



Review

Fluorescence methods for lipoplex characterization

Catarina Madeira ^{a,b,*}, Luís M.S. Loura ^{c,d}, Maria R. Aires-Barros ^{a,b}, Manuel Prieto ^{e,**}^a Department of Bioengineering, Instituto Superior Técnico, Universidade Técnica de Lisboa, Portugal^b IBB-Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Universidade Técnica de Lisboa, Portugal^c Faculdade de Farmácia, Universidade de Coimbra, Coimbra, Portugal^d Centro de Química de Coimbra, Universidade de Coimbra, Coimbra, Portugal^e Centro de Química Física Molecular and IN, Complexo I, Instituto Superior Técnico, Universidade Técnica de Lisboa, Portugal

ARTICLE INFO

Article history:

Received 3 May 2011

Received in revised form 29 June 2011

Accepted 15 July 2011

Available online 29 July 2011

Keywords:

Cationic lipids

Gene delivery

Lipoplex

Fluorescence techniques

FRET

Plasmid DNA

ABSTRACT

Since the first reported transfection studies using cationic liposomes in 1987, significant advances have been made on the understanding of the physical properties of DNA/cationic liposome complexes (lipoplexes) in order to improve their transfection efficiencies. In this review a critical survey of the biophysical techniques used in their characterization is presented, with an emphasis on fluorescence methodologies, namely FRET. It is shown that the use of FRET combined with state-of-the-art modeling and data analysis allows detailed structural information in conditions close to the *in vivo* utilization of these non-viral based vectors. We describe in detail the use of fluorescence-based methods in (i) the assessment of DNA–lipid interaction and kinetics of lipoplex formation; (ii) membrane mixing studies; (iii) characterization of lipoplex molecular structure through the determination of interlamellar distances; and (iv) qualitative and quantitative evaluation of DNA condensation by cationic liposomes. This review aims at providing a framework for future characterization studies of novel liposomal formulations as gene delivery carriers, taking advantage of more sensitive nucleic acid and lipid dyes concomitantly with increasingly sophisticated fluorescence techniques.

© 2011 Elsevier B.V. All rights reserved.

Contents

1. Introduction and scope	2694
2. Assembly of lipoplexes	2695
3. Techniques used in lipoplex biophysical and physical–chemical characterization	2696
4. Characterization of lipoplexes by fluorescence spectroscopy methods	2697
4.1. Lipid–DNA interaction and lipoplex formation	2697
4.2. Membrane mixing studies	2699
4.3. Characterization of lipoplex molecular structure using FRET	2700
4.4. DNA condensation assessment	2702
5. Concluding remarks.	2703
Acknowledgements	2703
References	2703

1. Introduction and scope

Gene therapy is a promising approach to the treatment of disease through the use of DNA-based vectors that allow targeting, delivery of DNA to cells, and expression of the therapeutic gene. Among the non-viral vectors, cationic liposomes are the most widely used chemical-based DNA delivery systems, not only in basic research [1], but also in clinical trials [2]. Indeed, recent data showed that in 6.4% of gene therapy clinical trials worldwide, cationic liposomes are used as vectors, for the treatment of vascular and monogenic diseases though the majority of these trials target several types of cancer (source

* Correspondence to: C. Madeira, IBB-Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, IST, Av. Rovisco Pais, 1049-001, Lisboa, Portugal. Tel.: +351 218419594; fax: +351 218419062.

** Correspondence to: M. Prieto, Centro de Química Física Molecular, Complexo I, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal. Tel.: +351 218419248; fax: +351 218464455.

E-mail addresses: catarina.madeira@ist.utl.pt (C. Madeira), manuel.prieto@ist.utl.pt (M. Prieto).

www.wiley.co.uk/genmed/clinical). On the other hand, each year several new cationic lipid-based transfection reagents to transfer DNA into mammalian cells are launched into the market for basic research, and most of them focus on the transfection of hard to transfect cells. In fact, earlier studies about the interaction of cationic lipids with DNA, mainly encompassing an extensive physical chemical characterization [3], gave rise to excellent current transfection agents that are very efficient regarding gene transfer into mammalian cells in vitro [4]. However, most of those successful formulations have shown to be inappropriate for in vivo or ex vivo gene delivery of certain somatic or stem cells known to be difficult to transfect. For these applications, cationic liposomes are still associated with low transfection efficiencies [5,6], although their advantages over viral vectors such as non-immunogenicity, low toxicity, and possibility of large-scale production, keep researchers questing for novel cationic lipids (reviewed elsewhere [6]) and more efficient formulations to transfect hard to transfect cells.

Many efforts have been made to fully characterize cationic liposome–DNA complexes (lipoplexes), because this is the only way to understand, improve, and control their transfection efficiency. In 1987, it was reported for the first time that plasmid DNA and cationic liposomes aggregate due to electrostatic attractive forces, forming small complexes able to transfer DNA to the cells [7]. The ability of DNA and oligonucleotides to induce lipid mixing, the electrostatic properties of the lipoplexes during and after their formation, the DNA accessibility to DNase I after complexation with lipids, the size and zeta potential of the lipoplexes, and the encapsulation efficiency of the lipid vector are some of the parameters considered in the characterization of lipoplexes. Importantly, back in 2003 different techniques were used to propose a mechanism for lipoplex formation, and it was verified that upon DNA and cationic liposomes contact, around 20min is sufficient to obtain stable complexes [8,9]. The unveiling of their microscopic arrangement, a multilamellar structure of lipid bilayers with sandwiched DNA, with a constant interlayer spacing invariant with the charge ratio and depending on cationic liposome formulations [10,11], is still considered a major breakthrough in this field. To date, the most commonly used techniques to characterize lipoplexes are fluorescence-based techniques and their main advantages are related to the possibility of using DNA and lipid concentrations similar to those used to transfect cells in vitro, ex vivo or in vivo, and mainly because it is possible to monitor

these complexes' behavior under physiological conditions. Throughout this review the usefulness of the techniques used over the years in lipoplex characterization is discussed. Nevertheless, we will focus mainly on the use of fluorescence-based techniques for lipoplex characterization, detailing their use in the assessment of (i) kinetics of lipoplex formation and DNA–lipid interaction; (ii) membrane mixing studies; (iii) lipoplex molecular structure through the determination of interlamellar distances and (iv) DNA condensation, as schematically shown in Fig. 1.

2. Assembly of lipoplexes

Liposomes used in lipoplexes are mainly constituted by cationic lipids. The term is often used in a loose sense, referring to all cationic amphiphiles, including cationic cholesterol and bile salt derivatives, as well as other micelle- or bilayer-forming cationic amphiphiles [12]. Cationic lipids are composed of three main elements: a hydrophobic tail, a linker and a cationic head group. Some of the most representative cationic lipids for gene transfection are 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP), 2,3-dioleoyloxypropyl-1-trimethylammonium bromide (DOTMA), 2,3-dioleoyloxy-*n*-(2-(spermincarboxamido)ethyl)-*n*,*n*-dimethyl-1-propaniminium penta-hydrochloride (DOSPA), *N*-(2-hydroxyethyl)-*n*,*n*-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium (DMRIE), dioctadecylamido-glycylspermine (DOGS) and 3β(*N*,*N*-dimethylaminoethane)carbonyl cholesterol (DC-CHOL) [6]. A modification of the chemical nature of each lipid component has led to the synthesis of new liposomal formulations with different and better abilities for transfection [6]. Most cationic liposomal formulations require the inclusion of a neutral lipid, frequently named helper lipid, in order to increase transfection efficiency [13]. The most commonly used neutral lipids are 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and cholesterol (Chol). DOPE is often considered to be a fusogenic lipid, due to its ability to adopt an inverted hexagonal phase in aqueous environment, thus promoting membrane fusion [14]. The incorporation of helper lipids into the cationic membranes results in increased conformational disorder of the apolar region and further dehydration of the interfacial region, which decreases with the increase of lipoplex lipid/DNA charge ratio (+/−) [15]. Changes in the hydration of the DNA bases were also observed as the molar ratio of helper lipid in the membranes was increased [16] and different helper lipids bind DNA

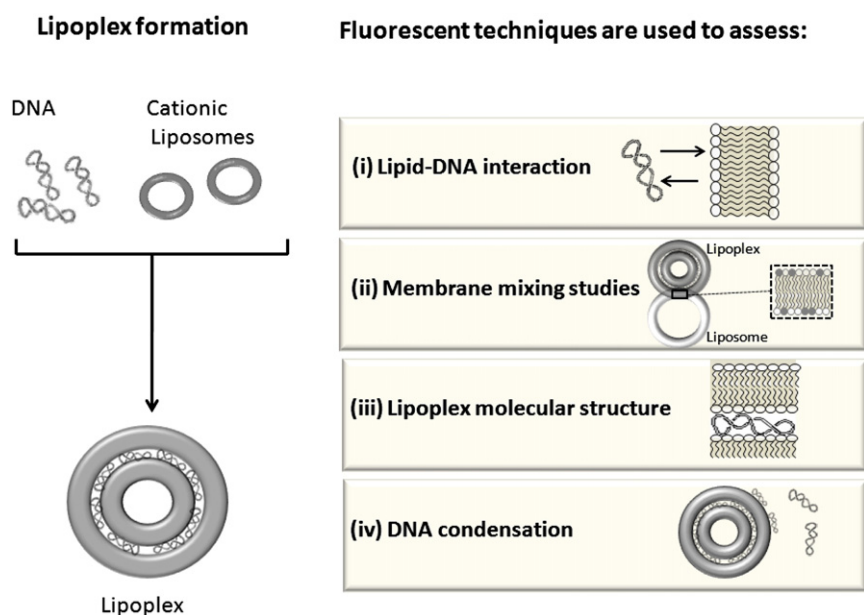


Fig. 1. Cationic liposomes and DNA are driven by electrostatic interactions to form lipid–DNA complexes or lipoplexes. Fluorescence-based techniques have been used to assess lipid–DNA interaction and membrane mixing during lipoplex formation, to characterize lipoplex molecular structure after formation and under physiological conditions, and also to evaluate the level of DNA condensation by the lipids.

differently [17,18]. On the other hand, in terms of DNA condensation by cationic lipids, it was verified that double-charged lipids are more efficient than single-charged ones [19].

It is well established that formation of lipid-based DNA delivery systems is a consequence of a self-assembly process triggered by electrostatic interactions between positively charged lipids and negatively charged DNA (Fig. 1) [20,21], and their kinetic and thermodynamic factors are discussed below. Common features to all lipoplex systems, which are characterized in most preparation methods and buffer conditions, are the size and zeta potential dependence on lipoplex charge ratio, showing a maximum and a change of sign (respectively) near charge neutrality [22].

Upon DNA contact with liposome vesicles, DNA-induced membrane fusion occurs, as indicated by lipid mixing studies [23] and electron microscopy [24]. The condensation of DNA molecules within the lipids, which occurs next, has been monitored by several methodologies [25–28] and predicted by modeling [29]. A three step mechanism of lipoplex formation was proposed, in which kinetic data was considered. In a first step, electrostatic interaction drives the coverage of one DNA strand initially with a single vesicle, which occurs in the millisecond time scale. Next, an unstable intermediate with a locally cylindrical structure is formed, which evolves to a multilamellar structure on a time scale of minutes [8]. Interestingly, a similar formation mechanism was previously proposed, based on different techniques, and the same type of structures were visualized by Atomic Force Microscopy [30].

