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REVIEW

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Cationic liposome/DNA complexes: from structure to interactions with cellular membranes

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Abstract Gene-based therapeutic approaches are based upon the concept that, if a disease is caused by a mutation in a gene, then adding back the wild-type gene should restore regular function and attenuate the disease phenotype. To deliver the gene of interest, both viral and nonviral vectors are used. Viruses are efficient, but their application is impeded by detrimental side-effects. Among nonviral vectors, cationic liposomes are the most promising candidates for gene delivery. They form stable complexes with polyanionic DNA (lipoplexes). Despite several advantages over viral vectors, the transfection efficiency (TE) of lipoplexes is too low compared with those of engineered viral vectors. This is due to lack of knowledge about the interactions between complexes and cellular components. Rational design of efficient lipoplexes therefore requires deeper comprehension of the interactions between the vector and the DNA as well as the cellular pathways and mechanisms involved. The importance of the lipoplex structure in biological function is revealed in the application of synchrotron small-angle X-ray scattering in combination with functional TE measurements. According to current understanding, the structure of lipoplexes can change upon interaction with cellular membranes and such changes affect the delivery efficiency. Recently, a correlation between the mechanism of gene release from complexes, the structure, and the physical and chemical parameters of the complexes has been established. Studies aimed at correlating structure and activity of lipoplexes are reviewed herein. This is a fundamental step towards rational design of highly efficient lipid gene vectors.

Keywords Gene therapy · Gene delivery · Cationic liposomes · DNA · Lipoplexes · Cellular lipids

Introduction

The transfer of genetic material into cells, both in vitro and in vivo, is essential for studying gene function and conducting gene therapy (Both et al. 2011; Denèfle 2011; Kay 2011; Melief et al. 2011). Two principal delivery systems include viral and nonviral vectors. Viral techniques use several classes of viruses as a tool for gene delivery such as retrovirus, adenovirus, adeno-associated virus, and herpes simplex virus. Viral vectors are most effective, but their application is hampered by detrimental side-effects. Tragic events associated with viral gene therapy (Hacein-Bey-Abina et al. 2003; Woods et al. 2006) led to reconsideration of this approach and suggested that design of virus-like nonviral vectors is an urgent task for the accomplishment of gene therapy (Hart 2010; Glover 2011). Among potential gene nanocarriers, cationic liposomes (CLs) made of cationic and zwitterionic "helper" lipids have emerged worldwide as the most prevalent synthetic carriers (Felgner et al. 1987; Gregoriadis 2001; Wasungu and Hoekstra 2006; Karmali and Chaudhuri 2007; Montier et al. 2008; Labas et al. 2009; Balazs and Godbey 2011). CLs are nonimmunogenic, nontoxic, and easy to produce, and have

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the potential to transfer large pieces of DNA into cells. By adjusting the molar fraction of zwitterionic lipid in the bilayer,Φ = zwitterionic/total lipid (mol/mol), CLs of varying membrane charge density are routinely prepared (Behr 1994; Farhood et al. 1995; Zuidam and Barenholz 1997; Koltover et al. 1999; Caracciolo et al. 2003; Ewert et al. 2005; Hirsch-Lerner et al. 2005; Karmali and Chaudhuri 2007). When mixed with DNA, CLs form stable complexes (lipoplexes) that can deliver DNA into cells by binding electrostatically to surface proteoglycans (Mislick and Baldeschwieler 1996). Despite the several advantages of lipoplexes over viral vectors, their transfection efficiency (TE) remains low compared with those of engineered viral vectors. This is due to lack of knowledge about the interactions between complexes and cellular components. Thus, understanding transfection-related mechanisms is a very active area of research (Simoes et al. 1999; Hafez et al. 2001; Zuhorn and Hoekstra 2002; Zuhorn et al. 2002; Gonçalves et al. 2004a, b; Rejman et al. 2004; Tandia 2005; Kirkham and Parton 2005; Khalil et al. 2006; Boktov et al. 2007; Hoekstra et al. 2007; Ma et al. 2007; Ondrej et al. 2007; Caracciolo et al. 2008a, 2009; Resina et al. 2009; Le Bihan et al. 2011; Ruthardt et al. 2011). A viewpoint now emerging is that a critical factor in lipid-mediated gene transfection (lipofection) is the structural and phase evolution of lipoplexes upon interaction and mixing with cellular anionic lipids (ALs; Koynova et al. 2005, 2006; Caracciolo et al. 2007a, b, c; Koynova and MacDonald 2007; Koynova 2008; Pozzi et al. 2009). Such structural rearrangement is supposed to play a central role in the DNA escape process, i.e., in how DNA dissociates from lipoplexes and is released into the cytoplasm and, eventually, into the nucleus. Unbinding of DNA from CLs is thought to result from charge neutralization by cellular anionic lipids (Xu and Szoka 1996; Zelphati and Szoka 1996; Caracciolo et al. 2007c). Indeed, addition of negatively charged liposomes to lipoplexes results in dissociation of DNA from the lipid. A noteworthy suggestion is that the structure of lipoplexes changes dramatically upon interaction with cellular lipids and that these changes are critical for efficient delivery. In the last decade, the application of high-resolution synchrotron small-angle X-ray scattering (SAXS) has helped in linking the nanostructure of lipoplexes with their biological activity (Kreiss et al. 1999; Zuhorn and Hoekstra 2002; Caracciolo et al. 2003; Tarahovsky et al. 2004; Hoekstra et al. 2007; Koynova et al. 2006; Wang et al. 2006; Marchini et al. 2010, 2011). This fundamental knowledge needs to be incorporated into the design of next-generation lipoplexes for enhanced transfection. In this review, we describe recent works that have elucidated the relationship between structural and phase changes of lipoplexes upon interaction with biomembranes, and their TE.

Materials

Cationic lipids

Two main cationic lipids for transfection are used: 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP) and 3β[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol). DOTAP, first synthesized about 20 years ago (Leventis and Silvius 1990), contains a quaternary amine head-group linked to a glycerol backbone with two oleoyl chains. It is fully protonated at physiological pH (Zuidam and Barenholz 1998), so more energy is needed to separate the DNA from the lipoplex (Zabner et al. 1995). DC-Chol, first synthesized by Gao and Huang (1991), consists of a cholesterol moiety connected by an ester bond to a hydrolyzable dimethylethylenediamine. Cholesterol was chosen for its biocompatibility and the stability it imparts to lipid membranes. In addition, DC-Chol was found to be fourfold less cytotoxic than commercial Lipofectin (Invitrogen, USA) in several cell lines (Gao and Huang 1991). Too strong lipid–DNA interactions can result in DNA entrapment, thus DOTAP and DC-Chol are often used in combination with a zwitterionic helper lipid (Simberg et al. 2004).

Zwitterionic lipids

Most liposomal formulations used for gene delivery consist of a combination of charged lipids and zwitterionic "helper" lipids (Hui et al. 1996). The neutral helper lipids often used are dioleoylphosphatidylethanolamine (DOPE), which is the most widely used one, and dioleoylphosphatidylcholine (DOPC) (Behr 1994; Farhood et al. 1995; Mok and Cullis 1997; Zuidam and Barenholz 1998; Maitani et al. 2007). Results have shown that use of DOPE in place of DOPC as the helper lipid yields higher transfection efficiencies in many cell types, most likely due to a phase transition to an inverted hexagonal structure that is ruled by DOPE at low pH (Farhood et al. 1995). Less frequently, dipalmitoylphosphatidylethanolamine (DPPE; Koynova and MacDonald 2003; Zuhorn et al. 2005), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC; Caracciolo et al. 2007b; Rodríguez-Pulido et al. 2009), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC; Koynova et al. 2005; Caracciolo et al. 2007b), and dipalmitoylphosphatidylcholine (DPPC; Mortazavi et al. 2007; Samadikhah et al. 2011) have also been used.

