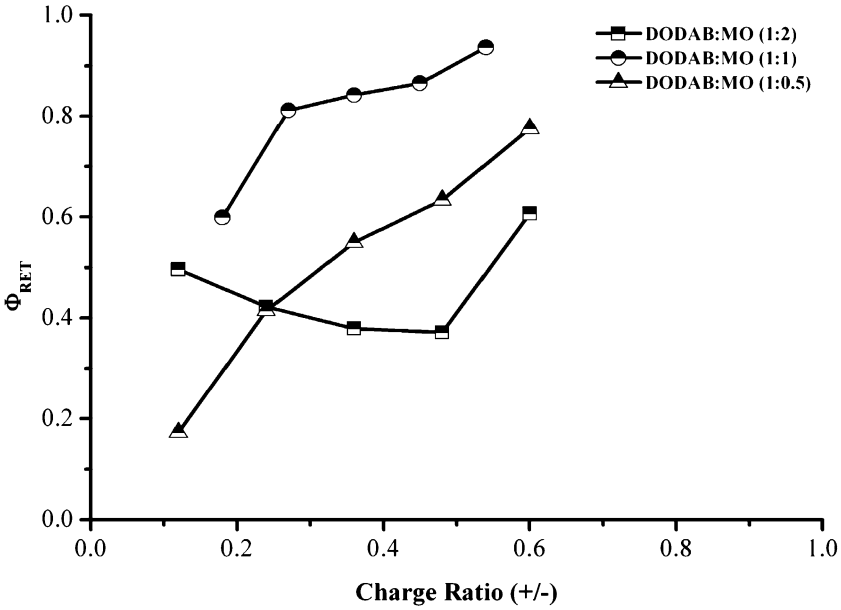

Fig. 6 Variation of fluorescence resonance energy transfer efficiency (Φ_{FRET}) between DPH-HPC (donor) and EtBr (acceptor) with charge ratio (+/-) increase, for the titration of different DODAB:MO systems presenting different molar ratios (*squares* (1:2), *circles* (1:1) and *triangles* (1:0.5)) to salmon sperm DNA solution (20 μg/ml). Φ_{FRET} represents the ratio between DPH-HPC emission (λ_{exc}=390 nm) in the presence of EtBr ($I_{F(DA)}$) and DPH-HPC emission (λ_{exc}= 390 nm) in the absence of EtBr ($I_{F(D)}$)

based on fluorescence energy transfer (FRET). For these experiments, the choice of the donor-acceptor pair was based in the fact of the emission spectra of the probe DPH-PE strongly overlaps the EtBr absorption spectrum, which is an essential FRET requirement (Fig. 5).

Figure 6 shows the efficiency of resonance energy transfer for DODAB:MO (1:2), (1:1) and (1:0.5) at different charge ratios (+/-). We observed a very significant enhancement in Φ_{FRET} when MO is increased from DODAB:MO (1:0.5) to DODAB:MO (1:1). As referred previously, this increase corresponds to the appearance of inverted lipoplexes structures. These are much more compact than the sandwich type ones [6], which leads to a lower mean donor-acceptor distance. As in the previous methods, an inversion occurs in the DODAB:MO (1:2) systems. This could be explained by the solubilization of the DPH-HPC in the mentioned plain DODAB/MO inverted structures.

Conclusions

Our results demonstrate that the inclusion of monoolein in the cationic liposomes formulation significantly modifies the rate of DNA complexation, being DODAB:MO (1:1) the system with higher DNA condensation efficiency. The 90° SLS and FRET assays suggest the existence of inverted structures due to the presence of monoolein, which may be of importance to the transfection process. These inverted structures can be different from the usual hexagonal morphology because the MO has a tendency to form inverted cubic phases [19]. In further work, we will try to observe these inverted structures using structural characterization techniques. The existence of proposed inverted structures without DNA would also be confirmed. Cell transfection efficiency and citotoxicity studies are currently underway.

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