The first visualization of lipoplexes was accomplished by electron microscopy, and soon their morphology was found to be dependent on incubation time and DNA concentration, and at longer incubation periods and higher concentration, larger and denser structures were visualized [31]. Shortly after, another group suggested that the DNA molecules were entrapped between lipid bilayers in clusters of aggregated multilamellar structures [32], and changes in DNA conformation upon lipid condensation were observed [33–35]. The use of diffraction techniques confirmed the existence of these lipid-enriched lipoplex structures. Several groups, using different formulations, verified that lipoplexes were constituted by fully condensed DNA molecules, as parallel helices, sandwiched between lipid bilayers [10,11,36,37], and concomitantly, theoretical models were also developed [38,39]. Lipoplexes with DOPE, at a molar fraction higher than 0.75, showed a different arrangement of DNA molecules within lipid bilayers, in which cylinders consisting of DNA were coated with a lipid monolayer arranged on a hexagonal lattice, frequently identified

as an inverted hexagonal structure [40]. Valuable information was obtained with diffraction techniques, and the effect of specific parameters on lipoplex microstructure has been studied, such as the size of plasmid DNA [41], the liposomal formulation [10,42], the charge ratio [10], and the ionic strength of the buffer [43,44]. According to X-ray data, lamellar distance does not depend on lipoplex charge ratio [10]. However, for identical lipid formulation, different values of interhelical spacing of DNA molecules were obtained for different charge ratios. In fact, negatively charged complexes have a higher DNA packing density than isoelectric complexes, whereas positively charged ones have a lower packing density [31,43,45]. Structure–activity relationships have been considered in an effort to rationalize lipoplex behavior. However, it is known that one specific liposomal formulation can efficiently transfect *in vitro* a certain type of cells and not others, and a much more complex combination of parameters is required for rationalization of *in vivo* or *ex vivo* studies.

3. Techniques used in lipoplex biophysical and physical–chemical characterization

The use of different techniques in lipoplex characterization has been essential to the understanding of their formation, ability of DNA condensation, macroscopic and microscopic structure and also, in specific cases, to predict their behavior *in vivo*. Table 1 summarizes the information obtained from each technique in lipoplex studies. Considering the DNA amount frequently used in transfection protocols, 1–2 µg by well (*in vitro*), 20–50 µg/animal (*in vivo*) or 50–150 µg/mL by dose (in clinical trials), one can verify that several techniques used in lipoplex characterization are sensitive to these concentration ranges. Nevertheless, in the analysis of lipoplexes by transmission electron microscopy or diffraction techniques, a previous sample treatment and higher concentrations of DNA (100–5000 µg/mL) are usually required. However, important information about lipoplex structure was and still is retrieved from these techniques. Lipoplex macro-structure, with lower DNA amounts (0.01–50 µg/mL) can be visualized by atomic force microscopy. In fact, microscopy and diffraction techniques are extremely powerful in the macro- and micro-structural study of biological systems, respectively, and when they were applied to lipoplexes, important features of these complexes were unveiled. With the exception of these techniques, all the others mentioned in Table 1 allow the physical–chemical characterization of lipoplexes in solution, with similar DNA concentrations to those used in transfection protocols, which is extremely advantageous. Moreover, fluorescence spectroscopy-based techniques

Table 1
Specific information derived from each technique in lipoplex characterization studies.

Technique	DNA (µg/mL)	Structural or physical–chemical properties of lipoplexes	Ref.
Light scattering	4–125	–Lipoplex size distribution	[22,121]
Electrophoretic mobility	4–125	–Surface charge (potential zeta) of lipoplexes	[28,79]
Agarose gel electrophoresis	1–250	–DNA integrity after lipoplex exposure to endonucleases	[17,23]
Microscopy	AFM	–Qualitative assessment of DNA condensation	[122]
	TEM	–Macroscopic structure of lipoplexes	[123]
	35–1000	–Qualitative assessment of DNA condensation	[123]
		–Lipoplex size	
Diffraction	100–5000	–Microscopic structure of lipoplexes, assessing interlamellar distances and mode of DNA packing	[10]
Calorimetry	0.01–1000	–Thermodynamic lipid–DNA binding constants	[8]
UV/VIS absorption	15–150	–DNA and lipid quantification	[124]
		–Melting profiles	
Circular dichroism	16–160	–DNA secondary structure upon condensation by liposomes	[49]
FT-IR	15–200	–Lipoplex state of hydration	[55,56]
Fluorescence	1.5–100	–Monitorization Lipid–DNA interaction	Section 4
		–Kinetics of lipoplex formation	
		–Membrane mixing studies	
		–Molecular structure of lipoplexes	
		–DNA condensation assessment	
NMR	–	–Distinguish between bilayer and nonbilayer arrangements upon DNA contact	[50,51]
ESR	350–1000	–Bilayer properties and changes upon DNA incorporation	[53,54]

AFM: atomic force microscopy; TEM: transmission electron microscopy.

have the additional advantage of allowing studies under physiological conditions without major interference of serum proteins.

DNA condensation ability by cationic liposomes has been assessed and confirmed by several techniques, such as light scattering, microscopy and fluorescence spectroscopy. Easy and common methods such as electrophoresis agarose gel allow a first approach to evaluate DNA condensation, and have been used to calculate encapsulation efficiency [17,23]. However, more rigorous encapsulation efficiencies of these vectors have been measured mainly by fluorescence methods, even in the presence of serum [46]. Lipoplex size is mainly determined by dynamic light scattering (DLS) and more rarely by microscopy, being an important parameter not only in transfection studies (in vitro and in vivo), but also as a measure of colloidal stability upon storage [47]. Calorimetry and again fluorescence spectroscopy have allowed monitoring lipoplex formation, from a thermodynamic, kinetic and electrostatic point of view [8,9,48]. Several techniques have been used to assess structural changes in DNA upon DNA–lipid contact, such as circular dichroism (CD) [49]. For studying the lipids, nuclear magnetic resonance (NMR) [50–52] or electron spin resonance spectroscopy (ESR) [53,54] has been used. Fourier transform infrared spectroscopy (FT-IR) [55,56] and several fluorescence methodologies described in detail in this review allowed to obtain structural information on both lipid and DNA.

Lipoplexes are usually prepared in low ionic or sugar solutions and there is a growing interest on their physical stability upon storage, which is obviously of paramount importance in the pharmaceutical area. Parameters such as size, surface charge, and state of dehydration upon liophilization are commonly monitored using DLS, electrophoretic mobility and recently FT-IR techniques, respectively, to assess lipoplex physical stability upon storage. While some other techniques may be very informative in terms of lipoplex structure, physical–chemical properties and formation under low ionic conditions, others have allowed their characterization under physiologic conditions, which could predict their behavior in vivo. In fact, several methodologies have been used, with success, in lipoplex physical–chemical characterization under physiological conditions, such as DLS, electrophoretic mobility, microscopy and several fluorescence methodologies, which will be detailed in the following sections.

4. Characterization of lipoplexes by fluorescence spectroscopy methods

Fluorescence spectroscopy has received increasing attention as a tool for lipoplex characterization, due to the use of more sensitive probes and possibility to operate under physiological conditions. In this section, several fluorescence based–methodologies in lipoplex characterization will be reviewed, underlining the obtained relevant information, shown schematically in Fig. 1.

4.1. Lipid–DNA interaction and lipoplex formation

The kinetics of lipoplex formation as a function of charge ratio and vesicle composition was followed by stopped–flow fluorescence studies, using ethidium bromide (EtBr) [8,9]. These authors observed that DNA association with cationic vesicles followed first–order reactions and occurred through a three–step mechanism. Firstly, DNA adsorbs onto the oppositely charged membrane surface in milliseconds, and thereafter, on a longer time scale (seconds), the formed DNA–lipid complex continues to aggregate and grow. A third step seems to be due to rearrangement of the complex with a further change of the DNA conformation. Interestingly, the time constants of the three steps were determined, and whereas those of the first and third steps seemed to be invariant with the charge ratio, the second increases with increasing charge ratios [9]. Similarly, Braun and co–workers examined the formation kinetics of two different lipoplex formulations. In separate experiments, these authors used high–affinity DNA dyes Hoechst 33258, a minor groove binding dye, or a bisintercalating dye YOYO–1 [57]. Fig. 2 shows stopped–

flow fluorescence data obtained with both dyes within DOTAP/DNA lipoplexes, where it is possible to verify that the initial phase of complexes formation (<100 ms) is characterized by an increase in fluorescence intensity which might reflect the initial binding of the lipids to DNA. According to the authors, this binding results in a reduction in the polarity of the environment of the bound dye and a consequent increase in fluorescent intensity. Using this methodology, these authors were able to resolve two sequential steps in the assembly of complexes that are assigned to binding/dehydration and condensation events, but with YOYO–1 it was not possible to fit the second constant rate (k_2) and calculate activation energy (E_a) from Arrhenius plots (Fig. 2) [57].

Fluorescence correlation spectroscopy is a highly versatile technique that can easily be adapted to solve specific biological questions, unraveling molecular interactions in vitro and in vivo [58]. Importantly, it gives information on molecular mobility and photophysical and photochemical reactions, by recording and correlating the fluorescence fluctuations of single labeled molecules through the exciting laser beam [58]. Moreover, using dual–color fluorescence cross–correlation, specific binding studies can be carried out. DNA and vesicle structure, as well as dynamics of lipoplex formation, were examined simultaneously using two–photon excitation fluorescence correlation spectroscopy (TPE–FCS) and two–photon excitation fluorescence cross–correlation spectroscopy (TPE–XCS) [59]. Despite using short oligonucleotides (40 bp), this study allowed the determination of the number of these molecules per lipid vesicle in the absence of aggregation, and a two–step mechanism of DNA condensation prior to vesicle aggregation was proposed [59]. To overcome the problems related to the fitting of autocorrelation curves when fluorescence bursts are present in FCS measurements, Margineanu and co–workers analyzed separately the baseline fluorescence levels and the fluorescence bursts in the same trace [60]. From the baseline levels, the number of free/bound DNA molecules and the presence of tens to hundreds of nanometer–sized lipoplexes were estimated using mathematical models. On the other hand, analysis of the fluorescent bursts provided an indication about the sizes of the lipoplexes, the number of DNA molecules in these aggregates, and the relative amount of lipids in each aggregate [60]. A detailed characterization of the composition and macromolecular organization structure of a ternary, targeted, lipopolyplex synthetic vector (LID complex) was accomplished [61]. These complexes are generally constituted by cationic lipids, plasmid DNA, and a cationic peptide component, which in this study had a dual function, containing both DNA–condensation and integrin–targeting sequences. Fluorophore–labeled lipid, peptide, and DNA components were used to formulate the vector, and the stoichiometry of the particles was established by fluorescence correlation spectroscopy (FCS). Fluorescence quenching experiments and freeze fracture electron microscopy were then used to demonstrate the arrangement of the lipid, peptide, and DNA components within the complex: the cationic portion of the peptide interacts with the plasmid DNA, resulting in a tightly condensed DNA–peptide inner core which is surrounded by a disordered lipid layer, from which the integrin–targeting sequence of the peptide partially protrudes [61]. In addition, the interaction of lipopolythiourea with DNA was investigated by FCS, and the influence of the lipid length and nature of the thiourea head on the thiourea/DNA interaction were studied [51]. FCS revealed a strong interaction between lipopolythiourea and DNA, occurring at 1 equivalent of a thiourea lipid by a DNA phosphate group, and leading to a condensed plasmid state [51].