Anionic cellular lipids

Dioleoylphosphatidylglycerol (DOPG) has a packing parameter (Israelachvili et al. 1977) less than 1 and tends to form flexible bilayers and vesicles (Koynova et al. 2006;

Caracciolo et al. 2007b; Pozzi et al. 2009). Dioleoylphosphatidylserine (DOPS) is used either alone (Koynova et al. 2005) or in mixtures with zwitterionic phosholipids (Koynova and MacDonald 2007; Pozzi et al. 2009; Rodowicz et al. 2010). Phosphatidic acids (PAs) are the acid forms of phosphatidates, a part of common phospholipids, major constituents of cell membranes. The lipid headgroup charge of PAs may be increased upon interaction with cholesterol and zwitterionic lipid species such as DOPE (Hafez et al. 2000; Kooijman et al. 2005). Dioleoyl phosphatidic acid (DOPA) has often been used as a DNA releaser from lipoplexes (Tarahovsky et al. 2004; Pozzi et al. 2009). DOPG, DOPS, and DOPA are common in the plasma membrane of mammalian cells.

DNA

SAXS experiments aimed at elucidating the nanostructure of lipoplexes require large amounts of DNA (typically, tenths of a milligram). Linear calf-thymus (CT) DNA, which is commercially available from various vendors, is the most widely used DNA for SAXS studies due to its reasonable price. Plasmids encoding for either Luciferase or green fluorescent protein (GFP) are ideal for cell transfections, while fluorescently labeled (Cy3) plasmid-DNA is commonly used in confocal laser scanning microscopy (CLSM). Even though the structure of lipoplexes containing linear or plasmid DNA is roughly the same (Caracciolo et al. 2008b), there is a possibility that the two architectures are processed differently by cells.

Formation of lipoplexes

The molecular events occurring when DNA interacts with CLs have been intensely studied over the last two decades. This has been inspired by the deep conviction that the mechanism of formation of the complexes strongly affects their final physicochemical properties that control, in turn, their biological activity. Moreover, mechanistic knowledge of lipoplex formation is a first and necessary step towards understanding how lipid/DNA complexes disassemble in the cytoplasm and release their gene payload. This is unanimously considered as a milestone for the future development of efficient gene delivery systems. Mixing of DNA and CLs results in spontaneous formation of supramolecular aggregates (Felgner et al. 1987; Gao et al. 2010) whose final size (100 nm to 1 μm) is strictly dependent on the methods of preparation (Ulrich 2002). Several studies have investigated the kinetics of lipoplex formation, and the existence of a multistep mechanism is well accepted (Pozharski and Macdonald 2005, 2007). In the first step, typically occurring within milliseconds (Barreleiro and Lindman 2003; Barreleiro et al. 2002), the negatively charged phosphate groups of DNA interact with the cationic lipid head-groups by electrostatic interactions (Cherstvy 2007; Rodríguez-Pulido et al. 2009). The dominant interactions in the complexes are electrostatic because of the huge charge densities of the complexing species. However, it is now well recognized that Coulomb attractions are not enough to explain the complex formation and its thermodynamic stability, because highly charged macromolecules in solution are surrounded by oppositely charged counterions. In the case of planar surfaces (lipid bilayers) or long cylindrically shaped macromolecules (DNA), nonlinear Poisson–Boltzmann theory predicts that counterions are largely confined to the immediate vicinity of their surface, the phenomenon recognized as counterion condensation (Manning, 1969, 1978; Le Bret and Zimm 1984). Upon complexation, counterions are released from both DNA and lipids in a one-to-one ratio, while counterions of excess material remain tightly bound to cationic membranes (Gao et al. 2010). The resulting entropy increase is large ($\approx k_{\mathrm{B}}T$ per released counterion), and it has been identified as the driving force for the formation of lipoplexes (Koltover et al. 1999). Both theory and experiment (Wagner et al. 2000) show large release of counterions. As a consequence, lipoplexes are particularly stable at the isoelectric point with one cationic lipid per negatively charged nucleotide base. On the other hand, overcharging of lipoplexes as a function of cationic lipid/DNA charge ratio, ρ, has been extensively investigated, and overcharged lipoplexes have frequently been observed experimentally (Koltover et al. 1999; Caracciolo et al. 2002, 2003, 2006b). The lipoplex is one-phase complex close to the isoelectric point, i.e., $\rho \approx 1$, while strong intermembrane electrostatic repulsions let the system split into complex plus excess lipid for $\rho > 1$. On the other hand, DNA–DNA repulsions force the lipoplex to separate into complex plus excess DNA for $\rho < 1$. Despite its good soundness, such an electrostatic explanation has clear shortcomings; For instance, several authors have pointed out that, at $\rho = 1$, DNA is not completely protected by lipids and is free in solution. In most cases, for complete DNA protection, charge ratios $\rho > 1$ are necessary (Gonçalves et al. 2004a, b; Madeira et al. 2003, 2007; Henriques et al. 2009). In recent experiments aimed at investigating the role of membrane charge density of CLs on their DNA-binding capacity, evidence supporting this statement has unambiguously been provided. The most unprecedented result was that lipoplexes containing excess cationic charge do coexist with unbound plasmid DNA (Caracciolo et al. 2006c; Marchini et al. 2009a). Since assuming the coexistence of free DNA with DNA-free CLs is not convincing, a persuasive explanation is that the surface area of cationic membrane was not enough to complex all the DNA (Marchini et al. 2009a). This

suggestion has been confirmed by experimental findings showing that CLs with distinct membrane charge density and charge ratio but with similar lipid surface area, A, exhibit the same DNA binding ability (Marchini et al. 2009a; Caracciolo et al. 2010a). This will be explained in detail in the following section "Inverted hexagonal-phase lipoplexes". Many authors have emphasized the special role played by DNA molecules in the formation of lipoplexes as well. The most conceivable mechanism postulates that DNA adsorbing to one side of the lipid bilayer provokes an asymmetry in the packing pressure between the outer and inner leaflets of the lipid bilayer, leading to destabilization of the membrane (Huebner et al. 1999; Kennedy et al. 2000; Hayes et al. 2001). This leads to local adhesion and fusion of liposomes which allow robust lipid mixing. This suggestion has been demonstrated by recent SAXS experiments (Caracciolo et al. 2005a, b) in which DNA was added to mixed lipid dispersions containing two distinct populations of binary cationic liposomes (indicated as "A" and "B" cationic liposomes in the following text). After interaction, only mixed A-B-DNA lipoplexes were found to exist. Indeed, no Bragg peaks of A-DNA or B-DNA lipoplexes were observed on the SAXS pattern of the A-B-DNA mixture. These results unambiguously implied fusion of A and B vesicles. Remarkably, the quaternary lipoplex exhibited membrane charge density, bilayer thickness, and DNA packing density midway between those of A and B membranes. Upon fusion of A and B liposomes, lateral diffusion of lipid molecules in the membrane plane leads to a two-dimensional (2D) spatial distribution of the lipid species, being, on average, uniform. In fact, lipid mixing entropy (May and Ben-Shaul 2004) in multicomponent aggregates was identified as the driving force for the formation of thermodynamically stable complexes (Caracciolo et al. 2006a). The special role played by DNA molecules in the self-assembly of lipoplexes has been further supported by recent SAXS experiments comparing equilibrium structures of "directly" and "indirectly" formed lipoplexes (Caracciolo et al. 2006b). By using a two-step process, consisting of adding excess material to preformed neutrally charged lipoplexes, it has been shown that excess DNA can enter early-formed lipoplexes while excess lipid can not. DNA, being a single molecule, does not face the constraints of lipids. Positive electrostatic charges of both liposomes and early-formed lipoplexes generate a repulsive barrier which overcomes short-range attractive van der Waals forces and prevents membrane aggregation and fusion.