Lipid–DNA interaction has also been studied using membrane dyes. Comparing the fluorescence behavior of probes with different locations in the membrane, such as 1,6–diphenylhexatriene (DPH; close to the hydrophobic tail) and trimethylammonium–DPH (TMA–DPH; close to water/lipid interface) it was demonstrated that more striking changes were obtained with the latter upon DNA contact, suggesting that major DNA–lipid interactions occur at the lipid/water interface [62]. The use of a pH–sensitive fluorophore (hydroxycoumarin or HC), in combination with Gouy–Chapman calculations, allowed the characterization of

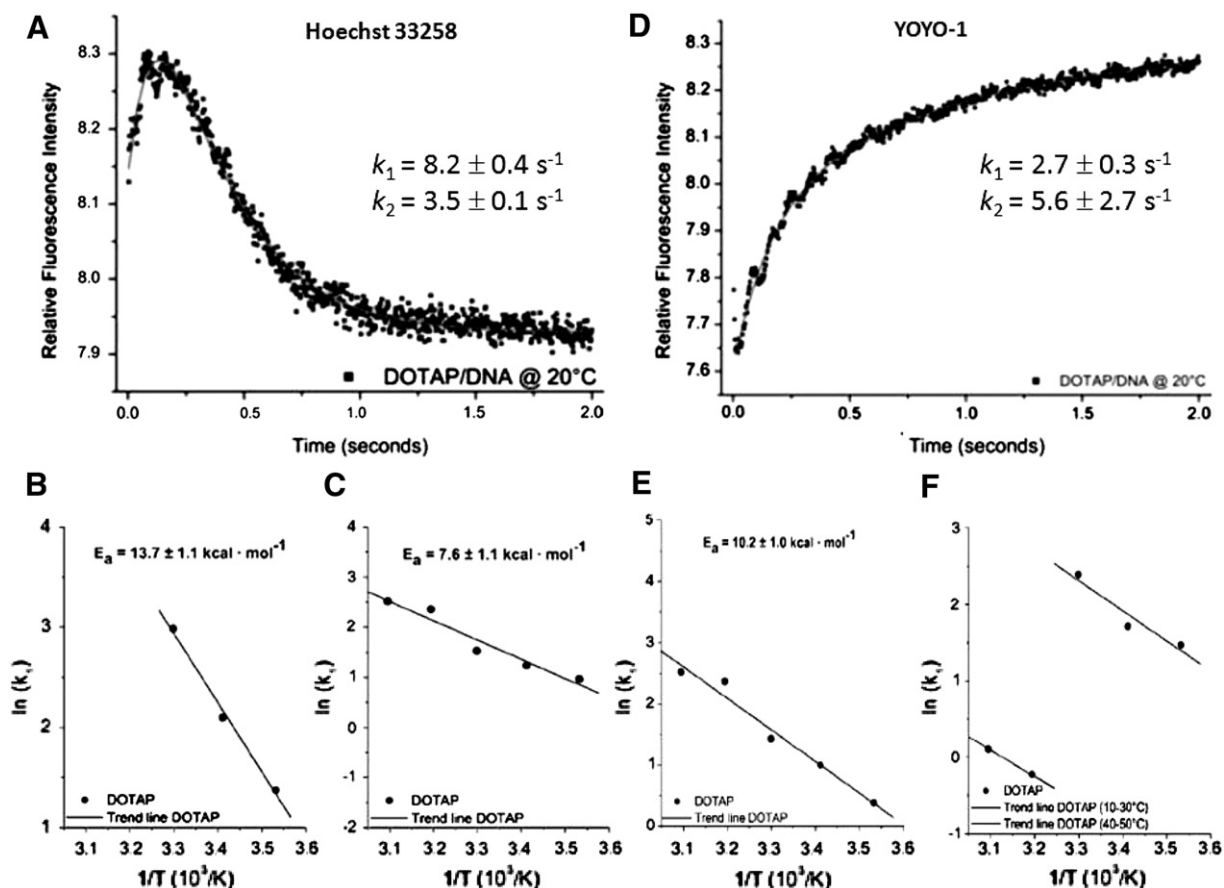


Fig. 2. Stopped-flow fluorescence data acquired for DOTAP/DNA lipoplexes with DNA previously labeled with Hoechst 33258 (1:150 dye/bp) (A–C) and YOYO-1 (1:50 dye/bp) (D–F). A biexponential function was fitted to the data and two rate constants (k_1 and k_2) were obtained (A, D). Arrhenius plots of the first (B, E) and second (C, F) rate constants derived from biexponential fits of the stopped flow fluorescence of DOTAP/DNA labeled with each dye, and respective activation energy (E_a). Adapted from [58]. Copyright Biophysical Society 2005.

electrostatic parameters of cationic lipids complexed with DNA at the water–lipid interface [63–65]. The relation between electrostatics, hydration, and state of aggregation was studied using not only the dyes referred above (TMA-DPH and HC in particular), but also labeled oligonucleotides, with which characterization of their electrostatics before and after lipid interaction was possible [65].

Fluorophores absorb light along a particular direction with respect to the molecular axis. Fluorescence emission from samples excited with polarized light may also be polarized. Fluorescence anisotropy r is a measure of emission polarization, defined by $r = (I_{VV} - G I_{VH}) / (I_{VV} + 2G I_{VH})$, where $G = I_{HV} / I_{HH}$, and the different intensities are the vertical (v) and horizontal (h) components of the fluorescence emission with excitation vertical (I_{VV} and I_{VH} , respectively) and horizontal (I_{HV} and I_{HH} , respectively) to the emission axis. The extent of rotation of the molecule during the excited-state lifetime determines its polarization or anisotropy. In aqueous nonviscous solutions, most fluorophores typically display anisotropies near zero. This is because in fluid solutions the molecules are able to rotate extensively in 50–100 ps and during the typical lifetime excited-state (1–10 ns) the molecules can rotate many times. As a consequence the orientation of the polarized emission is randomized. The opposite occurs in highly viscous media, where anisotropy may approach the limiting value for non-oriented samples of rotationally frozen molecules with collinear absorption and emission dipoles ($r = 0.4$). Accordingly, fluorescence anisotropy is used as a measurement of membrane fluidity. As the membrane becomes more fluid, the rotation of an excited fluorophore placed within the lipid bilayer will increase, and accordingly, anisotropy decreases [29]. Membrane fluidity was determined in several DOTAP analogs through DPH anisotropy measurements, and a correlation with transfection

efficiency was established [66]. The association of DNA with cationic liposomes gave rise to lower values of anisotropy, revealing a more fluid membrane than that of the liposomes in the absence of DNA [29]. Indeed, the fluidity of lipoplex membranes increases as the cationic lipid content decreases, and is also related to better transfection results [67]. Interestingly, Rajesh et al. verified that minor structural variations in cationic amphiphiles can profoundly influence DNA-binding characteristics, membrane rigidity, membrane fusogenicity, cellular uptake, and consequently gene delivery efficacies of cationic liposomes [68]. Moreover, by anisotropy measurements of liposomes embedding a membrane dye (DPH), it was possible to detect clear differences on membrane rigidity between liposomes constituted by cationic amphiphiles only differing on the orientation of their linker ester functionality. These authors found that more rigid vesicles are less fusogenic, originating lower cellular uptake of DNA [68]. Other works also describe that lipoplexes constituted by lipids having a saturated lipid chain exhibit a more pronounced anisotropy value, thus presenting a more rigid and higher viscosity structure, than those having unsaturated lipid chains [69]. Anisotropy values of fluorescent cyanine dyes within DNA associated with cationic liposomes at different charge ratios have also been measured [70,71], and although the anisotropy profile of benzothiazolium, 2,2'-[1,3-propanediylbis(dimethyliminio)-3,1-propanediyl-1(4H)-pyridinyl-4-ylidene]methylidene]]bis [3-methyl]-, tetraiodide (BOBO-1) within lipoplexes has a subtle perturbation in the electroneutrality region, it remains constant at ~ 0.2 at lower or higher charge ratios. These results are a clear evidence of the presence of dye within DNA even at higher charge ratios [71], which constitutes an advantage in Förster resonance energy transfer (FRET) measurements to study DNA–lipid interactions or lipoplex structure [70,71].

Förster Resonance Energy Transfer (FRET) is an important process that occurs in the excited state whenever the emission spectrum of a fluorophore, called the donor, overlaps with the absorption spectrum of another molecule, called the acceptor. The FRET rate from a donor to an acceptor depends on i) the distance between the donor (D) and acceptor (A) molecules, ii) the overlapping extension of acceptor absorption spectra and donor emission spectra, iii) the donor quantum yield and iv) the relative orientation of the acceptor and donor transition dipoles [72]. The transfer efficiency (E) is the fraction of photons absorbed by the donor that are transferred to the acceptor and is given by:

$$E = \frac{R_0^6}{R_0^6 + r^6} = 1 - \left(\frac{I_{DA}}{I_D} \right) \quad (1)$$

This is the most popular equation used to measure distances from FRET [73] because the relative fluorescence intensity of the donor, in the absence (I_D) and presence (I_{DA}) of acceptor is easily measured in the laboratory. E is strongly dependent on distance when the donor–acceptor molecules distance (r) is near the Förster distance (R_0), and when r equals R_0 the transfer efficiency is 50%.

In 1998 Szoka and co-workers reported a FRET-based assay using a dye on the DNA and another on the lipid, in order to assess oligonucleotide/liposome interaction in the presence of several components of serum [74]. Using oligonucleotides labeled with fluorescein (as donor) and fluorescent lipid 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (N-Rh-PE) (as acceptor) labeled DOTAP liposomes, these authors suggested that heparin and oleic acid destabilize lipoplexes. However, the pre-incubation of lipoplexes in increasing amounts of BSA decreases the referred instability, protecting the complex from dissociation. Due to a high background fluorescence of serum, these authors did not perform this assay in its presence [74]. A few years later, the use of probes with different emission wavelengths allowed the development of a FRET-based assay in the presence of serum, even though both dyes were DNA intercalators [75] or DNA covalently-labeled fluorophores [76]. Table 2 shows all the donor and acceptor dyes used in lipoplex FRET assays, using one dye on the DNA and the other on the lipid, and the respective aim of study. Indeed, FRET is a promising tool in the scope of biophysical and structural characterization of the lipoplexes, allowing to monitor not only of DNA–lipid interaction [77–81] but also of DNA condensation [26,46,71,82,83] and lipoplex structure [46,71,82,84–86], as discussed in the following sections.

FRET was used to monitor interactions between Cy3-labeled plasmid DNA and NBD-labeled cationic liposomes [87] and the data

showed that binding of cationic liposomes to DNA occurs immediately upon mixing (within 1 min), though FRET efficiencies do not stabilize for 1–5 h [87]. Interestingly, higher transfection efficiencies were obtained with lipoplexes incubated for 1 h, instead of 1 min or 5 h, and faster stabilization was obtained with higher charge ratio lipoplexes [87]. These authors verified that lipoplexes formulated with DOPE were more resistant to high ionic strength than complexes formulated with cholesterol. For DOTAP-DOPE/DNA lipoplexes at DOTAP/DNA charge ratios of 0.5 and 1, low concentrations of NaCl (0.01–0.5 M) increase FRET efficiency, while higher concentrations decrease it, suggesting that low NaCl concentrations enhance lipid–DNA interactions, and high salt concentrations weaken the association between lipid and DNA [87], as expected.

The aforementioned studies were all carried out in saline buffer. However, depending on the nature of DNA and lipid dyes, FRET may also be suitable to study DNA–lipid interaction under serum conditions [46,79,81], which is a great advantage in lipoplex characterization studies. Indeed, the confirmation of protein binding to the lipoplexes external layer without either destabilizing the multilamellar structure [46] or dissociation [79,81] was previously accomplished using FRET. Simberg et al. presented clear evidence that lipoplex–serum interaction was strongly dependent on helper lipid and ionic strength, and compared the behavior of DOTAP/DOPE and DOTAP/Chol lipoplexes. These authors verified by FRET that upon serum contact the lipoplexes seemed to lose integrity, although no lipid–DNA dissociation was detected. Indeed, as shown in Fig. 3, after addition of excess of serum to those lipoplexes there was no increase in fluorescence intensity at 520 nm and no decrease at 580 nm. Moreover, the emission at 520 nm showed a significant decrease, which could be ascribed to changes in the probe environment upon the addition of serum. Besides observing high level of aggregation which hampered the transfection efficiency, no other structural changes in serum were observed for cholesterol-based lipoplexes [79]. Interestingly, other authors reported that the increase of cholesterol content led to an increased serum stability, and DOTAP:Chol (mol/mol 1:4)/DNA with charge ratio of 4 was the most stable formulation in serum of all the formulations examined, maintaining lipid–DNA interactions, not aggregating and exhibiting high in vitro transfection efficiency in 50% (v/v) serum [81].