Equilibrium structures of lipoplexes

Synchrotron SAXS was used to elucidate the structure of CL–DNA complexes at the angstrom scale (Fig. 1). The existence of two "canonical" packing orders has been revealed: hexagonal (Koltover et al. 1998; Smisterová et al. 2001; Zuhorn et al. 2002, 2005; Krishnaswamy et al. 2006; Wang et al. 2006; Marchini et al. 2009b) and lamellar (Rädler et al. 1997; Koltover et al. 1999, 2000; Lin et al. 2000; Caracciolo et al. 2002, 2003), identical to the symmetries of the hexagonal (H_{II}) and lamellar (L_α) liquid-crystalline phases of pure lipid assemblies. The observed structures have thus been named L_α^C and H_{II}^C phases (Fig. 1, insets of bottom and top panel, respectively). Statistical-mechanical models have shown that the self-assembled liquid-crystalline L_α^C and H_{II}^C phases observed experimentally are equilibrium phases of lipoplexes (May and Ben-Shaul 1997, 2004; Bruinsma 1998; Harries et al. 1998; May et al. 2000). Less frequently, bilayer cubic arrays are seen in lipoplexes internalized in cells (Zabner et al. 1995).

Inverted hexagonal-phase lipoplexes

In certain lipoplex formulations, DNA rods are intercalated within the aqueous tubes of an inverted hexagonal lipid matrix. This arrangement of lipoplexes is identified by their

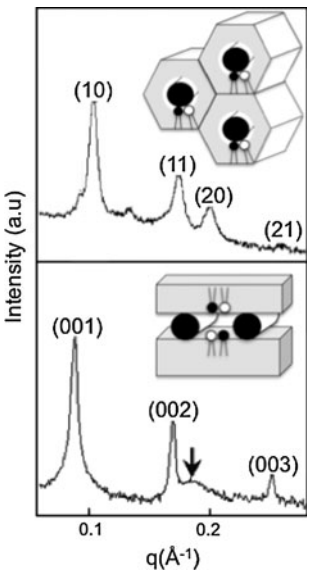

Fig. 1 *Top panel* SAXS pattern of DOTAP-DOPE/DNA lipoplexes ($\Phi = 0.7$, $\rho = 1$). Bragg peaks could be indexed to a two-dimensional hexagonal lattice with unit cell $a = 67.5$ Å according to the equation $q_{hk} = \frac{4\pi}{\sqrt{3}a}\sqrt{h^2 + hk + k^2}$. The inset shows the structure of inverted hexagonal lipoplexes with DNA rods located within aqueous channels. *Bottom panel* SAXS pattern of DC-Chol-DOPC/DNA lipoplexes ($\Phi = 0.5$, $\rho = 1$). Equally spaced Bragg reflections (labeled "*00n*") are due to the multilamellar periodicity along the normal to lipid bilayers, $d = 2\pi/q_{001}$, which is the sum of the membrane thickness plus the thickness of the water region occupied by DNA molecules ($d_W \approx 25$ Å). The structure of multilamellar complexes is shown in the *inset*

synchrotron SAXS pattern with Bragg peaks fitting the ratio $1:\sqrt{3}:\sqrt{4}:\sqrt{7}:2$ etc. according to the equation $q_{hk}=\frac{4\pi}{\sqrt{3}a}\sqrt{h^2+hk+k^2}$, where a is the unit cell spacing. As an example, a SAXS pattern of DOTAP-DOPE/DNA lipoplexes ($\Phi=0.7$, $\rho=1$) is shown in Fig. 1 (top panel). The symmetry of this structure is identical to that of the inverse-hexagonal, H_{II}, phase of pure (i.e., DNA-free) lipids. A pictorial representation of the H_{II}^{C} complex is given in Fig. 1 (top panel, inset). Due to the rigid constraints on the lipid chain packing in the H_{II} phase, the diameter of the water canals just slightly larger than the diameter of the DNA rods. Strong electrostatic attractions between the phosphate charges on the DNA surface and the surrounding cationic head-groups minimize the separation between the lipid and DNA surfaces, setting it to just one or two water molecule diameters. The spacing of the hexagonal DNA array, a, is thus fixed, corresponding to roughly twice the thickness of the lipid monolayer plus the diameter of the inverted micelle. Some earlier studies suggested that the inverted hexagonal phase leads to more efficient TE than the lamellar phase does (Koltover et al. 1998). However, recent experiments disputed this suggestion, providing considerable evidence against a direct general correlation between lipoplex structure and transfection efficiency (Caracciolo et al. 2003; Koynova et al. 2006).

Lamellar phase lipoplexes

Synchrotron SAXS has revealed that the most abundant nanostructure of lipoplexes is the so-called lamellar L_{α}^{C} phase, a lamellar stack of lipid bilayers intercalating monolayers of parallel DNA rods (Fig. 1, bottom panel, inset). In the SAXS patterns of lamellar lipoplexes a set of equally spaced Bragg reflections (labeled "*00n*" in Fig. 1, bottom panel) is seen. As an example, we report the SAXS pattern of DC-Chol-DOPC/DNA lipoplexes ($\Phi=0.5$, $\rho=1$; Fig. 1, bottom panel). The lamellar repeat distance, $d=2\pi/q_{001}$, is the sum of the membrane thickness plus the thickness of the water region occupied by DNA molecules ($d_W\approx 25$ Å). While d can change with lipid composition, d_W is almost constant in lamellar complexes. In addition to the sharp reflections due to multilamellar periodicity along the normal to lipid bilayers, a low-intensity broad peak is usually observed (marked by an arrow, Fig. 1, bottom panel). Such a peak, usually referred to as the "DNA peak," is due to in-plane packing of DNA rods. The "DNA peak" is broader than the lamellar ones because the two-dimensional (2D) smectic liquid crystal is less stable against thermal disorder than the three-dimensional (3D) smectic of the bilayer–DNA (Salditt et al. 1997). The one-dimensional (1D) correlation length extends over about ten DNA molecules, as evidenced from line-shape analysis of SAXS data (Salditt et al. 1997).

Less frequently, coexistence of inverted hexagonal and lamellar phase has been reported (Wang et al. 2006; Marchini et al. 2009b). While SAXS patterns show the unambiguous coexistence of hexagonal and lamellar phases, the exact mode of topological coexistence is still controversial. Recent calculations of electron density profiles from high-resolution SAXS patterns seem to suggest that both phases do contain DNA. This observation might be relevant for transfection, since lipoplexes with both phases have often been found to be more efficient than single-phase ones (Wang et al. 2006; Koynova 2008; Marchini et al. 2009b).

One-dimensional DNA packing within lamellar lipoplexes

The distance, d_{DNA}, between adjacent DNA strands in a given water gap has been found to depend on both the cationic lipid/DNA charge ratio, ρ, and the composition of the lipid layers (Φ; Koltover et al. 1999; Caracciolo and Caminiti 2004). This can clearly be seen in Fig. 2a, b, which show representative SAXS patterns of DOTAP-DOPC/DNA complexes with fixed Φ and varying ρ. The 1D array of DNA chains is affected by both Φ and ρ, with d_{DNA} ranging from approximately 25 Å, where the DNA rods are nearly touching, to about 60 Å (Fig. 2c). Noting that the radius of DNA is $R_D\approx 12.5$ Å, it follows that the minimum spacing between adjacent rods, $d_{DNA}=25$ Å, corresponds direct contact between DNA strands, with only a thin hydration shell separating two adjacent DNA strands. Remarkably, all the d_{DNA} distributions can be rescaled onto universal curves when plotted against γ/X_{DNA}, where γ is the ratio between the area of cationic membranes and that occupied by DNA molecules and X_{DNA} is the molar fraction of DNA condensed by lipids (Fig. 2d). This means that complexes with very different membrane charge density (Φ) and charge ratio (ρ) but with similar lipid surface area exhibit the very same DNA 1D packing density (i.e., the same d_{DNA}; Marchini et al. 2009a; Caracciolo et al. 2010a). As a consequence, the interfacial area of lipid membranes has been identified as a universal parameter regulating DNA condensation behavior in lamellar lipoplexes. In the first step, as far as DNA is not completely protected by lipids, the spacing between DNA chains changes almost linearly with γ and is set by the geometrical constraint of the interfacial area of lipid membranes available for DNA condensation. When all of the DNA is incorporated within the complex, the repulsive interaction between cationic membranes is the physical constraint that sets an upper limit on the DNA packing density. This revised idea of DNA adsorption onto the cationic

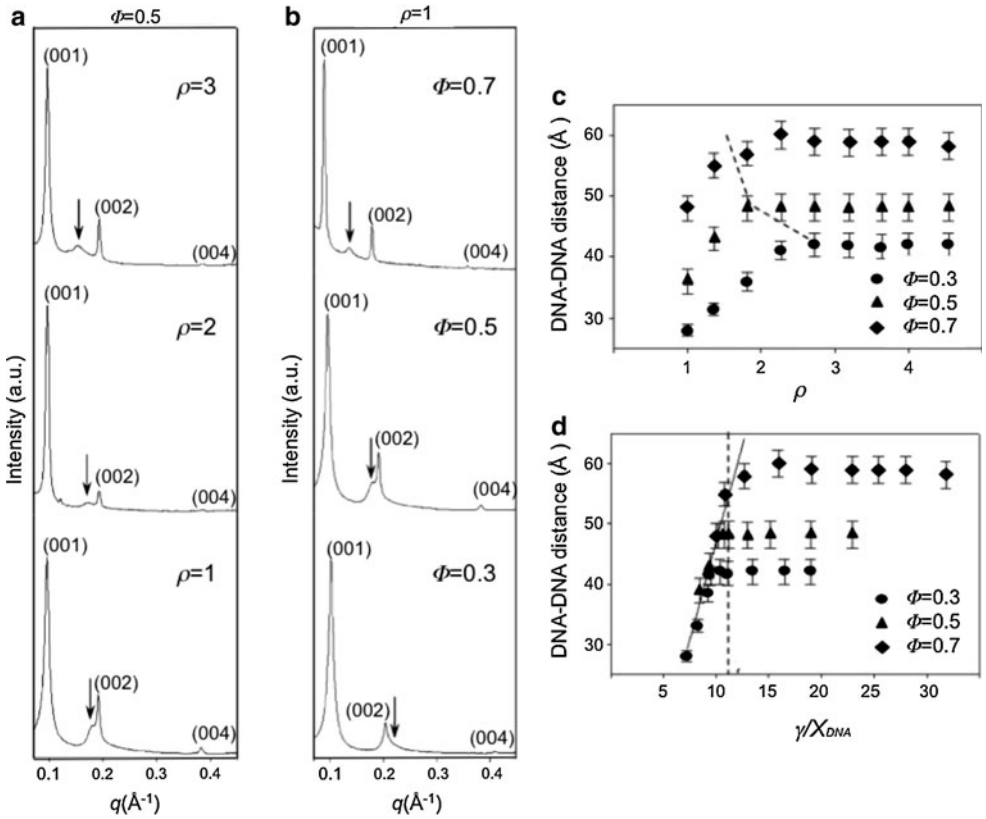

Fig. 2 **a** SAXS patterns of DOTAP-DOPC/DNA lipoplexes at $\Phi = 0.5$ as a function of cationic lipid/DNA charge ratio ρ. The diffuse broader peak (marked by an *arrow*) results from one-dimensional ordering of the DNA sandwiched between the lipid bilayers. **b** SAXS patterns of isoelectric DOTAP-DOPC/DNA lipoplexes ($\rho = 1$) as a function of neutral/total lipid molar fraction Φ. The DNA peak is marked by an *arrow*. **c** $d_{DNA}(\rho)$ curves for DOTAP-DOPC/DNA lipoplexes with different Φ. *Dashed lines* are a guide to the eye that links Φ saturation values. **d** When plotting d_{DNA} against γ/X_{DNA}, all the data points collapse onto a single curve (where γ is the ratio between the area of cationic membranes and that occupied by DNA molecules, and X_{DNA} is the molar fraction of DNA condensed by lipids). *Solid line* is the best linear fit to the data. *Dashed line* is the lowest γ/X_{DNA} value for which d_{DNA} plateaus

membranes of lipoplexes is in very good agreement with recent findings showing that less cationic lipid is required to prepare lipoplexes from plasmid DNA than linear DNA (Muñoz-Úbeda et al. 2011).

Lastly, it is noteworthy to remember that DNA molecules can arrange into a rectangular columnar superlattice between lipid bilayers in the low-temperature gel phase of lipoplexes (Artzner et al. 1998; Koynova and MacDonald 2004; McManus et al. 2004). In a single case, observation of transbilayer correlation of the DNA ordering in the liquid-crystalline lipid phase has been reported (Caracciolo et al. 2007d).

In summary, the capability to adjust the DNA-to-DNA spacing is a unique property of L_{α}^{C} lipoplexes. In addition to clarifying the mechanism of DNA condensation on lipid membranes, 1D DNA packing has successfully been used to investigate the structural changes of lipoplexes upon interaction with cellular lipids. This point is discussed in the next paragraph.

Interaction of lipoplexes with cellular lipids

It has been hypothesized that the structural evolution of lipoplexes upon interaction with cellular anionic lipids is a controlling factor in lipid-mediated DNA delivery. To test this suggestion, lipofection efficiency is compared with the the structural evolution and phase behavior of lipoplexes in mixtures with several anionic membrane lipids. Model membranes used in SAXS studies are listed below.