4.2. Membrane mixing studies

FRET studies have been used in lipoplexes to confirm lipid mixing upon DNA addition since the early characterization studies [23]. In a common experimental design, two liposome populations are prepared. In one of them, liposomes are labeled with two membrane probes, one acting as donor (often N-NBD-PE) and the other as acceptor (often N-Rh-PE), whereas the other population is prepared without

Table 2

Lipoplex formulations, donor and acceptor dyes used in FRET assays, with one dye on the lipid and the other on the DNA.

Liposomal formulation	Membrane dye	DNA dye	Objective of study	Ref.
Saline Buffer	DODAB:MO	DPH-HPC (d)	EtBr (a)	Lipoplex micro-structure [84]
	DOTAP	N-Rh-PE (a)	Fluorescein* (d)	DNA–lipid interaction [77]
	DOTAP,DDAB; DOPE, DOPC	BODIPY-PE (a) BODIPY-PC (a)	Hoechst 33258 (d) Cy3* (d)	Lipoplex micro-structure [86]
	DOTAP/DOPE	NBD-PE (d)	Cy3* (a)	DNA–lipid interaction [80]
	DOTAP	BODIPY-PC (a)	BOBO-1 (d)	Lipoplex micro-structure DNA condensation [71,82]
	EDOPC and derivatives	Rh-DOPE	YOYO-1	DNA release [83]
	Serum	DOTAP/Chol DOTAP/DOPE	LRPE (a)	CF (d)
DOTAP/Chol		NBD-PE (d)	Cy3* (a)	DNA–lipid interaction [81]
DOTAP/DOPE				
DOTAP/DOPC				
DOTAP		BODIPY-PC (a)	BOBO-1 (d)	Lipoplex micro-structure DNA condensation [46]

(a) Acceptor; (d) donor; * covalently labeled; DDAB: dimethyldioctadecyl ammonium bromide; DODAB: dioctadecyldimethylammonium bromide; DOPC: dioleoylphosphatidylcholine; EDOPC: dioleoyl-O-ethylphosphatidylcholine; MO: monoolein; BODIPY-PE: 2-(4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphoethanolamine; BODIPY-PC: 2-(4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine; DPH-PC: 2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine; LRPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl); NBD-PE: 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl); CF: carboxyfluorescein; YOYO-1: (1,19-[1,3-propanediylbis[(dimethyliminio)-3,1-propanediyl]]bis[4-[(3-methyl-2(3H)-benzo xazolylidene)methyl]]-tetraideide).

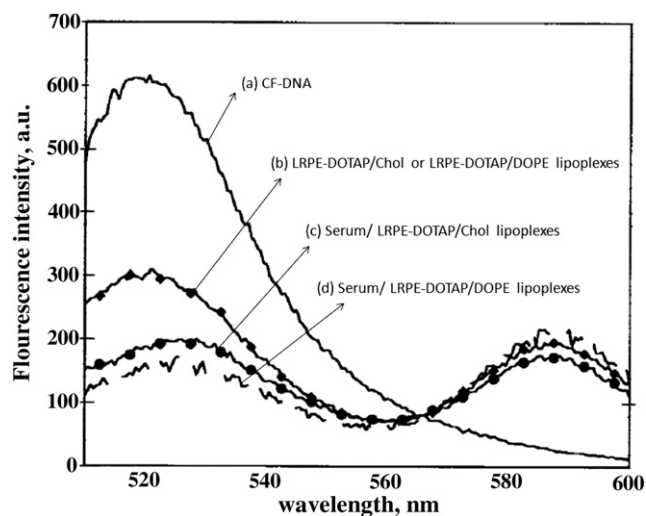


Fig. 3. Effect of serum addition to lipoplexes constituted by lissamine rhodamine-PE (LRPE)-labeled liposomes and carboxyfluorescein (CF)-labeled DNA monitored by FRET. This research was originally published in The Journal of Biological Chemistry. See Ref. [79] © the American Society for Biochemistry and Molecular Biology.

fluorescent probes. Due to energy transfer in labeled liposomes, the fluorescence coming from donors in the doubly-labeled liposomes is substantially reduced. After DNA addition, fluorescence intensity increases as a consequence of the mixing between labeled and non-labeled liposomes, which leads to a dilution of acceptors and an average increase in donor–acceptor distances. The lipid mixing percentage, upon DNA addition, is calculated considering as maximal the fluorescence obtained in the presence of detergent [88]. This method confirmed that DNA induces membrane fusion depending on charge ratio [89], and maximal lipid mixing is achieved for charge ratios near neutrality [88,90–92]. The effect of ionic strength and lipoplex mode of preparation in DNA–liposome interactions was monitored by this methodology, in parallel with isothermal titration calorimetry and size measurements, and a lipoplex formation mechanism was proposed [91]. Interestingly, particle growth, membrane mixing and vesicle rupture all proceed immediately when DNA is added to liposomes, whereas the referred occurrences are delayed until approximately equal numbers of positive and negative charges are present in the case of reverse addition [91].

The presence of serum also induces a certain extent of lipid mixing in several liposomal formulations [93,94]. Indeed, using a FRET assay, Tandia et al. confirmed that the ability of lipoproteins to inhibit the transfection efficiency of lipoplexes was well correlated with their ability to undergo lipid mixing with the cationic lipid bilayer [95]. When incorporating lysophosphatidylcholine into diC14-amidine-containing lipoplexes, their capacity to undergo lipid mixing with lipoproteins was completely abolished, and this allowed them to reach high transfection efficiency in the presence of the latter. On the other hand, the incorporation of DOPE into DOTAP/DNA lipoplexes activated lipid mixing with the lipoproteins, and was shown to be detrimental toward the transfection activity of these lipoplexes [95].

During lipoplex internalization, lipid mixing also occurs, especially between lipoplexes and endosome membranes [96], and some authors have suggested this step as a crucial barrier to overcome during lipid-based gene delivery [97]. Membrane fusion studies encompassing a FRET methodology similar to the above mentioned are frequently used to simulate these processes in vitro. Indeed, a multi-step assay was performed to model the sequential steps involved in transfection, using anionic liposomes [92], and also to assess fusogenic properties of novel lipids, frequently correlating their findings with transfection efficiency [68,69,83,96,98,99]. Higher gene transfection efficiencies have been correlated with increased ability of lipids to fuse with endosome-like membranes [68,96].

4.3. Characterization of lipoplex molecular structure using FRET

The microscopic structure of stable lipoplexes has been mainly studied by small-angle X-ray scattering (SAXS) [10,37,100]. With this technique, it was possible to determine for the first time the interlamellar distances in different lipoplex formulations in water or saline buffers [10]. Fluorescence spectroscopy, through the use of FRET methodology, has also provided valuable information about lipoplex microscopic structure by using a dye on the liposomes and another on the DNA. With this methodology, inter-fluorophore distances are associated with interlamellar spacing, with the advantages of allowing measurements in solution under serum conditions, mimicking physiological medium, and using lower amounts of lipid and DNA.

Application of FRET to retrieve meaningful structural information in lipoplexes is not a trivial matter. Indeed, in the particular case of lipoplexes, and as previously described for membranes [101–103], instead of an isolated donor/acceptor pair at a single defined distance, there is a distribution of donor and acceptor molecules in three-dimensional space or in a plane, and the donor fluorescence decay becomes complex and dependent on the acceptor concentration surrounding the donors. Middaugh and co-workers used FRET theoretical models to calculate inter-fluorophore distances in lipoplexes under low and high ionic buffer conditions [86]. These authors assumed that FRET between a DNA-bound donor fluorophore and the acceptor-labeled lipid bilayer can be modeled as a point donor and an infinite plane of randomly distributed acceptors. Unreasonably high DNA–lipid spacing was recovered at lower charge ratios and for high ionic strength solutions, most probably because these authors did not consider the existence of donor molecules which are not transferring energy [86]. When those isolated molecules are considered, using models that assume the possibility of non-equivalent donor populations [103], it is possible to determine not only inter-fluorophore distances (which do not vary with the charge ratio, as previously shown from SAXS [45]), but also the amount of DNA molecules not covered by the liposomes, and consequently encapsulation efficiencies (as detailed in the following section) [71].

Madeira et al. used FRET to characterize the structure of DNA/DOTAP complexes, for varying DNA plasmid size, buffer ionic strength, and both in the presence and absence of helper lipid (DOPE) and serum [46,71,82]. For this purpose, a multilamellar model was considered for lipoplexes, as suggested from previous findings [10,11]. In this specific geometry with DNA sandwiched between adjacent lipid bilayers as shown in Fig. 4, transfer occurs from one donor molecule (restricted to a plane) to acceptor molecules randomly distributed in two adjacent parallel planes. Two distinct possibilities are shown schematically in Fig. 4: either the donor is a labeled phospholipid and the acceptor is a DNA-intercalating probe (Fig. 4A) or the reverse arrangement is used (Fig. 4B).

Strictly, for each bilayer, there should be two planes of acceptor molecules (one for each bilayer leaflet), and the distances between those planes and that of the donors should not coincide, because the fluorophores in the labeled phospholipids are not expected to be located at the exact center of the bilayer (that is, strictly, one should have $d_1 \neq d_2$ in Fig. 4). However, for low-polarity acyl-chain labeled lipids such as used in the above mentioned works [71] the difference in transverse location for chromophores belonging to labeled lipid molecules in opposing leaflets of the same bilayer is small for FRET purposes ($\ll R_0$) and was neglected (that is, one can take $d \approx d_1 \approx d_2 \approx$ half the multilamellar repeat distance in Fig. 4). FRET from each donor to the two closest acceptor planes was considered. For a multibilayer structure, a second set of two acceptor planes would be located at $\approx 3d$. However, the contribution of FRET to this plane (and further planes) of acceptors would be much smaller and effectively masked by FRET to acceptors located at $\approx d$. The basic equation for the decay of the donor in the presence of a plane of acceptors, which assumes low density of excited acceptors, no energy migration among

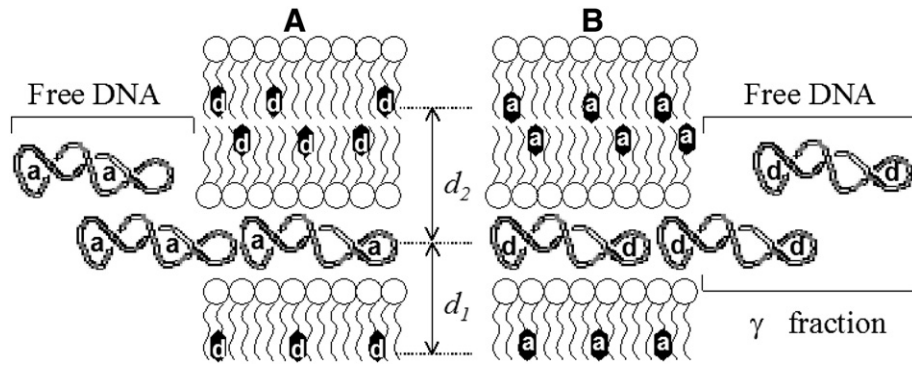


Fig. 4. Schematic representation of the lipoplex multilamellar structure with the fluorescent probes within DNA and lipid. A) Acceptor (a) on the DNA and donor (d) on the lipid. B) Acceptor (a) on the lipid and donor (d) on the DNA.

Reprinted with permission from [71]. Copyright Biophysical Society 2003.

donors, no translational diffusion of probes during the donor excited state lifetime, uniform distribution of acceptors, a single Förster distance R_0 value for all donor–acceptor pairs, and probe dimensions $\ll R_0$ is given by [102]:

$$i_{DA}(t) = i_D(t) \cdot \exp\left(-\frac{2C}{\Gamma(2/3)b} \int_0^1 \frac{1 - \exp(-tb^3\alpha^6)}{\alpha^3} d\alpha\right) \quad (2)$$

where

$$C = \Gamma(2/3) \cdot n \cdot \pi \cdot R_0^2 \tau_D^{-1/3} \quad (3)$$

In these equations, τ_D is the donor lifetime in the absence of acceptor, $i_D(t) = \exp(-t/\tau_D)$ is the donor decay in the absence of acceptor, n is the acceptor surface density (number of molecules/unit area), Γ is the complete gamma function, R_0 is the Förster distance, and $b = (R_0/d)^2 \tau_D^{-1/3}$. For donors with nonexponential decay (as often is the case), $i_D(t)$ should be the experimental decay law (sum of exponentials) and τ_D should be replaced by the average lifetime in the definition of b (e.g. [103]). For the purpose of theoretical computation of the decay, n is easily calculated using

$$n = 2 \times (\text{dye : lipid mole ratio}) / (\text{area per lipid molecule}) \quad (4)$$

The factor 2 reflects the fact that, in a multilayer geometry, the available surface area is only half of the product of the number of total lipid molecules times the area per lipid.