Model biomembranes

Anionic membranes of increasing complexity, both natural and synthetic, have used as model biomembranes: (1) Anionic membrane lipids such as DOPG, DOPA, DOPS, and cardiolipin; (2) Mimicking membrane (MM) anionic lipid mixtures. MM mixtures are typically made of anionic and zwitterionic lipids (DOPC, DOPE) at anionic/zwitterionic weight ratio 8:2, which is the approximate ratio of

plasma, endosomal, and nuclear membranes. A typical example is given by the MM1 lipid mixture made of DOPC, DMPC, DOPE, and DOPS (26:26:27:21, w/w; Koynova 2008). MM have been also prepared by lowering the anionic/zwitterionic weight ratio, as in the case of the MM2 lipid mixture made of DOPC:DOPE:DOPG (33:33:33, w/w) membranes (Pozzi et al. 2009); (3) Natural lipid extracts. Polar and total lipid extracts from bovine liver, heart, and brain can be purchased from Avanti Polar Lipids (Alabaster, AL).

Change in DNA packing intensity upon interaction with cellular lipids

Although the unbinding of DNA from CLs is not completely understood, it is thought to result from charge neutralization by cellular anionic lipids (Xu and Szoka 1996). Indeed, addition of negatively charged liposomes to lipoplexes results in dissociation of DNA from the lipid. A noteworthy suggestion is that the structure of lipoplexes changes radically upon interaction with cellular lipids (Caracciolo et al. 2007a) and that these changes are critical for endosomal escape (Minchin and Yang 2010). To mimic lipoplex–cell interaction, the most common approach consists in mixing lipoplexes with negatively charged anionic lipids (DOPG, DOPA, DOPS). SAXS experiments have revealed the existence of two structural regimes of lipoplex/AL mixtures (Caracciolo et al. 2007a, b; Pozzi et al. 2009): (1) at low anionic/cationic charge ratio, R, (typically $R < 1$), the multilamellar structure of lipoplexes is almost unchanged, while the 1D DNA lattice is largely perturbed; (2) for $R > 1$, the DNA–DNA in-plane correlation is lost and the lipoplex multilamellar structure starts to be disintegrated by anionic lipids. It has been shown that the release of DNA from lipoplexes occurs at low anionic/cationic charge ratios (Koynova et al. 2005; Caracciolo et al. 2007a). Thus, typical features of the first regime (termed the "DNA packing dilution regime") are discussed here, while the next section is dedicated to discussion of the structural changes of the multilamellar structure of lipoplexes. A typical example of the weakening of DNA–DNA interactions at low R is given in Fig. 3, which shows SAXS patterns of DC-Chol-DOPE/DNA lipoplexes upon interaction with DOPA in the $0 < R < 0.5$ range of the anionic/cationic charge ratio. At $R = 0$ (i.e., no anionic lipid added), the sharp peaks labeled at q_{00l} arose from the lamellar periodicity along the normal to the lipid bilayer, $d = 69.1$ Å. The DNA strands formed a 1D ordered array with interaxial spacing, $d_{DNA} = 35.1$ Å. The (001) peaks move only slightly toward smaller q, within this region of R, while the DNA peak (arrows) shifts over a wide range related to a variation in d_{DNA} from closed packed at 35.1 Å ($R = 0$) to extremely large at 53 Å ($R = 0.5$). This finding indicates

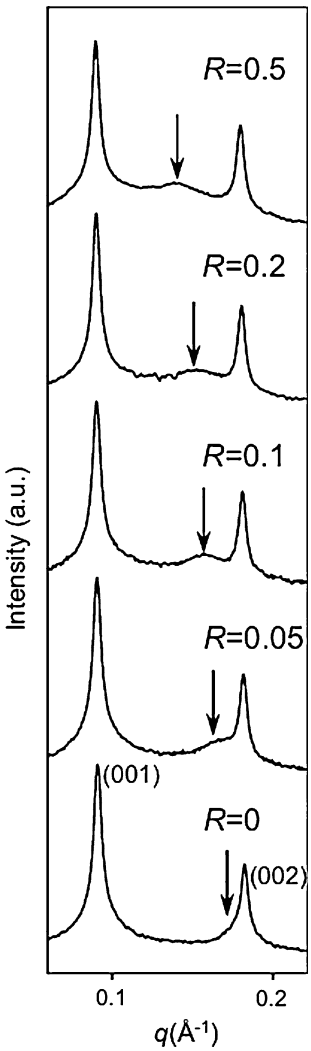

Fig. 3 SAXS patterns of mixed DC-Chol-DOPE/DNA/DOPA systems as a function of increasing anionic/cationic charge ratio R. Curves are arbitrarily shifted for clarity. As the mobile DNA peak (marked by an *arrow*) clearly shows, the one-dimensional DNA in-plane *rod* lattice is diluted by anionic lipids. For $R > 0.5$, the DNA peak was not seen in the X-ray pattern

that the 1D in-plane rod DNA lattice was progressively diluted by anionic lipids. For $R > 0.5$, the DNA peak was not detected from the X-ray pattern. In principle, this finding may imply that DNA release from lipoplexes leads to the complete loss of short-range order in the DNA–DNA correlations. Even though the exact explanation is currently under debate, the existence of a threshold $R = R_{DNA}$ (typically, $R_{DNA} < 1$), for which the correlation between DNA strands is lost, is unambiguous. The physical meaning of our results is therefore that dilution of the 1D DNA lattice is most likely to occur at the early steps of interaction before the lipoplex structure begins to be destroyed by cellular lipids. Such tendency is distinctive of any lipoplex formulations and may be related to varying levels of TE.

Unprecedented SAXS data (Pozzi et al. 2009) also clarified that the ability of a given AL to disorder the 1D DNA lattice depends on the nature of lipid molecules forming L_{α}^{C} complexes. It is currently believed that, in cells, lipoplexes may interact with a number of cellular membranes and that DNA may be gradually released after a number of multiple interactions. We address this issue below.

Lipoplex–cellular lipids interaction: the "structural stability" concept

Upon further addition of ALs ($R > 1$), the structure of lipoplexes is progressively disintegrated. Synchrotron SAXS is the most powerful technique to provide insights into the structural changes of lipoplexes/AL mixtures on the nanoscale; For instance, the evolution of the average domain size of the multilayers and the DNA arrays, L_m, can be estimated using the Debye–Scherrer relation $L_m = 2\pi/\Delta q$, where Δq is the full-width at half-maximum (FWHM) of the (001) diffraction peak in q space. For a proper calculation, Δq must be calculated as $\Delta q = \sqrt{\mathrm{FWHM}_{\mathrm{exp}}^2 - \mathrm{FWHM}_{\mathrm{beam}}^2}$, where $(\mathrm{FWHM})_{\mathrm{exp}}$ is the experimental width of the (001) diffraction peak and $(\mathrm{FWHM})_{\mathrm{beam}}$ is the width of the intrinsic instrumental resolution function. In most lipoplexes, L_m is about 2,500–3,000 Å with no AL added ($R = 0$), while it monotonically decreases with increasing R (Caracciolo et al. 2007a). This finding suggests that ALs gradually destabilize the multilamellar nanostructure of lipoplexes. Moreover, we found that the structural stability of lipoplexes upon interaction with ALs was strictly dependent on the lipid composition of lipid/DNA complexes. A persuasive example is given in Fig. 4, where the SAXS patterns of DOTAP-DOPC/DNA (left panel) and DC-Chol-DOPE–DMPC/DNA (right panel) lipoplexes upon interaction with anionic DOPG are reported. In the SAXS pattern of DOTAP-DOPC/DNA/DOPG mixtures, diffraction maxima of the L_{α}^{C} phase were visible up to $R = 1$. For $R > 1$, the system underwent a phase transition where disintegration of the initial structure occurred. SAXS experiments allow the identification of the precise anionic/cationic charge ratio R^* that is indispensable to fully solubilize the lipoplex structure ($R^* \approx 5$ for DOTAP-DOPC/DNA lipoplexes). In the regime of phase coexistence ($1 < R < R^* \approx 5$), the SAXS patterns of the DOTAP-DOPC/DNA/DOPG mixture are increasingly dominated by the lamellar phase of pure DOPG (data not reported). Further increase in the anionic lipid fraction ($R > 5$) leads to the third one-phase stage, where the system was identified as being in the lamellar phase of pure DOPG. Figure 4 (right panel) shows the SAXS patterns of DC-Chol-DOPE-DMPC/DNA/DOPG complexes as a function of increasing R. As evident, these lipoplexes were much more resistant than DOTAP-DOPC/DNA complexes. Indeed, at $R = 20$, the lamellar DC-Chol–DOPE–DMPC/DNA complexes still resisted disintegration by DOPG, as shown by the presence of the (001) (indicated by an arrow) and (002) Bragg peaks. In summary, SAXS studies clarified that, while all lipoplexes exhibit the same general phase behavior, relevant differences in their tendency to be destroyed by anionic lipids can be detected. In other words, when mixed with a specific AL, each lipoplex formulation exhibits its own anionic/cationic charge ratio at which the lamellar structure of lipoplexes is completely disintegrated, i.e., a specific R^* value. Thus, SAXS data indicate that the releasing activity of a given AL depends on the nature of the cationic component of the lipoplexes.

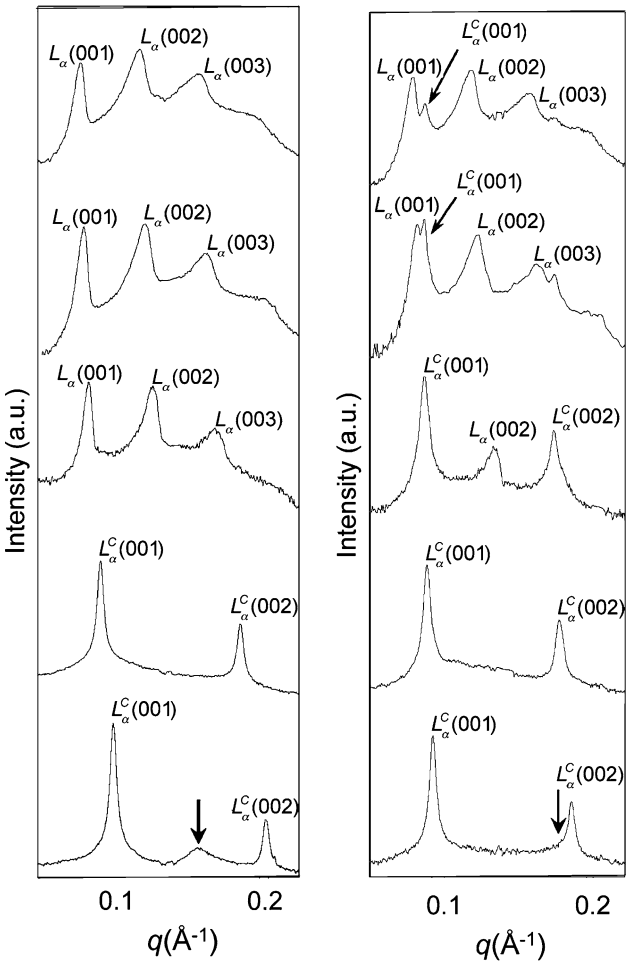

Fig. 4 SAXS patterns of DOTAP-DOPC/DNA (*left panel*) and DC-Chol-DOPE-DMPC/DNA (*right panel*) lipoplexes ($\Phi = 0.5$; $\rho = 3.2$) as a function of increasing anionic/cationic charge ratio, R

Remarkably, a clear correlation between the structural stability of lipoplexes upon interaction with cellular lipids and their TE has recently been established (Caracciolo et al. 2007a, b). In principle, the correlation between the structural stability of lipoplexes and their transfection activity may be ascribed to facilitated or impeded DNA release

(Koynova et al. 2006). To correlate structural changes of lipoplexes with TE behavior, the first step was to verify the existence of a correlation between disintegration of lipoplex structure and DNA release. Electrophoresis experiments have shown (Caracciolo et al. 2007a) that unstable lipoplexes rapidly release DNA at low R, while most stable complexes (such as the DC-Chol-DOPE-DMPC/DNA system) retain DNA up to high R. Therefore, the results can be generalized as follows: the higher the structural stability of complexes upon interaction with cellular membranes, the lower the extent of DNA release. Taken together, SAXS and electrophoretic data have revealed the existence of a general rule: DNA release is complete at $R \approx R^*$, as determined by SAXS. Since DNA release is a rate-limiting step for transfection, this observation let us theorize that the structure and TE of lipoplexes could strictly be related to each other. When plotted against R^*, it becomes obvious that TE data collapse onto a single quasi-Gaussian curve (Fig. 5), suggesting the existence of three TE regimes. In the first regime (Fig. 5, regime A), one finds complexes that are easily solubilized by anionic lipids. Such complexes are poorly efficient (TE $\approx 10^7$ relative light unit (RLU)/mg protein). In regime B, lipoplexes exhibit the highest efficiency ($10^8 <$ TE $< 10^9$ RLU/mg protein). In regime C, corresponding to highly stable complexes, again low efficiency is observed. Regime B appears therefore to be the "region of optimal stability." The bell-shaped curve of TE versus R^* is clearly seen in the cases of NIH 3T3, ovarian CHO, and tumoral A17 cell lines (Fig. 5, top, middle, and bottom panels, respectively). The universal behavior found in Fig. 5 let us put forth the "structural stability concept" that can be summarized as follows: Unstable lipoplexes rapidly release their gene payload with the consequence that free DNA is digested by DNAase. In contrast, most stable complexes entrap DNA within endosomal structures (Fig. 5, regime C). In both cases, transfection does not result in expression. "Optimally stable" lipoplexes (Fig. 5, regime B) are the appropriate compromise between this interplay, most probably due to a gradual DNA release in the cytoplasm. The structural stability concept has been confirmed by confocal laser scanning microscopy (Caracciolo et al. 2009; Marchini et al. 2011). When cells are treated with unstable complexes such as DOTAP-DOPC/DNA complexes, naked DNA outside the cells is often detected. This finding indicates that destabilization of unstable lipoplexes may even occur at the early steps of internalization (e.g., at the cell surface), thus leading to poor DNA uptake. On the other hand, the most stable lipoplexes enter cells, traffic towards the nucleus, but show a clear perinuclear accumulation. "Optimally stable" lipoplexes are largely internalized within cells and release efficiently their DNA cargo both in the cytoplasm and in the nucleus (Caracciolo et al. 2009).