Eqs. (2)–(4) are also valid for FRET to two opposing equivalent acceptor planes, as in Fig. 4B, but, in this case, the acceptor surface density should be further doubled. Eq. (2), as it stands, is only valid for significant excess of cationic lipid, leading to essentially no unbound DNA. In these conditions, for the arrangement depicted in Fig. 4B, all DNA-located donors have acceptors in their vicinity and are available for energy transfer. In the described studies, it was verified that for the charge ratio (+/–) DOTAP/DNA = 2 in the studied systems, there is already a small but significant fraction of unbound DNA [46,71], which implies the existence of donor molecules isolated from acceptors. To take this into account, the donor fluorescence decay law should allow for a fraction γ of molecules which decay is unaffected by the acceptors. If the decay of donors intercalated in lipid-bound DNA in the absence of acceptors ($i_D(t)$) differs from that of donors in unbound DNA ($i_{D0}(t)$), then Eq. (1) should be rewritten as

$$i_{DA}(t) = (1-\gamma)i_D(t) \exp\left(-\frac{2C}{\Gamma(2/3)b} \int_0^1 \frac{1 - \exp(-tb^3\alpha^6)}{\alpha^3} d\alpha\right) + \gamma i_{D0}(t) \quad (5)$$

For the charge ratio (+/–) DOTAP/DNA < 1, whereas Eq. (5) is valid for the arrangement described in Fig. 4B, there is a major difference in FRET geometry for the arrangement of Fig. 4A: donors (labeled phospholipids) are located close to the center of the bilayer, and acceptors (DNA-intercalated probes) are inside the DNA helix. Because of the excess of DNA in this system, a significant amount of DNA molecules are not involved in the complexes, and only a fraction f of acceptors will be available for transfer. The decay law (neglecting isolated donors – it is assumed that all bilayer-located donors have DNA in their vicinity, i.e., there are no lipid molecules outside lipoplexes) is now,

$$i_{DA}(t) = i_D(t) \cdot \exp\left(-\frac{2fC}{\Gamma(2/3)b} \int_0^1 \frac{1 - \exp(-tb^3\alpha^6)}{\alpha^3} d\alpha\right) \quad (6)$$

In all cases, the theoretical expectation, which is computed numerically from Eq. (7) below, can be compared with the experimental FRET efficiency data.

$$E = 1 - \frac{\int_0^\infty i_{DA}(t) dt}{\int_0^\infty i_D(t) dt} \quad (7)$$

Using this methodology, and using DNA intercalator probe BOBO-1 as FRET donor and membrane probe BODIPY-PC as FRET acceptor (arrangement of Fig. 4B), we were able to assess differences in lipoplex microscopic structure when varying charge ratio, helper lipid, plasmid size [82] and ionic strength of the solution and also under serum conditions [46]. When longer plasmids are used (~6.0 kb), larger donor–acceptor distances were obtained [82] when compared to smaller plasmids (~3.0 kb) [71]. This result was at variance with what was verified by other authors using SAXS, who did not detect any alterations in the microscopic structure of lipoplexes constituted by plasmids with sizes ranging from 1 to 52 kb [41]. In agreement with other authors who used a similar FRET methodology, we verified that the presence of DOPE did not affect interlamellar distances [82,86]. Interestingly, in physiological saline solutions with added serum, a multilamellar arrangement of the lipoplexes is maintained at least for 2 h, with reduced spacing distances between the FRET probes, relative to those in low ionic strength medium (30 Å vs 42 Å) [46].

Even though a full exploitation of the technique's potential requires careful modeling and data analysis using a kinetic formalism that accounts for the underlying donor and acceptor distribution, FRET may still be used on a more basic, qualitative level. FRET efficiency between DPH-PC and EtBr within lipoplexes provided qualitative information about lipoplex micro-structure, mainly relying on comparisons between different lipoplex formulations [84]. The authors of this report observed a very significant enhancement in FRET efficiency when the monolein (MO) amount is increased from DODAB:MO (1:0.5) to DODAB:MO (1:1), which may be related to the appearance of inverted lipoplex

structures, and consequently to a lower mean donor–acceptor distance [84] when compared to other lipoplex formulations.

4.4. DNA condensation assessment

DNA condensation by the liposomes is more commonly assessed in qualitative terms by fluorescence methodologies and mainly by dye–exclusion assays. Two decades after the first experiment to evaluate DNA condensation by cationic lipids using this type of experiment [23], it is still widely used to compare the efficiency of DNA condensation of novel cationic lipid formulations, due to its simplicity. The fluorescent probes more frequently used in these methodologies are EtBr [19,83,88,104–111] and quinolinium, 4-[(3-methyl-2(3H)-benzothiazolylidene)methyl]-1-[3-(trimethylammonio)propyl]-, diiodide (TO-PRO-1) [112–114]. When intercalated in DNA, these dyes display considerable fluorescence intensity, but upon DNA condensation by cationic liposomes, the probes are displaced from DNA and their fluorescence intensity is drastically reduced. At low charge ratios (DNA excess) high fluorescent intensities are measured, which drastically decrease, in a sigmoid profile, with the complete coverage of DNA by the liposomes (near neutral charge ratio). In addition, the DNA condensation is evaluated based on the dye exclusion percentage upon addition of cationic liposomes or polymers. However, according to Eastman et al., the obtained sigmoid profiles depend on probe concentration, and when using high EtBr concentrations a linear fluorescence decrease upon increasing charge ratio is obtained [28]. In a variation of the usual method, encapsulation efficiencies were recently calculated using the referred methodology after application of a spectral deconvolution procedure [84]. A method based on the covalent attachment of fluorescent probes to DNA (fluorescein or rhodamine) was proposed to evaluate a quantitative assessment of DNA condensation [27]. Assays based on the accessibility of DNA (after condensation by the liposomes) to fluorescent probes (PicoGreen, for example) also provide a qualitative approach to compare liposomal formulations' condensation ability and stability in several ionic and physiological conditions [115,116]. An interesting study comparing twenty nucleic acid stains, feasible to be used in DNA condensation by non-viral gene delivery systems, was reported [117]. In this study, dye characteristics such as efficacy of yielding fluorescent DNA-spectra and photo bleaching of DNA–dye complexes, were considered before DNA condensation assessment and a novel method to quantitatively evaluate the DNA condensation was proposed. Moreover, several DNA intercalators, differing in their intercalative position (major or minor groove) were also used to compare the interaction of various cationic lipids and polymer (polyethyleneimine (PEI)) with DNA in terms of regional displacement of the dyes, structural changes of DNA upon condensation and polycation-mediated changes in solvent accessibility of the minor groove [118]. Fluorescence studies concerning the structure and dynamics of condensed DNA probed by the dimeric cyanine dye

YOYO-1, also gave further insights about the information which may be retrieved from fluorescence measurements using this bis-intercalator-type of DNA probe [78,85,119].

FRET has also been used to quantify the amount of unbound DNA upon lipoplex formation, frequently related to the encapsulation efficiency of a given lipoplex formulation. The FRET models described in the previous sub-section allow not only the monitoring of DNA–lipid distances, but also the quantification of unbound DNA in lipoplexes with lower charge ratios [46,71,82]. Because FRET between two probes, one in the DNA and the other in the liposomes, is a direct measure of DNA/liposome contact, it is intrinsically more powerful than dye exclusion measurements. The latter appears to be simple on the surface, but their quantitative interpretation is in fact dependent on an accurate description of the probe distribution between DNA and the aqueous medium, which is generally lacking. The superiority of FRET is especially visible when it is conjugated with accurate modeling of its kinetics. The methodology developed by us to assess the liposome complexation efficiency of a specific lipid/DNA formulation can be described as follows: (i) A FRET assay with the absence of free DNA (confirmed by agarose gel electrophoresis, and so $\gamma = 0$ was fixed) was analyzed to recover $d = R_0 b^{-1/2} \tau^{1/6}$ as the sole parameter using Eq. (5) (Fig. 5A); (ii) Fixing d to the value recovered in (i), FRET assays of lipoplexes with lower charge ratios (with free DNA) enabled the recovery of the γ values (Fig. 5B–C); (iii) The liposome complexation efficiency (C.E.) was calculated for each case by $C.E. = (1 - \gamma) \times 100$ [46,71,82].

From the data shown in Fig. 5 it is possible to verify that lipoplexes constituted by DOTAP and plasmid DNA ~3 kb size has 50% of uncondensed DNA at charge ratio 0.5 (Fig. 5C) and 20% at charge ratio 2 (Fig. 5B) [71]. In this way, and considering a different lipoplex formulation, mainly using a larger plasmid DNA (6 kb), we verified that DOTAP liposomes have a degree of complexation of DNA of 60% at charge ratio (+/−) = 1 in low ionic solutions. When these lipoplexes are directly prepared in phosphate buffered saline solution (PBS), 23% of DNA is covered by the liposomes, whereas lipoplexes prepared in low ionic solution followed by addition of PBS have 53% of protected DNA for the same charge ratio [46]. On the other hand, and according to our results, complexation was virtually complete at (+/−) = 5 in all the studied conditions, with complexation efficiencies ranging from 95% to 100%. A similar behavior was observed with other dyes, in a qualitative FRET assay, using NaCl concentrations ranging from 100 to 500 mM [87]. According to our FRET results, and considering charge ratio (+/−) = 2, the presence of helper lipid (DOPE) increased in about 15% the amount of covered DNA, and, for negatively charged lipoplexes ((+/−) = 0.5), an increase in plasmid size from 2.7 to 6 kb led to a decrease in complexation efficiency of DOTAP, from 50% to 25% [82].

FRET methodologies have provided further insights into lipoplex stability in serum, by either allowing quantification of DNA that is

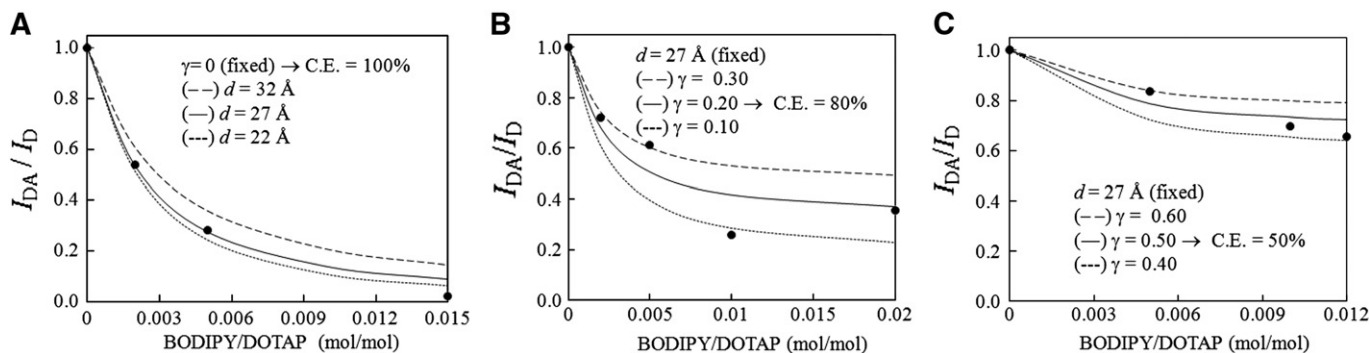


Fig. 5. FRET quenching ratios, $I_{DA}/I_D = 1 - E$, for BOBO-1/BODIPY pairs in DOTAP/DNA complexes with charge ratios (+/−) of 4 (A), 2 (B), and 0.5 (C). Experimental data (●); Fitting curves (— —); (—); (— — —) using Eqs. (5) and (7). The assumed fitting parameters of γ and d are shown inside the graphs. Adapted from [71]. Copyright Biophysical Society 2003.

released from the lipoplex upon serum contact [46,86] or qualitative assessment of its release by monitoring the decrease of FRET efficiency [76,79,87,120]. We observed that addition of serum within lipoplexes previously incubated in PBS leads to a displacement of less than 10% of DNA, this being more pronounced for a charge ratio of 0.5 [46]. In lipoplexes with lower charge ratios, degradation of DNA by endonucleases upon contact with serum is probable. At higher charge ratios, similar complexation efficiencies were obtained when compared with the corresponding lipoplex formulation prepared in low ionic strength solutions and incubated in PBS [46].