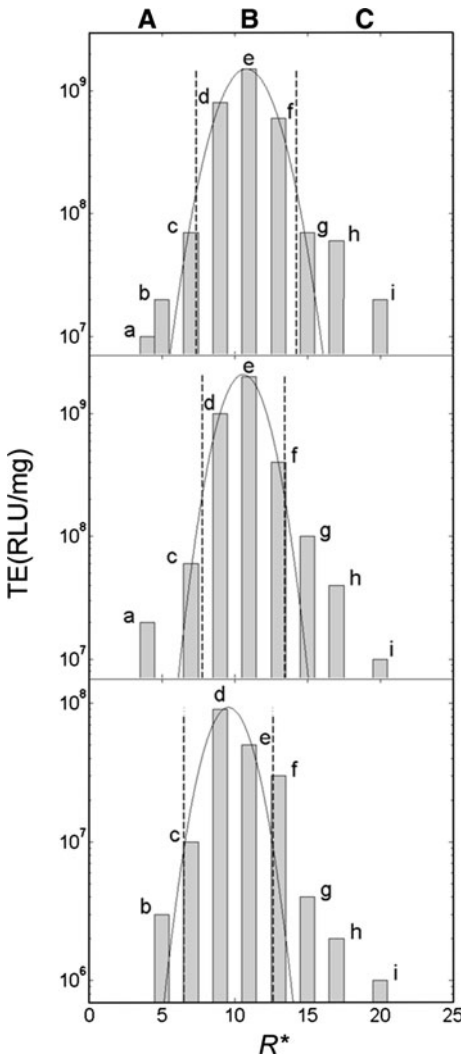

Fig. 5 TE in RLU/mg of cellular proteins plotted as a function of the charge ratio indispensable to fully solubilize the lipoplex structure R^*. R^* values refer to disintegration of lipoplexes by DOPG for the cell lines: NIH 3T3 (*top panel*), CHO (*middle panel*), and A17 (*bottom panel*). DOTAP-DOPC (*a*); DC-Chol-DMPC (*b*); DOTAP-DOPC (1:2) (*c*); DOTAP-DC-Chol-DOPC-DOPE (*d*); DOTAP-DC-Chol-DOPC-DOPE-DLPC-DMPC (2:2:1:1:1.1) (*e*); DC-Chol-DOPE (*f*); DOTAP-DOPC-DLPC (2:1:1) (*g*); DOTAP–DLPC (*h*); DC-Chol-DOPE-DMPC (2:1:1) (*i*). Where not specified, lipid species are in equimolar ratio. *Solid lines* are the best Gaussian fits to TE data. *Vertical dashed lines* identify three regimes of stability: low (*A*), optimal (*B*), and high (*C*). Boundaries of the regimes were identified by R^* values for which TE(R^*) = $(TE)_{max}/10$; i.e., TE is one order of magnitude lower than the maximum value $(TE)_{max}$

Lastly, a comment on the relation between the structural stability of lipoplexes and the membrane charge density of AL is mandatory. To better simulate the conditions that exist when lipoplexes interact with cellular lipids, MM lipid blends are used, such as MM1 and MM2 lipid mixtures. SAXS has demonstrated the existence of a strict correlation between the charge density of membranes containing a specific AL species (such as DOPG or DOPS)

and their ability to destabilize the structure of lamellar lipoplexes: the higher the charge density of anionic membranes, the higher their ability to solubilize the nanostructure of a given lipoplex formulation. SAXS experiments also provided evidence that, for a given MM mixture, the solubilization ranking of lipoplexes strongly depends on the shape coupling between the lipoplex and anionic cellular lipid AL used. This observation is consistent with the "phase evolution concept" previously suggested by Koynova et al. (2005). This concept is addressed in the next paragraph.

Lipoplex–cellular lipids interaction: the "phase evolution" concept

Over the last decade, several investigators have performed combined SAXS and TE investigations aimed at elucidating the relationship between the phase evolution of lipoplexes upon interaction with cellular lipids and their TE. The most relevant results have been obtained by Koynova and coworkers and have recently been reviewed (Koynova 2008). Remarkably, correlation between the delivery efficiency of lipoplexes and the mesomorphic phases they adopt when mixed with cellular lipids has been established. Highly efficient lipoplex formulations form phase of high negative interfacial curvature (bilayer cubic, inverted hexagonal, micellar cubic) when mixed with ALs. Thus, lipoplexes that are prone to transform into nonlamellar phases when mixed with ALs would very easily release DNA, which is the likely reason for their high TE. However, although the phase preferences of the anionic/cationic lipid mixtures might well be expected to influence the DNA-releasing efficiency, the existence of a direct general correlation still remains controversial (Pozzi et al. 2009). To investigate the phenomenon, we mixed numerous lipoplex formulations DOPG, DOPS and DOPA. When lipoplexes were mixed with DOPG and DOPS, all of them retained their lamellar structure up to complete disintegration. On the other hand, upon interaction with DOPA, all the lipoplex formulations exhibited a lamellar–hexagonal phase change. An example is given in Fig. 6a, b, where the SAXS patterns of DOTAP-DOPC/DNA/DOPA and DC-Chol-DOPE/DNA/DOPA mixtures as a function of *R* are shown. Remarkably, the phase transition rates depend on the lipid composition of the lipoplexes. This can be seen in Fig. 6c, d, where it is clear that the extension of the monophasic and biphasic regions of the phase diagram of lipoplex/DOPA mixtures largely depend on the lipid composition. As a general rule, DOPA holds the potential to solubilize lipoplexes rich in DOPE much more easily than those rich in phosphatidylcholines (PCs). Conversely, it was shown that DOPG and DOPS express greater ability to destroy the initial structure of complexes rich in PCs (see