The stability in physiological medium of lipoplexes was evaluated by detecting the conformational change of complexed plasmid DNA labeled simultaneously with fluorescein (donor) and X-rhodamine (acceptor) through FRET [76]. These authors verified that lipoplexes had two phases of dissociation in 1% serum. When the concentration of fetal bovine serum (FBS) was increased to 5% and further, the first phase of dissociation became prominent, resulting in a lower emission intensity ratio within a shorter time period than in the medium with 1% serum.

Barenholz and co-workers investigated the mechanism of interaction between fluorescently labeled lipoplexes and fluorescently labeled serum proteins. FRET measurements *in vitro* indicated that serum proteins interact instantly and closely with the DOTAP/cholesterol lipoplexes [120]. Moreover, these authors verified that DNA, heparin, anionic eggphosphatidylcholine/eggphosphatidylglycerol liposomes, or the polycation poly-L-lysine all efficiently prevented interaction between serum proteins and the lipoplexes. On the other hand, serum proteins did not interact with zwitterionic eggphosphatidylcholine liposomes or free DNA, but needed DOTAP to mediate such interaction [120]. When comparing serum and heparin, these authors detected that the latter produces much more dramatic effects on the lipoplex integrity, surface potential, and transfection efficiency than either albumin or whole serum [120].

Besides stability in serum, the extent of DNA release from novel lipoplex formulations after addition of negatively charged membrane lipids was found to correlate with the extent of transfection [83]. These works aimed at studying the feasibility of synthetic cationic phosphatidylcholines, with different hydrophobic moieties, to efficiently release DNA into the cytoplasm after cellular uptake. To shed light on the extent of DNA unbinding from lipoplexes, the authors used fluorescence-based methodologies, mainly an EtBr exclusion assay and FRET. Interestingly, formulations with higher transfection activity (such as EDOPC/1,2-diphytanoyl-*sn*-glycero-3-ethylphosphocholine (40:60) and 1-oleyl-2-decanoyl-*sn*-glycero-3-ethylphosphocholine) gave higher percentage of DNA unbinding (31.2 and 68.1%, respectively), compared with that of pure EDOPC (11.5%) [83].

5. Concluding remarks

After a brief survey of biophysical techniques suited to the study of lipoplex characterization, this review focuses on the contributions from fluorescence methods. Whereas it is beyond doubt that diffraction techniques and electron microscopy were of paramount importance in the establishment of the multilamellar model of lipoplex structure, fluorescence offers significant advantages, namely the possibility of carrying studies in solution and in the presence of serum, in concentrations close to that used for *in vivo* transfection, and they also allow the determination of the degree of DNA protection for a given system. Although fluorescence has been used since the earliest studies, the use of more sophisticated approaches, coupled with elaborate physical models (namely FRET and FCS) keeps it at the forefront of biophysical tools useful for lipoplex characterization.

Acknowledgements

CM acknowledges funding from *Compromisso para a Ciência* 2008. M.P. and L.M.S.L. acknowledge funding by FEDER (COMPETE pro-

gram), and by FCT (Fundação para a Ciência e a Tecnologia), project references PTDC/QUI-BIQ/099947/2008, PTDC/QUI-BIQ/112067/2009 and FCOMP-01-0124-FEDER-010787 (FCT PTDC/QUI-QUI/098198/2008), respectively.

References

- [1] Y. Seow, M.J. Wood, Biological gene delivery vehicles: beyond viral vectors, *Mol. Ther.* 17 (2009) 767–777.
- [2] L. Xu, T. Anchordoquy, Drug delivery trends in clinical trials and translational medicine: challenges and opportunities in the delivery of nucleic acid-based therapeutics, *J. Pharm. Sci.* 100 (2011) 38–52.
- [3] N.M. Rao, V. Gopal, Cell biological and biophysical aspects of lipid-mediated gene delivery, *Biosci. Rep.* 26 (2006) 301–324.
- [4] B. Dalby, S. Cates, A. Harris, E.C. Ohki, M.L. Tilkins, P.J. Price, V.C. Ciccarone, Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications, *Methods* 33 (2004) 95–103.
- [5] N.M. Rao, Cationic lipid-mediated nucleic acid delivery: beyond being cationic, *Chem. Phys. Lipids* 163 (2010) 245–252.
- [6] T. Montier, T. Benvegno, P.A. Jaffres, J.J. Yaouanc, P. Lehn, Progress in cationic lipid-mediated gene transfection: a series of bio-inspired lipids as an example, *Curr. Gene Ther.* 8 (2008) 296–312.
- [7] P.L. Felgner, T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northrop, G.M. Ringold, M. Danielsen, Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure, *Proc. Natl. Acad. Sci. U.S.A.* 84 (1987) 7413–7417.
- [8] P.C.A. Barreleiro, R.P. May, B. Lindman, Mechanism of formation of DNA–cationic vesicle complexes, *Faraday Discuss.* 122 (2003) 191–201.
- [9] P.C.A. Barreleiro, B. Lindman, The kinetics of DNA–cationic vesicle complex formation, *J. Phys. Chem. B* 107 (2003) 6208–6213.
- [10] J.O. Rädler, I. Koltover, T. Salditt, C.R. Safinya, Structure of DNA–cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes, *Science* 275 (1997) 810–814.
- [11] D.D. Lasic, H. Strey, M.C.A. Stuart, R. Podgornik, P.M. Frederik, The structure of DNA–liposome complexes, *J. Am. Chem. Soc.* 119 (1997) 832–833.
- [12] P.L. Felgner, Y. Barenholz, J.P. Behr, S.H. Cheng, P.R. Cullis, L. Huang, J.A. Jessee, L. Seymour, F.C. Szoka, A.R. Thierry, E. Wagner, G. Wu, Nomenclature for synthetic gene delivery systems, *Hum. Gene Ther.* 8 (1997) 511–512.
- [13] J.H. Felgner, R. Kumar, C.N. Sridhar, C.J. Wheeler, Y.J. Tsai, R. Border, P. Ramsey, M. Martin, P.L. Felgner, Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations, *J. Biol. Chem.* 269 (1994) 2550–2561.
- [14] D.C. Litzinger, L. Huang, Phosphatidylethanolamine liposomes – drug delivery, gene-transfer and immunodiagnostic applications, *Biochim. Biophys. Acta* 1113 (1992) 201–227.
- [15] D. Hirsh-Lerner, Y. Barenholz, Hydration of lipoplexes commonly used in gene delivery: follow-up by laurdan fluorescence changes and quantification by differential scanning calorimetry, *Biochim. Biophys. Acta* 1461 (1999) 47–57.
- [16] S. Choosakoonkiang, C.M. Wiethoff, T.J. Anchordoquy, G.S. Koe, J.G. Smith, C.R. Middaugh, Infrared spectroscopic characterization of the interaction of cationic lipids with plasmid DNA, *J. Biol. Chem.* 276 (2001) 8037–8043.
- [17] S. Even-Chen, Y. Barenholz, DOTAP cationic liposomes prefer relaxed over supercoiled plasmids, *Biochim. Biophys. Acta* 1509 (2000) 176–188.
- [18] C.A.H. Prata, Y. Li, D. Luo, T.J. McIntosh, P. Barthelemy, M.W. Grinstaff, A new helper phospholipid for gene delivery, *Chem. Commun.* (2008) 1566–1568.
- [19] Z. Hyyonen, A. Plotniece, I. Riene, B. Chekavichus, G. Duburs, A. Urtti, Novel cationic amphiphilic 1,4-dihydropyridine derivatives for DNA delivery, *Biochim. Biophys. Acta* 1509 (2000) 451–466.
- [20] D.D. Lasic, *Liposomes in Gene Delivery*, CRC Press, New York, 1997.
- [21] L. Huang, M.-C. Hung, E. Wagner, *Non Viral Vectors for Gene Therapy*, Academic Press, San Diego, CA, 1999.
- [22] E. Lai, J.H. van Zanten, Real time monitoring of lipoplex molar mass, size and density, *J. Control. Release* 82 (2002) 149–158.
- [23] H. Gershon, R. Ghirlando, S.B. Guttman, A. Minsky, Mode of formation and structural features of DNA–cationic liposome complexes used for transfection, *Biochemistry* 32 (1993) 7143–7151.
- [24] S. Huebner, B.J. Battersby, R. Grimm, G. Cevc, Lipid–DNA complex formation: reorganization and rupture of lipid vesicles in the presence of DNA as observed by cryoelectron microscopy, *Biophys. J.* 76 (1999) 3158–3166.
- [25] A. Rodriguez-Pulido, E. Aicart, O. Llorca, E. Junquera, Compaction process of calf thymus DNA by mixed cationic–zwitterionic liposomes: a physicochemical study, *J. Phys. Chem. B* 112 (2008) 2187–2197.
- [26] D. Lleres, E. Dauty, J.P. Behr, Y. Mely, G. Duportail, DNA condensation by an oxidizable cationic detergent. Interactions with lipid vesicles, *Chem. Phys. Lipids* 111 (2001) 59–71.
- [27] V.S. Trubetskoy, P.M. Slattum, J.E. Hagstrom, J.A. Wolff, V.G. Budker, Quantitative assessment of DNA condensation, *Anal. Biochem.* 267 (1999) 309–313.
- [28] S.J. Eastman, C. Siegel, J. Tousignant, A.E. Smith, S.H. Cheng, R.K. Scheule, Biophysical characterization of cationic lipid:DNA complexes, *Biochim. Biophys. Acta* 1325 (1997) 41–62.
- [29] A. Rodriguez-Pulido, A. Martin-Molina, C. Rodriguez-Beas, O. Llorca, E. Aicart, E. Junquera, A theoretical and experimental approach to the compaction process of DNA by dioctadecyldimethylammonium bromide/zwitterionic mixed liposomes, *J. Phys. Chem. B* 113 (2009) 15648–15661.
- [30] V. Oberle, U. Bakowsky, I.S. Zuhorn, D. Hoekstra, Lipoplex formation under equilibrium conditions reveals a three-step mechanism, *Biophys. J.* 79 (2000) 1447–1454.