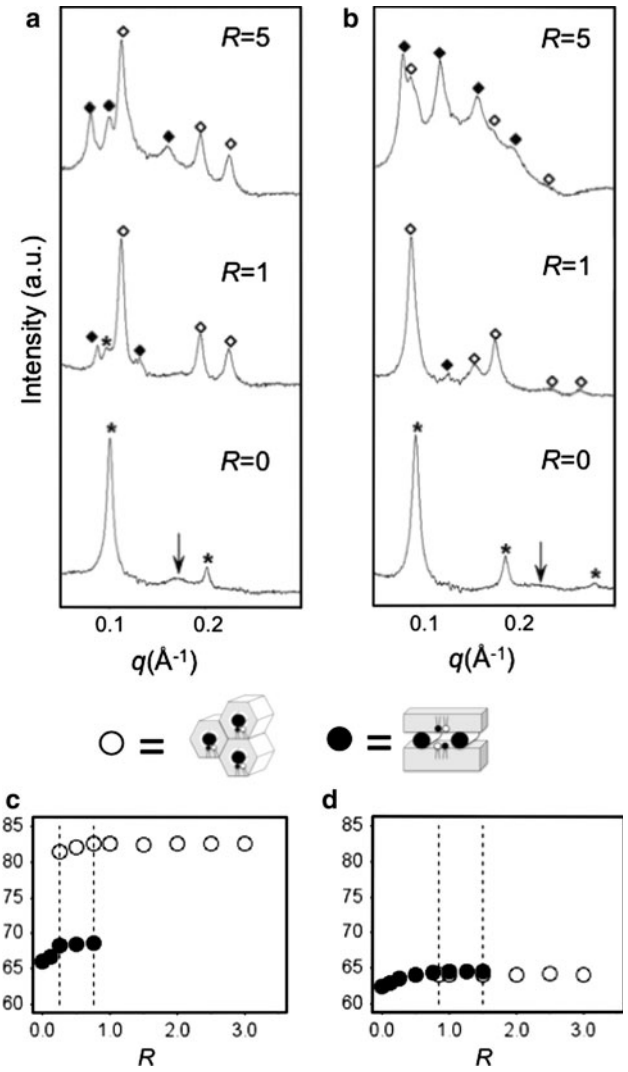

Fig. 6 SAXS patterns of DOTAP-DOPC/DNA (*a*) and DC-Chol-DOPE/DNA (*b*) lipoplexes ($\Phi = 0.25$) upon interaction with increasing amounts of DOPA. Bragg peaks arising from the lamellar structure of pure lipoplexes are indicated with *asterisks*, while the DNA peak is marked by an *arrow*. Diffraction maxima of hexagonal complexes are indicated with *white diamonds* and reflections of pure DOPA with *black diamonds*. Phase diagrams of DOTAP-DOPC/DNA and DC-Chol-DOPE/DNA lipoplexes as a function of the anionic/cationic charge ratio, *R*. *Dashed lines* separate regions occupied by lamellar lipoplexes, coexisting lamellar and hexagonal lipoplexes, and hexagonal lipoplexes

'Lipoplex–cellular lipids interaction: the "structural stability" concept'). Because DOPE has a negative spontaneous curvature, while PCs are bilayer-forming lipids (for which the curvature is zero), we put forth the concept that ALs with a disposition to form nonlamellar phases (such as DOPA) solubilize more easily complexes made of lipids prone to form nonlamellar structures, while ALs with a tendency to form lamellar phases (such as DOPG and DOPS) express a greater ability to destroy the initial lamellar structure of lipoplexes rich in bilayer-forming

lipids. Such a general feature of lipoplex/AL mixtures suggests that the tendency of lipoplexes to be destroyed by ALs does depend on the shape coupling between the lipoplex and anionic membrane lipids. The lipid shape is a very useful concept in membrane biophysics, frequently used to describe the volume occupied by phospholipids (Israelachvili et al. 1977). Using this approach, phospholipids can be classed as cylinders (e.g., PC), cones (e.g., phosphatidylethanolamine, PE), and inverted cones (e.g., lysophosphatiodylcholine), depending on the relative volumes of their polar head-groups and fatty acyl chains. Among other influences, shape and packing constraints have a major effect on lipid mixing and fusion. According to such studies, SAXS data suggest that a low packing competition between lipoplex and ALs may result in the high incorporation efficiency of ALs within lipoplex membranes. Such a packing-dependent integration ability of ALs is therefore supposed to regulate the destabilization of the lipoplex structure resulting in DNA release. In a recent work (Kasson and Pande 2007) the ability of a lipid perturbant to compensate for lipid-packing mismatch has been invoked to rationalize its capacity to promote stalk formation, which is the commonly recognized initial fusion intermediate that leads to lamellar–hexagonal phase transition. In summary, lipids that are similar in terms of "molecular shape" (such as DOPA and DOPE) appear to have an enhanced ability to mix, therefore lowering the lamellar–nonlamellar phase-transition energetic barriers (Kasson and Pande 2007). The phase evolution concept (Koynova et al. 2005) has been successfully applied to explain the results of TE studies (Marchini et al. 2010; Cardarelli et al. 2012). The plasma membrane is considered as a patchwork of microdomains that are strongly regulated by lipid shape. The shapes of the volumes occupied by membrane lipids allow them to coordinately pack with other lipid types with "cognate" volume shapes, and this allows membrane curvature generation, which is a prerequisite for any endocytic pathway. Electrostatic attractions let the lipoplex approach the anionic surface of the cell made of lipid domains with size ranging from ~70 nm to 1 μm. Since lipoplexes are about 200–500 nm in diameter, each lipid/DNA complex could interact with one or two lipid platforms. Shape coupling between lipoplex and membrane lipids is expected to regulate the efficiency of lipoplex–membrane interaction, thus determining the success of internalization along the specific pathway associated with the activated membrane domain (or alternatively, the success of escape from endosomes; Fig. 7). Thus, cholesterol-rich lipoplexes could favorably interact with lipid rafts where sphingolipids and cholesterol pack tightly, excluding most phospholipids with unsaturated hydrocarbon tails. This is in agreement with results showing the reduction in TE produced by cholesterol depletion when cells are treated with cholesterol-containing lipoplexes. For the same reason, DOTAP-DOPC liquid-crystalline bilayers could favorably interact with fluid-phase domains that are richer in unsaturated lipids of similar shape. This is supported by TE results obtained after treatment cells with wortmannin (that inhibits fluid-phase macropinocytosis). In summary, efficiency of lipoplex–cellular interaction membranes is regulated by shape coupling between lipoplex and membrane lipids. This suggests that tailoring the lipoplex lipid composition to the patchwork-like membrane profile could be a successful strategy for coordinating the intracellular pathway activities of lipoplexes. Furthermore, adjusting the lipoplex lipid composition may provide a convenient approach for targeting extracellular receptors for therapeutic applications (Herringson et al. 2009).

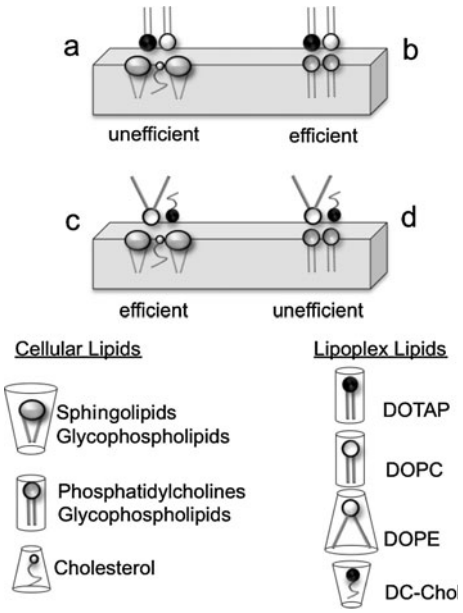

Fig. 7 Proposed mechanism of the interaction of lipoplexes with the plasma membrane of the cell. When lipoplexes made of cylinder-like fluid-phase lipids bind to lipid rafts (*a*), interaction is unfavorable and complexes accumulate at the plasma membrane, while binding to fluid microdomains results in efficient internalization (*b*). Lipoplexes made of cholesterol-like lipids and cone-shaped lipids preferentially interact with lipid rafts rich in cholesterol (*c*), whereas interaction with cylinder-shaped membrane domains is inefficient (*d*). This mechanism might also be relevant for interaction between lipoplexes and endosomal membranes

Conclusions and future studies

SAXS studies have helped to link the structure and activity of lipoplexes by clarifying several mechanisms responsible for gene transfection. This basic knowledge needs to be integrated into the design of next-generation lipoplexes for enhanced transfection. To bring lipoplexes to the forefront

of gene delivery, understanding how transfection mechanisms are affected by the "protein corona" worn by lipoplexes in vivo is an urgent task (Lynch et al. 2009; Caracciolo et al. 2010b; Walczyk et al. 2010; Monopoli et al. 2011a, b). Future work will be performed in this direction.

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