- [31] B. Sternberg, F.L. Sorgi, L. Huang, New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy, *FEBS Lett.* 356 (1994) 361–366.
- [32] J. Gustafsson, G. Arvidsson, G. Karlsson, M. Almgren, Complexes between cationic liposomes and DNA visualized by cryo-TEM, *Biochim. Biophys. Acta* 1235 (1995) 305–312.
- [33] T. Akao, T. Fukumoto, H. Ihara, A. Ito, Conformational change in DNA induced by cationic bilayer membranes, *FEBS Lett.* 391 (1996) 215–218.
- [34] N.J. Zuidam, Y. Barenholz, A. Minsky, Chiral DNA packaging in DNA–cationic liposome assemblies, *FEBS Lett.* 457 (1999) 419–422.
- [35] Z.L. Zhang, W.M. Huang, J.L. Tang, E.K. Wang, S.J. Dong, Conformational transition of DNA induced by cationic lipid vesicle in acidic solution: spectroscopy investigation, *Biophys. Chem.* 97 (2002) 7–16.
- [36] B. Pitard, O. Aguerre, M. Airiau, A.-M. Lachagès, T. Boukhnikachvili, G. Byk, C. Dubertret, C. Herviou, D. Scherman, J.-F. Mayaux, J. Crouzet, Virus-sized self-assembling lamellar complexes between plasmid DNA and cationic micelles promote gene transfer, *Proc. Natl. Acad. Sci. U.S.A.* 94 (1997) 14412–14417.
- [37] G. Caracciolo, R. Caminiti, F. Natali, A.C. Castellano, A new approach for the study of cationic lipid–DNA complexes by energy dispersive X-ray diffraction, *Chem. Phys. Lett.* 366 (2002) 200–204.
- [38] N.L. Dan, Multilamellar structures of DNA complexes with cationic liposomes, *Biophys. J.* 73 (1997) 1842–1846.
- [39] D. Harries, S. May, W.M. Gelbart, A. Ben-Shaul, Structure, stability, and thermodynamics of lamellar DNA–lipid complexes, *Biophys. J.* 75 (1998) 159–173.
- [40] I. Koltover, T. Salditt, J.O. Radler, C.R. Safinya, An inverted hexagonal phase of cationic liposome–DNA complexes related to DNA release and delivery, *Science* 281 (1998) 78–81.
- [41] P. Kreiss, B. Cameron, R. Rangara, P. Mailhe, O. Aguerre-Charriol, M. Airiau, D. Scherman, J. Crouzet, B. Pitard, Plasmid DNA size does not affect the physicochemical properties of lipoplexes but modulates gene transfer efficiency, *Nucleic Acids Res.* 27 (1999) 3792–3798.
- [42] J. Smisterová, A. Wagenaar, M.C.A. Stuart, E. Polushkin, G. Brinke, R. Hulst, J.B.F.N. Engberts, D. Hoekstra, Molecular shape of the cationic lipid controls the structure of cationic lipid/dioleoylphosphatidylethanolamine–DNA complexes and the efficiency of gene delivery, *J. Biol. Chem.* 276 (2001) 47615–47622.
- [43] I. Koltover, T. Salditt, C.R. Safinya, Phase diagram, stability, and overcharging of lamellar cationic lipid–DNA self-assembled complexes, *Biophys. J.* 77 (1999) 915–924.
- [44] E. Raspaud, B. Pitard, D. Durand, O. Aguerre-Charriol, J. Pelta, G. Byk, D. Scherman, F. Livolant, Polymorphism of DNA/multi-cationic lipid complexes driven by temperature and salts, *J. Phys. Chem. B* 105 (2001) 5291–5297.
- [45] J.O. Radler, I. Koltover, A. Jamieson, T. Salditt, C.R. Safinya, Structure and interfacial aspects of self-assembled cationic lipid–DNA gene carrier complexes, *Langmuir* 14 (1998) 4272–4283.
- [46] C. Madeira, L.M.S. Loura, M. Prieto, A. Fedorov, M.R. Aires-Barros, Effect of ionic strength and presence of serum on lipoplexes structure monitored by FRET, *BMC Biotechnol.* 8 (2008).
- [47] T.J. Anchordoquy, J.F. Carpenter, D.J. Kroll, Maintenance of transfection rates and physical characterization of lipid/DNA complexes after freeze-drying and rehydration, *Arch. Biochem. Biophys.* 348 (1997) 199–206.
- [48] P.C. Barreleiro, G. Olofsson, P. Alexandridis, Interaction of DNA with cationic vesicles: a calorimetric study, *J. Phys. Chem. B* 104 (2000) 7795–7802.
- [49] D. Simberg, D. Danino, Y. Talmon, A. Minsky, M.E. Ferrari, C.J. Wheeler, Y. Barenholz, Phase behavior, DNA ordering, and size instability of cationic lipoplexes – relevance to optimal transfection activity, *J. Biol. Chem.* 276 (2001) 47453–47459.
- [50] K.W.C. Mok, P.R. Cullis, Structural and fusogenic properties of cationic liposomes in the presence of plasmid DNA, *Biophys. J.* 73 (1997) 2534–2545.
- [51] T. Kral, J. Leblond, M. Hof, D. Scherman, J. Herscovici, N. Mignet, Lipopolythiourea/DNA interaction: a biophysical study, *Biophys. Chem.* 148 (2010) 68–73.
- [52] M. Scarzello, V. Chupin, A. Wagenaar, M.C.A. Stuart, J. Engberts, R. Hulst, Polymorphism of pyridinium amphiphiles for gene delivery: influence of ionic strength, helper lipid content, and plasmid DNA complexation, *Biophys. J.* 88 (2005) 2104–2113.
- [53] L. Ciani, S. Ristori, A. Salvati, L. Calamai, G. Martini, DOTAP/DOPE and DC-Chol/DOPE lipoplexes for gene delivery: zeta potential measurements and electron spin resonance spectra, *Biochim. Biophys. Acta* 1664 (2004) 70–79.
- [54] A. Salvati, L. Ciani, S. Ristori, G. Martini, A. Masi, A. Arcangeli, Physico-chemical characterization and transfection efficacy of cationic liposomes containing the pEGFP plasmid, *Biophys. Chem.* 121 (2006) 21–29.
- [55] S. Choosakoonkriang, C.M. Wiethoff, G.S. Koe, J.G. Koe, T.J. Anchordoquy, C.R. Middaugh, An infrared spectroscopic study of the effect of hydration on cationic lipid/DNA complexes, *J. Pharm. Sci.* 92 (2003) 115–130.
- [56] J. Liu, S. Guo, Z. Li, L. Liu, J. Gu, Synthesis and characterization of stearyl protamine and investigation of their complexes with DNA for gene delivery, *Colloid. Surf. B* 73 (2009) 36–41.
- [57] C.S. Braun, M.T. Fisher, D.A. Tomalia, G.S. Koe, J.G. Koe, C.R. Middaugh, A stopped-flow kinetic study of the assembly of nonviral gene delivery complexes, *Biophys. J.* 88 (2005) 4146–4158.
- [58] E. Hausteijn, P. Schuille, Fluorescence correlation spectroscopy: novel variations of an established technique, *Annu. Rev. Biophys. Biomol. Struct.* 36 (2007) 151–169.
- [59] D. Merkle, S.P. Lees-Miller, D.T. Cramb, Structure and dynamics of lipoplex formation examined using two-photon fluorescence cross-correlation spectroscopy, *Biochemistry* 43 (2004) 7263–7272.
- [60] A. Margineanu, S. De Feyter, S. Melnikov, D. Marchand, A. van Aerscht, P. Herdewijn, S. Habuchi, F.C. De Schryver, J. Hofkens, Complexation of lipofectamine and cholesterol-modified DNA sequences-studied by single-molecule fluorescence techniques, *Biomacromolecules* 8 (2007) 3382–3392.
- [61] M.F.M. Mustapa, P.C. Bell, C.A. Hurley, A. Nicol, E. Guenin, S. Sarkar, M.J. Writer, S.E. Barker, J.B. Wong, M.A. Pilkington-Miksa, B. Papahadjopoulos-Sternberg, P.A. Shamlou, H.C. Hailes, S.L. Hart, D. Zicha, A.B. Tabor, Biophysical characterization of an integrin-targeted lipopolyplex gene delivery vector, *Biochemistry* 46 (2007) 12930–12944.
- [62] D. Hirsch-Lerner, Y. Barenholz, Probing DNA–cationic lipid interactions with the fluorophore trimethylammonium diphenyl-hexatriene (TMADPH), *Biochim. Biophys. Acta* 1370 (1998) 17–30.
- [63] N.J. Zuidam, Y. Barenholz, Electrostatic and structural properties of complexes involving plasmid DNA and cationic lipids commonly used for gene delivery, *Biochim. Biophys. Acta* 1368 (1998) 115–128.
- [64] N.J. Zuidam, Y. Barenholz, Characterization of DNA–lipid complexes commonly used for gene delivery, *Int. J. Pharm.* 183 (1999) 43–46.
- [65] V.M. Meidan, J.S. Cohen, N. Amariglio, D. Hirsch-Lerner, Y. Barenholz, Interaction of oligonucleotides with cationic lipids: the relationship between electrostatics, hydration and state of aggregation, *Biochim. Biophys. Acta* 1464 (2000) 251–261.
- [66] A.E. Regelin, S. Fankhaenel, L. Gurtesch, C. Prinz, G. von Kiedrowski, U. Massing, Biophysical and lipofection studies of DOTAP analogs, *Biochim. Biophys. Acta* 1464 (2000) 151–164.
- [67] M. Munoz-Ubeda, A. Rodriguez-Pulido, A. Nogales, A. Martin-Molina, E. Aicart, E. Junquera, Effect of lipid composition on the structure and theoretical phase diagrams of DC-Chol/DOPE–DNA lipoplexes, *Biomacromolecules* 11 (2010) 3332–3340.
- [68] M. Rajesh, J. Sen, M. Srujan, K. Mukherjee, B. Sreedhar, A. Chaudhuri, Dramatic influence of the orientation of linker between hydrophilic and hydrophobic lipid moiety in liposomal gene delivery, *J. Am. Chem. Soc.* 129 (2007) 11408–11420.
- [69] L. Burel-Deschamps, M. Mevel, D. Loizeau, F. Ayadi, J.J. Yaouanc, J.C. Clement, P.A. Jaffres, P. Giamarchi, Fluorescence study of lipid-based DNA carriers properties: influence of cationic lipid chemical structure, *J. Fluoresc.* 18 (2008) 835–841.
- [70] C. Madeira, A. Fedorov, M.R. Aires-Barros, M. Prieto, L.M.S. Loura, Photophysical behavior of a dimeric cyanine dye (BOBO-1) within cationic liposomes, *Photochem. Photobiol.* 81 (2005) 1450–1459.
- [71] C. Madeira, L.M.S. Loura, M.R. Aires-Barros, A. Fedorov, M. Prieto, Characterization of DNA/lipid complexes by fluorescence resonance energy transfer, *Biophys. J.* 85 (2003) 3106–3119.
- [72] L.M.S. Loura, R.F.M. de Almeida, L.C. Silva, M. Prieto, FRET analysis of domain formation and properties in complex membrane systems, *Biochim. Biophys. Acta-Biomembr.* 1788 (2009) 209–224.
- [73] R.M. Clegg, *Fluorescence Imaging Spectroscopy and Microscopy*, Wiley, New York, 1996.
- [74] O. Zelphati, L.S. Uyechi, L.G. Barron, F.C. Szoka, Effect of serum components on the physico-chemical properties of cationic lipid/oligonucleotide complexes and on their interactions with cells, *Biochim. Biophys. Acta* 1390 (1998) 119–133.
- [75] M. Wong, S. Kong, W.H. Dragowska, M.B. Bally, Oxazole yellow homodimer YOYO-1-labeled DNA: a fluorescent complex that can be used to assess structural changes in DNA following formation and cellular delivery of cationic lipid DNA complexes, *Biochim. Biophys. Acta* 1527 (2001) 61–72.
- [76] K. Itaka, A. Harada, K. Nakamura, H. Kawaguchi, K. Kataoka, Evaluation by fluorescence resonance energy transfer of the stability of nonviral gene delivery vectors under physiological conditions, *Biomacromolecules* 3 (2002) 841–845.
- [77] O. Zelphati, C. Nguyen, M. Ferrari, J. Felgner, Y. Tsai, P.L. Felgner, Stable and monodisperse lipoplex formulations for gene delivery, *Gene Ther.* 5 (1998) 1272–1282.
- [78] D. Lleres, J.P. Clamme, E. Dauty, T. Blessing, G. Krishnamoorthy, G. Duportail, Y. Mely, Investigation of the stability of dimeric cationic surfactant/DNA complexes and their interaction with model membrane systems, *Langmuir* 18 (2002) 10340–10347.
- [79] D. Simberg, S. Weisman, Y. Talmon, A. Faerman, T. Shoshani, Y. Barenholz, The role of organ vascularization and lipoplex-serum initial contact in intravenous murine lipofection, *J. Biol. Chem.* 278 (2003) 39858–39865.
- [80] Y. Zhang, F. Schlachetzki, W.M. Partridge, Global non-viral gene transfer to the primate brain following intravenous administration, *Mol. Ther.* 7 (2003) 11–18.
- [81] Y. Zhang, T.J. Anchordoquy, The role of lipid charge density in the serum stability of cationic lipid/DNA complexes, *Biochim. Biophys. Acta* 1663 (2004) 143–157.
- [82] C. Madeira, L.M.S. Loura, M. Prieto, A. Fedorov, M.R. Aires-Barros, Liposome complexation efficiency monitored by FRET: effect of charge ratio, helper lipid and plasmid size, *Eur. Biophys. J. Biophys. Lett.* 36 (2007) 609–620.
- [83] L. Wang, R. Koynova, H. Parikh, R.C. MacDonald, Transfection activity of binary mixtures of cationic O-substituted phosphatidylcholine derivatives: the hydrophobic core strongly modulates physical properties and DNA delivery efficacy, *Biophys. J.* 91 (2006) 3692–3706.
- [84] J.P.N. Silva, P.J.G. Coutinho, M. Oliveira, Characterization of monoolein-based lipoplexes using fluorescence spectroscopy, *J. Fluoresc.* 18 (2008) 555–562.
- [85] J.P. Clamme, S. Bernacchi, C. Vuilleumier, G. Duportail, Y. Mely, Gene transfer by cationic surfactants is essentially limited by the trapping of the surfactant/DNA complexes onto the cell membrane: a fluorescence investigation, *Biochim. Biophys. Acta* 1467 (2000) 347–361.
- [86] C.M. Wiethoff, M.L. Gill, G.S. Koe, J.G. Koe, C.R. Middaugh, The structural organization of cationic lipid–DNA complexes, *J. Biol. Chem.* 277 (2002) 44980–44987.
- [87] Y. Zhang, W. Garzon-Rodriguez, M.C. Manning, T.J. Anchordoquy, The use of fluorescence resonance energy transfer to monitor dynamic changes of lipid–DNA interactions during lipoplex formation, *Biochim. Biophys. Acta* 1614 (2003) 182–192.
- [88] Y.H. Xu, S.W. Hui, P. Frederik, F.C. Szoka, Physicochemical characterization and purification of cationic lipoplexes, *Biophys. J.* 77 (1999) 341–353.

- [89] I. Jaaskelainen, B. Sternberg, J. Monkkonen, A. Urtti, Physicochemical and morphological properties of complexes made of cationic liposomes and oligonucleotides, *Int. J. Pharm.* 167 (1998) 191–203.
- [90] M.R. Almofti, H. Harashima, Y. Shinohara, A. Almofti, Y. Baba, H. Kiwada, Cationic liposome-mediated gene delivery: biophysical study and mechanism of internalization, *Arch. Biochem. Biophys.* 410 (2003) 246–253.
- [91] M.T. Kennedy, E.V. Pozharski, V.A. Rakhmanova, R.C. MacDonald, Factors governing the assembly of cationic phospholipid–DNA complexes, *Biophys. J.* 78 (2000) 1620–1633.
- [92] E.K. Wasan, P. Harvie, K. Edwards, G. Karlsson, M.B. Bally, A multi-step lipid mixing assay to model structural changes in cationic lipoplexes used for in vitro transfection, *Biochim. Biophys. Acta* 1461 (1999) 27–46.
- [93] S. Li, W.C. Tseng, D.B. Stolz, S.P. Wu, S.C. Watkins, L. Huang, Dynamic changes in the characteristics of cationic lipidic vectors after exposure to mouse serum: implications for intravenous lipofection, *Gene Ther.* 6 (1999) 585–594.
- [94] G. Nchinda, K. Überla, O. Zschörnig, Characterization of cationic lipid DNA transfection complexes differing in susceptibility to serum inhibition, *BMC Biotechnol.* 2 (2002) 1–10.
- [95] B.M. Tandia, C. Lonez, M. Vandenbranden, J.M. Ruyschaert, A. Elouahabi, Lipid mixing between lipoplexes and plasma lipoproteins is a major barrier for intravenous transfection mediated by cationic lipids, *J. Biol. Chem.* 280 (2005) 12255–12261.
- [96] M. Spelios, M. Savva, Novel N, N'-diacyl-1,3-diaminopropyl-2-carbamoyl bivalent cationic lipids for gene delivery – synthesis, in vitro transfection activity, and physicochemical characterization, *FEBS J.* 275 (2008) 148–162.
- [97] E. Yuba, C. Kojima, N. Sakaguchi, A. Harada, K. Koiwai, K. Kono, Gene delivery to dendritic cells mediated by complexes of lipoplexes and pH-sensitive fusogenic polymer-modified liposomes, *J. Control. Release* 130 (2008) 77–83.
- [98] B. Mui, Q.F. Ahkong, L. Chow, M.J. Hope, Membrane perturbation and the mechanism of lipid-mediated transfer of DNA into cells, *Biochim. Biophys. Acta* 1467 (2000) 281–292.
- [99] V.V. Kumar, C. Pichon, M. Refregiers, B. Guerin, P. Midoux, A. Chaudhuri, Single histidine residue in head-group region is sufficient to impart remarkable gene transfection properties to cationic lipids: evidence for histidine-mediated membrane fusion at acidic pH, *Gene Ther.* 10 (2003) 1206–1215.
- [100] T. Salditt, I. Koltover, J.O. Radler, C.R. Safinya, Self-assembled DNA–cationic-lipid complexes: two-dimensional smectic ordering, correlations, and interactions, *Phys. Rev. E* 58 (1998) 889–904.
- [101] B.K.-K. Fung, L. Stryer, Surface density determinations in membranes by fluorescence energy transfer, *Biochemistry* 17 (1978) 5241–5248.
- [102] L. Davenport, R.E. Dale, R.H. Bisby, R.B. Cundall, Transverse location of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene in model lipid bilayer membrane systems by resonance energy transfer, *Biochemistry* 24 (1985) 4097–4108.
- [103] L.M.S. Loura, A. Fedorov, M. Prieto, Fluid–fluid membrane microheterogeneity: a fluorescence resonance energy transfer study, *Biophys. J.* 80 (2001) 776–788.
- [104] T.J. Anchordoquy, S.D. Allison, M.d.C. Molina, L.G. Girouard, T.K. Carson, Physical stabilization of DNA-based therapeutics, *Drug Deliv. Today* 6 (2001) 463–470.
- [105] L.A. Wangerek, H.H. Dahl, T.J. Senden, J.B. Carlin, D.A. Jans, D.E. Dunstan, P.A. Ioannou, R. Williamson, S.M. Forrest, Atomic force microscopy imaging of DNA–cationic liposome complexes optimised for gene transfection into neuronal cells, *J. Gene Med.* 3 (2001) 72–81.
- [106] H. Faneca, S. Simoes, M.C. de Lima, Evaluation of lipid-based reagents to mediate intracellular gene delivery, *Biochim. Biophys. Acta* 1567 (2002) 23–33.
- [107] D. Lleres, J.M. Weibel, D. Heissler, G. Zuber, G. Duportail, Y. Mely, Dependence of the cellular internalization and transfection efficiency on the structure and physicochemical properties of cationic detergent/DNA/liposomes, *J. Gene Med.* 6 (2004) 415–428.
- [108] J. Biswas, A. Bajaj, S. Bhattacharya, Membranes of cationic gemini lipids based on cholesterol with hydroxyl headgroups and their interactions with DNA and phospholipid, *J. Phys. Chem. B* 115 (2011) 478–486.
- [109] R.L. Sheng, T. Luo, Y.D. Zhu, H. Li, A. Cao, Interactions of new synthesized fluorescent cationic amphiphiles bearing pyrene hydrophobe with plasmid DNA: binding affinities, aggregation and intracellular uptake, *Macromol. Biosci.* 10 (2010) 974–982.
- [110] A.L. Troutier-Thuilliez, J. Thevenot, T. Delair, C. Ladaviere, Adsorption of plasmid DNA onto lipid/polymer particle assemblies, *Soft Matter* 5 (2009) 4739–4747.
- [111] A. Aljaberi, P.S. Chen, M. Savva, Synthesis, in vitro transfection activity and physicochemical characterization of novel N, N'-diacyl-1,2-diaminopropyl-3-carbamoyl-(dimethylaminoethane) amphiphilic derivatives, *Chem. Phys. Lipids* 133 (2005) 135–149.
- [112] D.L. Reimer, Y.P. Zhang, S. Kong, J.J. Wheeler, R.W. Graham, M.B. Bally, Formation of novel hydrophobic complexes between cationic lipids and plasmid DNA, *Biochemistry* 34 (1995) 12877–12883.
- [113] Y.P. Zhang, D.L. Reimer, G. Zhang, P.H. Lee, M.B. Bally, Self-assembling DNA–lipid particles for gene transfer, *Pharm. Res.* 14 (1997) 190–196.
- [114] P. Harvie, F.M.P. Wong, M.B. Bally, Characterization of lipid DNA interactions. I. Destabilization of bound lipids and DNA dissociation, *Biophys. J.* 75 (1998) 1040–1051.
- [115] M.E. Ferrari, D. Rusalov, J. Enas, C.J. Wheeler, Trends in lipoplex physical properties dependent on cationic lipid structure, vehicle and complexation procedure do not correlate with biological activity, *Nucleic Acids Res.* 29 (2001) 1539–1548.
- [116] M.E. Ferrari, D. Rusalov, J. Enas, C.J. Wheeler, Synergy between cationic lipid and co-lipid determines the macroscopic structure and transfection activity of lipoplexes, *Nucleic Acids Res.* 30 (2002) 1808–1816.
- [117] C. Welz, A. Fahr, Spectroscopic methods for characterization of nonviral gene delivery systems from a pharmaceutical point of view, *Appl. Spectrosc. Rev.* 36 (2001) 333–397.
- [118] C.M. Wiethoff, M.L. Gill, G.S. Koe, J.G. Koe, C.R. Middaugh, A fluorescence study of the structure and accessibility of plasmid DNA condensed with cationic gene delivery vehicles, *J. Pharm. Sci.* 92 (2003) 1272–1285.
- [119] G. Krishnamoorthy, G. Duportail, Y. Mély, Structure and dynamics of condensed DNA probed by 1,1'-(4,4,8,8-tetramethyl-4,8-diazaundecamethylene)bis[4-[[3-methylbenz-1,3-oxazol-2-yl]methylidene]-1,4-dihydroquinolinium]tetraiodide fluorescence, *Biochemistry* 41 (2002) 15277–15287.
- [120] D. Simberg, A. Weiss, Y. Barenholz, Reversible mode of binding of serum proteins to DOTAP/cholesterol lipoplexes: a possible explanation for intravenous lipofection efficiency, *Hum. Gene Ther.* 16 (2005) 1087–1096.
- [121] P.C. Ross, S.W. Hui, Lipoplex size is a major determinant of in vitro lipofection efficiency, *Gene Ther.* 6 (1999) 651–659.
- [122] F. Sakurai, R. Inoue, Y. Nishino, A. Okuda, O. Matsumoto, T. Taga, F. Yamashita, Y. Takakura, M. Hashida, Effect of DNA/liposome mixing ratio on the physicochemical characteristics, cellular uptake and intracellular trafficking of plasmid DNA/cationic liposome complexes and subsequent gene expression, *J. Control. Release* 66 (2000) 255–269.
- [123] S. Weisman, D. Hirsch-Lerner, Y. Barenholz, Y. Talmon, Nanostructure of cationic lipid–oligonucleotide complexes, *Biophys. J.* 87 (2004) 609–614.
- [124] Y.S. Tarahovsky, V.A. Rakhmanova, R.M. Epan, R.C. MacDonald, High temperature stabilization of DNA in complexes with cationic lipids, *Biophys. J.* 82 (2002) 264–273.